Role of IL-12B Promoter Polymorphism in Adamantiades–Behcet's Disease Susceptibility: An Involvement of Th1 Immunoreactivity against Streptococcus Sanguinis Antigen

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Adamantiades–Behcet's disease (ABD) is a chronic inflammatory multisystem disorder. Although the precise etiology is unclear, high prevalence of human leukocyte antigen (HLA)-B51 predisposition and predominantly involved T-helper type 1 cells (Th1)-type proinflammatory cytokines and extrinsic Streptococcal infection suggest a substantial association with an immunogenetic basis and strengthens the hypothesis that IL-12, a potent inducer of Th-1 immune reaction, is a putative candidate in its pathogenesis. These clinicopathological findings led us to examine interleukin 12 p40 (IL-12B) promoter polymorphism, for which the 4-base pair (bp) heterozygous insertion has been shown to affect the gene transcription and subsequent protein production. We analyzed IL-12B promoter genotypes in 194 Japanese subjects (92 with ABD and 102 normal controls) by PCR-based restriction enzyme digestion. The frequency of the insertion heterozygosity was significantly higher in patients than in controls (49/92, 53.3% vs 39/102, 38.2%, respectively). Comparing these with HLA haplotype data, this trend was more significant in HLA-B51-negative patients (29/42, 69.0% vs 20/50, 40.0%; $P = 0.005$). As assessed by semiquantitative reverse transcription-PCR and ELISA, stimulation with Streptococcal antigens specifically increased expression of IL-12 p40 mRNA and protein, in conjunction with IL-12 p70 induction, in peripheral blood mononuclear cells from heterozygous patients. Our results provide evidence for anti-bacterial host response toward Th1-immunity mediated by IL-12 in patients with ABD, and the possible insight into the genetic susceptibility that is independent of HLA background.

INTRODUCTION

Adamantiades–Behcet's disease (ABD) is a chronic inflammatory disorder characterized by oral and genital ulcerations, various cutaneous manifestations, and ocular involvement (McCarty et al., 2003). Owing to these widespread symptoms overlapping medical specialities, the disease is sometimes difficult to diagnose, although several clinical criteria are helpful. The clinical course of mucocutaneous manifestations may be protracted with only limited symptomatic relief from topical ointment or anti-inflammatory agents. Systemic immunosuppressive therapy (e.g. corticosteroid, oral cyclosporine, and colchicine) may be required for severe disease with vascular, ophthalmologic, neurologic, or gastrointestinal involvements. Thus, ABD represents morbidity and mortality, and is also an important disease process to investigate.

Clinical and epidemiological studies have suggested that the condition is underestimated and may have a prevalence of more than 1 in 10,000 with almost equal sex predilection, although this trend varies considerably in other geographic penetration (Kurokawa et al., 2004). ABD can occur at any age, but the highest peak ranges from adolescence to middle age. The etiology of ABD is poorly understood, but accumulating evidence suggests an autoimmune and genetic basis: for example, the disease is the most common in the Mediterranean, the Middle East, and the Far East countries, along with the historically Silk road. In these areas, they have reported an increased incidence of human leukocyte antigen (HLA)-B51 in ABD patients than in healthy controls (59.4 vs 13.6%; Mizuki et al., 2001). This genetic predisposition is irrelative with Caucasian population who lives in Western

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countries (25%; Jung *et al.*, 1976; Chamberlain, 1977; Lehner *et al.*, 1982). As transgenic studies for HLA molecule are not currently available to reproduce ABD manifestations in mice (Takeno *et al.*, 1995), additional hits other than HLA restriction mechanism may be implicable as a pathogenic importance of the disease.

