Transfection

The *IL-12B* promoter–reporter construct was transfected in RAW264. The cells were collected, resuspended at a concentration of 3 × 10^6 cells / 10 ml in an 80 mm^2 flask, and incubated for 24 hr at 37℃ in a 5% CO_2 atmosphere. DNA (reporter construct 12 μg, CMV control vector 3 μg) was resuspended in 600 μL of OPTI-MEM (Gibco BRL, Grand Island, NY, USA) in a 15 mL Falcon tube, to which 15 μL of lipofectamine 2000 (Gibco BRL, Grand Island, NY, USA) was added; the mixture was incubated for 20 min at 37℃ in a 5% CO_2 atmosphere. The cells were washed once, resuspended by 6 mL of DMEM (Gibco BRL, Grand Island, NY, USA) with 10% FCS and 600 μL of OPTI-MEM including the reporter construct in an 80 mm^2 flask, and incubated for 24 hr at 37℃ in a 5% CO_2 atmosphere. The cells were treated with recombinant murine IFN-γ (1,000 U/mL; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 6 mL of the same medium for the stopping lipofectamine reaction for 24 hr before the addition of LPS (1 μg/mL) (Sigma, St. Louis, USA). After the stimulation with LPS for 24 hr, cells were harvested and lysed by cell lysis buffer.[17] The lysates were used for luciferase assay with a PicaGene Dual SeaPansy Luminescence kit (Toyo

Ink Mfg. Co., Ltd., Tokyo, Japan).

Statistical analyses

The significant difference between two groups was analyzed by the Mann-Whitney *U* test. The significant differences among three groups were analyzed by the Bonferroni/Dunn test. The frequency in *IL-12B* promoter polymorphism between the asthmatic patients and the controls was compared using the chi-square test for independence. Hardy-Weinberg equilibrium was determined be means of chi-square test for independence. Statistical significance was assumed for p-values less than 0.05.

RESULTS

Detection of IL-12B polymorphisms

Exons 1-8 including parts of the introns and region 3 kb upstream from the transcriptional start site of *IL-12B* were amplified by PCR. Several polymorphisms have been reported in *IL-12B*.[8, 9] We found three novel polymorphisms. The T/C allele (NP2) existed at -2403 from the transcriptional start site of *IL-12B*, and the T/G (NP15) and C/T (NP16) alleles existed in introns 4 and 6 (Fig. 1). We investigated the frequency of *IL-12B* polymorphisms and plasma IL-12 concentrations in 30 subjects. Although the frequencies of all *IL-12B* polymorphisms were not significantly different between the allergic patients and the controls, plasma IL-12 concentrations in the subjects with the homozygotes for the $^{-2703}$CTCTAA /$^{-2403}$T allele (haplotype 1) were significantly different from those in the subjects with homozygotes for the $^{-2703}$GC / $^{-2403}$C allele (haplotype 2) in NP1+2 (data not shown). Therefore, we investigated that whether there is an association between NP1+2 and asthma, and whether NP1+2 affect IL-12 production.

Association between IL-12B promoter polymorphism and asthma

NP1 existed at -2703 from the transcriptional start site in *IL-12B*. We also identified NP2 at -2403 from the transcriptional start site, and this polymorphism was linked to NP1. We designated the homozygotes for haplotype 1, heterozygotes, and homozygotes for haplotype 2 as genotypes 1.1, 1.2, and 2.2, respectively. The frequencies of *IL-12B* promoter polymorphisms in 189 samples are shown in Table 2. Allele frequency of *IL-12B* promoter polymorphism did not deviate from expected Hardy-Weinberg equilibrium ($P>0.1$). The frequency of a 1.1 genotype in the asthmatic patients was significantly higher than that in the controls ($P<0.001$).

Plasma IL-12 concentrations in the controls and asthmatic patients

As shown in Figure 2a, plasma IL-12 concentrations in the asthmatic patients were significantly lower than those in the controls. The mean±SD of plasma IL-12 concentrations in the asthmatic patients and controls were 136.8±62.0 pg/mL and 232.0±84.0 pg/mL ($P<0.001$).

Association between IL-12B promoter polymorphism and plasma IL-12 concentrations

We examined plasma IL-12 concentrations in each genotype in both the controls and asthmatic patients. Plasma IL-12 concentrations in the subjects with a 1.1 genotype were 153.1±70.0 pg/mL, which were significantly lower than those in the subjects with a 2.2 genotype (218.6±85.1 pg/mL) (P<0.001; Fig. 2b). Since reduced IL-12 production in the asthmatic patients might be affected by many factors such as some genetic effects and environmental factors, we also examined plasma IL-12 concentrations in each genotype only in the controls. Plasma IL-12 concentrations in the controls with a 1.1 genotype were 205.2±63.8 pg/mL, which were significantly lower than those in the controls with a 2.2 genotype (255.8±70.8 pg/mL) (P<0.05; Fig. 2c).

