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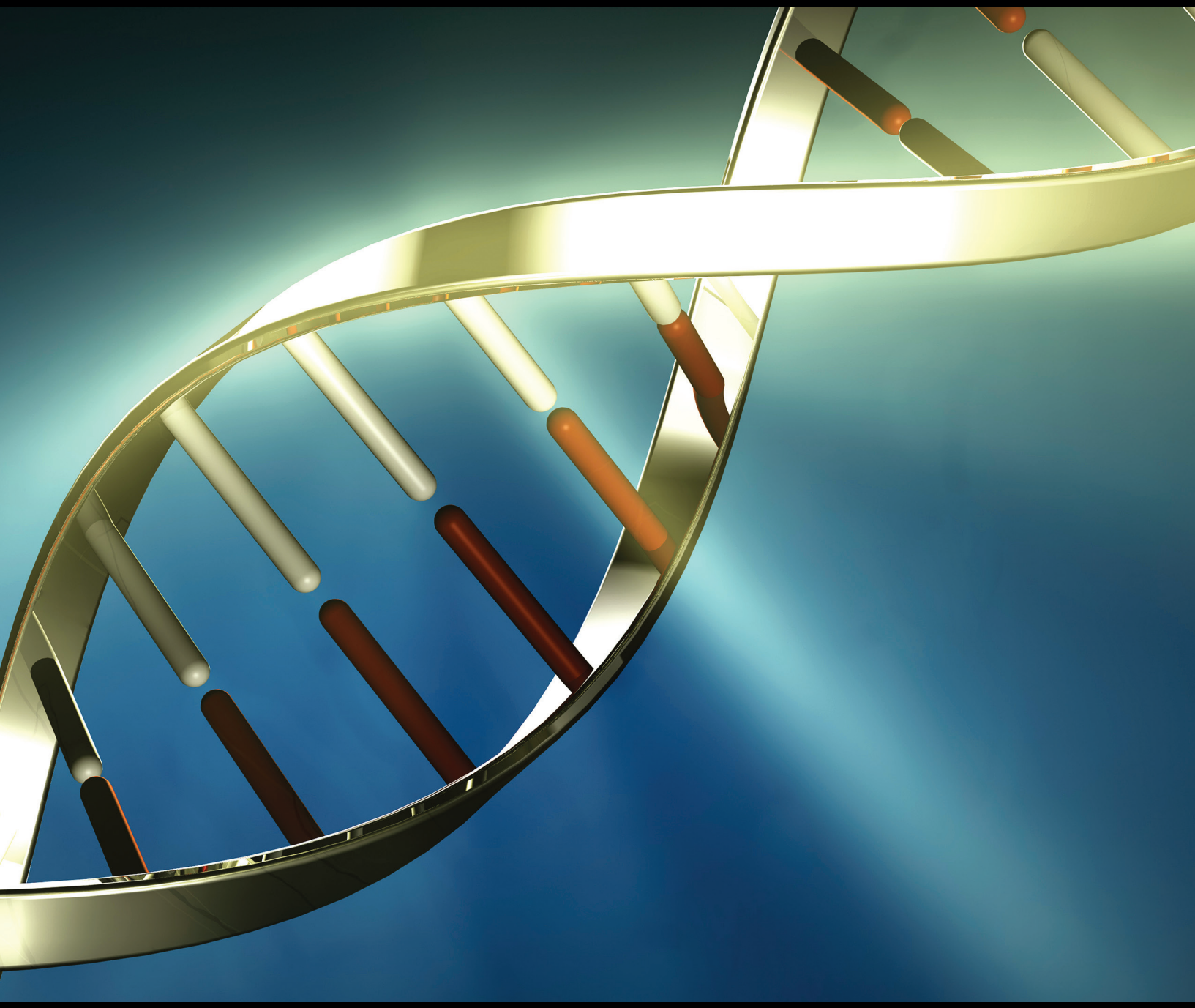
# Immune Reactions in Behçet's Disease

Guest Editors: Fumio Kaneko, Dongsik Bang, Rafi Haner Direskeneli, Shigeaki Ohno, and Yoshiaki Ishigatsubo



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# Editorial Board

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*Editorial*

# Immune Reactions in Behçet's Disease

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Behçet's disease (BD) is a systemic and chronic inflammatory disorder which mainly involves mucocutaneous, ocular, vascular, gastrointestinal, and/or central nervous system organs in association with some genetic background as the intrinsic factors. Generally, the disease starts with oral recurrent aphthous stomatitis (RAS) in young-aged generation and develops to the systemic recurrent inflammation. BD patients are considered to be mainly distributed from the countries around the Mediterranean, old Silk-Road, and Korea to Japan. Historically, there was the antiquity description by Hippocrates and the disease was precisely reported by Turkish dermatologist, Hûlusi Behçet, as a trisymptom complex in 1937. BD pathogenesis is still obscure and classified as an autoimmune and recently in kind of autoinflammatory disorders. To clarify the genetics, epidemiology, diagnosis, pathogenesis, and treatment, a number of investigative studies must be addressed. In this special issue, we approached the clinicopathology of BD in aspects of the quality of life (QOL) in the oral health, a trial of new diagnostic ways utilized by the hypersensitivity to oral streptococci, infectious immunology in correlation with heat shock protein (HSP), T-helper 1(Th1), and Th17 cell responses in the inflammatory lesions and abnormal immune response to herpes simplex virus (HSV).

One of the papers of this issue addresses oral health QOL (OHQOL) in comparison with BD patients and non BD patients having RAS as controls, because RAS is sometimes seen in even healthy children and adults. However, OHQOL of BD patients is worse in their life activities because of more frequent and long involvements though it is not correlated with HLA-B51 gene. Another paper describes that BD patients have hypersensitivity to streptococcal group bacteria. To make a diagnosis for BD, observation of the clinical manifestations and mysterious "Pathergy test" by a thick stick-like 20G syringe needle are conventionally performed. However, the positive rate by the stick test is low in BD patients lately and the diagnostic value for BD is suspected. Then, the authors indicate the high diagnostic value by a fine stick with self-saliva, because oral streptococci are found to be included. The oral streptococci are considered to be one of the extrinsic triggering factors for BD patients with or without HLA-B51 gene. The authors speculate the relationship between the oral immune reaction and the systemic manifestations. In other papers, the following immunological phenomena are described. HSP60/65, which might be derived from mycobacterium and/or streptococci infection and are considered to work as a scavenger for the damaged tissues, might play an important role in innate immunological reactions in BD pathogenesis. They are also speculated to transfer some antigenic peptides to the antigen presenting cells (APCs) through toll-like receptors (TLRs) which activate specific T-cells and enhance MHC-peptide

complexes. The mechanism of both Th1 and Th17 cells was influenced by interferon-$\gamma$, which produce interleukin 17 (IL-17) found in autoimmune disorders, is addressed in BD lesions. In another paper, the authors review the role of HSV in the immunopathogenesis of the HSV-induced mouse model because HSV DNA is detectable from the lesions of BD patients with the clinical evidence supporting the role of HSV infection.

The 5 papers propose the clinical characteristics and pathogenesis of BD which have been better clarified. The real pathogenesis of BD still remains to be obscure as far as the questions are unclear in the correlation between the intrinsic factors like genetic background and the extrinsic triggering factors. However, the number of BD patients seems to be decreasing and the clinically severe cases seen before have been released by the recent excellent immunological treatments.

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*Research Article*

# One-Year Period Prevalence of Oral Aphthous Ulcers and Oral Health-Related Quality of Life in Patients with Behçet's Disease

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The aim of this study was to investigate the 1-year period prevalence of oral aphthous ulcers (OAUs) and their association with oral health-related quality of life (OHQOL) in patients with Behçet's disease (BD) and in the general population. In this cross-sectional study, 675 patients with Behçet's disease (BD group) and 1,097 males and females in the Japanese general population (control group) completed both questionnaires on their OAU status during the prior year and the General Oral Health Assessment Index (GOHAI). In the BD group, 84% of patients reported experiencing an OAU during the previous year, and the mean number of OAUs/year was 13. In the control group, 31% of individuals experienced an OAU during the previous year, and the mean number of OAUs/year was one. Multivariate analysis indicated that both BD patients (OR, 6.2; 95% CI, 4.8–8.0) and controls (OR, 2.6; 95% CI, 2.0–3.5) who had OAUs at least twice per year were more likely to have GOHAI scores below the norm than were controls who had fewer than two OAUs per year. The association between HLA-B*51 and OAUs remains unknown. The presence of OAUs has a negative effect on the OHQOL of patients with BD.

## 1. Introduction

Behçet's disease (BD) is a multisystem disease characterized by recurrent oral and genital ulcers and relapsing uveitis with mucocutaneous, articular, neurological, urogenital, vascular, intestinal, and pulmonary manifestations. Most patients with BD experience recurrent aphthous stomatitis (RAS), which is often the initial feature of the disease [1]. The prevalence of RAS has been reported to vary between 1% and 66% among adults [2, 3]. BD patients may suffer more RAS events in daily life than do individuals in the general population, although the descriptive data on RAS are insufficient.

Previous studies have detailed the impact of BD, including its symptoms and disease activity, on quality of life (QOL)

[4–13]. Blackford et al. [4] found that oral and genital ulcerations in particular affected patients' personal relationships. BD patients with ocular involvement have been reported to be susceptible to anxiety and depression [5]. Arthritis had a significant effect on both the pain level and QOL of patients with BD, leading to an increased risk of mental distress, including anxiety or depression [8].

Oral health status is closely associated with QOL [14]. Tabolli et al. [15] showed that oral mucosal conditions could have a radical effect on oral health-related QOL (OHQOL). Mumcu et al. [9] reported that BD patients with active oral ulcers reported relatively poor OHQOL compared with ulcer-free patients. OHQOL was worse in BD patients than RAS patients, whereas BD patients exhibited a prolonged healing time for oral ulcers, and RAS patients showed an increased frequency of oral ulcers [9].

The extant data are insufficient for clarifying the effects of oral aphthous ulcers (OAUs) on the OHQOL of patients with BD. To our knowledge, no study has analyzed a large dataset including a control group from the general population. Therefore, the aim of this study was to investigate the annual prevalence of OAUs and their association with OHQOL in BD patients and to compare them with those in the general Japanese population.

## 2. Materials and Methods

*2.1. Study Subjects.* Two groups of subjects participated in this study, patients with BD (BD group) and individuals representative of the general population (control group). For the BD group, a cross-sectional study was performed of all members of the Japanese Association for Patients with Behçet's Disease between October 2004 and January 2005. The control group was selected via stratified, multistaged random sampling from 200 locations in Japan. Stratification was performed according to area and municipality based on national census figures. To obtain first-stage sampling units, municipalities were sampled for each stratum. For second-stage sampling, survey units were randomly sampled for each stratum. To obtain third-stage sampling units, individuals from the Basic Resident Registry who were eligible for participation in the survey and resided within the survey areas were systematically sampled at regular intervals. The cross-sectional study was performed in February 2006.

Questionnaires were administered to 2,400 potential control subjects and 1,358 BD patients aged 15–79 years and 17–91 years, respectively. The Ethics Board of iHope International approved this study protocol in October 2004, and the Ethics Board of the Nagoya University School of Medicine approved the study protocol in December 2005.

*2.2. Instruments.* The questionnaire packet included items regarding oral health status, current number of teeth, and the Japanese version of the General Oral Health Assessment Index (GOHAI). The GOHAI contains 12 questions, each of which is scored between 1 and 5; total scores range from 12 to 60—a higher total score indicates a better OHQOL.

The GOHAI, an instrument designed to assess OHQOL, was originally developed in the United States for use among elderly individuals [16]. The Japanese version of the GOHAI questionnaire has been adapted for and validated in the Japanese population [17]. The national norms for Japanese are available online [18]. This index defines health-related QOL as a person's assessment of how the following affects his or her well being: functional factors, psychological factors, social factors, and the experience of pain or discomfort. When the assessment is focused on orofacial concerns, OHQOL is assessed [19].

Patients in the BD group self-reported disease information. They were evaluated for disease severity according to the criteria proposed by the Behçet's Disease Research Committee of Japan in 2003 [20].

*2.3. Data Analysis.* Differences in GOHAI scores and in the frequency and duration of OAUs were analyzed using $t$-tests. For purposes of data analysis, GOHAI scores were treated as dichotomous categorical outcome variables. Group (i.e., control with or without OAUs, BD with or without OAUs) was included as an independent variable. Covariates included age and sex. Age-adjusted and multivariate-adjusted logistic regression models were used to assess the association between BD, experiencing OAUs at least twice per year ("with OAUs" was defined as ≥2 outbreaks during the past year), and the risk of scoring below the national norms on the GOHAI (GOHAI score = 53.1). Responses were analyzed after excluding subjects with missing data related to their OAU 1-year period prevalence, GOHAI scores, or BD diagnosis.

Mean values are reported with standard deviations (SDs). Odds ratios are reported with 95% confidence intervals (95% CIs). All $P$-values were two sided, and $P < 0.05$ was considered to indicate statistical significance. Analyses were performed using the IBM SPSS Statistics software (version 20 for Windows; IBM Corporation, New York, NY, USA).

## 3. Results

*3.1. Response Rates and Compositions of Final Groups.* Responses were obtained from 1,170/2,400 potential control subjects (response rate = 49%) and 883/1358 patients with BD (response rate = 65%). Analyses were limited to data from subjects aged 20–79 years because most respondents (1,122/1,170 controls, 867/883 BD patients) were in this age range. Both sexes were well represented in both groups. The final data analysis was conducted after 192 patients and 25 controls were excluded due to incomplete data. The mean age of the final control group (50.8 ± 15.7 years) differed significantly from that of the final BD group (55.5 ± 12.5 years; $P < 0.001$). The demographic characteristics of the BD group and those of participants in the National Survey of BD patients in 2002 [21] were similar. The mean ages of males were 50.2 and 47.8 years and those of females were 51.3 and 51.3 years in the BD group and the National Survey, respectively. The respective male: female ratios were 1.07 and 0.93.

Table 1: Characteristics of study participants in the final analysis ($n$ = 1,772).

| Variable | BD patients | | Controls | |
|---|---|---|---|---|
| | $n$ | (%) | $n$ | (%) |
| Sex | | | | |
| Male | 349 | (51.7) | 532 | (48.5) |
| Female | 326 | (48.3) | 565 | (51.5) |
| Age | | | | |
| 20–29 | 22 | (3.3) | 109 | (9.9) |
| 30–39 | 62 | (9.2) | 195 | (17.8) |
| 40–49 | 112 | (16.6) | 200 | (18.2) |
| 50–59 | 183 | (27.1) | 231 | (21.1) |
| 60–69 | 212 | (31.4) | 206 | (18.8) |
| 70–79 | 84 | (12.4) | 156 | (14.2) |
| Disease severity | | | | |
| No symptoms | 89 | (13.2) | | |
| I | 283 | (41.9) | | |
| II | 131 | (19.4) | | |
| III | 31 | (4.6) | | |
| IV | 91 | (13.5) | | |
| V | 17 | (2.5) | | |
| Unknown | 33 | (4.9) | | |
| Drug treatment | | | | |
| Colchicines | 217 | (33.8) | | |
| Steroids | 201 | (31.0) | | |
| Immunosuppressant | 56 | (8.4) | | |
| Other(s) | 152 | (23.3) | | |

The mean disease duration for BD patients was 22 ± 12 years (range, 1–50 years). The most common drug treatments used by BD patients were colchicines and steroids. Detailed data on the demographic characteristics of both groups and the disease severity of and drug treatments used by BD patients are presented in Table 1.

*3.2. Characteristics of the 1-Year Period Prevalence of OAUs.* We found that 83.9% of BD patients and 31.0% of controls, regardless of age, had OAUs during the prior year. The mean number of OAUs per year was 12.7 (SD = 31.5) in BD patients and 1.1 (SD = 2.4) in the control group. BD patients experienced OAUs significantly more frequently than did controls (Table 2).

Among patients with BD, more males than females in all age groups, with the exception of 40–59 years, reported having had OAUs. We found a significant difference between males and females in the 1-year period prevalence of OAUs only among those aged 30–39 years ($P = 0.042$). The mean number of persons having OAUs decreased significantly with age among males ($P = 0.007$). In the control group, more females than males in all age groups, except those aged 20–29 and 60–69 years, reported having had OAUs. The mean number of persons having OAUs decreased significantly with age among both males and females ($P < 0.001$). Detailed data on the frequency of OAUs in each group according to sex and age are presented in Table 2.

