in control of mitogenic signals activated by EGF[36]. That the *O. viverrini* ES product markedly stimulated the expression of this gene represents the possibility that this parasitic product stimulates cell proliferation *via* EGF-mediated signal transduction cascade. There are 4 main pathways induced by EGF, including the activations of Ras/Raf/ERK, phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ (PLC-γ)/protein kinase C (PKC) and c *jun*-N-terminal kinase (JNK). These pathways lead to different cellular actions[37]. Only the Ras/Raf/ERK and JNK-transduced signal transduction pathways involve cell proliferation. In this study, the expression of *h ras* and *raf* 1 was slightly up-regulated by *O. viverrini* ES product, indicating *O. viverr*ini ES product stimulates cell proliferation *via* other pathways rather than the common pathway of Ras/Raf/ERK. The up-regulated expression of *pk*C represents the possibility of cell proliferation activated by *O. viverrini* ES product *via* the EGF-stimulated PKC signal transduction pathway. This is opposed to the data that *pk*C-mediated signal transduction pathway stimulated by EGF does not involve in control of cell proliferation[37]. Moreover, the array data showed the increased expression of JNK (2.78-fold, data not shown) represents the possibility that EGF-associated JNK pathway may be the pathway activated by *O. viverrini* ES product.

Taken all together, *O. viverrini* ES product activates cell proliferation *via* either TGF-β- or EGF-mediated signal transduction pathways. These two signal pathways have been proved to be strongly correlated with cancer development[37,38]. TGF-β also mediates tumor-promoting effects, through differential effects either on tumor or stromal cells[38]. Its signaling pathway is being evaluated as a prognostic or predictive marker for cancer patients. Over-expression of EGF has been found in many cancer types[37]. The roles of TGF-β and EGF in cholangiocarcinogenesis need further investigations. Regardless of the exact signal transduction pathway stimulated by *O. viverrini* ES product, the induction of fibroblast cell proliferation and the accumulation of extracellular matrix (i.e. collagen) have been proven to be associated with the fibrogenetic process[13]. The increased expression of collagen type I, III, and IV (between 2-3-fold increase) was observed in cDNA array analysis (data not shown). This supports the hypothesis that *O. viverrini* ES product can stimulate cell proliferation and collagen production leading to the fibrosis formation. Since the fibrosis has been detected in cholangiocarcinoma in both animal models and human[9-11], it is possible that *O. viverrini* ES product-induced fibrosis may be one component in cholangiocarcinogenesis. Moreover, it may be proposed that the ES product-induced fibrosis may act synergistically with the immunological response against the parasites to promote CC.

In summary, this study demonstrates the gene expression profile obtained from cDNA array analysis to determine the possibility of *O. viverrini* ES product as the growth factor in induction of fibroblast cell proliferation. Signal transduction-related genes may have a significant expression increment in cells exposed to this parasitic product. Among this set of genes, *tgfβ* 1*i*4 and *eps* 8 were found to have the strongest expression levels with corroboration data obtained by both cDNA array and RT-PCR. These two genes are the specific signal transduction molecules for TGF-β- and EGF-stimulated pathways. It may therefore be proposed that *O. viverrini* ES product activated cellular proliferation *via* the pathways of these growth factors. The issues of whether one type of growth factor is capable to cross-stimulate the two pathways or two types of growth factors are available in the *O. viverrini* ES product, needs future experiments to be clearly elucidated. This report is an important resource to indicate the mechanism of *O. viverrini* ES product-stimulated fibroblast cell proliferation *via* TGF-β- or EGF-stimulated signal transduction pathways. Since the activated fibroblast has been shown to initiate non-tumorigenic epithelium to carcinoma[16], *O. viverrini* ES product-stimulated fibroblast may play a part to change bile duct epithelium into CC. The ongoing research in understanding the signaling pathways stimulated by *O. viverrini* ES product and roles of fibroblasts in the development of CC will provide a novel target for chemoprevention and treatment of fibrosis in this cancer which may delay the formation of CC.

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Collaborative Action of NF-κB and p38 MAPK Is Involved in CpG DNA-Induced IFN-α and Chemokine Production in Human Plasmacytoid Dendritic Cells[1]

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CpG DNA induces plasmacytoid dendritic cells (pDC) to produce type I IFN and chemokines. However, it has not been fully elucidated how the TLR9 signaling pathway is linked to these gene expressions. We examined the mechanisms involving the TLR9 and type I IFN signaling pathways, in relation to CpG DNA-induced IFN-α, IFN regulatory factor (IRF)-7, and chemokines CXCL10 and CCL3 in human pDC. In pDC, NF-κB subunits p65 and p50 were constitutively activated. pDC also constitutively expressed IRF-7 and CCL3, and the gene expressions seemed to be regulated by NF-κB. CpG DNA enhanced the NF-κB p65/p50 activity, which collaborated with p38 MAPK to up-regulate the expressions of IRF-7, CXCL10, and CCL3 in a manner independent of type I IFN signaling. We then examined the pathway through which IFN-α is expressed. Type I IFN induced the expression of IRF-7, but not of IFN-α, in a NF-κB-independent way. CpG DNA enabled the type I IFN-treated pDC to express IFN-α in the presence of NF-κB/p38 MAPK inhibitor, and chloroquine abrogated this effect. With CpG DNA, IRF-7, both constitutively and newly expressed, moved to the nuclei independently of NF-κB/p38 MAPK. These findings suggest that, in CpG DNA-stimulated human pDC, the induction of IRF-7, CXCL10, and CCL3 is mediated by the NF-κB/p38 MAPK pathway, and that IRF-7 is activated upstream of the activation of NF-κB/p38 MAPK in chloroquine-sensitive regulatory machinery, thereby leading to the expression of IFN-α. *The Journal of Immunology,* **2006, 177: 4841–4852.**

Dendritic cells (DC)[3] consist of a heterogeneous population of APC that regulates immune responses. They are characterized by surface markers and cytokines induced in response to inflammatory stimuli, including the ligands for TLRs (1), which recognize distinct families of pathogenic products. Among DC, plasmacytoid DCs (pDC) are a unique population exhibiting plasmacytoid morphology (2). The biological distinction of pDC is that they produce a large amount of IFN-α through the ligation of TLR9 (3) with bacterial DNA or its synthetic counterpart, the so-called oligoDNA containing unmethylated CpG motifs (CpG DNA) (4–6). They also produce chemokines (7–9); through the interplay with the IFN-α, pDC participate not only in innate immunity but also in adaptive immunity (2).

Due to the identification of pDC and the discovery of microbial inducers of IFN-α, IFN-α has recently been recognized as a multifunctional cytokine (2, 10). IFN-α, as a member of the type I IFN-family, deliver signals to the classical type I IFN pathway via IFN-αβ receptor, and consequently the genes whose promoters carry IFN-stimulated response elements (ISRE) are transcribed. IFN regulatory factor (IRF) 7 is one of the genes up-regulated by type I IFN (11–13). In pDC, however, IRF-7 is constitutively expressed (14–17). Its association with MyD88 and TNF receptor-associated factor 6 (TRAF6) has recently been reported to be a prerequisite for the activation of the IFN promoter (18, 19).

Following ligation of TLR9, the adaptor MyD88 recruits signaling mediators to activate NF-κB (20). Therefore, most of the chemokines whose gene expression requires the activation of NF-κB could be induced through the TLR9 signaling in pDC. However, to our knowledge, there has not yet been any report that referred to the integral participation of NF-κB in the expression of IFN-α and chemokines in CpG DNA-stimulated human pDC. Recently, it has been demonstrated that, besides ISRE, the human IRF-7 gene promoter has a NF-κB binding site (21), and the activation of NF-κB is required for EBV latent membrane protein 1- and TNF-α-induced IRF-7 expression (21, 22). Then, we speculated that, in the end-point of TLR9 signaling, the activation of NF-κB could be involved in the up-regulation of the gene expression of IRF-7, subsequently of IFN-α, and that of other NF-κB-dependent chemokines in human pDC.