IL-12 production by Derf1-stimulated PBMCs in the controls and asthmatic patients

Since most asthmatic patients had positive CAP-RAST scores for D farinae (*Derf1*), their PBMCs were cultured with the specific antigen *Derf1* for 24 hr. IL-12 production by *Derf1*-stimulated PBMCs in the asthmatic patients (378.0±271.4 pg/mL) was

significantly lower than that in the controls (663.0±364.2 pg/mL) (P<0.001; Fig. 3a).

Association between IL-12B promoter polymorphism and IL-12 production by Derf1-stimulated PBMCs

We examined IL-12 production by *Derf1*-stimulated PBMCs in each genotype in both the controls and asthmatic patients. The subjects with a 1.1 genotype (347.2± 229.2 pg/mL) had a lower IL-12 production by *Derf1*-stimulated PBMCs than those with a 2.2 genotype (690.2±331.1 pg/mL) (P<0.001; Fig. 3b). In the controls, the subjects with a 1.1 genotype (412.2±315.0 pg/mL) had a lower IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype (807.2±292.2 pg/mL) (P<0.001; Fig. 3c). IL-12 production by *Derf1*-stimulated PBMCs positively correlated with IFN-γ production by IL-12-stimulated PBMCs (data not shown). Therefore, the subjects with a 1.1 genotype showed lower IL-12 and IFN-γ productions than those with a 2.2 genotype.

Effects of promoter polymorphism on promoter activity

To examine the functional activity of promoter polymorphism, we cloned the -2808/-2303 region of *IL-12B* into the PGV-P2 firefly luciferase reporter plasmid. The activity of the *IL-12B* promoter-reporter constructs was assessed in transient transfection assay using RAW264 cells. As shown in Figure 4, the luciferase activity of the PGV-P2 plasmid with the *IL-12B* promoter-reporter constructs was significantly higher than that of the PGV-P2 plasmid only. A significantly lower luciferase activity was observed for haplotype 1 construct than for haplotype 2 construct (32.8% decrease; P=0.0083). Furthermore, we constructed mutant vectors with homozygotes for mut-1 (the CTCTAA/C allele) and mut-2 (the GC/T allele). The luciferase activity of the construct with mut-2 was significantly lower than that of the construct with mut-1 and haplotype 2. This result indicates that *IL-12B* promoter polymorphism, that is, not the CTCTAA/GC allele but the T/C allele, has a major influence on the basal transcription rate of *IL-12B*.

DISCUSSION

IL-12 is a key mediator of immune responses. IL-12 is a heterodimeric molecule composed of two disulfide-linked subunits, a 35-kd subunit encoded by *IL-12A* on chromosome 3p12-q13.2 and a 40-kd subunit encoded by *IL-12B* on chromosome 5q31-33.[18] A previous study has reported the association between *IL-12B* and asthma as determined by a genome-wide search.[7] We sequenced exons 1-8 and region 3 kb upstream from the transcriptional start site of *IL-12B,* and found three novel polymorphisms. In nineteen polymorphisms, NP1+2 that existed in the promoter region were shown a significant difference between the asthmatic patients and the controls in the genome frequency analysis. The frequencies of *IL-12B* polymorphisms in the coding region (NP3 and NP19) are not significantly different between the controls and asthmatic patients (data not shown). This result is similar to the result of Noguchi *et al.*.[12]

Furthermore, the subjects with a 1.1 genotype had significantly lower plasma IL-12 concentrations and the lower IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype. IL-12 plays an important role in the inhibition of Th2

cytokine production and the promotion of IFN-γ production by binding to plasma membrane receptors on activated T cells or NK cells.[2] IFN-γ inhibits IgE synthesis by human PBMCs *in vitro*.[19-22] Reduced IL-12 production and IL-12-dependent IFN-γ concentrations have been reported in asthmatic patients.[23-25] Therefore, IL-12 was shown to be associated with atopic dermatitis and asthma.[26-28] According to our result, *IL-12B* promoter polymorphism is likely to be associated with asthma prevalence by reducing IL-12 production.