The mean duration of OAUs was 55.3 ± 76.4 days in the BD group and 4.7 ± 12.1 days in the control group. The duration of OAUs suffered by BD patients was substantially longer than was that experienced by members of the control group, irrespective of sex. The trends in duration for each age category were congruent with the frequency data. Detailed data on the frequency of OAUs in each group by sex and age are provided in Table 2.

Among patients with BD, more females than males in all age groups, with the exception of 30–39 years, reported OAUs of longer duration. We found no significant differences between males and females in all age groups, with the exception of 30–39 years ($t(60) = 2.520$, $P = 0.014$). A similar trend was observed in the control group, with more females than males in all age groups, with the exception of 20–29 years, reporting OAUs of longer duration. We found significant differences in OAU duration between males and females in the 20–29-year-old and 40–49-year-old groups ($t(107) = 2.078$, $P = 0.040$ and $t(198) = -2.554$, $P = 0.012$, resp.).

*3.3. Effect of OAUs on OHQOL.* The 1-year period prevalence of BD had a negative effect on the GOHAI scores of both males and females (both $P < 0.001$). The age-stratified analysis of mean GOHAI scores indicated that the trend observed in females with BD was similar to that in females in the control group. Males without BD had decreased GOHAI scores with age, but those in the 20–39-year-old and 70–79-year-old subgroups of BD patients had lower scores.

After adjusting for age and sex, BD patients with OAUs (where "with OAUs" was defined as ≥2 outbreaks in the last year) were more likely to have GOHAI scores below the norm. Detailed multivariate analysis results before and after stratification for sex and age are reported in Table 3. Stratified analyses by sex and age suggested that the adjusted odds for GOHAI scores below the norm were highest for female BD patients with OAUs who were 60–79 years, followed by female BD patients with OAUs who were 20–39 years. The adjusted odds among control participants with OAUs were higher than were those among BD patients without OAUs.

## 4. Discussion

In the present study, we confirmed that BD patients had a considerably higher frequency of OAUs and a lower OHQOL than the general population. Our sex-stratified analysis further revealed that OHQOL was particularly compromised among females with OAUs. This work corroborates the findings of a prior study indicating that BD patients had lower OHQOL than did healthy controls [9]. The strengths of our study were (1) the inclusion of more BD patients compared with previous studies, (2) the relatively high response rate for a mail-in survey, and (3) the inclusion of a control group drawn from the general population.

The reported 1-year period prevalence rates for RAS vary widely (5–60%), depending on the population examined, with a potential female predominance in adults [22–30]. According to a population study in the United States, only 1%

Table 2: One-year period prevalence, frequency, and mean total duration of OAUs during the past year in the general population (controls) and BD patients according to sex and age ($n$ = 1,772).

| Sex, age | No. BD patients[a]/no. controls[a] | BD patients | | | | | Controls | | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | One-year period prevalence, % | Mean no. OAUs | SD | Mean total duration, day | SD | One-year period prevalence, % | Mean no. OAUs | SD | Mean total duration, day | SD | One-year period prevalence | Mean no. OAUs | Mean total duration |
| Males | | | | | | | | | | | | | | |
| 20–29 | 11/64 | 81.8 | 24.4 | (30.9) | 38.3 | (38.6) | 54.7 | 2.8 | (4.0) | 13.1 | (21.7) | 0.091 | 0.043 | 0.059 |
| 30–39 | 25/93 | 100.0 | 32.0 | (52.5) | 111.0 | (104.7) | 38.7 | 1.3 | (2.2) | 6.5 | (14.2) | <0.001 | 0.007 | <0.001 |
| 40–49 | 61/90 | 90.2 | 16.1 | (47.3) | 60.5 | (91.0) | 25.6 | 1.1 | (2.9) | 3.0 | (8.5) | <0.001 | 0.016 | <0.001 |
| 50–59 | 102/111 | 80.4 | 9.0 | (11.8) | 47.4 | (68.9) | 23.4 | 0.6 | (1.6) | 2.3 | (5.6) | <0.001 | <0.001 | <0.001 |
| 60–69 | 109/110 | 67.0 | 10.2 | (35.8) | 39.7 | (68.1) | 16.4 | 0.6 | (2.5) | 1.6 | (4.8) | <0.001 | 0.006 | <0.001 |
| 70–79 | 41/64 | 73.2 | 6.2 | (10.4) | 34.6 | (66.3) | 15.6 | 0.3 | (0.9) | 1.5 | (5.9) | <0.001 | 0.001 | 0.003 |
| Total | **349/532** | **78.5** | **12.4** | **(33.1)** | **61.7** | **(77.2)** | **27.8** | **1.0** | **(2.6)** | **4.2** | **(11.5)** | **<0.001** | **<0.001** | **<0.001** |
| Females | | | | | | | | | | | | | | |
| 20–29 | 11/45 | 100.0 | 9.6 | (9.5) | 47.3 | (34.2) | 51.1 | 1.7 | (2.7) | 6.8 | (9.5) | 0.003 | 0.021 | 0.003 |
| 30–39 | 37/102 | 94.6 | 9.3 | (10.5) | 57.2 | (63.3) | 49.0 | 1.7 | (2.5) | 7.6 | (14.2) | <0.001 | <0.001 | <0.001 |
| 40–49 | 51/110 | 94.1 | 18.9 | (52.5) | 89.2 | (88.8) | 41.8 | 1.5 | (2.6) | 7.4 | (15.6) | <0.001 | 0.022 | <0.001 |
| 50–59 | 81/120 | 91.4 | 14.4 | (26.5) | 65.7 | (79.8) | 30.8 | 1.0 | (2.4) | 4.1 | (10.9) | <0.001 | <0.001 | <0.001 |
| 60–69 | 103/96 | 83.5 | 8.5 | (10.8) | 50.0 | (75.2) | 13.5 | 0.4 | (1.1) | 1.5 | (5.4) | <0.001 | <0.001 | <0.001 |
| 70–79 | 43/92 | 88.4 | 18.2 | (40.7) | 57.4 | (75.6) | 25.0 | 0.7 | (1.8) | 4.3 | (15.0) | <0.001 | 0.007 | <0.001 |
| Total | **326/565** | **89.6** | **13.0** | **(29.6)** | **61.7** | **(77.2)** | **34.0** | **1.1** | **(2.3)** | **5.2** | **(12.7)** | **<0.001** | **<0.001** | **<0.001** |

[a] All BD patients and controls were analyzed, regardless of the 1-year period prevalence of OAUs.

Table 3: Odds ratios (with 95% CI) for GOHAI scores lower than Japanese norms based on 1-year period prevalence of OAUs[a] in the past year for controls and BD patients.

| Group[a] | Total | | | Males | | | Females | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. with GOHAI scores higher than the norm/no. with those lower than the norm | Crude OR (95% CI) | OR[b] (95% CI) | No. with GOHAI scores higher than the norm/no. with those lower than the norm | Crude OR (95% CI) | OR[c] (95% CI) | No. with GOHAI scores higher than the norm/no. with those lower than the norm | Crude OR (95% CI) | OR[c] (95% CI) |
| All ages | | | | | | | | | |
| GP without OAUs | 523/319 | 1 | 1 | 268/153 | 1 | 1 | 255/166 | 1 | 1 |
| GP with OAUs | 107/148 | 2.3 (1.7–3.0) | 2.6 (2.0–3.5) | 47/64 | 2.4 (1.6–3.7) | 2.8 (1.8–4.3) | 60/84 | 2.2 (1.5–3.2) | 2.6 (1.8–3.9) |
| BD without OAUs | 87/59 | 1.1 (0.8–1.6) | 1.0 (0.7–1.4) | 60/38 | 1.1 (0.7–1.7) | 0.9 (0.6–1.4) | 27/21 | 1.2 (0.7–2.2) | 1.0 (0.6–1.8) |
| BD with OAUs | 113/416 | 6.0 (4.7–7.8) | 6.2 (4.8–8.0) | 73/178 | 4.3 (3.0–6.0) | 4.4 (3.1–6.1) | 40/238 | 9.1 (6.2–13.5) | 9.3 (6.4–13.6) |
| 20–39 years | | | | | | | | | |
| GP without OAUs | 131/55 | 1 | 1 | 69/27 | 1 | 1 | 62/28 | 1 | 1 |
| GP with OAUs | 65/53 | 1.9 (1.2–3.1) | 1.9 (1.2–3.1) | 33/28 | 2.2 (1.1–4.2) | 2.2 (1.1–4.3) | 32/25 | 1.7 (0.9–3.4) | 1.7 (0.9–3.4) |
| BD without OAUs | 3/4 | 3.1 (0.7–14.7) | 3.1 (0.7–14.2) | 1/1 | 2.6 (0.2–42.3) | 2.7 (0.2–44.6) | 2/3 | 3.3 (0.5–21.0) | 3.5 (0.6–22.3) |
| BD with OAUs | 19/58 | 7.3 (4.0–13.3) | 7.5 (4.0–13.8) | 10/24 | 6.1 (2.6–14.5) | 6.1 (2.6–14.4) | 9/34 | 8.4 (3.5–19.8) | 9.8 (4.1–23.7) |
| 40–59 years | | | | | | | | | |
| GP without OAUs | 212/123 | 1 | 1 | 112/57 | 1 | 1 | 100/66 | 1 | 1 |
| GP with OAUs | 35/61 | 3.0 (1.9–4.8) | 2.9 (1.8–4.8) | 10/22 | 4.3 (1.9–9.7) | 4.4 (1.9–9.9) | 25/39 | 2.4 (1.3–4.3) | 2.5 (1.4–4.6) |
| BD without OAUs | 32/21 | 1.1 (0.6–2.0) | 1.2 (0.7–2.2) | 26/13 | 1.0 (0.5–2.1) | 0.9 (0.4–1.9) | 6/8 | 2.0 (0.7–6.1) | 1.6 (0.6–4.8) |
| BD with OAUs | 63/179 | 4.9 (3.4–7.0) | 5.0 (3.4–7.1) | 41/83 | 4.0 (2.4–6.5) | 4.1 (2.5–6.7) | 22/96 | 6.6 (3.8–11.6) | 6.8 (3.9–11.9) |
| 60–79 years | | | | | | | | | |
| GP without OAUs | 180/141 | 1 | 1 | 87/69 | 1 | 1 | 93/72 | 1 | 1 |
| GP with OAUs | 7/34 | 6.2 (2.7–14.4) | 6.1 (2.6–14.3) | 4/14 | 4.4 (1.4–14.0) | 4.6 (1.4–14.7) | 3/20 | 8.6 (2.5–30.1) | 8.2 (2.3–28.9) |
| BD without OAUs | 52/34 | 0.8 (0.5–1.4) | 1.0 (0.6–1.6) | 33/24 | 0.9 (0.5–1.7) | 1.0 (0.5–1.8) | 19/10 | 0.7 (0.3–1.6) | 0.8 (0.4–1.8) |
| BD with OAUs | 31/179 | 7.4 (4.7–11.5) | 8.3 (5.3–13.0) | 22/71 | 4.1 (2.3–7.2) | 4.9 (2.7–8.9) | 9/108 | 15.5 (7.3–32.7) | 15.2 (7.5–30.6) |

[a]For purposes of analysis, the cutoff for "with OAUs" was ≥2 OAU outbreaks within the last year.

[b]Adjusted for age and sex.

[c]Adjusted for age.

of children had recurrent oral ulcers, but 35–40% may have had a history of RAS-like diseases, with ulceration beginning before 5 years of age [31]. Moreover, the prevalence of affected patients increased with age. However, a study of elderly dental patients in Thailand found RAS in only 0.7% of persons older than 70 years of age [14]. Because OAU status was self-reported in our study, we could not determine whether RAS was one form of BD symptoms. Nevertheless, it was clear that BD patients had a greater burden of suffering related to OAUs than did the general population.

Oral diseases give rise to significant morbidity, resulting in physical, social, and psychological consequences that affect QOL [9]. Oral health problems can result in pain and discomfort and lead to problems with eating, interpersonal relationships, appearance, and self-image. Pain is an important factor that can limit oral functions. Bernabé et al. showed that BD had a considerable impact on QOL as assessed by the generic questionnaire, the EuroQol (EQ-5D) [32].

Al-Omiri and colleagues recently found that patients with RAS reported higher levels of anxiety than did controls. Females (both patients and controls) had higher scores on the Hospital Anxiety and Depression Scale than did males [33]. Stress may trigger the mechanisms involved in the pathogenesis of recurrent OAUs, leading to pain and negative effects on daily activities, initiating a cycle in which stress leads to ulcers and ulcers lead to stress [33, 34]. Pain-related interference appears to be related to psychological rather than to physical characteristics [34]. Krisdapong et al. showed that the discomfort of RAS primarily affected eating and tooth brushing [35]. Our findings show that those in the control group who had OAUs had a lower OHQOL than did BD patients without OAUs. Indeed, recurrent OAUs may have a major negative impact on the OHQOL of not only patients but also of healthy individuals. Prevention of and treatment for OAUs may play an important role in maintaining or improving QOL, particularly in BD patients.

In our study, female BD patients with two or more OAUs within 1 year had almost double the risk for a subnormal GOHAI score than did their male counterparts. This difference should be evaluated further given that it persisted after multivariate adjustment. Gur et al. suggested that BD patients could benefit from psychoeducational or self-management interventions aimed at enhancing self-esteem and ability to manage their disease and its symptoms on a daily basis. They showed that such programs could improve patients' physical and psychological functioning [8].

Although an association between HLA-B*51 and RAS has been reported [36], the findings remain inconsistent [37, 38]. Oral streptococci have been suggested as important determinants of RAS, either as direct pathogens or as an antigenic stimulus culminating in the genesis of antibodies that may cross-react with keratinocyte antigenic determinants [39–41]. *Helicobacter pylori* [42, 43], herpes viruses [44–46], *Varicella zoster* virus [47], and cytomegalovirus [47, 48] have also been implicated in RAS. Our study could not analyze the associations between these factors and OAUs because of a lack of information.

This study had several limitations. First, the cross-sectional nature of the data restricts our ability to make inferences regarding the direction of associations. However, it is less reasonable to believe that the domains of QOL affect the 1-year period prevalence OAUs than vice versa. Second, we used a mail-in questionnaire for data collection, and the diagnosis of BD was self-reported. However, we analyzed the data from only those subjects who provided a confirmed year of BD diagnosis. It is unlikely that this practice resulted in misclassifications. Third, this study focused on the frequency of OAUs but not on their severity. Finally, it has been suggested that various physiological and biochemical factors are associated with recurrent aphthous ulcers (e.g., smoking and other lifestyle habits). These factors should be evaluated in greater detail according to the different clinical presentations of the disease.

In conclusion, our data indicate that having OAUs has a negative impact on OHQOL, particularly for female BD patients. Caring for OAUs may be a critical factor in maintaining and improving the QOL of BD patients. Associations between biological features, such as HLA-B*51, and the prevalence of OAUs remain unknown. A more detailed analysis including biological factors may reveal additional associations between OAUs and BD.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# A New Diagnostic Way for Behcet's Disease: Skin Prick with Self-Saliva

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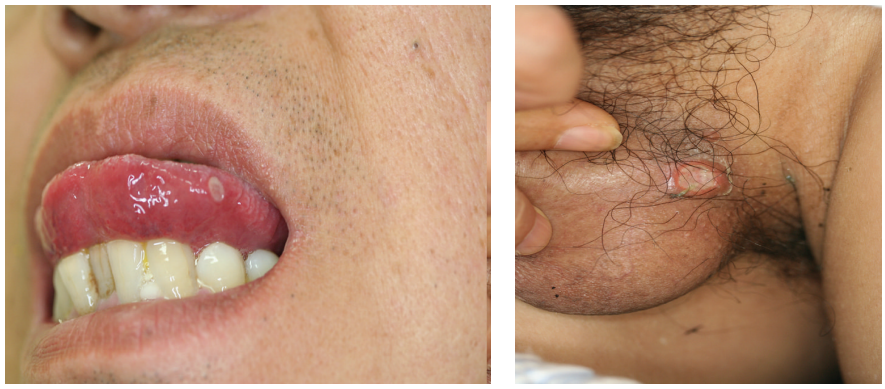
Behcet's disease (BD) is a mysterious multisystemic disorder characterized by recurrent involvement of mucocutaneous (including recurrent aphthous stomatitis; RAS), ocular, intestinal, vascular, and/or nervous system organs. Previously, the positivity of "pathergy test", which is one of the diagnostic examinations, was reported to be related to the possession of HLA-B51 gene in BD patients, even though the positivity is low and different from the countries. Here, instead of the ordinal pathergy test, we would like to propose the prick with self-saliva as a new diagnostic way for patients with RAS of BD based on the genetic intrinsic factors including HLA-B51 and extrinsic triggering factors. BD patients are considered to acquire the hypersensitivity against oral *streptococci* through the innate immune mechanism in the oral cavity. *Bes-1* gene and 65 kD of heat shock protein (HSP-65) derived from oral *S. sanguinis* are supposed to play important roles as extrinsic factors in BD pathogenesis. Although the prick positivity was not related to the possession of HLA-B51 gene, the method is suggested to be a significant way for BD diagnosis. The results also suggest that BD symptoms are due to the vascular immune responses by monocytes expressed oral streptococcal agents of the patients.