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[3] Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid DC; ISRE, IFN-stimulated response element; IRF, IFN regulatory factor; TRAF6, TNF receptor-associated factor 6; ODN, oligonucleotide; Act D, actinomycin D; CHX, cycloheximide; PDTC, pyrrolidinedithiocarbamate; CAPE, caffeic acid phenethyl ester; DEX, dexamethasone; PMX, polymixin B; BDCA, blood DC Ag; SR, scavenger receptor.

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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 GGGGGGGGGGAGCAT 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 × 10^6/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 palGACGA1010 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, palGACGA1010 up-regulated the expression of all of the genes with the exception of IFN-κ (Fig. 1*A*). 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. 1*B*. 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. 1*C*). 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 I 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 I IFN isotypes and confirmed that CXCL10 and CCL3 were expressed, as was IRF-7 that was used as a representative type I IFN-inducible gene (Fig. 2*A*). 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 expression between the culture with and without Ab was observed (Fig. 2*B*). Up-regulation of CXCL10 and CCL3 by CpG DNA thus appears to be initiated in a manner independent of the type I IFN

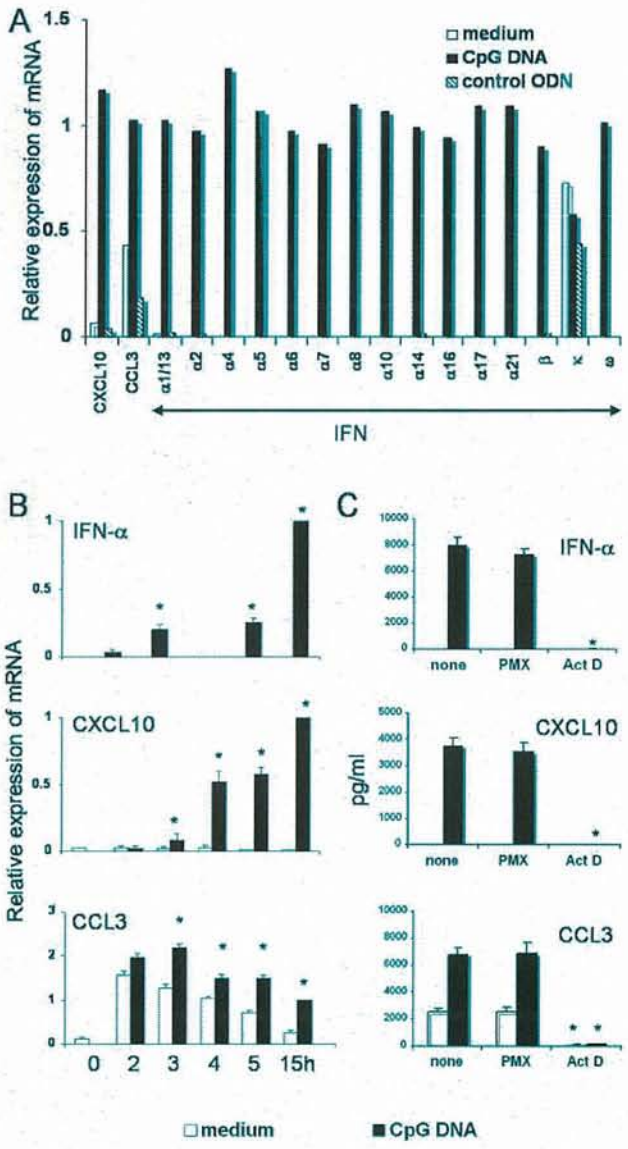

FIGURE 1. CpG DNA induces human pDC to express CXCL10 and CCL3, in addition to type I IFNs. *A*, pDC were cultured for 16 h with CpG DNA, control ODN, or medium alone, and RT-PCR was performed for the genes indicated. Data are from one of four separate experiments and shown as the mean relative expression levels in a triplicate assay. Three donors' pDC were used for one set of assay. The CpG DNA gene induction for chemokines and type I 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.

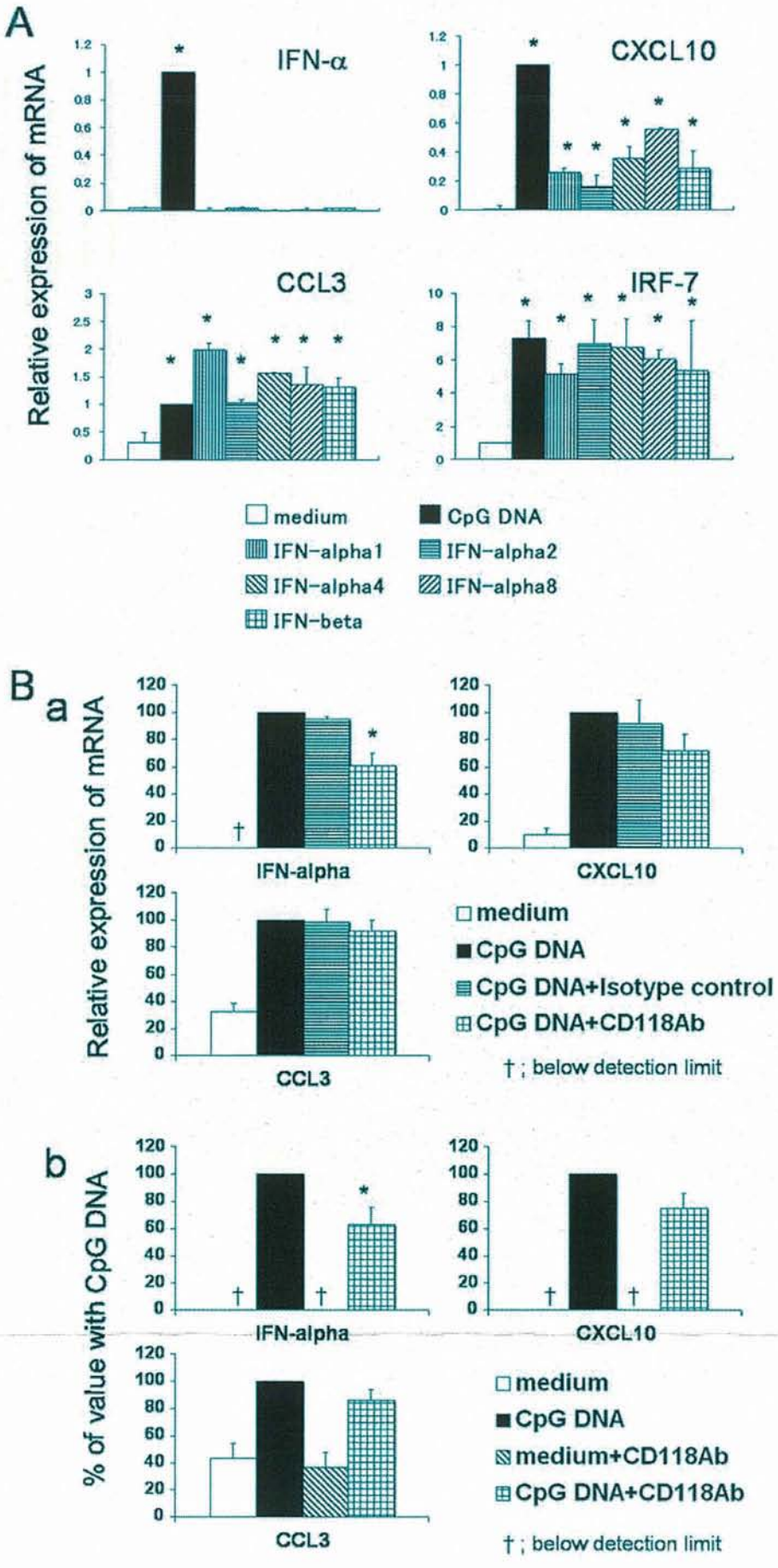

FIGURE 2. Type I IFNs induce pDC to express CXCL10 and CCL3, but not IFN-α, whereas CpG DNA induces them to express IFN-α, CXCL10, and CCL3 in the presence of CD118 Ab. *A*, pDC were cultured overnight with medium alone, CpG DNA, control ODN, or 1000 IU/ml IFN-α1, α2, α4, α8, or β, and the genes for 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. 2*B*. 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