From the clinical perspective, the disease activity of ABD has been known to correlate with bacterial infection, particularly Streptococci. The best examples for this notion come from the fact that Streptococcus (S.) Sanguinis was usually isolated from oral bacterial flora of the patients (Isogai *et al.*, 1990; Yoshikawa *et al.*, 1991), and some patients show a clinical improvement after tonsillectomy or administration of antibiotics targeting Streptococci (Kaneko *et al.*, 1997). Moreover, skin prick test using Streptococcal antigen, but not other common bacterial antigens, can reproduce erythema nodosum-like eruptions and oral aphthae, those of which are typical mucocutaneous manifestations of ABD (Kaneko *et al.*, 1985). A series of our recent investigation has also shown that irrespective of HLA-restricted antigen presentation, stimulation with S. Sanguinis antigen induced inflammatory cytokines from peripheral blood mononuclear cells (PBMCs) (Kaneko *et al.*, 1997; Lee *et al.*, 2003). Immunologically, high titers of circulating antibody to the membrane protein fractions (80–150 kDa) from the isolated Streptococcal strain, a KTH-1 line, were demonstrable in part of the patients (Yokota *et al.*, 1995). Thus, identification of new ABD susceptibility genes relevant to abnormal immunobiological response against Streptococcal antigens is an important challenge.

It has been shown that colonization with normal commensal bacteria promotes immune tolerance and deviation toward T-helper type 1 cells (Th1)-type response. IL-12 is a Th1-type proinflammatory cytokine, which is composed of two heterodimeric subunits, a 35-kDa chain encoded by p35 and a 40-kDa chain encoded by p40 (IL-12B). These two subunits represent a biologically active form. IL-12 is mainly produced by antigen-presenting cells, such as macrophages and monocytes, and plays a crucial role in the obligatory transformation of naïve T cells into Th1 cells (Trinchieri, 1993). Several reports have documented that PBMCs from ABD patients predominantly produce Th1 cytokines, mainly IFN-γ and tumor necrosis factor-α, and this response is more significant in the active clinical stage, suggesting that ABD can be categorized as a Th1-mediated disorder (Raziuddin *et al.*, 1998; Frassanito *et al.*, 1999). On this basis, IL-12B may be a potential candidate in the pathogenic importance of ABD. Owing to its fundamentally important biological action in the immune system, several studies have been reported so far to link between IL-12B polymorphism and other allergic or autoimmune disorders. Morahan *et al.* (2002) demonstrated a close association between the IL-12B promoter polymorphism and severity of children with asthma. They revealed that heterozygosity in the IL-12B promoter sequence reduced the gene transcription and also decreased IL-12 p70 secretion in PBMCs. Recently, a study examining the IL-12B promoter polymorphism in skin diseases has been initiated in psoriasis (Litjens *et al.*, 2004), although there was no

significant association between these. The molecular-based approaches may thus help provide some diagnostic advantages in the confusing clinical characteristics of ABD (i.e. mimicking disease such as Crohn's disease or symptoms that do not fulfill the clinical criteria of ABD), and more specifically understanding as yet unidentified immunopathogenesis of the disease, particularly Th1-predominant immunity and aberrant reactivity to Streptococcus antigens. In this study, therefore, we screened the same IL-12B promoter polymorphism in a large cohort of ABD to test the hypothesis that such a genetic background would affect abnormal immune response against Streptococcal antigen mediated by IL-12 production, and to correlate these with the immunopathogenic consensus so far reported in ABD.

RESULTS
Frequency of IL-12B promoter polymorphism
We initially determined HLA-B genotypes in all patients and comparative normal control subjects. The results showed a significantly higher incidence of HLA-B51 in ABD patients than in controls (50/92, 54.3% *vs* 13/102, 12.7%; $P < 0.001$), as were similar to the previous report (Yanagihori *et al.*, 2004).

Next, we examined a 4-base pair (bp) insertion polymorphism within IL-12B promoter region in the same cohort by a PCR-based restriction enzyme digestion. This assay allowed detection of two distinct bands, 79-bp, the common product, and either 206-bp (inserted allele) or 202-bp (non-inserted allele; Figure 1). Overall, the genotypic analysis revealed that our cohort has heterozygous (+/−) or homozygous states (−/− or +/+) of IL-12B promoter polymorphism. The frequencies of these allelic variations comprised 32.6% (−/−), 53.3% (+/−), and 14.1% (+/+) in ABD patients, whereas there were 43.1% (−/−), 38.2% (+/−), and 18.6% (+/+) in normal controls. The distribution of the IL-12B promoter genotypes did not deviate from the expected Hardy–Weinberg equilibrium. Although we found no differences in IL-12B promoter genotypes between these two groups (χ^2 test, $P = 0.145$), there were statistically significant differences between heterozygosity (53.3%) and homozygosity (46.7%) in ABD as compared to those in normal controls (38.2 and 61.8%, respectively; $P = 0.036$; Table 1).