## 1. Introduction

Behcet's disease (BD) [1] is a chronic multisystematic inflammatory disorder characterized by the recurrent involvement of mucocutaneous [oral and genital ulceration, erythema nodosum (EN)-like eruption, acne-like eruption, etc.], ocular, vascular, digestive, and/or nervous system organs. Although the actual etiology of BD is still unclear, the pathogenesis has been generally clearer by the etiological research based on the genetic intrinsic factors and immunological reactions to the extrinsic triggering factors in an environmental agent [2–14]. As one of the triggering factors, the oral unhygienic condition may be suspected, because periodontitis, decayed teeth, chronic tonsillitis, and so forth are frequently noted in the oral cavity of BD patients [9, 10]. The infectious triggering factors are suspected to be many organisms including streptococci, herpes simplex viruses (HSVs), *Saccharomyces fermentans, Borrelia burgdorferi, Helicobacter pylori, Escherichia coli, Staphylococcus aureus, Mycoplasma fermentans,* and *mycobacterium* [11].

The proportion of *Streptococcus sanguinis (S. sanguinis),* which was previously recognized as *species* of the *genus Streptococcus* named "*S. sanguis*," was significantly high in the oral bacterial flora of BD patients in comparison with those of healthy controls [12–14]. Most of the patients tend to acquire hypersensitivity against *streptococci* in their oral bacterial flora, as previously demonstrated that much stronger cutaneous reactions were seen by the prick with streptococcal antigen than those by "Pathergy test" [8, 9, 15, 16]. Non-BD patients with recurrent aphthous stomatitis (non-BD-RAS) were also having the hyperreactivity as reported by Graykowski et al. in the 1960s [17]. In vitro system, inflammatory cytokines, interleukin (IL)-6, and interferon (IFN)-$\gamma$ were produced from peripheral blood mononuclear cells (PBMCs) of BD patients, which were stimulated by streptococcal antigen [18], and the serum-antibody titers against *streptococci* were also elevated in BD patients [19]. The peptides of 65 kD of heat shock protein (HSP-65) derived from *streptococci* show considerable homology with those



(a) (b)

Figure 1: Oral aphtha and genital ulcerations seen in a male BD patient with neuropathy (55 M YY in Table 1). (a) Oral ulcer round and punched-out shaped on the tongue. (b) Genital ulcer shaped like oral ulcer.

of the human HSP-60 which appeared as counterpart after streptococcal infection [20–22].

Here, an attempt to review on the mucocutaneous manifestations clinically seen in BD patients was done in the connection with the genetic intrinsic and extrinsic triggering factors. We would like to take up a new diagnostic way for BD using self-saliva prick instead of the ordinal "Pathergy test" which seems to be low positive in BD patients.

## 2. Mucocutaneous Involvements

*2.1. Aphthous Ulceration.* RAS generally starts as an initial symptom in BD patients since their childhood and/or youth and other mucocutaneous symptoms follow after RAS [23–25] (Figures 1(a) and 1(b)). The oral aphthous ulceration punch-out shaped occurs with pain on the tongue, buccal mucosa, gingival, and lip and it continues around a week in BD patients. The clinical features of the oral ulcers is divided as minor, major, herpetiform, and the combined types depending on the lesional size and shapes. Non-BD-RAS is a very common disorder due to trauma, some viral and/or bacterial infections except for patients with BD, and other autoimmune diseases; because it is known that 20% of the general population is affected in the world [26]. On the other hand, nearly 100% of BD patients are associated with RAS as the initial symptom as aphthous ulceration. The biopsy specimen of aphthous ulcer lesion from a BD patient revealed a reaction—like the antibody dependent cell mediated cytotoxicity that the epithelial cells surrounded by neutrophils and lymphoid cells look like leaves falling down from the mucous epithelial layer (Figures 2(a) and 2(b)). These epithelial cells are stained with IgM and HLA-DR and are surrounded by T cells in the immunohistological findings and in addition antistreptococcal antibody was also stained on the cell membrane of the epithelium [15, 27]. However, it is histologically difficult to differentiate aphthous ulceration of BD patient from non-BD-RAS patients.

*2.2. Genital Ulcer.* The clinical features of genital ulceration are generally shaped as similar to oral aphthous ulceration in BD patients (Figure 1(b)) and in young female a genital ulceration suddenly occurs as the initial symptom of BD as Lipschutz ulceration [28], although it was reported to be related to Epstein-Barr viral infection [29]. About more than 50% of BD patients are found to be associated with genital ulceration (female, 55.5%; male, 58.7%); that is, ulcers occur on vulva (66.1%), vaginal mucosa (35.7%), anus (9.6%), cervix (4.1), and groin area (0.8%) in female patients and on the penis (46.5%), scrotum (38.5%), anus (9.2%), and groin area (5.0%) in male patients [23, 25].

*2.3. EN-Like Eruption.* More than 50% of BD patients is reported to be associated with EN-like eruption on the legs [23–25, 30], which relatively looks smaller indulation than that of non-BD patients (Figure 3(a)). The histology is generally vascular reaction infiltrated by lymphoid cells, so-called lymphocytic vasculitis, in the dermis and septal panniculitis in the subcutaneous fatty tissue (Figure 3(b)). In acute phase, however, vasculitis surrounded by neutrophils is also able to be recognized. Immunofluorescence technique revealed deposits of IgA, IgM, and complement in the vascular walls and the similar findings can be seen in the reactive site by pathergy test [31–33]. Streptococcal related materials can also be detected in the vascular walls by use of antistreptococcal antibody (Figure 3(c)) [9, 15, 27]. Recently, Cho et al. [34, 35] have demonstrated that IgA and IgM deposited at the lesional vascular walls targeted against human nuclear ribonucleoprotein (hnRNP) A2/B1 of the endothelial cells in BD patients whose serum IgA and IgM also reacted with *S. sanguinis* and HSP-65/60.

*2.4. Other Cutaneous Disorders.* Acne-like eruption due to perifolliculitis repeatedly appears on the upper body of BD patients and subcutaneous thrombophlebitis, so-called "thrombophlebitis migrans," is suddenly noticed on the lower extremities. Rarely, the follicular lesions may develop to a large ulceration like "pyoderma gangrenosum" on the extremities. Some male BD patients may have a sudden pain and edema of the scrotum due to epididymitis.

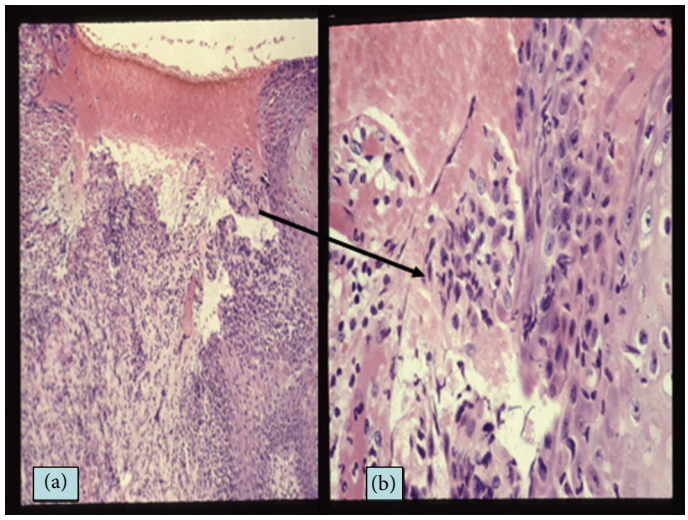



Figure 2: Histology of aphthous ulceration of a BD patient. (a) Aphthous ulcer of the lip defecting the epithelial layer (HE, ×100). (b) Magnified feature of the ulcer edge of the epithelial layer. The epithelial cells are surrounded by inflammatory infiltrates like "Rosetta formation."

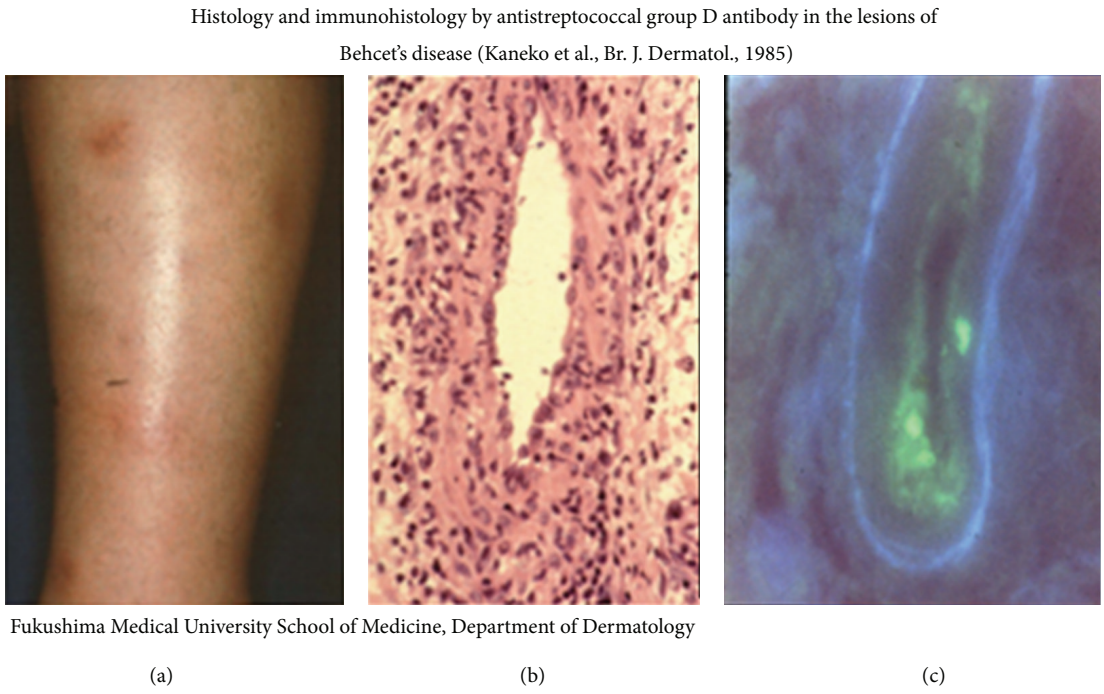



Figure 3: EN-like eruption and the histology and immunohistology. (a) EN-like eruption on the lower legs of a BD patient. (b) Vasculitis infiltrated by lymphoid cells and neutrophils (HE, 400x). (c) Deposits of streptococcal antigen adhering to the vascular wall (direct immunofluorescence, ×400).

## 3. Pathergy Test and Oral Streptococci

The diagnosis of BD is not thought to be difficult for the clinically typical cases who are based on the diagnostic criteria by Japanese and/or International Study Group [24, 36], except for the atypical cases without the main mucocutaneous symptoms including RAS. Pathergy test, which is a nonspecific cutaneous hypersensitive reaction showing around 2 mm pustule 24–48 h after 20 G syringe needle stick, has been thought to be helpful for making a diagnosis of BD for long time, because the phenomenon has been believed as a unique feature for BD. The reactive conditions seem to be varied by the technical method and generally the high positivity is found in Mediterranean and Middle East countries [30]. The reactivity of the "pathergy test" is suggested to be correlated with HLA-B51 in Mediterranean countries [37] and it is one of diagnostic criteria by International Study Group of BD [36]. On the other hand, in the Japanese BD diagnostic criteria "pathergy test" is considered as one of the diagnostic references [23, 24]. However, its positivity in BD

Table 1: Self-salivary prick test in patients with aphthous ulceration and controls.

| Patients | Age, sex (initials) | Prick test (after 48 h) | Small pustule | SS-prick | CS-prick |
|---|---|---|---|---|---|
| Neuro BD | 55 M YY | 11 × 15 mm | + | nd | – |
| Incomplete | 33 F AT* | 22 × 22 | – | – | – |
| | 26 F MN* | 10 × 10 | + | + dot | – |
| | 27 M TG | 11 × 12 | + | + dot | – |
| | 47 M YT | 10 × 13 | + | + dot | – |
| | 36 F AN* | 5 × 10 | – | nd | – |
| | 46 M KH | 10 × 10 | – | – | – |
| | 17 F YT | 5 × 5 | – | – | – |
| Complete | 23 M OO* | 10 × 10 | + | – | – |
| Recurrent aphthous stomatitis (non-BD RAS) | 24 F YN | 8 × 10 mm | – | – | – |
| | 28 F YS | 8 × 4 | – | – | – |
| | 32 F YN | – | – | nd | – |
| | 29 M ON | – | – | nd | – |
| | 28 F MS | 3 × 5 | – | – | – |
| Disease controls non-BD EN | 39 F KY | – | – | nd | – |
| | 61 F MF | – | – | — | – |
| Viral aphthosis (3) | | – | – | nd | – |
| Healthy controls (6) | | – | – | – | – |

BD: Behcet's disease; EN: erythema nodosum; F: female; M: male; dot: small spot; +: positive; −:negative. S-prick: prick with self-saliva; SS-prick: prick with sterilized self-saliva; CS: prick with saline; nd: not done. The clinical type of BD is followed by the Japanese BD classification. *Same cases in Table 2.

patients seems to be chronologically lower to less than 40% of BD patients seen in 2007s, though more than 70% of the patients exhibited positive to the pathergy test in 1970s. The positivity by the test is also different from the prevalence in the countries, as mentioned [38–40]. It is of interest that the surgical cleaning of the forearm before needle prick reduced the prevalence of the "pathergy reaction" [41], suggesting that the positive reaction might be a cutaneous response to some bacteria living on the surface of the skin. In our all cases shown in Tables 1 and 2 none of cutaneous reactions were found 24–48 hours after venipuncture for the clinical examinations using syringe with 22 G needle, because, before the venipuncture, their forearm was cleaned.

As it is known that many kinds of bacteria are contained in our saliva, we tried to incubate saliva form a BD patient using Mitis-Salivarius (MS) agar which streptococci are selectively grown. The result showed many oral streptococci grew up from pure saliva (Figure 4(a)) and that no bacteria grew from the sterilized saliva by use of a syringe micromembrane filter (Figure 4(b)). Then, instead of conventional "pathergy test," we tried to prick with self-saliva in which oral bacteria including streptococci are ordinary contained to the forearms of BD patients for diagnosis using a Lancetter with a tiny stick (OY ALGO AB Espoo/Esbo, Sweden) because the patients have hypersensitivity to oral streptococci, as described previously. The results revealed more than 90% of 10 BD patients showed erythematous reaction by stick with self-saliva and that a tiny spot or no reaction was seen by the prick with microfilter-sterilized saliva and control saline (Figure 5, Table 1) [42]. The results also suggest that oral streptococci are playing an important role in the pathogenesis of the RAS of BD patients and that the salivary prick is able to make a differentiation of BD from non-BD disorders. The reaction and severity to self-saliva prick was not related to the possession of the HLA-B51 gene in BD patients (Table 2).