Since the IL-12 levels measured during an exacerbation of asthma or bacterial/viral infection are analyzed in relation to the polymorphism, the functional consequences of the polymorphism cannot be fully explored. Hence, we investigated the functional activity of *IL-12B* promoter polymorphism. The transcriptional activity of the construct with a 1.1 genotype was lower than that of the construct with a 2.2 genotype. The transcriptional activity with mut-2 was significantly lower than that with mut-1 and haplotype 2. These results indicate that *IL-12B* promoter polymorphism, not NP1 but NP2, reduces the *IL-12B* transcriptional activity and IL-12 production. Since the NF-IL6 binding site in the *IL-12B* promoter region is shown to

be in the -2405 to -2397 area of the 5'-upstream region, the T/C allele at this point may affect *IL-12B* transcriptional activity.[29] The transcriptional activity of the 296-bp construct from the transcriptional start site was reduced to half compared with that of the 3.3-kb construct.[17] This result shows that there are significant binding sites influencing the transcriptional activity from -296 to -3.3 kb of the transcriptional start site. The sequence near the T/C allele may be one of the binding sites affecting *IL-12B* transcriptional activity.

Morahan et al.[13] reported the association between asthma severity and a 4-bp microinsertion, which exists at a region 3 kb upstream from the transcriptional start site of *IL-12B*. Since we identified only NP1 around the region 3 kb upstream from the transcriptional start site, the 4-bp microinsertion is likely to be NP1. According to their report, heterozygosity for *IL-12B* promoter polymorphism is associated with asthma severity, reduced *IL-12B* transcription level and decreased IL-12 secretion. Inconsistent results may have occurred due to differences of methods and population. In another study, Morahan *et al.* also reported that a 1.1 genotype is associated with mortality from cerebral malaria and with reduced production of nitric oxide in

Tanzanian children, and that the *IL-12B* mRNA expression in the subjects with a 1.1 genotype is lower than that in the subjects with a 2.2 genotype. These results are consistent with our results.

Khoo *et al.*[14] have recently shown that *IL-12B* promoter polymorphism is not associated with asthma prevalence, but that there is an association was between 1.1 genotype and elevated serum IgE levels in male subjects, and reduced pulmonary function in female subjects in childhood. In our data, the subjects with a 1.1 genotype had high IgE levels, however, there was no correlation with sex (data not shown). The subjects with a 1.1 genotype had reduced IL-12 production and *IL-12B* transcriptional activity compared with those with a 2.2 genotype. Therefore, homozygosity for haplotype 1 may elevate serum IgE by reducing IL-12 production.

In conclusion, the frequency of homozygosity for haplotpe 1 in asthmatic patients was significantly higher than that in controls. The subjects with a 1.1 genotype had reduced plasma IL-12 concentrations and the IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype. The *IL-12B* transcriptional activity was reduced by the $^{-2403}$T allele, not by the $^{-2703}$CTCTAA allele. Hence, *IL-12B*

promoter polymorphism ($^{-2403}$T/C) can be a risk factor for the development of asthma.

REFERENCES

1. Chan SH, Perussia B, Gupta JW *et al.* Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 1991; **173**: 869-879.

2. Chan SH, Kobayashi M, Santoli D, Perussia B, Trinchieri G. Mechanisms of IFN-gamma induction by natural killer cell stimulatory factor (NKSF/IL-12). Role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J. Immunol.* 1992; **148**: 92-98.

3. Daniels SE, Bhattacharrya S, James A *et al.* A genome-wide search for quantitative trait loci underlying asthma. *Nature* 1996; **383**: 247-250.

4. Mitsuyasu H, Izuhara K, Mao XQ *et al.* Ile50Val variant of IL4R alpha upregulates IgE synthesis and associates with atopic asthma. *Nat. Genet.* 1998; **19**: 119-120.

5. Rosa-Rosa L, Zimmermann N, Bernstein JA, Rothenberg ME, Khurana Hershey GK. The R576 IL-4 receptor alpha allele correlates with asthma severity. *J. Allergy Clin. Immunol.* 1999; **104**: 1008-1014.

6. Heinzmann A, Mao XQ, Akaiwa M *et al.* Genetic variants of IL-13 signalling and

human asthma and atopy. *Hum. Mol. Genet.* 2000; **9**: 549-559.

7. Yokouchi Y, Nukaga Y, Shibasaki M *et al.* Significant evidence for linkage of mite-sensitive childhood asthma to chromosome 5q31-q33 near the interleukin 12B locus by a genome-wide search in Japanese families. *Genomics* 2000; **66**: 152-160.

8. Pravica V, Brogan IJ, Hutchinson IV. Rare polymorphisms in the promoter regions of the human interleukin-12 p35 and interleukin-12 p40 subunit genes. *Eur. J. Immunogenet.* 2000; **27**: 35-36.