Upon combining with the data of HLA-B51 genotypes in ABD patients, the HLA-B51-negative patients had a significantly higher incidence of IL-12B promoter heterozygosity in comparison with HLA-B51-positive patients (29/42, 69.0% *vs* 20/50, 40.0%, respectively; $P = 0.005$; Table 2).
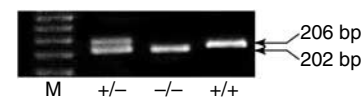

Figure 1. **Detection of IL-12B promoter polymorphism.** The PCR products from amplification using IL-12B promoter-specific primers were electrophoresed on 4% high-resolution agarose gels and visualized with ethidium bromide staining. The insert and non-inserted alleles were designated ''+'' and ''−'', respectively; three different allelic variations for IL-12B promoter, homozygous (−/− and +/+) and heterozygous (+/−), are indicated. M, 20-bp molecular weight markers.

Table 1. IL-12B promoter genotypes in ABD patients and normal controls

Genotype of IL-12B promoter	ABD (*n*=92)	Control (*n*=102)	χ^2	*P*	OR (95% CI)
Homozygous (genotype +/+ or −/−)	43 (46.7%)	63 (61.8%)	4.4	0.036	1.8 (1.0–3.3)
Heterozygous (genotype +/−)	49 (53.3%)	39 (38.2%)			

OR, odd ratio; CI, confidence intervals.

Table 2. Frequencies of IL-12B promoter genotypes in HLA-B51-positive and -negative patients

Genotype of IL-12B promoter	ABD HLA-B51		χ^2	*P*	OR (95% CI)
	Positive (*n*=50)	Negative (*n*=42)			
Homozygous (genotype +/+ or −/−)	30 (60.0%)	13 (31.0%)	7.7	0.005	3.3 (1.4–7.9)
Heterozygous (genotype +/−)	20 (40.0%)	29 (69.0%)			

OR, odd ratio; CI, confidence intervals.

Production of IL-12 p40/p70 subunits

To address *in vitro*/*in vivo* abnormal reactivity against Streptococcal antigens in ABD patients (Kaneko *et al.*, 1997, 2003), we determined IL-12 p40 and p70 production levels in response to KTH-1 (uncommon serotype 1, strain 113-20) S. Sanguinis antigen and lipopolysaccharide (LPS; Figure 2). For this assay, our cohort (*n* = 194) was classified into four different subgroups by means of genotypic variations of IL-12B promoter polymorphism: homozygous disease (*n* = 43) and normal (*n* = 63), and heterozygous disease (*n* = 49) and normal (*n* = 39). As assessed by IL-12 subunit-specific ELISA, our time-course experiment revealed that the steady-state level of IL-12 p40 production from PBMCs in ABD patients did not differ from that in normal (data not shown). By stimulation with 1 μg/ml S. Sanguinis antigen, however, we found approximately 2–2.5-fold increase of IL-12 p40 production from PBMCs in ABD patients with IL-12B promoter heterozygosity when compared to other three subgroups (repeated measure analysis of variance (ANOVA), P = 0.0011; *post hoc* test, P < 0.05). The response was evident at day 1 after the stimulation and reached a plateau up to day 7 culture. Stimulation with LPS showed a similar trend in all four subgroups, although we failed to obtain a statistical significance other than at day 5 culture.

