

Ltd., Tokyo, Japan). Furthermore, we constructed the mutant vector, which changed the T allele in the reporter vector with a 1.1 genotype into the C allele (mut-1). Circular DNA, which was the vector with a 1.1 genotype, was amplified using the sense primer 5'-GACAAGTGATTTCACTGCGGGAAGACAATTCAGAGC-3', and the anti-sense primer 5'-GCTCTGAATTGTCTTCCCGAGTGAAATCACTTGTC-3' with native Pfu DNA polymerase (Stratagene, California, USA). These primers were oligonucleotides with one base mismatch (underline). The conditions for the PCR were 95°C for 1 min, 55°C for 1 min, and 68°C for 10 min. The DNA sequence of PCR product was confirmed with an ABI 3100 DNA sequencer. We constructed another mutant vector, which changed the C allele in the reporter-vector with a 2.2 genotype into the T allele (mut-2) by the same method. The vector with a 2.2 genotype was amplified using the sense primer 5'-GACAAGTGATTTCACTGTGGGAAGACAATTCAGAGC-3', and the anti-sense primer 5'-GCTCTGAATTGTCTTCCACAGTGAAATCACTTGTC-3'.

### *Transfection*

The *IL-12B* promoter–reporter construct was transfected in RAW264. The cells were collected, resuspended at a concentration of  $3 \times 10^6$  cells / 10 ml in an 80 mm<sup>2</sup> flask, and incubated for 24 hr at 37°C in a 5% CO<sub>2</sub> 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°C in a 5% CO<sub>2</sub> 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<sup>2</sup> flask, and incubated for 24 hr at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells were treated with recombinant murine IFN- $\gamma$  (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.<sup>17</sup> 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 by 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*.<sup>8, 9</sup> 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 <sup>-2703</sup>CTCTAA / <sup>-2403</sup>T allele (haplotype 1) were significantly different from those in the subjects with homozygotes for the <sup>-2703</sup>GC / <sup>-2403</sup>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  $\pm$  SD of plasma IL-12 concentrations in the asthmatic patients and controls were  $136.8 \pm 62.0$  pg/mL and  $232.0 \pm 84.0$  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  $153.1 \pm 70.0$  pg/mL, which were significantly lower than those in the subjects with a 2.2 genotype ( $218.6 \pm 85.1$  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  $205.2 \pm 63.8$  pg/mL, which were significantly lower than those in the controls with a 2.2 genotype ( $255.8 \pm 70.8$  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 ( $378.0 \pm 271.4$  pg/mL) was

significantly lower than that in the controls ( $663.0 \pm 364.2$  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 ( $347.2 \pm 229.2$  pg/mL) had a lower IL-12 production by *Derf1*-stimulated PBMCs than those with a 2.2 genotype ( $690.2 \pm 331.1$  pg/mL) ( $P < 0.001$ ; Fig. 3b). In the controls, the subjects with a 1.1 genotype ( $412.2 \pm 315.0$  pg/mL) had a lower IL-12 production by *Derf1*-stimulated PBMCs than those with a 2.2 genotype ( $807.2 \pm 292.2$  pg/mL) ( $P < 0.001$ ; Fig. 3c). IL-12 production by *Derf1*-stimulated PBMCs positively correlated with IFN- $\gamma$  production by IL-12-stimulated PBMCs (data not shown). Therefore, the subjects with a 1.1 genotype showed lower IL-12 and IFN- $\gamma$  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.<sup>18</sup> A previous study has reported the association between *IL-12B* and asthma as determined by a genome-wide search.<sup>7</sup> 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.*<sup>12</sup>

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- $\gamma$  production by binding to plasma membrane receptors on activated T cells or NK cells.<sup>2</sup> IFN- $\gamma$  inhibits IgE synthesis by human PBMCs *in vitro*.<sup>19-22</sup> Reduced IL-12 production and IL-12-dependent IFN- $\gamma$  concentrations have been reported in asthmatic patients.<sup>23-25</sup> Therefore, IL-12 was shown to be associated with atopic dermatitis and asthma.<sup>26-28</sup> 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.<sup>29</sup> 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.<sup>17</sup> 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.*<sup>13</sup> 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.*<sup>14</sup> 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 haplotype 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 <sup>-2403</sup>T allele, not by the <sup>-2703</sup>CTCTAA allele. Hence, *IL-12B*

promoter polymorphism (<sup>-2403</sup>T/C) can be a risk factor for the development of asthma.

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TABLE 1. Sequence of oligonucleotides for PCR.

Primer	Sequence	Primer	Sequence	position
1S	5'-GAGAAGCATTGAGAAGCTCT-3'	1A	5'-GTCCCACTTCACAATCCAGA-3'	promoter
2S	5'-GTTTGTGACGACACCTTCT-3'	2A	5'-GGAACAGGGCTCTGAATTGT-3'	promoter
3S	5'-GACAAGTGATTTCACTGCCG-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'-GCAGGTACATGTTCTGTTTC-3'	6A	5'-GGTTCTTCCCAAGTCAGAGA-3'	promoter
7S	5'-GCCAAGATGGGTGTAATA-3'	7A	5'-GAGGAGGAACATAGACATC-3'	promoter
8S	5'-GCATCTCCATCTCCTTCTT-3'	8A	5'-GCACACTAACGGTTTCTACA-3'	exon1
9S	5'-GGCTTAAAGGGCCAAGT-3'	9A	5'-AGGGAGCACTATCCCTCAGC-3'	intron1-1
10S	5'-ATGTTATCTCATTGCCTTTC-3'	10A	5'-AAGTGTTCTGAAACCACTG-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'-GCATCTCAAACCTTTTGAC-3'	exon3-1
14S	5'-GTGACACCCTGAAGAAGAT-3'	14A	5'-GAGGCTAAGCATTGACTG-3'	exon3-2
15S	5'-GATAGTGTACTCTGCAC-3'	15A	5'-GCTGAGAAACCAGAGCAGTT-3'	exon4
16S	5'-TACTTCTGCTGACACCACTA-3'	16A	5'-GAACTAGGATCAAATTGTATAC-3'	intron4-1
17S	5'-GGTTACATAATCATATGTA-3'	17A	5'-GTTAGGATTCAGGTGTGAG-3'	intron4-2
18S	5'-GTCCAGAGACATGTAAGTGC-3'	18A	5'-GAGATGATGCTTGCAACCA-3'	exon5
19S	5'-GCATCTCTCAGATCCTGCAA-3'	19A	5'-GCACCTGAATCACTTCTTAC-3'	exon6
20S	5'-GCTAGAAAGATGAAAGCTGG-3'	20A	5'-GTTTCTGATTCTGGCAACTG-3'	exon7
21S	5'-TAGCTCATCTTGAGCGAAT-3'	21A	5'-GCTTGCCAGAGGCTTCTTG-3'	intron7
22S	5'-GCAAGCTTGCAGGACTCAGA-3'	22A	5'-GATGGATCAGGTCATAAGAG-3'	exon8-1
23S	5'-GCCAGGATGTATGGAATGTT-3'	23A	5'-GACAGGTCTCATTCTGTCA-3'	exon8-2
24S	5'-GCCTAGGTGACAGAATGAGA-3'	24A	5'-GCAAGCAGAGTACTCAAATC-3'	exon8-3