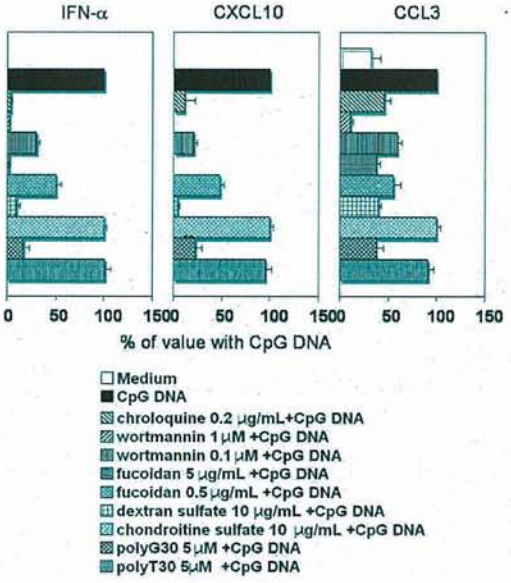

FIGURE 3. SR-A ligand-sensitive pathway and endosomal maturation are required for the CpG DNA-induction of CXCL10 and CCL3 as it is for IFN-α induction. pDC were cultured for 12–18 h with medium or CpG DNA in the presence or absence of chloroquine (0.2 μg/ml), wortmannin (1 and 0.1 μM), fucoidan (5 and 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. 4*A*, 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. 4*B*). 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. 5*A*). 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. 5*B*). 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. 5*C*). The modest increment of the binding activities by CpG DNA stimulation may be accounted for by their constitutive activation.

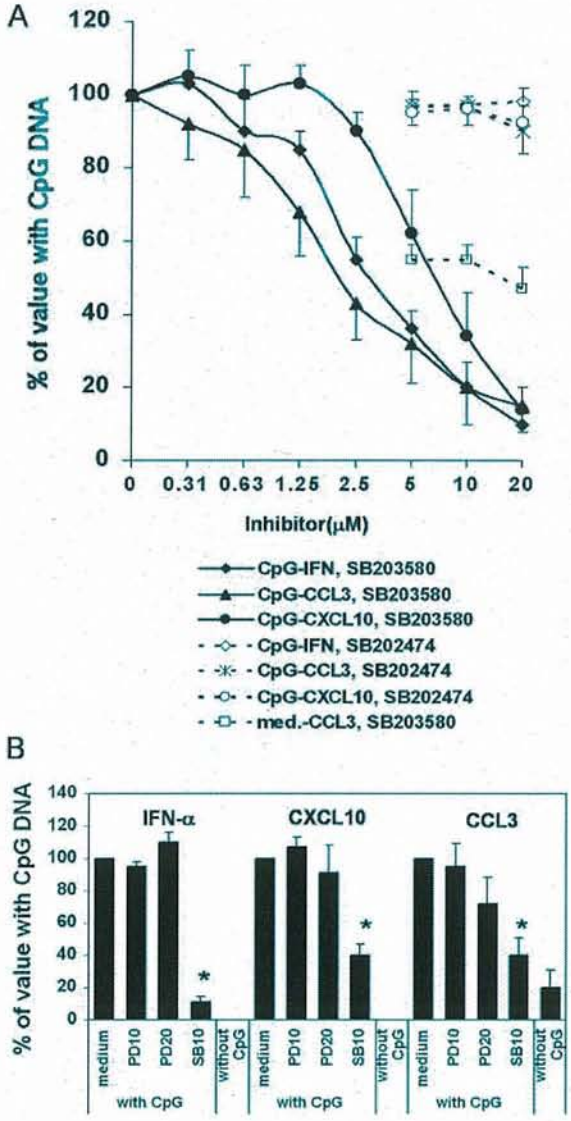

FIGURE 4. The p38 MAPK pathway involved in the induction of IFN-α is also required for that of CXCL10 and CCL3. *A*, pDC were precultured for 1 h with or without various concentrations of SB208530 or SB202474, and cultured for 14 h with medium (med.) or CpG DNA (CpG). The amounts of IFN-α, CXCL10, and 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 inhibitor-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. 5*C*). Type I IFN induced IRF-7 expression in pDC (Fig. 2*A*), and this pathway was in part involved in the augmentation of IFN-α production by CpG DNA (Fig. 2*B*). 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. 5*D*, *left*). This was also the case for CXCL10 (Fig. 5*D*, *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. 5*A*). 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 8*B*). 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. 6*A*, 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. 6*B*, *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. 6*B*, *right*). These results suggest