Looking at the production levels of IL-12 p70, there was a tiny but similar response seen in IL-12 p40 induction (Figure 3). By stimulation with S. Sanguinis antigen, we found approximately 2–4-fold increase of IL-12 p70 production from PBMCs in ABD patients with IL-12B promoter heterozygosity, but did not in other three subgroups (repeated measure ANOVA, P = 0.0001; *post hoc* test, P < 0.05). Interestingly, the mean level of S. Sanguinis-induced IL-12 p70 decreased in a time-dependent manner, suggesting the feedback autoregulation in IL-12 p70 production. In contrast, stimulation with LPS did not provide any significant results in IL-12 p70 production among all four subgroups, further implicating the IL-12 subunit-dependent induction by Streptococcal antigen. These results suggest that the IL-12B
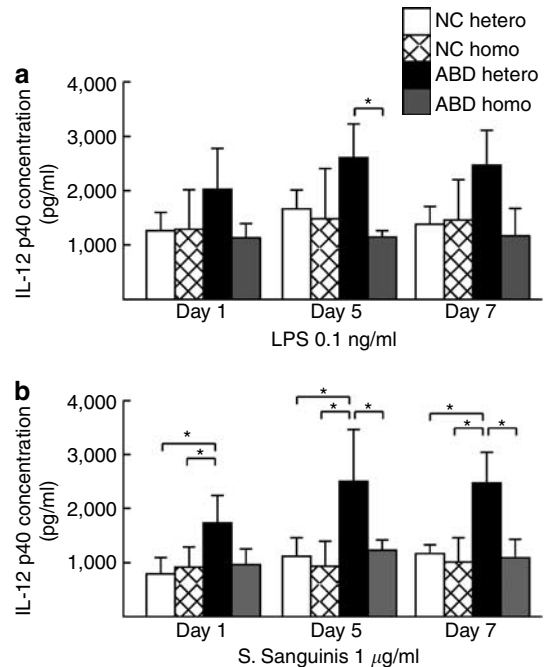

Figure 2. **IL-12 p40 production from LPS- and S. Sanguinis antigen-stimulated PBMCs.** After PBMCs were stimulated with 0.1 ng/ml LPS (**a**) and 1 μg/ml KTH-1 S. Sanguinis antigen (**b**) for the indicated periods (day 1, 5, and 7), the culture supernatants were analyzed with IL-12 p40-specific ELISA. The cells were taken from four different subgroups in terms of IL-12B promoter genotypes; normal control with heterozygosity (white columns) and homozygosity (crosshatched columns), and ABD patients with heterozygosity (black columns) and homozygosity (gray columns). Repeated measure ANOVA was used to compare the IL-12 p40 production levels between S. Sanguinis antigen-stimulated PBMCs (P = 0.0011) and LPS-stimulated PBMCs (P = 0.0518), whereas *post hoc* test to compare between four subgroups. Asterisks indicate the statistical significance with *post hoc* tests lower than P < 0.05.

promoter heterozygosity specifically affects its gene transcription and protein production of IL-12 p40/p70 subunits from patients' PBMCs in response to S. Sanguinis antigen, and
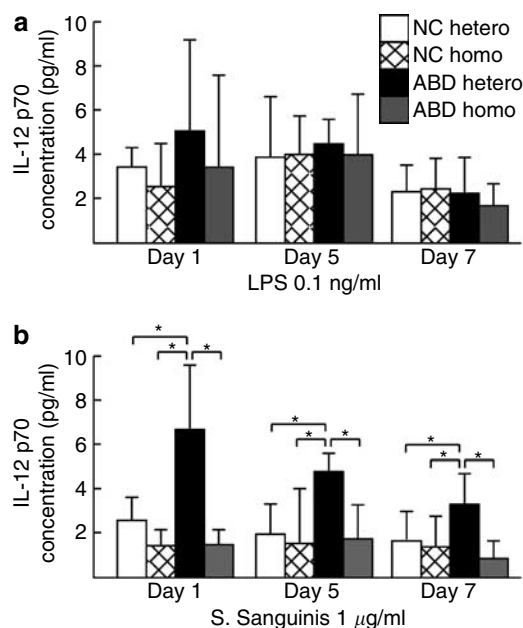
Figure 3. **IL-12 p70 production from LPS- and S. Sanguinis antigen-stimulated PBMCs.** After PBMCs stimulated with 0.1 ng/ml LPS (**a**) and 1 μg/ml KTH-1 S. Sanguinis antigen (**b**) for the indicated periods (day 1, 5, and 7), the culture supernatants were analyzed with IL-12 p70-specific ELISA. The cells were taken from four different subgroups in terms of IL-12B promoter genotypes; normal control with heterozygosity (white columns) and homozygosity (crosshatched columns), and ABD patients with heterozygosity (black columns) and homozygosity (gray columns). Repeated measure ANOVA was used to compare the IL-12 p70 production levels between S. Sanguinis antigen-stimulated PBMCs ($P = 0.0001$) and LPS-stimulated PBMCs ($P = 0.913$), whereas *post hoc* test to compare between four subgroups. Asterisks indicate the statistical significance with *post hoc* tests lower than $P < 0.05$.

that this response is in part mediated by antigen-specific regulation.