## 4. HLA Genotyping of BD and Streptococcal Infection

HLA-B51 is supposed to be a highly associated genetic marker of BD patients from many different ethnic groups including European, Mediterranean, and Asian people and BD has several unique epidemiologic features from Southern Europe to Japan along "the old silk route" [2, 4, 5, 44]. The appearance of BD lesions is not directly correlated with HLA-B51 in the immunological background of the patients, but it was recently found that HLA-B51-restricted cytotoxic T lymphocytes (CTLs) and $\gamma\delta$T cells played some roles in correlation with the stressed target tissues expressing major histocompatibility complex class I-related gene A (MICA) in BD pathogenesis. When the transmembrane-MICA located nearly at the HLA-B51 gene is expressed preferentially on epithelial and endothelial cells by stress, they seem to be the candidates for the HLA-B51-restricted CTLs response and MICA expressed on the stressed epithelium and endothelium which are considered to be the ligand for activating natural killer (NK) cells with NKG2D molecule, $\gamma\delta$T cells, and $CD8^+$ T cells as CTLs [45]. Regarding NK cell activation, inhibitory CD34/NKG2A and activating CD94/NKG2C molecules are

Table 2: Self-salivary prick test in BD patients with or without HLA-B51.

| Type of BD (Japanese classification) | Patients (initials) | Prick test (mm) | | | HLA-B51 |
|---|---|---|---|---|---|
| | | S | SS | CS | |
| Complete type | 23 M OO* | 10 | — | – | + (B51) |
| Incomplete type | 40 M HG | 10 | 7 | – | + (B51, 01, 01) |
| | 31 F MA | 30 | 7 | – | + (B51, 40) |
| | 42 F MK | 7 | 4 | – | + (B51, 46, DR4, 8) |
| | 34 F MY | 26 | 5 | – | – (B35, 48) |
| | 36 F AN* | 10 | – | – | – (B44, 03, 01) |
| | 33 F YK | 10 | – | – | – |
| | 37 F AT* | 22 | – | – | – (B40, 48) |
| | 30 F MN* | 10 | – | – | – |
| | 35 M YI | 10 | nd | – | – (B15, 35) |
| | 37 F HT | 4 | – | – | – (B40, 44) |
| | 36 M MK | 4 | 3 | – | – (B35, 44) |
| | 35 M KF | 7 | 2 | – | – (B46, 54) |
| | 35 F YO | 14 | – | – | – (B07, 02, 01) |

F: female; M: male; S: self-saliva; SS: filtered sterilized saliva; CS: control saline; +: positive; −: negative; nd: not done. *Same cases in Table 1.

alternatively expressed on NK, $CD4^+CD8^+$ T cells, as indicating an imbalance in cytotoxic activity in BD patients [46], although the function of NK cells is supposed to be down-regulated in the active stage and to be up-regulated in the remission of BD patients [47]. The excessive $CD4^+$ T cells activated by inflammatory cytokines including interferon (IFN)-$\gamma$, IL-12, and IL-23 were altered to Th17 cells and IL-17 which might be released from them in the BD lesions [48].

It is considered that HSP-65/60 derived from microorganism including *S. sanguinis* and from human tissues, which is detected in the oral mucosal and skin lesions of BD patients [20, 21], also becomes a stress-inducible factor in connection with MICA*009 expression. Generally, antigen presenting cells (APCs), which produce IL-12 in correlation with Th1 type immune-reaction, are thought to be activated in BD patients with HLA-B51 in active stage, as indicated by Yasuoka et al. [45]. However, we have obtained the results that PBMCs from BD patients without HLA-B51 gene can be significantly stimulated by *S. sanguinis* antigen in the expression of IL-12p40 mRNA and increasing of protein level in connection with IL-12p70 (70 kDa composed of p35 and p40 subunits) rather than those of the patients with HLA-B51 [51]. It has been suggested that antibacterial host response in T cell type immunity mediated by IL-12 is much stronger in HLA-B51-negative BD patients. The skin response severity by the prick with oral streptococci of self-saliva seemed to be unrelated to the HLA-B51 gene as seen in Table 2.

## 5. Hypersensitivity against *S. sanguinis*

Generally, the oral health is impaired in BD patients [8–13], which seems to be associated with the disease severity [10]. Although there are a number of the triggering factors for BD in environmental agent, the predisposition of BD patients may be correlated with streptococcal infection as one of the factors, because the uncommon serotype oral *S. sanguinis* is

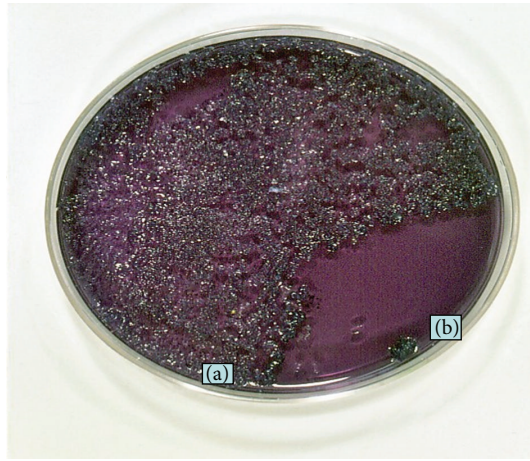



Figure 4: Incubation of saliva of a BD patient using MS (mitis and salivarius) agar in which oral streptococci are selectively grown. (a) Oral streptococci grew from saliva in 5 day. (b) Area of sterilized saliva using syringe micromembrane filter.

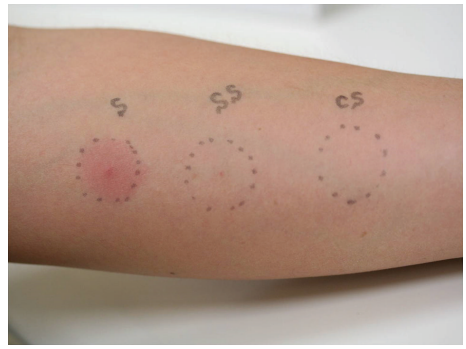



Figure 5: Prick test with self-saliva using Lancetter (33 F AT in Table 1). The skin reactions were observed 48 hours after prick. S: self-saliva; SS: sterilized saliva using syringe-filter with 0.2 $\mu$m pores; CS: control saline.

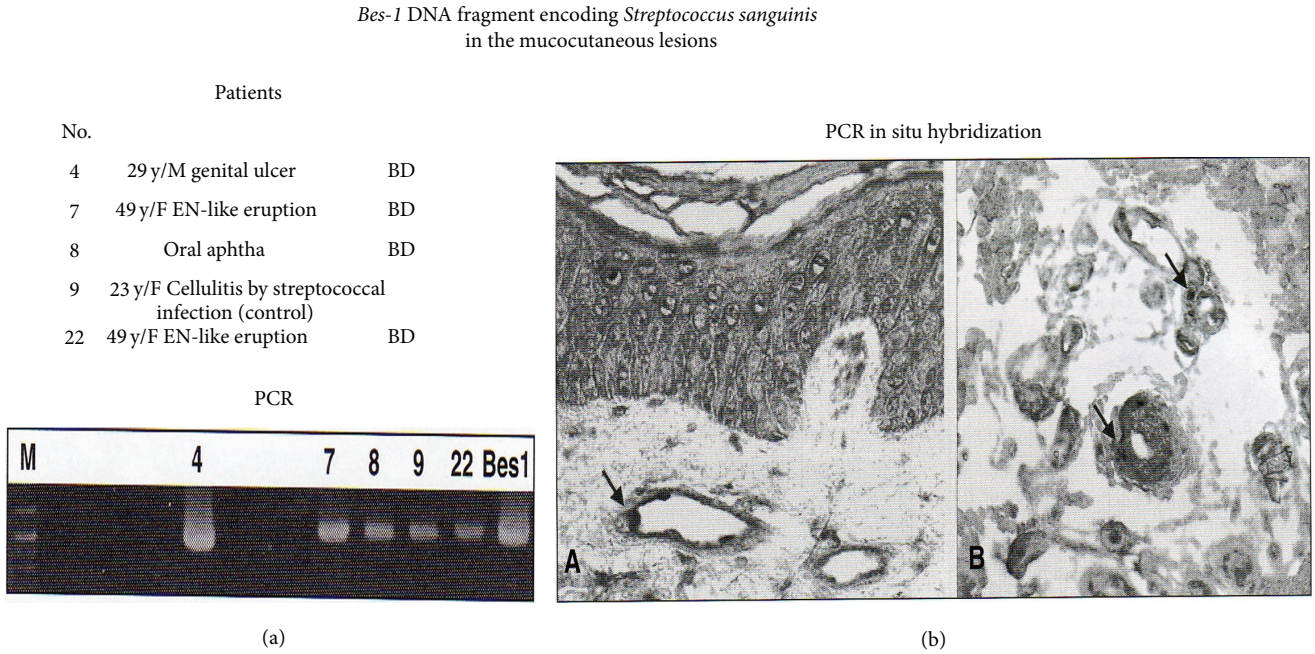



Figure 6: *Bes-1* gene expression in the mucocutaneous lesions of patients with Behcet's disease (BD) [43]. (a) Three of 11 BD patients were positive for *Bes-1* DNA in the lesions including aphthous and genital ulcerations and erythema nodosum (EN)-like eruption by amplified polymerase chain reaction (PCR) using the primers: *Bes-1-1* (5′-TAATAACCCTGACCAAGCCTA-3′) and *Bes-1-2* (5′-CCCTTTCAAAAGTCATAAATC-3′) encoding *S. sanguinis*. (b) In these positive lesions, *Bes-1* DNA was also detected in the cytoplasm of monocytes adhering to the vascular walls and infiltrated around the vessels by PCR in situ hybridization.

significantly increased in BD patients compared with healthy and disease controls [11–15]. The antibodies against *S. sanguinis* in sera from BD patients showed cross reactivity with the some synthetic peptides of HSP-65 derived from *S. sanguinis* [52–54]. The patients show strong delayed type cutaneous hypersensitivity reactions against streptococcal antigens in skin tests [8, 9, 15] and sometimes the BD symptoms were provoked by skin injection of the antigens [16]. Because aphthous ulceration can be also induced by a prick with streptococcal antigen on the oral mucous membrane of a BD patient [9], the appearance of aphthous ulceration is considered to be based on the hypersensitive reaction against *S. sanguinis* which may be traumatically penetrated into the oral membrane of BD patients. Isogai et al. [53] demonstrated that the symptoms mimicking BD appeared in germ-free mice when *S. sanguinis* from BD patients was inoculated into their oral tissue damaged by heat shock and/or mechanical stress. This report suggests that the immunization with *S. sanguinis* through the oral membrane route elicits BD-like symptoms in the animal model as seen in BD patients who carry *S. sanguinis* as the pathogenic microorganism in their oral cavity. In order to find polymerase chain reaction (PCR) targeting *Bes-1* gene in BD lesions using 2 distinct primer sets (peptides, 229–243, and 373–385) encoding *S. sanguinis* (serotype KTH-1) which was prepared by Yoshikawa et al. [54], we recognized that *Bes-1* DNA was present in various mucocutaneous lesions including oral and genital ulcerations and EN-like lesions. The PCR in situ hybridization also revealed that *Bes-1* DNA was expressed in the cytoplasm of inflammatory infiltrated monocytes adhering the vascular walls in mucocutaneous lesions (Figure 6) [43]. In contrast, we failed to detect DNAs of HSV-1, HSV-2, cytomegalovirus, human herpes virus (HHV)-6, and HHV-7 in the lesions by PCR [55], although HSV infection has been speculated as etiologically important since the report of Behcet [1]. Interestingly, the amino acid sequence of the peptides of *Bes-1* (229–243 and 373–385) shows more than 60% similarity to the human intraocular ganglion peptide, *Brn-3b* which is a subfamily of POU (pit-Oct Unc) domain factors containing *Brn-3a* and *Brn-3c* [56]. The peptide of *Bes-1* (229–243) was also found to be correlated with the peptide of HSP-60 (336–351) [54]. Recently, it has been found that the peptide of *Bes-1* (337–385) stimulated PBMCs of BD patients which produced IFN-$\gamma$ and IL-12, though the cellular proliferation of the stimulated PBMCs was not observed [57]. These results suggest that *Bes-1* derived from oral *S. sanguinis* might be an inducer for the possible retinal and neural involvement in BD patients.

## 6. HSPs and BD Pathogenesis

Antibodies against the HSP peptides derived from bacteria including *S. sanguinis* are found in aphthous ulceration and serum of BD patients [58], though HSP specific antibodies and T cells are considered to play a complicated role in the pathogenesis of human autoimmune diseases [59]. It is speculated that HSPs trigger both innate and adaptive immune mechanisms in BD. On the other hand, the therapeutic approaches involving HSP immunomodulation may be available as "oral toleration" using the peptide of HSP (336–351)

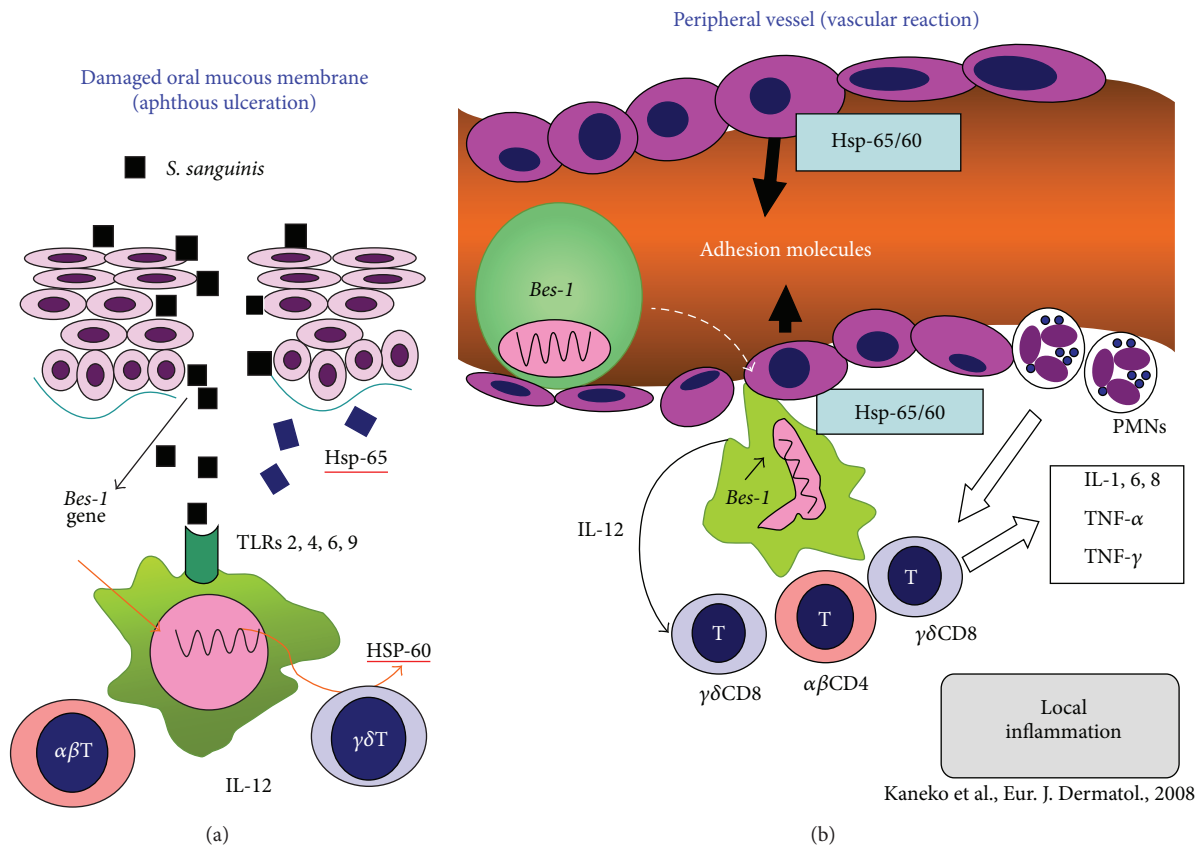



FIGURE 7: Hypothesis of the mechanisms in the appearance of various lesions of BD patients [49, 50]. (a) The antigen presenting cells (APCs) (macrophages and/or dendritic cells) immunized by *S. sanguinis* agents though TLRs in the oral cavity might be carried to the peripheral regions. (b) If the APCs in the blood flow adhered to the impaired and/or MICA and adhesion molecules expressed endothelial cells of vascular wall, the immunological reaction might be appeared as BD lesion.

linked to recombinant cholera toxin B for BD patients with advanced uveitis, as demonstrated by Stanford et al. [60]. In order to understand the suppressive mechanisms of the cytokine production in PBMCs from active BD patients, we tried to find the binding sites of the peptides on monocytes by cDNA chips (Gene Chip; Human Genome) using NOMO-1 cells (human macrophage cell line) activated by *S. sanguinis* antigen and they were incubated with the peptides. It was found that although the expression of IL-8, IL-16, IL-13R, and IL-17R was decreased after incubation with HSP-65 peptides (LO1 and UK), respectively, LO2 (480–499) did not decrease IL-8 production. CD58 (lymphocyte function-associated antigen-3) molecule and/or FK506 binding protein were highly expressed on the cell membrane by LO1 (249–264) and UK (311–326) [61, 62].