9. Huang D, Cancilla MR, and Morahan G. Complete primary structure, chromosomal localisation, and definition of polymorphisms of the gene encoding the human interleukin-12 p40 subunit. *Genes. Immun.* 2000; **1**: 515-520.

10. Morahan G, Huang D, Ymer SI *et al.* Linkage disequilibrium of a type 1 diabetes susceptibility locus with a regulatory IL12B allele. *Nat. Genet.* 2001; **27**: 218-221.

11. Tsunemi Y, Saeki H, Nakamura K *et al.* Interleukin-12 p40 gene (IL12B) 3'-untranslated region polymorphism is associated with susceptibility to atopic dermatitis and psoriasis vulgaris. *J. Dermatol. Sci.* 2002; **30**: 161-166.

12. Noguchi E, Yokouchi Y, Shibasaki M *et al.* Identification of missense mutation in

the *IL12B* gene: lack of association between *IL12B* polymorphisms and asthma and allergic rhinitis in the Japanese population. *Genes. Immun.* 2001; **2**: 401-403.

13. Morahan G, Huang D, Wu M *et al.* Association of *IL-12B* promoter polymorphism with severity of atopic and non-atopic asthma in children. *Lancet* 2002; **360**: 455-459.

14. Khoo SK, Hayden CM, Roberts M *et al.* Associations of the *IL12B* promoter polymorphism in longitudinal data from asthmatic patients 7 to 42 years of age. *J. Allergy Clin. Immunol.* 2004; **113**: 475-481.

15. Matsui E, Kaneko H, Teramoto T *et al.* Reduced IFN-γ production in response to IL-12 stimulation and/or reduced IL-12 production in atopic patients. *Clin. Exp. Allergy* 2000; **30**: 1250-1256.

16. Watanabe M, Kaneko H, Shikano H *et al.* Predominant expression of 950delCAG of IL-18R alpha chain cDNA is associated with reduced IFN-gamma production and high serum IgE levels in atopic Japanese children. *J. Allergy Clin. Immunol.* 2002; **109**: 669-675.

17. Ma X, Chow JM, Gri G *et al.* The interleukin 12 p40 gene promoter is primed by

interferon gamma in monocytic cells. *J. Exp. Med.* 1996; **183**: 147-157.

18. Sieburth D, Jabs EW, Warrington JA *et al.* Assignment of genes encoding a unique cytokine (IL12) composed of two unrelated subunits to chromosomes 3 and 5. *Genomics* 1992; **14**: 59-62.

19. Coffman RL, Ohara J, Bond MW, Carty J, Zlotnik A, Paul WE. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* 1986; **136**: 4538-4541.

20. Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 1987; **236**: 944-947.

21. Finkelman FD, Katona IM, Mosmann TR, Coffman RL. IFN-gamma regulates the isotypes of Ig secreted during *in vivo* humoral immune responses. *J. Immunol.* 1988; **140**: 1022-1027.

22. Pene J, Rousset F, Briere F *et al.* IgE production by normal human B cells induced by alloreactive T cell clones is mediated by IL-4 and suppressed by IFN-gamma. *J. Immunol.* 1988; **14**: 1218-1224.

23. van der Pouw Kraan TC, Boeije LC, de Groot ER *et al.* Reduced production of IL-

12 and IL-12-dependent IFN-gamma release in patients with allergic asthma. *J. Immunol.* 1997; **158**: 5560-5565.

24. Plummeridge MJ, Armstrong L, Birchall MA, Millar AB. Reduced production of interleukin 12 by interferon gamma primed alveolar macrophages from atopic asthmatic subjects. *Thorax* 2000; **55**: 842-847.

25. Shikano H, Kato Z, Kaneko H *et al.* IFN-gamma production in response to IL-18 or IL-12 stimulation by peripheral blood mononuclear cells of atopic patients. *Clin. Exp. Allergy* 2001; **31**: 1263-1270.

26. Wills-Karp M. IL-12/IL-13 axis in allergic asthma. *J. Allergy Clin. Immunol.* 2001; **107**: 9-18.

27. Kondo N, Matsui E, Kaneko H *et al.* Atopy and mutations of IL-12 receptor beta 2 chain gene. *Clin. Exp. Allergy* 2001; **31**: 1189-1193.

28. Kondo N, Matsui E, Kaneko H *et al.* RNA editing of interleukin-12 receptor beta2, 2451 C-to-U (Ala 604 Val) conversion, associated with atopy. *Clin. Exp. Allergy* 2004; **34**: 363-368.