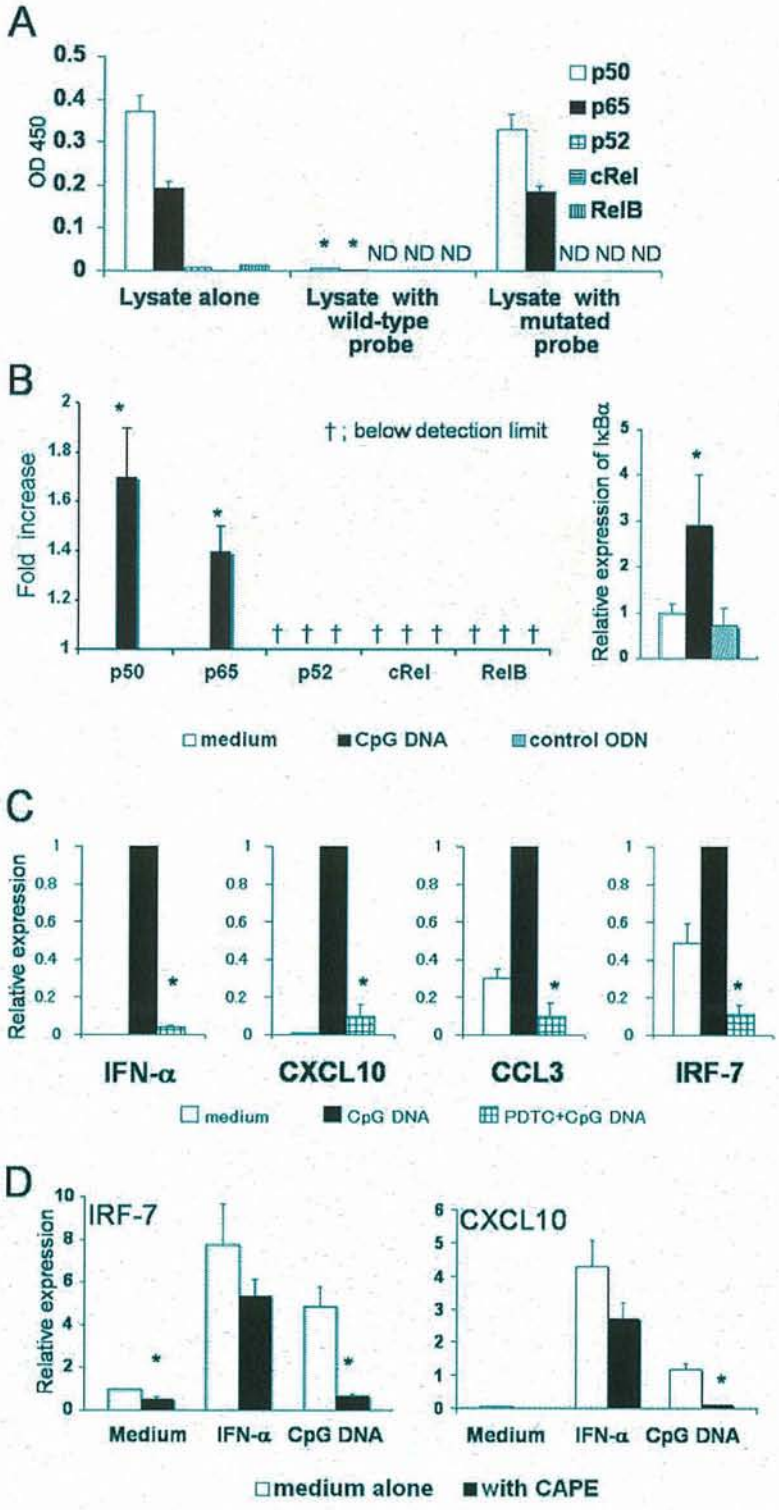

FIGURE 5. NF-κB p65 and p50 are constitutively activated in pDC, and their activities are up-regulated by CpG DNA. *A*, Different donors' pDC were pooled, and their nuclear proteins were analyzed in triplicate for the binding activity of NF-κB family members using TransAM NFκB Kits. Values (mean ± SD) 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. 7*A*). However, the data shown in Figs. 2*B* and 5*D* suggest that type I 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

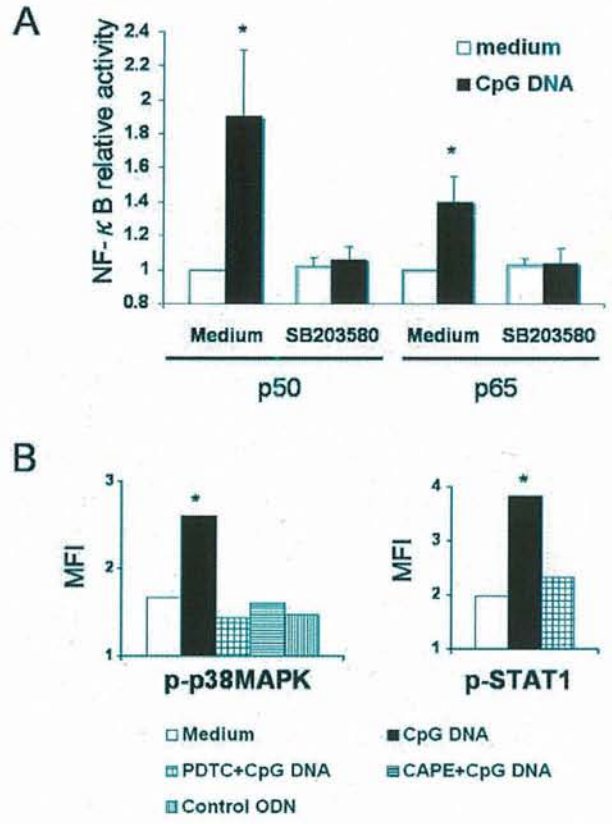

FIGURE 6. CpG DNA causes collaborative activation in p38 MAPK and NF-κB. *A*, pDC were treated for 1 h with or without 10 μM SB203580, and cultured for 3 h with or without CpG DNA. The activities of NF-κB p50 and p65 were analyzed as described in Fig. 5. Data are the mean ± SE of four separate experiments, 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.

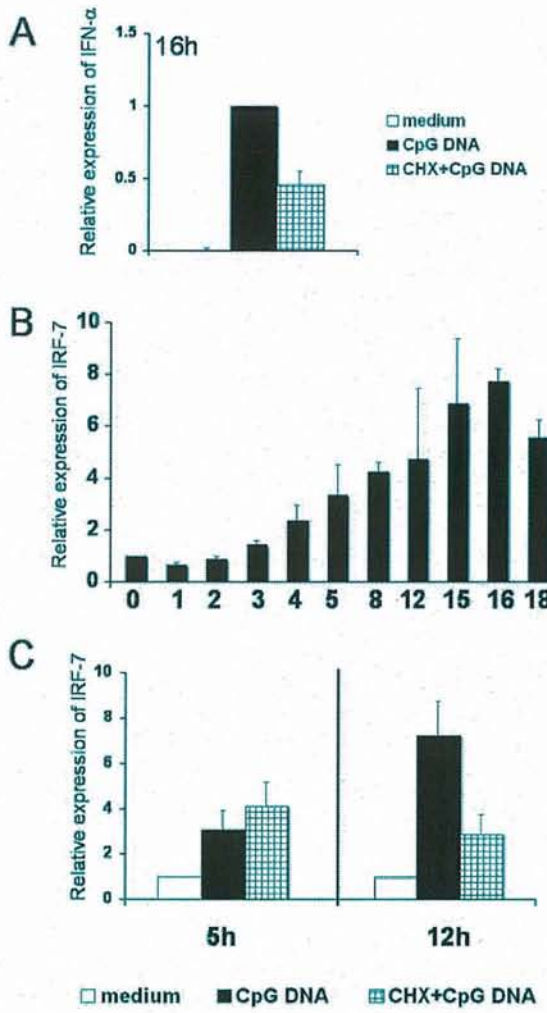

FIGURE 7. CpG DNA can up-regulate the expression of IRF-7 independently of protein synthesis. *A*, pDC were preincubated with or without 1 μg/ml CHX, which we confirmed blocked the CpG DNA-induced production of IFN-α, CXCL10, and CCL3, and cultured for 16 h with CpG DNA or medium alone. The expression 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.

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 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