## 7. Toll-Like Receptor (TLR) Expression in the Innate Immunity

Regarding the recognition system for the microorganism antigens in humans, 10 numbers of TLR family are supposed to act as innate immune receptors by binding of particular structures present on bacteria, viruses, fungi, and so forth [63]. Although generally TLRs are weakly detectable in various human tissues with varying levels, the TLR expression of the organs involved in immune response and exposed to environment is found to be significantly stronger [64]. TLR-3 [ds RNA] and TLR-6 [mycoplasma, staphylococci, etc.] are also reported to be enhanced in expression on neutrophils and monocytes of BD patients, when stimulated by HSP-60 and *S. sanguinis* antigen [65]. In the oral ulcer lesion, expression of TLR-9 [unmethylated CpG DNA, bacteria, and virus] has been found recently [66]. These findings suggest that innate immune system contributes the acquisition of hypersensitivity against oral *S. sanguinis* as the extrinsic factor in the pathogenesis of BD.

## 8. Oral Aphthous Ulceration and Systemic Symptoms

BD symptoms are characterized by vascular involvements showing swollen endothelial cells of the microarteries infiltrated by inflammatory monocytes and a few neutrophils histologically, as so-called "vascular reaction" seen in EN-like eruption and other lesions [15, 31, 67, 68]. The strong hypersensitivity reaction against *S. sanguinis* agents [8, 9, 15, 16, 18] which might be gained by antigen present cells (APCs)

through the innate immune mechanism can be suspected as the extrinsic triggering factor in the pathogenesis of BD. In the treatment by antibiotics for the involvement of oral *S. sanguinis*, especially minocycline, which not only reduces the growth of streptococci but also suppresses IL-1$\beta$ and IL-6 production from T cells inflamed, was clinically effective for aphthous ulceration, acne-like eruption and EN-like lesion in BD patients [9]. Other study also showed that combination therapy, colchicine and benzathine penicillin, was effective to suppress BD symptoms compared to colchicine monotherapy [69, 70]. Although Kaneko et al. [49] and others [5–7] have already reviewed on the role of infectious agents in BD pathogenesis, we also dare to propose the hypothesis that after *Bes-1* gene taken in the cytoplasm of APCs through the TLRs in the oral cavity, the APCs, which are expressing the streptococcal antigen, produce HSP-65/60, as demonstrated by Deniz et al. [58]. If these APCs are curried in the blood flow to the impaired and/or MICA expressed endothelium of the vessels in correlation with HSP-65/60, VEGF, adhesion molecules, and so forth, BD lesions might be induced by the "vascular reaction" and/or "lymphocytic vasculitis" as the immunological reaction by the APCs expressing *S. sanguinis* antigen. Then, the relationship between oral ulceration and the systemic symptoms might be considered as illustrated in Figure 7. From the viewpoint, it is considerable that the positivity of the prick with self-saliva is high for BD patients [49, 50, 62]. So, we would like to propose a new diagnostic way for BD and differentiation from non-BD patients and/or non-BD RAS patients.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# Innate and Adaptive Responses to Heat Shock Proteins in Behcet's Disease

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Behcet's disease (BD) is a systemic, chronic inflammatory disorder with both innate and adaptive immune responses. Heat shock proteins (HSP) are highly conserved molecules in different species with scavenger activity and involved in correct folding of newly synthesized proteins. T and B cell responses against HSPs are observed in BD patients in both $\alpha\beta$ and $\gamma\delta$ T-cell populations. 60-kD HSP (HSP60) is also shown to be recognized by pattern recognition receptors such as toll-like receptors (TLR) and is suggested to be an endogenous "danger" signal to the immune system with rapid inflammatory cytokine releases and enhancement of adaptive Th1-type responses. Elucidating the exact role of HSPs in BD pathogenesis might pave the way to less toxic therapeutic approaches to BD, such as antibacterial therapies and immunomodulation.

## 1. Introduction

Behcet's disease (BD) is a systemic, chronic inflammatory disorder with a diverse spectrum of clinical manifestations including mucocutaneous, ocular, vascular, gastrointestinal, musculoskeletal, and central nervous system involvement [1, 2]. A complex genetic background leading to a proinflammatory, innate-immune system derived activation perpetuated by adaptive immune responses against environmental and autoantigens is accepted as the main pathogenic mechanism in BD [3, 4].

Microbial infection has been implicated in the development of BD since its initial description in 1937 by Hulusi Behcet. Four principal hypotheses have been suggested: (i) bacterial, with *Streptococci* in the foreground, (ii) viral, (iii) indirectly via heat shock proteins (HSP), and (iv) crossreactive or molecular mimicry etiologies [5].

Clinical observations such as increased oral manifestations after dental manipulations, streptococcal hypersensitivity in skin tests, dominance of atypical streptococcus species in BD patients' oral flora, and recent reports of beneficial antibacterial therapy put forward the role of *Streptococcia* in BD [2, 6–8]. As a wide variety of *Streptococcia* (*sanguis, salivarius,* etc.) are implicated, antigens common to various species are logical candidates of immune stimuli in BD [9].

## 2. Heat Shock Proteins: Adaptive Responses

Heat-shock proteins are a group of intracellular proteins which have scavenger roles for other intracellular proteins under denaturating stress conditions such as infections, hypoxia, trauma, and toxic drugs [10, 11]. Significant sequence homology exists between the mammalian and microbial HSPs (mycobacterial and streptococcal HSP65s have over 90% and human HSP60 over 50% homology) [6], shown recently also with bioinformatic approaches [12]. In addition to their physiological roles, they are implicated in the pathogenesis of various immune-mediated disorders such as infections (tuberculosis and chlamydia), autoimmune diseases (rheumatoid arthritis and multiple sclerosis), vascular thrombosis (atherosclerosis), and malignant disorders [13].

HSP60 with a molecular mass of 60 kD is mainly expressed in mitochondria. However, during stress, an intracellular redistribution of HSP60 and cell surface expression is reported. HSP65 is also expressed on monocytes after IFN-$\gamma$ stimulation and on T-cells going apoptosis [14]. Local HSP60 overexpression is present in oral ulcers of both patients with recurrent oral ulcer patients and BD [15]. Similarly HSP was present more in BD in the epidermal regions of active skin lesions such as erythema nodosum and papulopustules [16]. Increased expression is also observed in intestinal BD lesions

[17]. Serum levels of HSP60 were investigated in one study and was higher in BD; however, its level did not correlate with disease activity [18].

A *molecular-mimicry* based pathogenic mechanism for HSPs in BD is first suggested by Lehner et al. that human HSP-responsive T-cells stimulated by microbial counterparts (*cross-reactivity*) might trigger T-cell activation and memory responses [6]. First supporting evidence for this hypothesis is the identification of anti-HSP65 antibodies cross-reactive with oral mucosal homogenates and oral *Streptococcia* [19]. Four epitopes of mycobacterial HSP65 (amino acid sequences 111-25, 154-72, 219-33, and 311-26) and their human counterparts with 50–80% homology were recognized to be immunodominant antigens for T-and B-cell responses in BD in studies from UK, Japan, and Turkey [20–23]. PPD and HSP65 specific long-term T-cell lines (mainly TCR $\alpha\beta$+CD4+ or CD8+) are also highly reactive to human HSP60-derived peptides in both BD patients and healthy controls showing that these self-reactive T-cells are escaping central tolerance and are present in the peripheral repertoire [24]. However, most PPD-stimulated lines responded to epitope 425-41 of HSP60 in BD patients (an epitope not described in primary cultures), whereas epitope 336-51 dominated in controls. The reaction pattern changes with HSP60 stimulation, which drives a dominant 336-51 response in both groups. This observation suggested that differential epitope recognition of the immune system associated with the balance of microbial versus human HSP expressions might determine the level of pathogenic self-reactivity in BD.

Although some "*in vitro*" data implicating a Th2 activation is reported, as most other vasculitides, BD is mainly a Th1/Th17 type disorder with interleukin-2 (IL-2), IL-12, interferon-$\gamma$ (IFN-$\gamma$), and IL-17 cytokine profile. In this context, stimulation of peripheral blood mononuclear cells (PBMC) with human HSP60 peptide 336-51 produced IFN-$\gamma$, tumor-necrosis factor-$\alpha$ (TNF-$\alpha$), and IL-12, whereas Th2 cytokines IL-4 and IL-10 suppressed the proliferative responses in BD [17, 25].

## 3. $\gamma\delta$-T-Cells and HSPs

$\gamma\delta$ T-cells are a minor T-cell population (1–10% of PB T-cells) that express T-cell receptors (TCRs) comprised of $\gamma$ and $\delta$ heterodimers [26]. V$\gamma$9$\delta$2+ T-cells, a major subset of $\gamma\delta$ T-cells in the PBMCs, recognize nonpeptide antigens produced by bacteria. $\gamma\delta$ T-cells have important roles in immunity as a "*first line of defence*" against microorganisms, surveillance against tumors, and possibly in modulating autoimmune responses [27]. Whereas B cells and $\alpha\beta$ T-cells are commonly thought to contribute primarily to the antigen-specific effector and memory phases of immunity, $\gamma\delta$ T-cells are distinct in that they combine conventional adaptive features (inherent in their T-cell receptors and pleiotropic effector functions) with rapid, innate-like responses [28].

Peripheral blood $\gamma\delta$ T-cells are observed to be elevated in most, but not all studies in BD [29–32]. These $\gamma\delta$ T-cells are associated with active disease and have higher expression of CD29, CD69, and production of IFN-$\gamma$ and TNF$\alpha$ [33]. Whereas PB $\gamma\delta$ T-cells are mainly V$\delta$2+, local fluids such as bronchoalveolar lavage and cerebrospinal fluid are dominated by V$\delta$1+ T-cells. Maybe more significant is the local $\gamma\delta$ T-cell presence in active BD lesions where HSP65 expression is upregulated, with possible HSP-$\gamma\delta$ T-cell interactions [16].

$\gamma\delta$-T-cell activation is also shown with oral flora extracts which might contain HSPs as antigens [30]. HSP-derived peptide responsive T-cells were mainly of $\gamma\delta$ T-cell subset in UK, whereas CD4+ T-cells are reported from Japan and Turkey [34]. However, in contrast to these data, no response to HSP60 is observed in any T-cell line derived from intraocular fluid of uveitis patients with BD, whereas nonpeptide prenyl pyrophosphate reactive $\gamma\delta$ T-cells were present [35].

## 4. HSPs and Antibody Responses

Similar to T-cell studies, "*cross-reactivity*" is also demonstrated for anti-HSP60 antibodies. Both antistreptococcal and antiretinal HSP60 antibodies are elevated in BD patients' sera with uveitis [36]. With competitive ELISA, both antigens inhibit the binding of anti-HSP60 antibodies to each other. Increased anti-HSP65 antibody responses are also present in the cerebrospinal fluid (CSF) of neuro-BD patients with parenchymal involvement [37]. Similarly, optical densities obtained from ELISAs against the recombinant human hnRNP-A2/B1, which is shown to be expressed in endothelial cells and is a target antigen of anti-endothelial cell antibodies (AECA) in BD, correlated with those against the recombinant streptococcal hsp65 [38].

## 5. Animal Models

In an animal model with subcutaneous HSP inoculation, human HSP derived, immunodominant peptides caused an experimental uveitis without other symptoms of BD in rats [39]. Oral administration of peptides also induced uveitis in contrast to most models of *"oral tolerance"* where mucosal immune encounter with pathogenic antigens suppress the immune activity. Heat-shock to oral mucosa also increases *S. sanguis* colonisation, oral inflammatory cytokine expressions (IL-2, IL-6, IFN-$\gamma$, and TNF-$\alpha$), and mild iridocyclitis in mice, implying that stress might be crucial for the breakdown of mucosal defences and anti-HSP reactivity [40].

## 6. Other HSPs and BD

$\alpha$B-crystallin is a small stress protein constitutively abundant in vertebrate eye lens and found in several other organs including skeletal muscle, kidney epithelial cells, and glia cells of central nervous system [41]. Serum and CSF IgG and serum IgM antibody responses to $\alpha$B-crystallin are shown to be elevated in neuro-BD patients. When responses were subclassified according to the type of neuro-BD, similar to anti-HSP65 responses, patients with parenchymal neuro-BD had higher CSF IgG responses to $\alpha$B-crystallin compared to neuro-BD group with intracranial hypertension (vascular

involvement). CSF IgG responses to HSP65 and $\alpha$B-crystallin showed a significant correlation with each other, possibly due to similar immune mechanisms driving both autoantibody responses in the CSF. Another recent study, screening with a protein macroarray also led to the identification of stress-induced-phosphoprotein-1 (STIP-1) as an antigenic target for antineuronal antibodies in BD [42].

Elevated anti-HSP70 antibody levels are also observed in patients with BD [43, 44], but not in all studies [45]. However, when free serum HSP70 levels are investigated in the same samples, no correlation is observed between free serum HSP70 and anti-HSP70 antibodies [44, 45]. This observation points to an important difficulty in HSP hypothesis: the role of HSPs in tissue selectivity. HSPs are expressed by all cells under suitable stress conditions, whereas BD involves a limited number of tissues. This selectivity can be explained by differences in local HSP expressions (not reflected in PB), such as preferential HSP expression of the skin and retina.

## 7. Pattern Recognition Receptors and HSPs: Activation of the Innate System Directly

With its autoinflammatory features, innate immune activation through pattern recognition receptors, NODs, and inflammasome-associated mechanisms are implicated in BD pathogenesis [46, 47]. In addition to being processed and presented to $\alpha\beta$ and $\gamma\delta$ T-cells by monocyte-macrophages and stimulating classical, adaptive T-cell responses, HSPs might also activate innate immune mechanisms directly in BD. Recent studies have suggested that HSP60 serves as a *"danger signal"* to the innate immune system [48]. Macrophages, endothelial, and smooth muscle cells were found to elicit a proinflammatory response when incubated with HSP60, releasing IL-6, IL-12, IL-15, and TNF-$\alpha$ and upregulating adhesion molecule expressions such as E-selectin, VCAM-1, and ICAM-1 [49]. The proinflammatory response to HSP60 is similar in kinetics and extent to lipopolysaccharide (LPS) stimulation. In early studies, HSP60 is shown to activate mononuclear cells through CD14 which is a high affinity receptor of bacterial LPS on cell membranes. However, later on CD14 is shown to be a coreceptor for a novel molecule of innate immunity, toll-like receptor-4 (TLR4), activating p38 mitogen-activated protein kinase and NF-$\kappa$B [50]. TLRs are evolutionarily conserved, germline encoded receptors that recognize specific molecular patterns associated with microorganisms [51]. There are currently 13 known TLR members with ligands representing unique products of microbial metabolism such as LPS, peptidoglycan, flagellin, or hypomethylated CpG DNA motifs. Activation of the toll system is suggested to induce dendritic cell (DC) maturation, causing elevated major Histocompatibility Complex (MHC) and costimulatory molecules (CD80 and CD86). Expression of various cytokines such as IL-12, which direct Th1 differentiation by DCs are also associated with TLR signaling. HSP60 is one of the first autoantigens shown to activate the toll system through TLR2 and TLR4 [52]. HSPs released from necrotic (*but not apoptotic*) cells are observed to activate DCs, and HSP60 is shown to induce DC maturation with increased MHC class II, CD40, CD54, and CD86 expressions and allogeneic T-cell proliferation with a Th1 bias [53]. HSP60 is also found to rapidly activate the mitogen-activated protein kinases p38, c-Jun N-terminal kinase, extracellular signal-regulated kinase, and NF-$\kappa$B in DC. These data support a new model of immunity depending on "*danger*" signals such as HSP60, postulated by Matzinger who suggests that the immune system mainly responds to substances that cause "*damage*," rather than the classical theory of those that are simply *"foreign"* [54].