Expression of IL-12 p40 mRNA in PBMCs stimulated by S. Sanguinis antigen

We examined the levels of IL-12 p40 mRNA (Figure 4). In PBMCs from all four IL-12B genotypes, the steady-state levels of IL-12 p40 mRNA were not detectable even at day 5 culture, whereas we found the marked expression by stimulation with S. Sanguinis antigen. This induction was most prominent in PBMCs from ABD patients with IL-12B promoter heterozygosity as compared to other three subgroups. There was a similar response in LPS-stimulated PBMCs, with lesser induction levels of IL-12 p40 mRNA (data not shown).

DISCUSSION

In this study, we have elucidated the characteristic gene polymorphisms of the proinflammatory cytokine that is possibly relevant to the immunopathogenic importance of ABD. Our genotyping study using a large cohort ($n = 194$) showed that heterozygosity for the 4-bp insertion polymorphism within IL-12B promoter region contributes to the susceptibility of ABD. More specifically, this allelic variation is closely
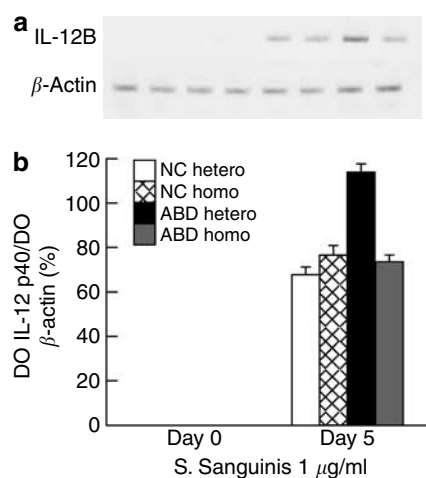

Figure 4. **Semi-quantitative reverse transcription-PCR analysis of IL-12 p40 mRNA expression.** Expression levels of IL-12 p40 mRNA in S. Sanguinis-stimulated PBMCs at day 0 and day 5 were determined by semiquantitative reverse transcription-PCR. For normalization of the mRNA expression levels, β-actin mRNA was used as an internal control. A representative of three independent experiments is shown (**a**). The results are expressed as the ratios of optical densities (OD) to β-actin bands, and represent the mean value ± SD of three experiments (**b**); normal control with heterozygosity (white columns) and homozygosity (crosshatched columns), and ABD patients with heterozygosity (black columns) and homozygosity (gray columns).

associated with increased production of IL-12 p40/p70 in PBMCs from ABD patients in response to the common bacterial antigens. The most pronounced reaction was obtained in the patients' PBMCs upon stimulation with KTH-1 S. Sanguinis antigen. These results indicate the involvement of differential immune response against environmental microorganisms in ABD. This observation extends our previous findings demonstrating that oral bacterial flora of ABD patients consists of Streptococci (Kaneko *et al.*, 1985; Yokota *et al.*, 1995), and that Bes-1 (encoding KTH-1 S. Sanguinis antigen correlated with ABD) DNA fragment can be detected in erythema nodosum-like eruption and oral aphthous lesions in ABD (Tojo *et al.*, 2003). This combined evidence may thus help illustrate the extrinsically pathogenic relevance of Streptococcal antigens mediated by IL-12 in ABD.

Interestingly, the IL-12B promoter polymorphism was mostly found in HLA-B51-negative patients. Given the fact that Japanese ABD patients have considerably higher prevalence of HLA-B51 than that of other West European countries (Jung *et al.*, 1976; Chamberlain, 1977; Lehner *et al.*, 1982), it is possible that the IL-12B promoter heterozygosity is not associated with HLA predisposition. This is in agreement with the recent reports suggesting the involvement of HLA-dependent and -independent genes in susceptibility of ABD (Boiardi *et al.*, 2001; Salvarani *et al.*, 2002, 2004).