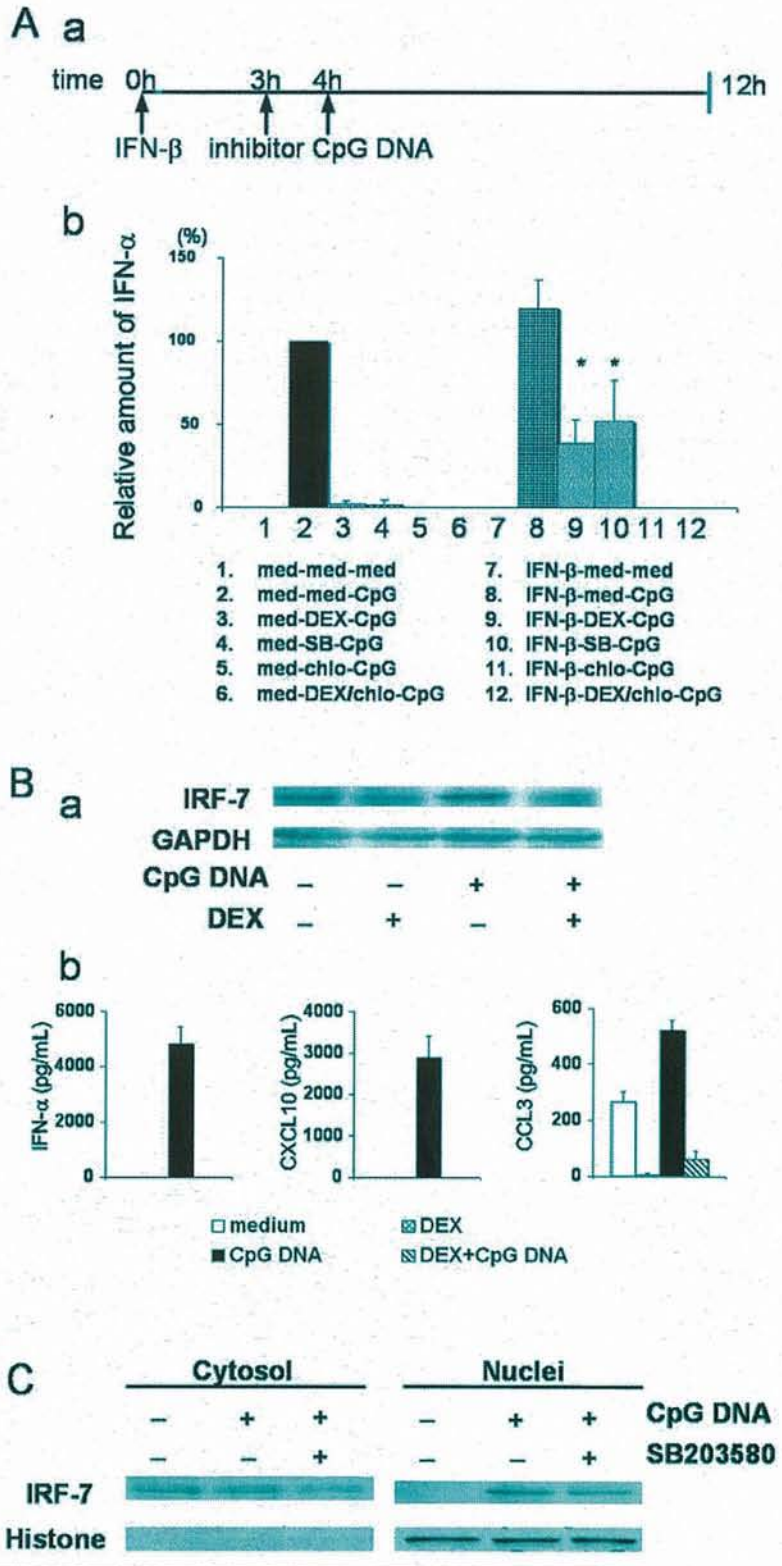

FIGURE 8. Machinery sensitive to chloroquine but not to NF-κB/p38 MAPK is involved in the expression of IFN-α via the activation of IRF-7. *A*, pDC were precultured for 3 h with or without 200 IU/ml IFN-β, and then for 1 h with DEX (0.5 μM), SB203580 (SB, 20 μM), chloroquine (chlo, 0.2 μg/ml), DEX + 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-αε 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.

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.

8A). 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. 8*C* 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. 2*A*, 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 8*B*); IRF-7 was directly induced by CpG DNA (Fig. 7*C*), which activated NF-κB (Fig. 5*B*); and pDC failed to up-regulate IRF-7 when pretreated with NF-κB inhibitor (Figs. 5, *C* and *D*, and 8*B*). 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. 5*C*). 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. 2*A*), all seem to be mediated by the activation of NF-κB (Fig. 5*C*) 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. 5*D*). It is not clear at present whether the signal transduction

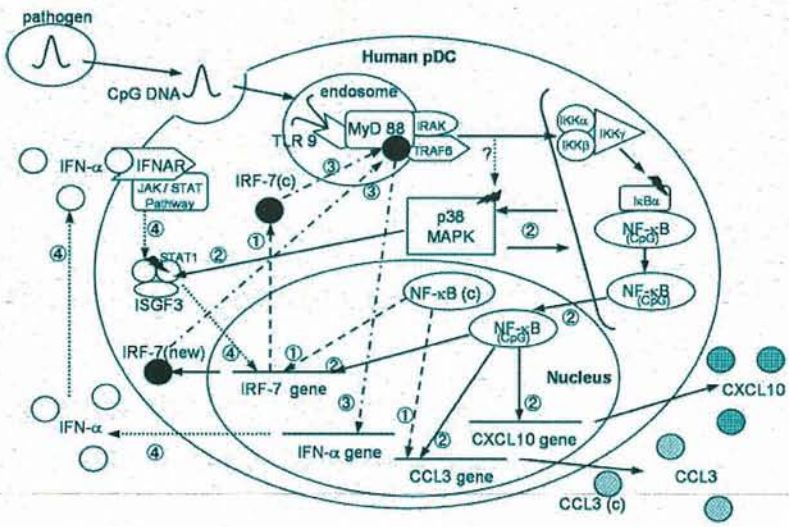

FIGURE 9. Proposed pathways which lead to the expression of IFN-α, CXCL10, and CCL3 following stimulation of human pDC with CpG DNA. The signaling pathway depicted is based on the finding of the current and previous studies (14). ①, Human pDC express constitutively activated NF-κB (NF-κB(c)), which would 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. 6*B*), 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. 7*C*), 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-dependent 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. 2*A* and 5*D*), and neutralization of IFN-αβ receptor decreased IFN-α production (Fig. 2*B*). However, this cascade was mostly conducted in a NF-κB-independent way (Fig. 5*D*). 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 palGACGA1010 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.

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Disclosures

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ORIGINAL PAPER

ADAM33 polymorphisms are associated with asthma susceptibility in a Japanese population

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Clinical and Experimental Allergy,

Summary

Background Asthma is the most common chronic disorder in childhood, and asthma exacerbation is an important cause of childhood morbidity and hospitalization. Asthma is believed to be a complex disorder involving genetic and environmental factors, and several asthma susceptibility loci have been identified through genome-wide screening. A disintegrin and metalloprotease 33 (*ADAM33*) was the first asthma susceptibility gene to be discovered by positional cloning in 2002.

Objective The aim of the present study was to investigate whether single-nucleotide polymorphisms (SNPs) in *ADAM33* are associated with childhood asthma in the Japanese population.

Methods Twenty-three *ADAM33* SNPs were genotyped by fluorescence correlation spectroscopy with the use of DNA from 155 families (538 members) identified through children with atopic asthma. The transmission disequilibrium test (TDT) was performed for family-based association study.

Results TDT revealed that minor alleles of S+1, ST+4, and T2 SNPs were over-transmitted to asthma-affected offspring ($P < 0.05$). According to the haplotype TDT, no haplotype of *ADAM33* was transmitted preferentially to asthmatic offspring.

Conclusion Our results confirm the involvement of *ADAM33* in the development of childhood asthma among the Japanese.

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Introduction

Asthma is the most common chronic disorder in childhood, and asthma exacerbation is an important cause of childhood morbidity and hospitalization. Overall, asthma affects nearly 155 million individuals worldwide [1].

Atopy, which is characterized by increased levels of IgE against common environmental allergens, is considered the strongest predisposing factor for asthma. Family and twin studies have shown that atopy runs in families [2–4]. More than 90% of children with asthma develop specific IgE against the house dust mites, and dust-mite allergy is strongly associated with asthma [5, 6]. However, only a small subset of subjects with dust-mite allergy develops asthma [7], suggesting that other factors are necessary for the development of asthma. Another characteristic feature of asthma is bronchial hyper-responsiveness (BHR). BHR involves altered airway structure and wall thickening, and thickened asthmatic airways account for a large proportion of cases of BHR and excessive airway narrowing. Previous studies have shown that BHR is inherited independently of atopy [8, 9], suggesting that genes involved in BHR differ from those involved in atopy. These data suggest that genes involved in BHR in addition to genes for atopy play important roles in the development of asthma.