In PBMNC analysis, TLR4 expression is shown to be increased in BD patients [55]. In this study, TLR4 levels negatively correlated with heme oxygenase HO-1 (an inducible heme-degrading enzyme that is induced by various stresses) which suppress inflammatory responses. Monocytes of active BD patients also showed higher expressions of TLR2 and TLR4 in PBMNC analysis [56]. *In vitro* analysis, in this study, showed that vitamin D(3) dose-dependently suppress the protein and mRNA expressions of TLR2 and TLR4. In contrast to these studies, TLR6 expressing granulocytes in BD patients was significantly decreased, which enhanced after stimulation with HSP60 and streptococcal extracts [57]. TLR2 and 4 mRNA are also shown to be increased in the intestinal lesions of BD patients and colocalize with HSP60 and IL-12 suggesting that HSP60 may activate Th1 cells through TLRs [58]. Recent genetic data may also implicate the role of TLRs in BD pathogenesis. Genetic associations with TLR2 and TLR4 are shown in some but not all studies [59–62]. A recent, most comprehensive study from China with >800 patients confirmed the role of TLR2 polymorphisms in ocular BD [63]. A recent large study also demonstrated that rare, low-frequency nonsynonymous variants of TLR4 are shown to be increased in BD patients [64].

## 8. HSPs and Other Immune Mechanisms

A final possible role of HPSs is their adjuvant function. In addition to self-presentation discussed previously, HSPs, as molecular chaperones, might transfer antigenic peptides to *"professional"* APCs which then activate specific T-cells or enhance the presentation of MHC-peptide complexes by poorly immunogenic tumor cells. Deficiencies in HLA class I expression on tumor cells are proposed as a mechanism to interfere with the antitumor cytotoxic T-cell responses (CTL). HSP65 transfected clones of melanoma cell lines exhibit significantly increased levels of HLA class I expression and are effectively lysed by alloreactive CTL [65]. Similarly, increased HSP60 expression of APCs may help antigen presentation by BD-associated HLA-class I molecule HLA-B51 to the effector T-cells and enhance pathogenic immune responses. Although an association of anti-HSP60 responses and HLA-B51 is not previously demonstrated, HSP-HLA interactions require further studies.

It was also demonstrated that both HSP65 and HSP70 upregulate CD8+ T-cell derived $\beta$-chemokine expressions (RANTES, MIP-1$\alpha$, and MIP-1$\beta$) both directly and also as an adjuvant linked to peptides indirectly [66]. This stimulation of innate immunity might drive adaptive responses and

attract APCs (dendritic cells and macrophages) and effector T-cells.

## 9. Specificity of Anti-HSP Responses

T and B cell responses against HSPs are observed in diverse inflammatory disorders. Whether these responses are specific to different disorders or are present as a part of a nonspecific autoimmunity is currently unknown. Some studies (other than BD) using peptide epitopes suggest that adaptive responses can be specific against different T and B cell epitopes. A cross-reactive antimycobacterial HSP65 peptide (aa 91-105) is shown to be specific to recurrent oral ulcer patients compared to HC [67]. Tolerogenic peptides of human HSP60 are also reported only in juvenile idiopathic arthritis cases but not in healthy or diseased controls [68].

## 10. Possible Therapeutic Approaches

Elucidating the exact role of HSPs in BD pathogenesis might pave the way to less toxic therapeutic approaches with HSPs. Immunomodulation with HSPs is demonstrated by "*oral tolerisation*" with peptide 336-51 linked to cholera toxin B subunit, first in an animal model and later in uveitis patients [13, 69]. Similarly, treatments aiming to suppress oral colonization with *Streptococci* leading to less bacterial HSP load might also be effective as adjuvant therapies to immunosuppressives and deserve further studies [70]. Other possible mechanisms of HSP-associated therapeutic approaches may be RNA interference [71], HSP inhibition with synthetic inhibitors [72], inhibition of HSP-ligand interactions [73], or antisense oligonucleotides targeting HSPs [74].

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*Review Article*

# Skewed Helper T-Cell Responses to IL-12 Family Cytokines Produced by Antigen-Presenting Cells and the Genetic Background in Behcet's Disease

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Behcet's disease (BD) is a multisystemic inflammatory disease and is characterized by recurrent attacks on eyes, brain, skin, and gut. There is evidence that skewed T-cell responses contributed to its pathophysiology in patients with BD. Recently, we found that Th17 cells, a new helper T (Th) cell subset, were increased in patients with BD, and both Th type 1 (Th1) and Th17 cell differentiation signaling pathways were overactivated. Several researches revealed that genetic polymorphisms in Th1/Th17 cell differentiation signaling pathways were associated with the onset of BD. Here, we summarize current findings on the Th cell subsets, their contribution to the pathogenesis of BD and the genetic backgrounds, especially in view of IL-12 family cytokine production and pattern recognition receptors of macrophages/monocytes.

## 1. Introduction

Behcet's disease (BD) is a systemic inflammatory disease, characterized by recurrent signs and symptoms of oral aphthosis, genital ulcers, skin lesions, and uveitis. BD is not chronic inflammatory disease, but patients with BD suffer from recurrent attacks of acute and self-limiting inflammation. Repeated attacks of uveitis can lead to blindness.

The etiology of BD is largely unknown and skewed T-cell responses are associated with the development and maintenance of BD [1]. Excessive cytokine production by Th1 cells was reported using immunohistochemistry [2, 3] and intracellular cytokine staining [4, 5]. Th1 dominance was observed in BD uveitis [6] and stomatitis as well [7]. We reported excessive Th1 cell infiltration in BD skin and intestinal lesions but interleukin- (IL-) producing T cells were rarely detected [8–10]. T cells and peripheral blood mononuclear cells (PBMC) from patients with BD responded to KTH1 antigens of *Streptococcus sanguinis* in oral cavity of patients with BD and produced interferon $\gamma$ (IFN$\gamma$) and IL-12 [11].

Recently, Th1/Th2 paradigm was challenged by the discovery of various subsets of Th cells, such as Th17 cells and regulatory T (Treg) cells [12] (Figure 1). Researchers showed that Th cell differentiation in each subset was closely related and sometimes converted into another subset in response to environmental signals both in peripheral blood and in organs [13]. Recent studies on innate immune system suggested that antigen-presenting cells (APC) stimulated with pattern-recognition receptors (PRR) and corresponding ligands regulated Th cell differentiation by cytokine production [14].

In this review, we summarize current understanding of Th cell responses to IL-12 family cytokines produced by APC through PRR in patients with BD. We also review recent findings on the disease susceptibility genes in BD and human autoimmune diseases, which regulate immune functions.

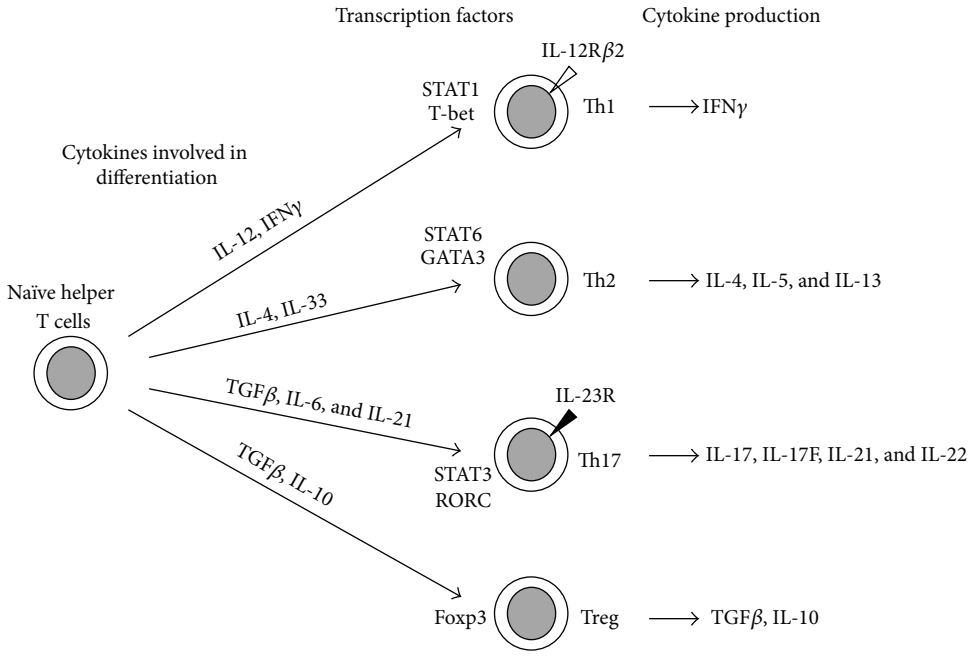


Figure 1: Current view of helper T (Th) cell subsets in humans [12]. Naïve Th cells differentiate into several Th cell subsets in the presence of appropriate cytokines. In response to the cytokines, the corresponding signaling molecules and transcription factors are expressed to regulate lineage commitments. Th1 and Th17 cells require IL-12 and IL-23 for their expansion, respectively. TGF$\beta$: transforming growth factor $\beta$, STAT: signal transducer and activator of transcription 3, GATA: GATA transcription factor, RORC: retinoic-acid-receptor-related orphan receptor c, and Foxp3: forkhead box P3.

## 2. Th1 Cells, Th17 Cells, Treg Cells, and IL-12 Family Cytokines

Th17 cells produce a number of proinflammatory cytokines, including IL-17, IL-17F, IL-21 and IL-22. IL-6, IL-21, and transforming growth factor (TGF)$\beta$ were reported to play a role in the differentiation of Th17 cells which proliferated in the presence of IL-23 (Figure 1) [12]. Treg cells control T-cell immune responses and also need TGF$\beta$ for their differentiation (Figure 1) [12]. TGF$\beta$ activates Smad pathway and activated Smad protein leads to forkhead box P3 (Foxp3) expression which is a master gene of Treg cells [15]. In the presence of TGF$\beta$, IL-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway plays a critical role in the induction of retinoic-acid-receptor-related orphan receptor c (RORC) expression which is a master gene of Th17 cells [16]. The two Th cell subsets require a common stimulation of TGF$\beta$ for the cell differentiation, but the resultant cells show opposite immune function in the presence or absence of IL-6.

As mentioned above, Th17 cells require IL-23 for the proliferation and survive, while Th1 cells require IL-12 for the differentiation (Figure 1). Recently, some researchers revealed that IL-12, IL-23, IL-27, and IL-35 are heterodimeric and share the subunits (Figure 2) and named them IL-12 family cytokines [17, 18]. IL-23 is composed of p19 and p40 subunits, IL-12 is composed of p35 and p40 subunits, IL-27 is composed of p28 and Epstein-Barr-virus-induced gene 3 (Ebi3) subunits, and IL-35 is composed of p35 and Ebi3 subunits. The 4 cytokines require each corresponding receptor which also shares components for the function (Figure 2). For example, IL-12 receptor (IL-12R) and IL-23 receptor (IL-23R) share IL-12R $\beta$1 subunit (IL-12R$\beta$1), and IL-12R and IL-35R share IL-12R $\beta$2 subunit (IL-12R$\beta$2). It is thought that the 4 cytokines have overlapping but distinct effect on T cells with corresponding Janus kinase (JAK)-STAT signaling pathway. The experimental data demonstrated a functional spectrum from proinflammatory to inhibitory in Th cell differentiation (Figure 2). IL-12 and IL-23 are produced by activated dendritic cells and macrophages and induce inflammation through Th1 and Th17 differentiation, respectively. IL-23 phosphorylates STAT1, 3, 4, and 5, but STAT4 activation, which is essential to produce IFN$\gamma$, is not strong compared to that in IL-12 stimulation [19]. IL-27 is secreted from APC and produces IL-10 secreting Th cells through STAT1 and 3 phosphorylation [20]. IL-35 is mainly produced by Treg cells, amplifies IL-35-producing Th cells, and induces T-cell arrest through STAT1 and 4 heterodimer's in mice [21], but the function in humans is still controversial [22].

Moreover, IL-6 and IL-11, both of which being single-molecule cytokines, need gp130 for their signal transductions in Th cell differentiation [23]. The concept of IL-12 family cytokine spectrum is simple, but physiological condition of the spectrum is supposed to be complicated. The relationship

between the spectrum and TGF$\beta$ expression remains largely unclear.

## 3. Th17 Cells, Treg Cells, and Tissue Damage

Excessive expressions of Th17-related cytokines were found in psoriasis [26], rheumatoid arthritis [27], multiple sclerosis [28], and inflammatory bowel diseases [29]. Recently, several studies have demonstrated that Th17 cell phenotype was not fixed *in vitro* and *in vivo* and Th17 cells turned into IFN$\gamma$ expressing Th17 cells and subsequently into nonstandard Th1 cells (Figure 3) [24, 25]. These two types of cells were thought to be more pathogenic and have higher affinity for inflammatory lesions than original Th17 cells [30–34]. IFN$\gamma$-expressing Th17 cells were found in several human autoimmune diseases such as Crohn's disease [30], psoriasis [31], multiple sclerosis [32], and juvenile idiopathic arthritis [33, 34].

Skewed Treg cell function was reported in many research articles of human autoimmune diseases [35]. Recent study revealed that there were differences in cell fate and functional stability between thymus-derived (t)Treg cells and periphery-induced (p)Treg cells [36]. tTreg cells had more effective functional stability, whereas pTreg cells were not stable in peripheral environment and converted into effector Th cells [37]. Epigenomic changes in Treg cells were suggested to regulate the Treg cell stability [38].

## 4. Th17 and Treg Cell Involvement in BD

It is generally thought that Th17 effector function is increased and Treg cell function is decreased in patients with BD. Overexpression of RORC mRNA [39, 40], underexpression of Foxp3 [41, 42], and high frequencies of Th17 cells [39–41, 43] were reported in patients with BD. Th17 cells were found in skin lesions [39, 40] and brain inflammatory lesions [41]. We recently reported that TGF$\beta$/Smad signaling pathway of mononuclear cells was overactivated in patients with BD [44]. We also reported the possibility that Th cells in patients with BD showed higher sensitivity to IL-23 and IL-12, and produced more IFN$\gamma$ and IL-17, as compared with normal controls [40]. We observed Th1, Th17, and IFN$\gamma$-expressing Th17 cells simultaneously in one skin specimen obtained from erythema-nodosum-like lesion of BD (Figure 4). We speculate that both Th17 cells and Treg cells and the plasticity play a crucial role in the pathogenesis of BD.

## 5. Pathogen/Damage-Associated Molecular Patterns (PAMP/DAMP) and Toll-Like Receptors (TLR)

Phagocytes were thought to be activated by various pathogens and pathogen-derived antigens in innate immune responses. Recent studies provided evidence for the existence of specific receptors on the phagocytes against the microbial antigens where they were named pattern-recognition receptors (PRR). The receptors are not rearranged even with adaptive immune system and recognize bacterial and viral pieces, known as pathogen-associated molecular patterns (PAMP). PAMP are indispensable parts of the microbes, such as lipopolysaccaride (LPS), peptidoglycan, bacterial DNA/heat shock proteins (HSP) and viral DNA/RNA [45]. Interaction between PRR and PAMP and subsequent induction of innate immune function are highly conserved among species [46]. Phagocytes with PRR recognition produced proinflammatory cytokines and upregulated major histocompatibility complex (MHC) proteins for the promotion of adaptive immune function [47].

Toll-like receptors (TLR) are transmembrane glycoproteins and called membrane-associated PRR. Ten functional human TLR have been identified [48]. TLR1, TLR2, TLR4, TLR5, and TLR6 were expressed on phagocyte cell surfaces and TLR3, TLR7, TLR8, and TLR9 localized within intracellular vesicles. It was shown that cell surface TLR recognized cell membrane-type PAMP, such as LPS and peptidoglycan, and intracellular TLR recognized nucleic-acid-type PAMP [49].