The IL-12B promoter polymorphism has recently been shown to affect the gene transcription, although the precise molecular mechanism is unknown. The heterozygous state was associated with disease severity of child patients with atopic/non-atopic asthma, as was mediated by decreased production of IL-12 mRNA and protein in PBMCs upon

stimulation with LPS and IFN-γ (Morahan *et al.*, 2002). As assessed by reverse transcription-PCR and ELISA, however, we found the opposite responses; stimulation with S. Sanguinis antigen or LPS induced IL-12 p40 gene transcription and protein production, as well as increased IL-12 p70 production, in patients' PBMCs with IL-12B promoter heterozygosity. The increased IL-12 levels continued for up to 7 days. Nevertheless, there was a concomitant decrease of the mean levels in IL-12 p70 production. Thus, it is likely that IL-12B promoter heterozygosity contributes to not only ABD susceptibility but also loss of feedback inhibition of the IL-12 p40 gene expression.

Genome-wide linkage analysis for ABD has well been documented in many ethnic groups. Among these, the most disease-susceptible genes include HLA-B51 and major histocompatibility complex class I chain-related gene A, locating a 46-kilobase centromeric portion to the HLA-B gene (Ota *et al.*, 1999). While comparing between ethnically matched populations, ABD patients tend to have a high prevalence of a triplet repeat polymorphism within the transmembrane region of major histocompatibility complex class I chain-related gene A gene (Mizuki *et al.*, 1997). Both of these genes are clustered on chromosome 6. Contrary to this genetic implication, IL-12 p40 gene locates on chromosome 5q31.1–q33.1, and thus far, there are no available data for linkage equilibrium with chromosome 5 or even the region that is at close proximity to this locus in ABD. Moreover, our recent investigation demonstrated less association between 1188A/C single-nucleotide polymorphism within 3′-untranslated region of IL-12B, which is highly detected in autoimmune-based diseases, and ABD susceptibility. The discrepant results between the genome-wide microsatellite mapping and individual gene polymorphisms are in accordance with other chronic inflammatory and allergic disorders, that is, psoriasis, asthma, and atopic dermatitis (Tsunemi *et al.*, 2002; Randolph *et al.*, 2004). Despite their highly inherited condition, these diseases have been considered to be associated pathogenically with a variety of extrinsic and intrinsic factors, such as bacterial and viral infections, and stress (Gul, 2001; Kaneko *et al.*, 2003). It may therefore not be surprising that epidemiological and genetic approaches to address the miscellaneous clinical presentation of ABD have imperfectly covered important areas of its pathogenesis. Another explanation for the discrepancy would include differences in the genetic backgrounds between the investigated populations, as well as differences in the design and setting of the study. Therefore, investigations regarding a large cohort multicenter study for analyzing the qualification of disease-susceptible genes and identification of symptom-specific gene polymorphisms are needed to understand the immunogenetic nature of ABD.

A further intriguing finding in our study is that the IL-12B promoter heterozygosity accounts for the overproduction of IL-12 p40/p70 in response to antigenic microorganisms. Apart from epigenetic DNA modification (i.e. methylation or acetylation), there are no proper explanations for ''heterozygote sufficiency'' at the gene expression. Hypothetically, these disease-susceptible polymorphisms would be in linkage equilibrium with each of the promoter alleles, and would rather appear in patients with homozygosity. For this phenomenon, one may speculate that the heterozygous allele potentially carries impaired stability of the gene transcription. Once upon stimulation with antigenic microbial organisms – especially Streptococcal antigens – intensity of signal transductions and/or induction rates for unstabilized IL-12 p40 gene expression may be abruptly overdriven in immune cells. Besides, it should be noted that normal control subjects with IL-12B heterozygosity did not show such anti-bacterial immune responses. As Streptococcal antigens and LPS are originated constitutively from the common bacterial flora in human, the development of ABD manifestations may need further additional background, for example, more characteristic predisposition of HLA-B51 genotypes (B*5101–5121) or other inflammation-mediated gene polymorphisms, notwithstanding that a number of molecular studies for discovering such candidate genes in ABD susceptibility have not yet provided the novel genetic implication other than HLA and major histocompatibility complex class I chain-related gene A genes (Mizuki *et al.*, 1992; Yanagihori *et al.*, 2004). As the magnitude of these associations may differ among the individual genes, we need to ascertain whether these disease-related genes may act independently or together with, or even just simply modify the clinical course of the disease. It is therefore conceivable that a certain combination of several genes responsible for ABD pathophysiology confers imbalance of anti-bacterial host defence, and eventually elicit abnormal immune response against antigenic microorganisms, particularly Streptococcus antigens. The chronicity of these subsequent reactions may cause the constitutive shift to Th-1 autoimmunity, thereby contributing to the development of ABD symptoms and/or exacerbation of the disease.