Asthma is believed to be a complex disorder involving genetic and environmental factors, and several asthma susceptibility loci have been identified through genome-wide screening [10–16]. A gene encoding a disintegrin and metalloprotease 33 (ADAM33) was the first asthma

susceptibility gene to be discovered by positional cloning in 2002 [17]. Since 2002, a number of replication studies have been conducted in diverse ethnic populations and in patients with different clinical manifestations. Some single-nucleotide polymorphisms (SNPs) in *ADAM33* have been associated with asthma, but others showed conflicting results (reviewed in reference [9]). Recently, Blakey et al. [18] performed a meta-analysis for *ADAM33* and asthma and reported that the F+1 and ST+7 variants in *ADAM33* were significantly associated with asthma in case–control and family-based studies. The role of ADAM33 in the development of asthma remains unclear, but the accumulating data indicate that ADAM33 may be involved in airway remodelling and may determine lung function throughout life [9]. To date, most data available for *ADAM33* and asthma have been obtained from Caucasians, and few data on *ADAM33* SNPs associated with asthma and atopic disease are available for Asian populations [19, 20].

The aim of the present study was to determine whether *ADAM33* SNPs are associated with the development of asthma in the Japanese. We genotyped previously reported *ADAM33* polymorphisms and examined possible associations of these polymorphisms with atopic asthma in Japanese families identified through asthmatic children.

Materials and methods

The probands were mite-sensitive asthmatic children who visited the Paediatric Allergy Clinic of the University Hospital of Tsukuba. A full verbal and written explanation of the study was given to all family members interviewed, and 155 families (538 members), including 47 families used for our genome-wide screening [16], gave informed consent and participated in this study.

Each family member was questioned regarding allergic symptoms and underwent a physical examination performed by a participating paediatrician. Asthma was diagnosed in subjects according to the criteria of the National Institutes of Health, USA [21]. Patients had to satisfy the following criteria: (1) two or more episodes of wheezing and shortness of breath during the past year and (2) reversibility of the wheezing and dyspnoea, either spontaneously or by bronchodilator treatment. Methacholine challenge testing was not performed because of the low ages of the asthmatic patients in the families; however, asthma was diagnosed differentially in the affected children by participating physicians or paediatricians who had treated the children for more than 2 years. Because wheezing is often associated with viral respiratory infection in young children, we excluded children younger than 3 years of age [22]. The young adult patients included in this study had chronic asthma since childhood. Total serum IgE levels and IgE levels specific for *Dermatophagoides farinae* (Df) were determined with the Pharmacia CAP System (Uppsala, Sweden). To achieve homogeneity of the asthmatic patients, we selected families having more than one asthmatic child with a Df RAST score > 3. The clinical characteristics of the families are shown in Table 1.

Table 1. Clinical details of the asthmatic families

No. of families	155
No. of affected children	228
Mean age (years ± SD)	10.1 ± 5.8
Total IgE (IU/mL ± SD)	1254, 35–19 000
Df-RAST IgE (UA/mL ± SD)	63, 3.9–100
No. of parents	310
Mean age (years ± SD)	40.2 ± 7.1
Total IgE (mean IU/mL, range)	287, 0–5200
Df-RAST IgE (mean UA/mL, range)	8.5, 0–100

Values < 0.34 and > 100 for Df RAST IgE were expressed as 0 and 100, respectively, in this study.

DNA was extracted from peripheral blood leucocytes. Genotyping of 23 *ADAM33* SNPs was performed by fluorescence correlation spectroscopy (FCS) [23]. In brief, first-round PCR was performed to amplify a fragment containing an SNP site. The first-round PCR products were used for sequence-specific primer-polymerase chain reaction (SSP-PCR). In SSP-PCR, allele-specific primers, which differ in a single nucleotide at the 3′ end and are coupled with different fluorescence dyes (TAMRA and Cy5), were used for allele-specific amplifications. SSP-PCR with two allele-specific primers was performed competitively in a 10 μL reaction mixture consisting of 0.5 U AmpliTaq DNA Stoffel Fragment (Applied Biosystems, Foster City, CA, USA), 200 μM dNTP, 2.5 mM $MgCl_2$, 20 nM each primer, with 0.5 μL of first-round PCR products. Amplified products were subjected to FCS at 543 and 633 nm excitation wavelength with a single molecule fluorescence detection system (MF10, Olympus, Tokyo, Japan). Primers used for first-round PCR and SSP-PCR are listed in Table 2. Accuracy of genotyping was confirmed by the sequence of at least 16 unrelated individuals.

Statistics

Deviation from Hardy–Weinberg expectancy was examined with the χ^2-test. Differences in allele frequencies were analysed with the χ^2-test. The association between total serum IgE levels and genotypes was analysed by ANOVA and Wilcoxon's test. The transmission disequilibrium test (TDT) was performed with the ASPEX Program [24]. Quantitative TDT analysis for total serum IgE was performed with QTDT software [25]. Linkage disequilibrium (LD) between SNPs, as expressed by D prime, was calculated with Haploview software [26]. Haplotypes were inferred by using an accelerated EM algorithm [27], which was implemented in Haploview [26]. Haplotype association testing was performed with Haploview software [26]. Haplotype blocks were defined by the method of Gabriel et al. [28]. Statistical