TLR also recognize endogenous damage-associated molecular patterns (DAMP) which are secreted from severe damaged host cells caused by any environmental stress, such as microbial infection or injury. Self-DNA/RNA, high-mobility group box1 (HMGB1), a DNA-binding nuclear protein, and self-HSP are included in the DAMP. These molecules were reported to be rapidly released following unprogrammed cell death and activate PRR-expressing cells similar to the PAMP [50]. Major TLR, PAMP, and DAMP were summarized in Table 1. In PAMP, bacterial lipopeptides, HSP, and LPS were recognized by TLR1/TLR2/TLR6, TLR2/TLR4, and TLR4 with CD14, respectively [46]. Similar mechanisms were found in DAMP with self-lipoproteins, self-HSP, and HMGB1. Two major TLR signaling pathways were demonstrated, namely, myeloid differentiation primary response protein (MyD)88-dependent pathway and Toll/interleukin receptor 1 (TIR) domain-containing adaptor-inducing IFN$\beta$ (TRIF)-dependent pathway (Figure 5). With TLR stimulation, except TLR3, APC produced proinflammatory cytokines through MyD88 and activated mitogen-activated protein kinases (MAPK). APC produced type 1 IFN by utilizing of TRIF through TLR3 stimulation, an intracellular TLR [46].

## 6. Th Cell Differentiation through TLR Stimulation

Dendritic cells stimulated with TLR2 and TLR4 ligands produced IL-12 and IL-23 [51, 52]. APC secreted IL-27 through TLR3 and TLR4 signaling [53–55] and type 1 IFN enhanced the expression [53, 54]. It was found that each IL-12 family subunit (Figure 2) had an expression pattern in APC through TLR4 stimulation [55]. For example, APC expressed p19 during early phase for a short time and produced p35 and p40 continuously in later phase. P28 acted as an intermediary between them. These data suggest that TLR stimulation may play a role in autocrine activation of APC by type 1 IFN induction (Figure 5) and the APC regulate T-cell differentiation though IL-12 family cytokines in a time-dependent manner.

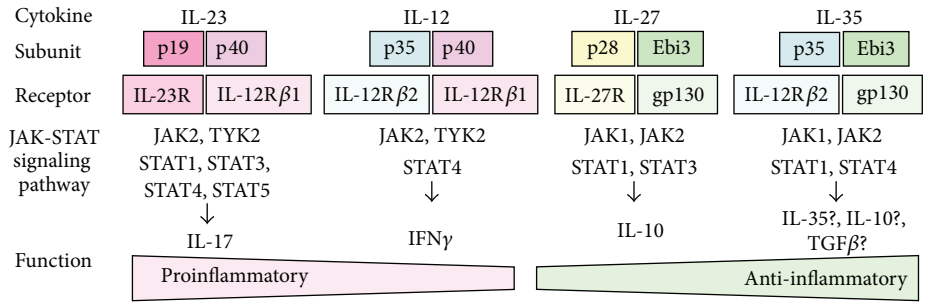


Figure 2: A schematic representation of IL-12 family cytokines and the corresponding receptors and JAK-STAT signaling pathways [16]. IL-12, IL-23, IL-27, and IL-35 are heterodimeric and share the subunits. The 4 cytokines require each corresponding receptor which also shares components for the function. It is thought that the 4 cytokines have overlapping but distinct effect on T cells with corresponding Janus kinase (JAK)-STAT signaling pathway. The experimental data demonstrated a functional spectrum from proinflammatory to inhibitory in Th cell differentiation. IL-12 and IL-23 are produced by activated dendritic cells and macrophages and induce inflammation through Th1 and Th17 differentiation, respectively. IL-27 is secreted from antigen-presenting cells and produces IL-10 secreting Th cells. IL-35 is mainly produced by Treg cells, amplifies IL-35 producing Th cells, and induces T-cell arrest.

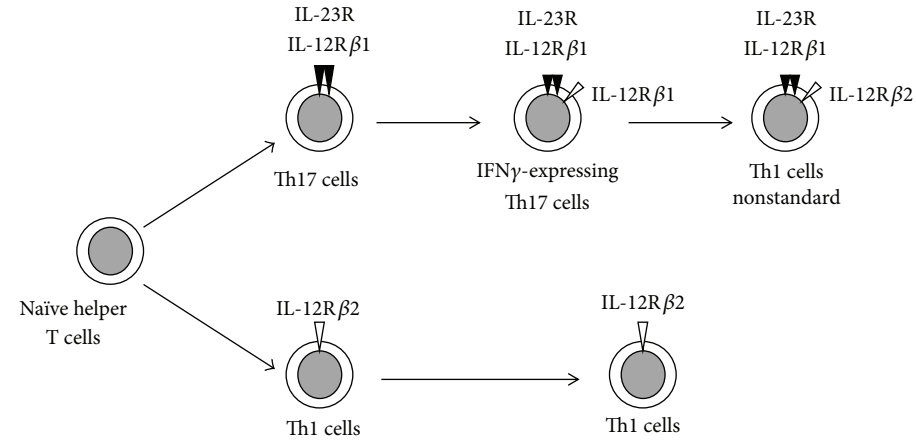


Figure 3: Th17 and Th1 cell differentiations and the phenotype plasticity [24, 25]. Th17 cell phenotype is not fixed *in vitro* and *in vivo* and Th17 cells can turn into IFN$\gamma$-expressing Th17 cells and subsequently into nonstandard Th1 cells. These two types of cells are thought to be more pathogenic and have higher affinity for inflammatory lesions than original Th17 cells.

Th cells are suggested to express TLR [14]. T-cell receptor (TCR) stimulation activates T cells by phosphorylation of extracellular signal-regulated kinases (ERK)1/2, both of which are subsets of MAPK family. TLR2 costimulation to the human TCR signaling promoted the phosphorylation and directly modulated the T-cell differentiation [56]. Several researchers demonstrated that TLR2 signaling without APC led to the induction of not only Th1 [57–59] and Th17 [60] cells but also Treg cells [57] in mouse experiments. Human naïve and Treg cells converted into Th17 cells with stimulation of TLR ligands [61]. In human infectious disease, TLR2 receptor on Th cells of patients with tuberculosis was overexpressed and its stimulation caused a marked activation of the cells [62]. In contrast, underexpression of TLR2 on Th cells and lower secretion of IFN$\gamma$ by TLR stimulation were observed in patients with filarial infection [63]. A possibility was considered that the repeated antigen exposure may explain the discrepancy [14].

Experimental approaches demonstrated various aspects of the relationship between TCR and TLR4 stimulation. TLR4 co-stimulation inhibited ERK1/2 phosphorylation of Th cells in mice [64] and TCR signaling with a pretreatment of LPS decreased activated MAPK [58]. TLR4 co-stimulation did not directly regulate Th cell differentiation, but selective deletion of TLR4 in Th cells decreased IFN$\gamma$ and IL-17 production at experimentally inflammatory sites [65].

These results suggest a need to assess the molecular relationship between MAPK/ERK and JAK/STAT signaling pathways in Th cell differentiation under both physiological and pathological conditions.

## 7. Possible Effects of HSP on Th Cell Activation as Both PAMP and DAMP

HSP are highly conserved and ubiquitously expressed proteins and function as an intracellular chaperonin for other proteins. An HSP was found as a remarkably increased factor in *Drosophila* salivary glands with "heat shock" in the first study. After numerous studies, subgroups of HSP were named

Table 1: TLR and corresponding PAMP and DAMP [46, 50].

| TLR | PAMP | DAMP |
|---|---|---|
| TLR1 | Bacterial lipopeptide | |
| TLR2 | HSP (mycobacteria, Chlamydia), LPS, bacterial lipopeptide, peptidoglycan | HSP, HMGB1, and lipoprotein |
| TLR3 | Viral RNA | Self-RNA |
| TLR4 | HSP (mycobacteria, Chlamydia), LPS | HSP60, HSP70, HMGB1, and lipoprotein |
| TLR6 | Bacterial lipopeptide | |
| TLR7 | Viral and bacterial RNA | Chromatin and ribonucleoprotein, self-DNA |
| TLR9 | Viral, bacterial and parasitic DNA | HSP, chromatin and ribonucleoprotein, and self-DNA |

TLR: Toll-like receptors; DAMP: damage-associated molecular patterns; PAMP: pathogen-associated molecular patterns; HSP: heat shock proteins; HMGB1: high-mobility group box1; LPS: lipopolysaccharide.

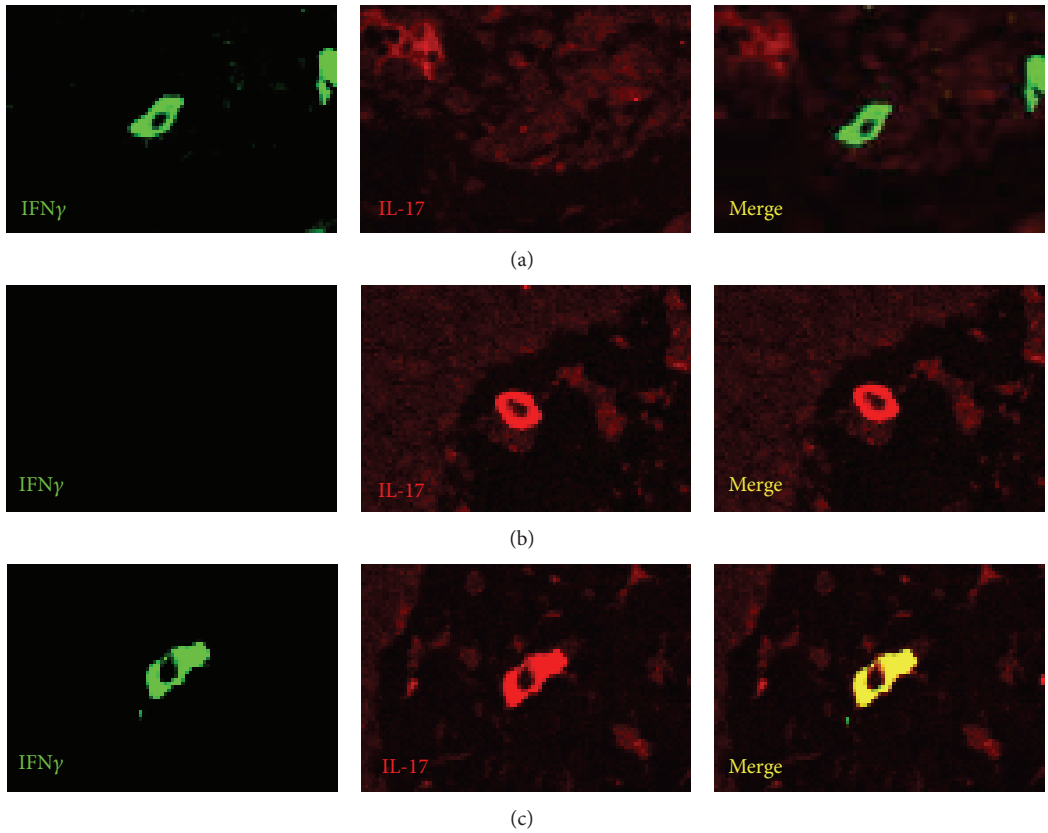


Figure 4: Immunofluorescence of Th1, Th17, and IFN$\gamma$-expressing Th17 cells in a BD skin lesion. (a) Th1 cell, (b) Th17 cell, and (c) IFN$\gamma$-expressing Th17 cell were simultaneously observed in one skin specimen obtained from erythema-nodosum-like lesion of BD.

for their molecular weights and subdivided into two major functional systems. HSP60-HSP10 system assisted the adequate protein folding and HSP70-HSP40 system was involved in the stability of cytosol peptides [66]. Significant sequence homology is found between mammalian and microbial HSP. For example, mycobacterial and streptococcal HSP65 have more than 90% homology, and mycobacterial HSP65 and human HSP60 have 42% homology [67].

It was suggested that HSP were secreted from both microbes and necrotic cells and were recognized by TLR2 and TLR4 [46]. In several studies, HSP were categorized into both PAMP and DAMP (Table 1) [50, 68]. Certainly, clinical studies demonstrated that HSP accumulation was promoted in the lesions of several human autoimmune diseases [69–72]. HSP peptide-specific T cells were found in patients with type 1 diabetes [73, 74], rheumatoid arthritis [75], and juvenile idiopathic arthritis [76]. Several experimental model studies of autoimmunity reported protective effects of HSP peptide by deletion of peptide specific T cells [77]. In fact, oral administration of an HSP peptide successfully increased Treg cells [75] and reduced disease activity in patients with rheumatoid arthritis [78].

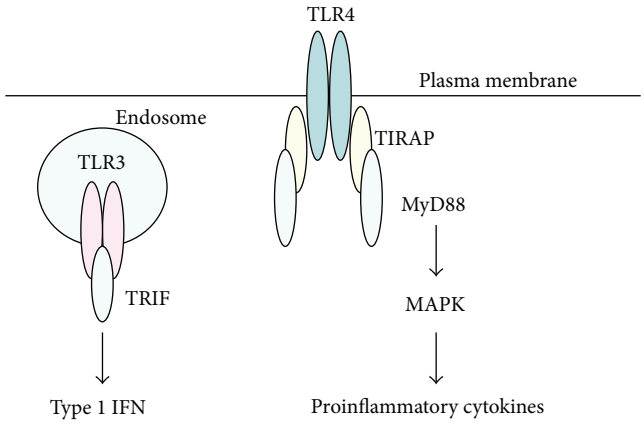


Figure 5: Two major TLR signaling pathways [48]. With TLR stimulation, except TLR3, APC produced proinflammatory cytokines through MyD88 and activated mitogen-activated protein kinases (MAPK). APC produced type 1 IFN by utilizing of TRIF through TLR3 stimulation, an intracellular TLR. TIRAP: Toll/interleukin 1 receptor (TIR) domain containing adaptor protein, MyD88: myeloid differentiation primary response protein 88, TRIF: TIR domain-containing adaptor-inducing IFN$\beta$, MAPK: mitogen-activated protein kinases, and IFN: interferon.

## 8. TLR and HSP Involvement in BD

Clinical studies demonstrated that both TLR and HSP expressions increased in patients with BD. Elevated gene expressions of TLR2 and TLR4 were found in peripheral blood monocytes [79], PBMC [80], polymorphonuclear leukocytes [80], bronchoalveolar lavage leukocytes [81], and oral mucosa [82] in patients with BD compared to normal controls. TLR2- and TLR4-positive cells in buccal lesions [83] and TLR6-positive polymorphonuclear leukocytes cultured with HSP60 [84] were significantly increased in patients with BD.

Several researchers observed massive expressions of HSP60 in BD skin [85] and oral ulcer lesions [86, 87]. HSP60 was expressed more diffusely [87] and intensely [85, 87] in BD lesions than those in other types of inflammation, such as oral lichen planus and recurrent aphthous stomatitis. Excessive T- and B-cell responses to major four peptides of *Mycobacterium tuberculosis* HSP65 and human counterparts of HSP60 were observed in patients with BD who lived in Europe, Far-Eastern Asia, and Middle East [10, 88–90].

We have found that TLR2 and TLR4 mRNA were expressed on ileocaecal ulcer lesions of BD, but less on unaffected sites of BD and on Crohn's disease lesions. IL-12 producing TLR2 positive macrophages located neighboring to T cells and HSP60 was expressed on the same region of the intestinal lesions [8, 9]. C-C-type chemokine receptor (CCR)5 and macrophage inflammatory protein (MIP)1$\beta$, a Th1 related chemokine receptor and its ligand, were detected in the intestinal lesions of BD and CCR5/MIP1$\beta$ interaction was thought to play a role in the migration of activated Th1 cells [9]. Moreover, we have reported that Th cells yielded proliferative responses to human HSP60 peptide in Japanese BD patients by a TCR V$\beta$ gene restricted antigen-driven process [90]. We suggest that TLR/HSP60 interactions induce destructive Th1-type responses at the intestinal lesion in patients with BD [91].

## 9. Genetic Variations of IL-12 Family Genes in BD and Human Autoimmune Diseases

Detailed analysis of comorbidity in dozens of human autoimmune diseases revealed the importance of treating the diseases as one group and suggested that there were several common etiopathologies among the diseases [92]. In the past decade, genetic clustering in the human autoimmune diseases has progressed with Genome-Wide Association studies (GWAS) to invest underlying genetic factors. Particulary, there have been noteworthy advances in the research of genetic variants in IL-12-family-related genes, which have shown major two subclusters, namely, Th17/Th1 cluster and Th1/IL-35 cluster (Figure 6) [93]. Th17/Th1 cluster was related to the polymorphisms of IL-23R and IL-12B and affiliated with inflammatory bowel diseases [94], psoriasis [95], ankylosing spondylitis [96], and rheumatoid arthritis [97]. Th1/IL-35 cluster was related to the polymorphisms of IL-12A and IL-12R$\beta$2 and affiliated with primary biliary cirrhosis [98] and Graves' disease (Figure 6) [99]. Several studies suggest that celiac disease [100] and multiple sclerosis [101] show both clusters' polymorphisms (Figure 6).