In view of the clinical perspective, our experimental data strongly support the previous observation that anti-bacterial agents targeting Streptococcus (i.e. tetracycline or macroride) aid the resolution of mucocutaneous symptoms of ABD (Kaneko *et al.*, 1997). Taken together, Mannon *et al.* (2004) have shown that administration of IL-12-specific monoclonal antibody was indeed effective for the treatment of Crohn's disease, which is often difficult to discriminate clinically from ABD. In a mouse model for Crohn's disease, injection of anti-IL-12 antibody can prevent the systemic inflammatory condition (Neurath *et al.*, 1995), implicating molecular basis of the new therapeutic application targeting IL-12 in ABD. Considering these therapeutic advancement, we propose that it is noteworthy to address the IL-12B promoter genotypes in individual ABD patients.

In conclusion, our data highlight the possible association between the 4-bp insertion polymorphism within IL-12B promoter and susceptibility of ABD in Japanese patients. The allelic heterozygosity can be responsible for abnormal IL-12 p40/p70 production against Streptococcal antigens. Our data are the first documentation for the underlying immunopathogenesis relevant to the predominant Th-1 immune response induced by IL-12 in ABD. Given the lack of published criteria for determining the clinical severity of ABD and a consider-

ably variety of medical management (i.e. various specialized consultations and treatments), our present study did not allow comparisons between the clinical presentation and responsiveness to the ongoing treatment and IL-12B promoter genotypes. Further investigations for linking these individual clinical aspects now need to be opened.

MATERIALS AND METHODS
Subjects
Participants were part of an ongoing study in a large community-based cohort organized by Ministry of Health, Labour and Welfare in Japan. In detail, 92 Japanese patients with ABD (52 males and 40 females, mean age 49.5 ± 18.3 years, range 21–77 years) were recruited from the Department of Dermatology, Fukushima Medical University Hospital (Fukushima, Japan) and the Department of Ophthalmology, Yokohama City University Hospital (Yokohama, Japan). All patients were diagnosed on the basis of the clinical criteria proposed by the International Study Group for Behcet's disease by at least two dermatologic and ophthalmologic specialists. They had no clinical overlap with other systemic inflammatory diseases or autoimmune disease. Of these ABD patients, 55 DNA samples had been shown previously to have no association with 1188A/C single-nucleotide polymorphism in IL-12B 3'-untranslated region (Yanagihori *et al.*, 2004). All clinical information was accurately recorded at the same time as blood samples were taken.

As a control, 102 healthy Japanese volunteers (54 males and 48 females, mean age 28.0 ± 7.8 years, range 19–62 years) with no history of autoimmune and allergic diseases were randomly chosen from our archival DNA samples. This study was conducted according to the Declaration of Helsinki Principles. The local ethical committees approved the design of our study. All subjects enrolled in this study gave their informed consent.

HLA genotyping and detection of IL-12B promoter polymorphism
Genomic DNA was obtained from whole peripheral blood cells by using a standard proteinase K digestion and subsequent phenol/chloroform extraction (Yanagihori *et al.*, 2004). The DNA samples were extracted just before the polymorphism detection procedure. HLA-B genotype was determined by a PCR-based sequencing as described previously (Yanagihori *et al.*, 2004).

For detection of the 4-bp insertion polymorphism within IL-12B promoter (GenBank AC011418), PCR was performed for DNA amplification. On the basis of the previously published data (Morahan *et al.*, 2002), the primers used were designed specifically for IL-12B promoter: forward, 5'-TCAGACACATTAACCTTGCA-3'; reverse, 5'-AGGTTCTAATGTGGTCATTG-3'.