Table 2. Primers used for single-nucleotide polymorphism (SNP) genotyping

SNPs	db SNP ID	First primer (F)	First primer (R)	Primer A(TAMRA)	Primer B(Cy5)
F1	rs3918392	ATACTGGGACTCGAGGCCTG	ACCCTAGTGGCCTGATCACC	ATC CCT GTG GCC ACA GGC	ATC CCT GTG GCC ACA GGC
F+1	rs511898	TGTACAGTTCCAGGTACTTCCG	AGATCTTTCGGATGGAGCAG	CGA GGC CTG TGA ATT CCC	CGA GGC CTG TGA ATT CCT
G − 1	rs2485700	TGTACAGTTCCAGGTACTTCCG	AGATCTTTCGGATGGAGCAG	TGA GCC CTG ACC ACC AAC	TGA GCC CTG ACC ACC AAT
L − 1	rs2280092	GGCGAGCCGACTTAACCTGG	TTTCCGAGTAACCTGGCGAGG	AGA GAA GCG CGG GGG TTG GG	AGA GAA GCG CGG GGG TTG GA
M+1	rs3918395	GGAGCCAGGCCTACCCAAGC	CCCTGGCCAGGTTAAGTCGG	GCC GGC TCC CAA GCT CCC	GCC GGC TCC CAA GCT CCA
Q − 1	rs612709	GAAGCTCCTGGGAAGGCATTC	TAGATGGCCAGGAAGTGACTTG	TTC AAA CGG CAA GGA GGA	TTC AAA CGG CAA GGA GGG
S1	rs3918396	AGGAGTAGGCTCAGGAAGCAGG	TGCAGGCTGAAAGTATGCCAG	GAG CAG AGG CAG CAG GAT	GAG CAG AGG CAG CAG GAC
S2	rs528557	CAGGAGTAGGCTCAGGAAGCAG	TGCACCTGCTCAGGACTCAG	CTG CCT CTG CTC CCA GGG	TGC TAA CTC TGC TCC CAG GC
S+1	rs2853209	GAAGTCAAGGGTGAGGCAGC	TGCTTCCTGAGCCTACTCCTG	GGC CTC CCA GTC AAG CGT	GGC CTC CCA GTC AAG CGA
ST+4	rs44707	TCCTAGGACAGCCATGCTTAG	TCAGATTGCAGTCCCTGTACC	CAG CAT TTG GGA ACT TCA AGT	AGC ATT TGG GAA CTT CAA GG
ST−7	rs574174	CAAAGAACCTTGGATGTCCG	TGTCTGACATAGCCTGGTCTTG	ACT GTC CCC ATC CCA TCC	ACT GTC CCC ATC CCA TCT
T1	rs2280091	GACTCAAGGTGACTGGGTGC	TAACCCACTCAGGATCACTGTG	GGG CGG CGT TCA CCC CAT	GGG CGG CGT TCA CCC CAC
T2	rs2280090	GACTCAAGGTGACTGGGTGC	TAACCCACTCAGGATCACTGTG	CTC ACC CAG GGG CCA GGA	CTC ACC CAG GGG CCA GGG
T+1	rs2280089	TGAATCCAGGTCCCTGCCTAC	TCTTATCACGTTGCTCAGCCC	GCT CAT GCC TCC TGC CTT	GCT CAT GCC TCC TGC CTC
T+2	rs630712	TGAATCCAGGTCCCTGCCTAC	TCTTATCACGTTGCTCAGCCC	GTG AAT ATG GTC AGC AGG AGA	TGA ATA TGG TCA GCA GGA GC
V − 3	rs628977	TTCTGGGACTGATGGTTTAT	GGCTCAGGTTATTATGGAAA	GTG TGG CAG IGA GCC CAC	GTG TGG CAG IGA GCC CAT
V − 2	rs628965	TTCTGGGACTGATGGTTTAT	GGCTCAGGTTATTATGGAAA	TGC AAG CTG TGT GGC AGA	TGC AAG CTG TGT GGC AGG
V − 1	rs543749	TTCTGGGACTGATGGTTTAT	GGCTCAGGTTATTATGGAAA	GGA GGA TAT GTT GTC CCC TAA G	AGG AGG ATA TGT TGT CCC CTA AT
V1	rs3918401	TTCTGGGACTGATGGTTTAT	GGCTCAGGTTATTATGGAAA	TCC TCA TTC TCA GCA GAT CAA	CTC CTC ATT CTC AGC AGA TCA T
V2	rs3918400	CCGTGGAAATTGCATGTAGG	TTAAAGACAGGTGGCCACTGAC	CCT GGA GTG GGT GGG TCG	CCT GGA GTG GGT GGG TCA
V3	rs677044	CCGTGGAAATTGCATGTAGG	TTAAAGACAGGTGGCCACTGAC	AGG AAC CCA GAG CCA CAT	AGG AAC CCA GAG CCA CAC
V4	rs2787094	GAGCTCCAGAACCTCAGGTG	CTACATGCAAATTTCCACGGAC	GAG TCC ACA CTC CCC TGG	GAG TCC ACA CTC CCC TGC
V5	rs3746631	GAGCTCCAGAACCTCAGGTG	CTACATGCAAATTTCCACGGAC	CTG GCT GGC CTC TGC AAG	CTG GCT GGC CTC TGC AAA

Table 3. Results of transmission disequilibrium test (TDT) in single single nucleotide polymorphisms (SNPs)

SNPs	db SNP ID	SNPs	Allele*	Allele frequencies				Transmited	Not Transmited	*P*-Values
				Present study	Van Eerdewegh et al. [17]	Cheng et al. [19]	Lee et al. [20]			
F1	rs3918392	A/G	G	0	0.032†			41	44	0.8
F+1	rs511898	C/T	T	0	0.35	0.38		95	102	0.64
G − 1	rs2485700	C/T	C	0	0.093†			50	44	0.58
L − 1	rs2280092	C/T	T	0	0.11	0.14		40	32	0.4
M+1	rs3918395	G/T	T	0	0.11			51	37	0.14
Q − 1	rs612709	A/G	A	0	0.15			40	49	0.25
S1	rs3918396	A/G	A	0	0.105†		0.02†	2	1	1
S2	rs528557	C/G	G	0	0.26	0.3		75	82	0.64
S+1	rs2853209	A/T	T	0	0.49			135	98	0.012
ST+4	rs44707	A/C	C	0	0.49			117	90	0.038
ST+7	rs574174	C/T	T	0	0.12			45	42	0.81
T1	rs2280091	A/G	G	0	0.11	0.15	0.08†	35	30	0.42
T2	rs2280090	A/G	A	0	0.094†	0.18		62	42	0.03
T+1	rs2280089	A/G	A	0	0.11	0.15		48	36	0.2
T+2	rs630712	A/C	C	0.27	0.117†			91	74	0.21
V − 3	rs628977	C/T	T	0	0.37			99	93	0.69
V − 2	rs628965	A/G	A	0	0.37			101	87	0.26
V − 1	rs543749	G/T	T	0	0.15		0.11	57	52	0.56
V1	rs3918401	A/T	T	0	0.035†			43	50	0.52
V2	rs3918400	A/G	A	0	0.037†			56	56	1
V3	rs677044	A/G	G	0	0.22			80	80	1
V4	rs2787094	C/G	G	0	0.233†		0.34	97	100	0.89
V5	rs3746631	C/T	C	0	0.037†			49	55	0.57

*Alleles are based on reference SNP number.

†Allele frequencies that are significantly different from those in the present study ($P < 0.05$).

significance was defined at the 5% level. The statistical power of the TDT for detecting a polymorphism that directly affected risk was calculated according to the method proposed by Risch and Merikangas [29].

Results

We genotyped 23 SNPs in *ADAM33*. The allele frequencies of the parental genotypes for all SNPs did not deviate from expected values based on Hardy–Weinberg equilibrium ($P > 0.05$). Minor allele frequencies of SNPs are shown in Table 3. Allele frequencies of nine SNPs in the present study differed significantly from those reported in Caucasians [17] ($P < 0.05$, Table 3). The allele frequencies of controls in another Japanese population [19] (Table 3) did not differ from those in the present study ($P > 0.1$), but the S1 and T1 allele frequencies in Koreans (Lee et al. [20], Table 3) differed significantly from those in the present study ($P = 0.005$ for S1 and $P = 0.005$ for T1). Although Koreans are considered genetically close to Japanese, our data indicate that there are considerable ethnic differences in *ADAM33* allele frequencies. The LD map constructed from 22 SNPs with minor allele frequencies > 0.05 is shown in the Fig. 1, as measured by D prime. The allele frequency of S1 was very low (0.003) in our population, so this SNP was dropped from the analysis. Tight LD was observed throughout the region. Haplotypes with frequencies > 0.01 are shown in Table 4.

The TDT revealed that minor alleles of S+1, ST+4 and T2 SNPs were over-transmitted to asthma-affected offspring ($P < 0.05$, Table 3). The statistical power of the TDT for *ADAM33* SNPs without S1 was more than 0.8 at the α level of 0.05 if the relative risk for asthma in those persons carrying a putative risk allele is 1.8 compared with that in persons without the allele. No association was observed between total serum IgE levels and any *ADAM33* SNP according to quantitative TDT analysis ($P > 0.05$). Mean log (total serum IgE) levels of parents with each genotype in a single SNP were compared, and no significant association was observed between groups ($P > 0.05$).

According to the haplotype TDT, no haplotype of *ADAM33* was transmitted preferentially to asthmatic offspring (Table 4). As shown in Fig. 1, ST+4, which was associated with the development of asthma ($P = 0.038$), was not included in any of the haplotype blocks.