29. Shirakawa F, Saito K, Bonagura CA *et al.* The human prointerleukin 1 beta gene

requires DNA sequences both proximal and distal to the transcription start site for tissue-specific induction. *Mol. Cell Biol.* 1993; **13**: 1332-1344.

30. Morahan G, Boutlis CS, Huang D *et al.* A promoter polymorphism in the gene encoding interleukin-12 p40 (*IL12B*) is associated with mortality from cerebral malaria and with reduced nitric oxide production. *Genes. Immun.* 2002; **3**:414-418.

TABLE 1. Sequence of oligonucleotides for PCR.

Primer	Sequence	Primer	Sequence	position
1S	5'-GAGAAGCATTCAGAAGCTCT-3'	1A	5'-GTCCCACTTCACAATCCAGA-3'	promoter
2S	5'-GTTTGTCAGCAGACCTTCCT-3'	2A	5'-GGAACAGGGCTCTGAATTGT-3'	promoter
3S	5'-GACAAGTGATTTCACTGCGG-3'	3A	5'-GGGCTAGTCCTATATGAAAG-3'	promoter
4S	5'-GGTATCCAGCTCTCTAACTC-3'	4A	5'-GACTTTGCCTTTTAGCCTTC-3'	promoter
5S	5'-GCAATCTGCTTTGTCCACTT-3'	5A	5'-GCTAAGAGGTATGCAAAGGT-3'	promoter
6S	5'-GCAGGTACATGTTCCTGTTC-3'	6A	5'-GGTTCTTCCCAAGTCAGAGA-3'	promoter
7S	5'-GCCAAGATGGGTGGTAAATA-3'	7A	5'-GAGGAGGGAACATAGACATC-3'	promoter
8S	5'-GCATCTCCATCTCCTTCCTT-3'	8A	5'-GCACACTAACGGTTTCTACA-3'	exon1
9S	5'-GGCTTAAAGGGGCCAAGT-3'	9A	5'-AGGGAGCACTATCCCTCAGC-3'	intron1-1
10S	5'-ATGTTATCTCATTGCCTTTC-3'	10A	5'-AAGTGGTTCTGAAACCACTG-3'	intron1-2
11S	5'-GTATCAGATGGCTTGCCTTA-3'	11A	5'-GTGCATGGTTGCCCATTTCA-3'	exon2
12S	5'-GGGAAGACTAAGCTCTACTG-3'	12A	5'-CAACGAACCAAGACTGTCAT-3'	intron2
13S	5'-GTCTTGTGCTGTTTGCAGTT-3'	13A	5'-GCATCTCCAAACTCTTTGAC-3'	exon3-1
14S	5'-GTGACACCCCTGAAGAAGAT-3'	14A	5'-GAGGCTAAGCATTCAGACTG-3'	exon3-2
15S	5'-GATAGTGTATCACTCTGCAC-3'	15A	5'-GCTGAGAAACCAGAGCAGTT-3'	exon4
16S	5'-TACTTCTGCTGACACCACTA-3'	16A	5'-GAACTAGGATCAAATTGTATAC-3'	intron4-1
17S	5'-GGTTACATAATCATATGTA-3'	17A	5'-GTTAGGATTTCAGGTGTGAG-3'	intron4-2
18S	5'-GTCCAGAGACATGTAAGTGC-3'	18A	5'-GAGATGATGCTTGTCAACCA-3'	exon5
19S	5'-GCATCTCTCAGATCCTGCAA-3'	19A	5'-GCACCTGAATCACTTCTTAC-3'	exon6
20S	5'-GCTAGAAAGATGAAAGCTGG-3'	20A	5'-GTTTCTGATTCTGGCAACTG-3'	exon7
21S	5'-TAGCTCATCTTGGAGCGAAT-3'	21A	5'-GCTTGCCAGAGGCTTTCTTG-3'	intron7
22S	5'-GCAAGCTTGCAGGACTCAGA-3'	22A	5'-GATGGATCAGGTCATAAGAG-3'	exon8-1
23S	5'-GCCAGGATGTATGGAATGTT-3'	23A	5'-GACAGGGTCTCATTCTGTCA-3'	exon8-2
24S	5'-GCCTAGGTGACAGAATGAGA-3'	24A	5'-GCAAGCAGAGTACTCAAATC-3'	exon8-3

TABLE 2. Genotype frequencies of *IL-12B* promoter polymorphism in asthmatic patients and controls.

Genotype	Control (n =78) (%)	Asthmatic patient (n =111) (%)
1.1	15 (17.9%)	34 (30.6%)
1.2	34 (43.6%)	63 (56.8%)
2.2	29 (37.2%)	14 (12.6%)
P -value		<0.001