The amplification conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, annealing temperature 56°C for 30 seconds, and 72°C for 30 seconds with a final extension for 10 minutes at 72°C. The reaction mixture contains 5 ng of individual genomic DNA as a template, 20 pmol of the primers, 2.5 mM MgCl$_2$, 1 mM dNTPs, and 1 U of *Taq* DNA polymerase (TaKaRa Bio Inc., Tokyo, Japan). To analyze the 4-bp insertion polymorphism, aliquots of PCR products (10 μl in each) were digested with 0.5 U *Hind*III (TaKaRa Bio Inc.) for at least 1 hour at 37°C, and were subsequently electrophoresed on 4% high-resolution agarose gel (Agarose-1000; Invitrogen Co., Carlsbad, CA) containing ethidium bromide to

visualize two distinct bands (Figure 1). To exclude the possibility of introducing PCR error and miscutting of the restriction enzyme, all steps were repeated at least twice. When we encountered unconvincing results in the above experiments, the PCR products were sequenced directly with an automated DNA sequencer CEQ2000XL (Beckman Coulter, Fullerton, CA).

Cell culture
PBMCs were taken from ABD patients ($n = 8$), in whom systemic corticosteroids and immunosuppressants have not yet been given, and age-matched normal healthy volunteers ($n = 9$). The cells were isolated from heparinized peripheral blood with a Ficoll Paque density-gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). The PBMCs obtained were adjusted to 5.0×10^5 cells/ml in an endotoxin-free RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum in a 24-well plate, and were then cultured with indicated doses of LPS (Sigma, St. Louis, MO), S. Sanguinis antigen from a KTH-1 line (kindly provided by Dr Oguma K, Okayama University, Okayama, Japan), or a medium alone for up to 7 days in a 5% CO_2-humidified incubator. After stimulation, the culture supernatant and cells were corrected at day 1, day 5, and day 7, and then stored at −70°C until the following assay. Optimal doses of the stimulants for cytokine production were determined in pilot experiments. The tested doses of LPS and S. Sanguinis antigen ranged from 0.1 to 10 and 100 to 1000 ng/ml, respectively.

IL-12 ELISA
To determine IL-12 p40 and p70 protein production in the culture supernatant, we performed ELISA with commercially available kits (IL-12 p40; R&D systems Inc., Minneapolis, MN; IL-12 p70, Biosource Europe SA, Fleurus, Belgium), following the manufacturer's instructions. The sensitive limits for quantitative determinations of IL-12 p40 and p70 were 15 and 0.5 pg/ml, respectively. Each sample was assayed in duplicate.

RNA extraction and semiquantitative reverse transcription-PCR
According to the previous study (Akiba *et al.*, 2002), total RNA was extracted from the PBMCs by using Isogen reagent (Nippon gene, Tokyo, Japan). Aliquots of DNase I (TaKaRa Bio Inc.)-treated total RNA (1 μg/reaction) was reverse transcribed with random primers (TaKaRa Bio Inc.) and Superscript II reverse transcriptase (Invitrogen). The final amount of RNA in each reaction was normalized using the amount of housekeeping gene β-actin mRNA. The cDNA obtained was amplified by PCR step using different sets of primers:
   for β-actin as an internal control,
      forward, 5'-GACTATGACTTAGTTGCGTTA-3',
      reverse, 5'-GCCTTCATACATCTCAAGTTG-3',
   for IL-12B,
      forward, 5'-TGGCCAGTACACCTGTCACAAAG-3',
      reverse, 5'-CAGCAGGTGAAACGTCCAGAATAA-3'.
The amplification conditions were optimized by different settings of PCR, and finally conducted with 35 cycles consisting 30 seconds at 94°C, 30 seconds at 62°C, and 30 seconds at 72°C. The PCR products were electrophoresed on 1.5% agarose gel and were then visualized by ethidium bromide staining. Intensity of each band was analyzed by a Luminous Imager densitometer (Aisin Seiki Co., Ltd, Aichi, Japan), and were expressed as the ratio of optical densities to

β-actin bands (Figure 4). The results were representative of three independent experiments.

Statistical analysis

Statistical significance ($P < 0.05$) was determined using StatView version 6 for Windows (SAS Institute Inc., Cary, NC). The distribution of the IL-12B promoter genotypes was compared using χ^2 test. Repeated measure ANOVA and *post hoc* tests were used to assess levels of IL-12 p40 and p70 production.

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