Discussion

In the present study, we observed that minor alleles of *ADAM33* SNPs were significantly over-transmitted to

atopic-asthma-affected offspring, confirming the previous studies showing that *ADAM33* is associated with asthma. The allele frequencies in the present study were similar to those reported by Cheng et al. [19] but differed significantly from those reported in Caucasians and Koreans. A lesser degree of difference in allele frequencies was detected between our subjects and reported Korean subjects than between our subjects and reported Caucasian subjects.

Correction for multiple tests, such as Bonferroni's correction, is used to exclude a high probability of false-positive results. However, the aim of the present study was to confirm associations between *ADAM33* polymorphisms and asthma in our population, and correction such as Bonferroni's correction seems to be too stringent. Moreover, SNPs were in tight LD in this population, and they are not independent of each other. We therefore applied *P*-values of 0.05 in the present study.

Table 4. Haplotypic transmission test

Haplotype		Frequencies	Transmitted/ not transmitted	*P*-value
Block 1				
V1, V2	AG	0.84	46.3 : 35.0	0.21
	TA	0.12	27.0 : 33.0	0.44
	AA	0.042	11.0 : 15.3	0.40
Block 2				
T+1, T+2, V − 3,	GACGG	0.38	66.1 : 79.5	0.27
V − 2, V − 1	GCTAG	0.26	59.1 : 53.0	0.57
	GACGT	0.15	37.0 : 36.0	0.91
	AACGG	0.12	32.6 : 24.2	0.26
	GATAG	0.077	19.0 : 26.2	0.28
Block 3				
ST+7, T1, T2	CAG	0.72	59.4 : 74.4	0.19
	CGA	0.14	41.1 : 29.9	0.18
	TAG	0.12	31.0 : 31.6	0.94
	CAA	0.021	8.9 : 4.3	0.21

ADAM33 SNPs have been shown to be associated with an accelerated decline in lung function of asthmatics followed up for over 20 years [30]. Moreover, Simpson et al. [31] reported that *ADAM33* SNPs were associated with reduced lung function at the age of 3 years (F+1) and with decreased FEV1 (F+1, M+1, T1, and T2), suggesting that reduced early-life lung function is in part genetically determined. In the present study, all cases were of childhood or child-onset asthma, supporting the idea that *ADAM33* SNPs may be associated with smaller airways.

The study by Van Eerdewegh was the first to show that *ADAM33* polymorphisms (Q − 1, S1, ST+4, ST+7, V − 1, and V4) and haplotypes were associated with asthma in Caucasian families [17]. A number of studies have been conducted to replicate the original findings, some confirming and others refuting the association of *ADAM33* with asthma [32–35]. A meta-analysis of *ADAM33* SNPs and asthma showed that F+1 and ST+7 were significantly associated with asthma in both TDT and case–control studies [18]. Most of the data in the meta-analysis were from Caucasian subjects; only one study of Asian origin [20] was included. In the present study, childhood asthma was associated with *ADAM33* SNPs, S+1, ST+4, and T2, but not with F+1 and ST+7. The

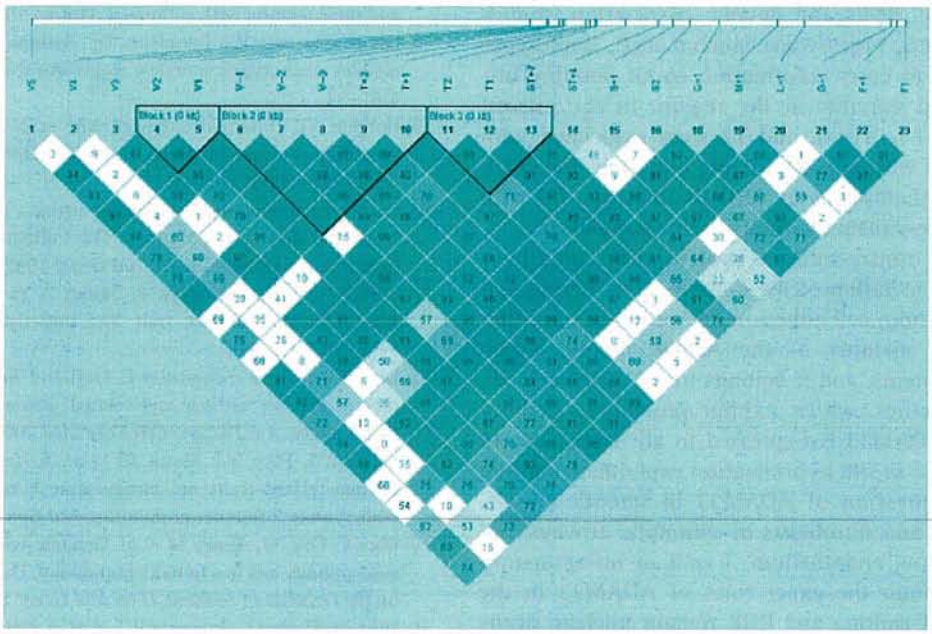

Fig. 1. Pairwise linkage disequilibrium (LD) between SNPs in *ADAM33* as measured by D prime in 538 subjects (155 families). LD is divided into three blocks. The D prime values for LD are colour-coded and the extent of red indicates the strength of LD.

genetic basis of asthma may differ between different ethnic groups; it is possible that a particular subset of SNPs may be a risk factor for asthma in Caucasians, and that a different subset may increase the risk in Asian populations.

Recently, Raby et al. performed a family-based association study for childhood asthma and *ADAM33* SNPs in 652 nuclear families. They showed that no single SNP was associated with childhood asthma in Caucasians but that a common haplotype of *ADAM33* was associated with the disease [35]. They also reported that T1 and T+1 were marginally associated with childhood asthma in Hispanics [35]. In previous association studies of *ADAM33* and asthma/asthma-associated phenotypes, no single *ADAM33* SNP was consistently associated with asthma or intermediate phenotypes. This may be because of allelic heterogeneity, a single disorder caused by different mutations within a gene. Another explanation is the difference in LD patterns between populations, which means that unobserved causal variants are the same in different populations, but that SNPs in strong LD with the variants differ between populations or that the disease-causing variants are in strong LD with an SNP in one ethnic population, but not in others. The allele frequencies in the present study were similar to those reported in another Japanese study [19] but differed significantly from those reported in Caucasians [17] and Koreans [20]. Suggesting that different genetic backgrounds may account for the discrepant results.

In recent years, much has been performed to catalogue millions of SNPs and haplotypes across diverse populations and to use these to identify subsets of highly informative tag SNPs for genetic association studies (HapMap projects, http://www.hapmap.org/). Haplotypes are considered to carry information about possibly unobserved causal variants in the region. In the present study, SNPs, S+1, ST+4, and T2 were associated with asthma but not with any haplotype, implying that S+1, ST+4, and T2 themselves or variants in strong LD with these are disease-causing variants in this population.

ADAM is a transmembrane protein that contains a disintegrin and metalloprotease domain, and, therefore, it potentially has both cell adhesion and protease activities. ADAM33 is a member of the ADAM family, which includes 35 proteins, and it belongs to the ADAM12, 15, and 19 subfamilies, which exhibit proteolytic activity [36]. Human ADAM33 is expressed in all tissues except liver [37], and an *in situ* hybridization experiment showed preferential expression of ADAM33 in smooth muscle, myofibroblasts and fibroblasts of asthmatic airways, but not in epithelium, endothelium, T cells or inflammatory cells [9]. Although the exact roles of ADAM33 in the development of asthma and BHR remain unclear, accumulated association data support the conclusion that ADAM33 is involved in asthma and/or BHR. Further studies to elucidate the functional role of ADAM33 are needed for a better understanding of the pathogenesis of asthma, which will in turn lead to new therapies for asthmatic patients.

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