A decade of GWAS was conducted for BD in Turkey [102–104], Japan [105, 106], China [107], Iran [108], and Korea [109]. Human leukocyte antigen (HLA)-B51 is the most strongly associated risk factor for BD by a meta-analysis of case control genetic association studies [110] and the GWAS data support the result [102, 103, 106]. Recent two major studies [103, 105] identified MHC class I locus, IL-10, and IL-23R-IL12RB2 as BD susceptibility genes. IL-10 is an inhibitory cytokine to both T cells and APC [111], and secreted from T cells under IL-27 stimulation, as it was previously mentioned in Section 2. IL-10 production of healthy donors' PBMC with a BD-associated allele was significantly decreased compared to that without the allele in the presence of LPS [103]. Other several studies reported that, adding to IL-10 [108] and IL-23R-IL12RB2 [108, 109], STAT4 [107, 109] and IL-17A [109] genes were associated with BD. These data indicated a possibility that BD was included in Th17/Th1 cluster according to the above-mentioned clustering analysis. The IL-12 family cytokine gene polymorphisms suggest that the function of each IL-12 family cytokine subunit molecule needs to be reinvestigated based on the clustering analysis in patients with BD.

## 10. Genetic Variations of TLR and HSP in BD and Human Immune Diseases

Researchers mentioned that TLR gene polymorphisms were associated with several allergic and inflammatory diseases [112–116]. Skewed monocyte and mononuclear cell responses in cytokine production against microbe extracts were found in atopic dermatitis and asthma patients with a TLR2 [112] and a TLR4 [113] polymorphisms, respectively.

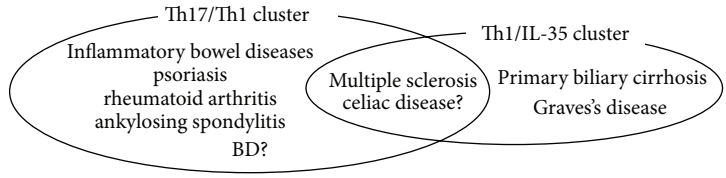


Figure 6: IL-12-family-cytokine-related genetic polymorphisms were found to be associated with several human immune diseases [44]. Th17/Th1 cluster was related to the polymorphisms of IL-23R and IL-12B and affiliated with inflammatory bowel diseases, psoriasis, ankylosing spondylitis, and rheumatoid arthritis. Th1/IL-35 cluster was related to the polymorphisms of IL-12A and IL-12R$\beta$2, and affiliated with primary biliary cirrhosis and Graves's disease. Several studies suggest that celiac disease and multiple sclerosis show both clusters' polymorphisms. Several Genome-Wide Association Studies identified IL-23R-IL12RB2, STAT4, and IL-17A as BD susceptibility genes and indicated a possibility that BD was including in Th17/Th1 cluster.

Several TLR gene polymorphism studies in patients with BD demonstrated no association with susceptibility to BD [117–124]. Recently, a targeted resequencing study was undertaken to detect rare genetic variants and, adding to IL-23R, TLR4 and nucleotide-binding oligomerization domain 2 (NOD2) genes, the latter of which was an intracellular PRR, were found to be associated with BD [125]. MyD88 adaptor-like protein (Mal), also known as TIR domain-containing adaptor protein (TIRAP, Figure 5), polymorphism was suggested to be associated with BD in UK [83]. TLR2 and TLR4 use TIRAP as an additional adaptor to recruit MyD88 [46]. The two studies offered new approaches for identifying BD susceptibility gene. Moreover, Killer cell lectin-like receptor subfamily C, member 4 (KLRC4) gene, a natural killer cell receptor, and endoplasmic reticular aminopeptidase 1 (ERAP1) gene, a major immunoregulatory molecule by peptide trimming inside the reticulum, were identified as BD susceptibility genes [102]. These analyses of gene polymorphisms in BD, with the high susceptibility of HLA-B51, indicated the importance of innate immune function as an effective therapeutic target in patients with BD. In fact, inhibitors of tumor necrosis factor $\alpha$, a downstream effector cytokine of MAPK signaling pathway in APC with TLR4 stimulation, remarkably ameliorated clinical symptoms in patients with BD [126, 127].

It was reported that HSP and the promoter gene polymorphisms were associated with Crohn's disease [128], bacterial sepsis [129], and multiple organ dysfunction after severe trauma [130]. HSP genes may serve as important factors for the detection of BD susceptibility gene.

## 11. Conclusions

We reviewed here current concept in Th cell differentiation and the functional/genetic contribution of the cells to the pathogenesis of BD. Skewed IL-12 family cytokine responses and related genetic variants were suggested to play a crucial role in the pathophysiological conditions in BD. Interestingly, dysregulation of Th17/Th1 cells and genetic variation in IL-12 gene family were found in several human autoimmune diseases. The existence of genetic variants both in innate and adaptive immune responses suggests that it is important to understand the molecular mechanical differences in the Th cell responses of BD between with and without APC of the patients with BD.

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*Review Article*

# Immunopathogenic Role of Herpes Simplex Virus in Behçet's Disease

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The role of viral infections, such as herpes simplex virus (HSV) infection, in the pathogenesis of Behçet's disease (BD) has been investigated for many years. HSV has been detected in peripheral blood leukocytes, saliva, and genital ulcers of patients with BD. Various cell adhesion molecules on cultured endothelial cells have been induced by HSV in a TNF-$\alpha$ dependent manner. In addition, a BD-like animal model was developed by inoculating ICR mouse earlobes with HSV, and antiviral treatment was effective in improving BD-like symptoms in this model. Still, there are several incompletely characterized proteins that possess antiviral properties and are being investigated as mediators of viral infection-related chronic inflammatory reactions. Although the role of HSV in the pathogenesis of BD remains to be fully established, recent research findings regarding HSV in BD have expanded our understanding of the disease and will hopefully lead to the development of more effective therapeutic agents in the near future.

## 1. Introduction: Historical Background

The role of viral infection in the pathogenesis of Behçet's disease (BD) was first suggested by Hûlusi Behçet, a Turkish dermatologist, in 1937 [1]. Early publications reported isolating virus from the ocular fluid, eye, and brain of patients with BD, but these findings were not initially confirmed by others [2–4]. With more recent advances in virology and immunology, DNA has been isolated in BD patients from various types of viruses, including herpes simplex virus (HSV), varicella zoster virus, cytomegalovirus, Epstein-Barr virus, human herpes virus 6 and 7, hepatitis virus, human immunodeficiency virus, and parvovirus B19 [5, 6]. Among these viruses, HSV is the leading candidate for playing a potentially key role in the pathogenesis of BD. *In situ* DNA-RNA hybridization techniques have demonstrated the presence of part of the HSV-1 genome in peripheral blood mononuclear cells of patients with BD [7]. Polymerase chain reaction (PCR) studies have confirmed the presence of a 211-base pair (bp) HSV-1 DNA fragment in the peripheral blood leukocytes of patients with BD [8] and demonstrated significantly greater quantities of HSV-1 DNA in the saliva, intestinal ulcers, and genital ulcers in BD patients than controls [9]. In addition, a BD-like animal model was developed by inoculating ICR mice with HSV [10, 11] and antiviral treatment was effective in improving BD-like symptoms in 40% of famciclovir treated BD mice [12].

Despite the aforementioned observations, the role of HSV in the pathogenesis of BD has not been firmly established, and the function of innate immunity and immunization treatment options remain to be elucidated. This review will discuss the current state of our knowledge regarding the role of HSV in BD and explore the possible future implications of this knowledge for the diagnosis and treatment of the disease.

## 2. Clinical Evidence Supporting the Role of HSV Infection and Detection of HSV in the Mucocutaneous Lesions in Behçet's Disease

BD is a recurrent, multisystemic inflammatory disease typically characterized by recurring oral aphthous ulcers, genital ulcers, ocular lesions, and cutaneous lesions and occasional articular, urogenital, vascular, gastrointestinal, and neurological involvement [13]. Oral ulcerations, the most common clinical manifestation of BD, include three patterns: minor ulcers, major ulcers, or herpetiform ulcers. The least common variety of oral aphthosis is herpetiform ulceration, which consists of numerous (up to 100) 2-3 mm lesions distributed throughout the oral cavity [14]. In common herpetic ulcers caused by HSV, the viral blisters quickly rupture, resulting in multiple small ulcers that often coalesce to form larger irregular ulcers [15]. The clinical similarities between herpetiform ulcers in BD and ulcers due to HSV infection suggest an etiologic role of HSV in BD and several studies have attempted to isolate HSV from the oral ulcers of patients with BD. HSV-1 DNA fragments have been detected by PCR [8] or *in situ* hybridization [7] in significant numbers in the peripheral blood leukocytes of patients with BD; however, viral DNA has not been detected in biopsy samples taken from oral ulcers, even in the presence of high anti-HSV-1 antibody concentrations in the peripheral blood of BD patients [8, 16]. The inability to detect viral DNA in tissue could be due to the viral DNA being present in small fragments rather than as an intact viral genome [8]. To further explore the role of HSV in the pathogenesis of BD, our group evaluated the presence of HSV DNA in saliva samples from 66 patients with the disease. The 289-bp band specific for HSV DNA was detected in DNA preparations from the saliva of 26 (39.4%) patients [9].

Although less common than oral lesions, patients with BD also often have genital lesions, which are characterized by ulcers. Clinically differentiating BD genital ulcers from HSV-induced ulcers (the most common type of genital ulcer in developed countries) is often difficult [17]. The clinical similarities suggest that HSV has a pathogenic role in the development of genital ulcers in BD. Moreover, HSV-1 DNA has been identified by PCR in biopsy samples obtained from genital ulcers of BD patients but not in biopsies from healthy controls [18].

Various cutaneous manifestations may be found in BD, including an erythema multiforme-like rash, which accounts for approximately 5% of BD skin lesions [19]. Conventional erythema multiforme is a hypersensitivity reaction associated with certain infections and medications. It can involve the oral, ocular, genital, upper respiratory, or pharyngeal mucosa [20]. HSV is the most common precipitating agent. HSV-associated erythema multiforme is usually limited to the oral mucosa, more specifically, the labial and buccal mucosa, nonattached gingivae, and vermillion lip [21]. Except for the occurrence of a prodromal episode of recurrent herpes infection in HSV-associated erythema multiforme, it is often difficult to distinguish this condition from the erythema multiforme-like lesions in BD. Because of this, many diagnostic criteria for BD do not include erythema multiforme-like rash as a disease-defining skin manifestation [22, 23]. Till now direct detection of HSV DNA from erythema multiforme-like lesions in BD has not been reported although the presence of HSV-1 and HSV-2 genomes in skin lesion from patients with BD was infrequently reported [24]. However, the clinical similarities between HSV-associated erythema multiforme and erythema multiforme-like lesions in BD are further evidence supporting an association between HSV and BD.

## 3. The Effect of HSV Infection on the Expression of Cell Adhesion Molecules in Cultured Human Dermal Microvascular Endothelial Cells

Cell-mediated immune responses to HSV have been implicated in the pathogenesis of various inflammatory diseases, including BD. HSV DNA and viral antigens are frequently detected in skin lesions of HSV-associated erythema multiforme [25], but it is unclear whether the pathologic changes in skin lesions are caused by the virus itself or by a secondary immunological reaction to a viral antigen. *In vitro* studies of cultured human dermal microvascular endothelial cells (HDMECs) have demonstrated that exposure to HSV-1 increased binding of T cells to HDMECs [26] and increased expression of cell adhesion molecules, such as CD54 (intercellular adhesion molecule-1 [ICAM-1]), vascular adhesion molecule 1 (VCAM-1), and E-selectin by HDMECs [26]. Similarly, incubation of HSV-infected peripheral blood mononuclear cells with uninfected HDMECs induced upregulation of CD54 and major histocompatibility complex class I molecules on HDMECs [27]. The binding of T cells to HDMECs was inhibited by anti-CD54, anti-interleukin (IL)-1$\alpha$, or anti-tumour necrosis factor (TNF)-$\alpha$ antibody [26]. These *in vitro* data suggest that IL-1$\alpha$ or TNF-$\alpha$ produced by HSV-infected HDMEC may function in the immunopathogenesis of BD. Thalidomide is a TNF-$\alpha$ inhibitor that is effective in both BD patients [28] and a BD-like mouse model [29]. The TNF-$\alpha$ blockers infliximab and etanercept have both been shown to reduce serum concentrations of soluble ICAM-1 and E-selectin in patients with arthritis [30, 31]. As well, a synthesized pyridine compound derivative (SK94) downregulated E-selectin, VCAM-1, and ICAM-1 in the HSV-induced BD mouse model [32]. Together with TNF-$\alpha$ inhibitors, targeting HSV-induced cell adhesion molecules on endothelial cells may thus be a promising therapeutic strategy for BD.

## 4. Behçet's Disease-Like Symptoms Induced by HSV in ICR Mice

To further explore the role of HSV in the pathogenesis of BD, we developed an HSV-induced BD mouse model. After inoculation of the scratched earlobe of 258 ICR mice with HSV type 1, 77 (29.8%) mice exhibited BD-like syndrome, defined by the presence of two or more BD symptoms. These symptoms and their overall frequency

were as follows: skin ulcers (57.1%); eye symptoms (39.0%); partial hair loss (33.8%); genital ulcers (19.5%); bullae (11.7%); arthritis (5.2%); gastrointestinal ulcer (5.2%); and tongue ulcers (3.9%). The ulcers, uveitis, and arthritis were clinically similar to those seen in patients with BD. Skin lesions stained with hematoxylin and eosin showed accumulations of perivascular inflammatory cells, and vasculitis was common in the intestinal, oral, ear lobe, and genital epithelial lesions [11]. More recently, $^{18}$F-fluorodeoxyglucose positron emission tomography showed the presence of symptomatic and asymptomatic knee joint inflammation in BD mice [33]. Although BD-like symptoms have been induced by inoculating the scratched earlobe of mice, direct injection of HSV in the perioral area, tongue, cornea, peritoneum, and footpad has not produced BD-like symptoms (unpublished data). HSV-induced BD-like symptoms are dependent on the species of mice: symptoms have been observed in 40%–50% of HSV-inoculated B10.BR, B10.RIII, and C57BL/6 mice but only 2% of C3H/He strain mice [34]. The function of HLA-G (Qa-2 in mice), which is immunosuppressive gene, was also decreased in HSV-induced BD mice [35]. The species-specificity and inherent abnormality of HLA molecules in this mice model are also similar to the presence of genetic predisposition in BD patients.

## 5. Expression of Th2 Cytokine Decreases the Development of and Improves Behçet's Disease-Like Symptoms Induced by Herpes Simplex Virus in Mice

Although BD-like symptoms have been induced by HSV inoculation of mice, viral infection alone is insufficient to explain the pathogenesis of BD, as no more than half of inoculated mice develop BD symptoms [11, 34]. To study the possible role of immunologic abnormalities in the development of BD-like symptoms induced by HSV inoculation of ICR mice, macrophages were depleted by intraperitoneal injection of liposome-encapsulated clodronate [36]. The incidence of BD-like symptoms was 28% in mice injected with HSV alone and 0% in mice that underwent macrophage depletion plus HSV inoculation. Macrophage depletion correlated with increased IL-4 expression in the mice spleens. When type 2 T helper cell (Th2) adjuvant ovalbumin (OVA)-alum was injected into mice with BD-like symptoms, cytokine IL-4 was upregulated and cutaneous symptoms improved. Adoptive transfer with splenocytes from OVA-alum-injected normal healthy mice into BD mice also resulted in improvement. These findings thereby suggest that upregulated Th2 cytokine expression can prevent or improve at least some BD-like symptoms. Nagafuchi et al. [37] also reported Th1 cells and the Th1-associated cytokines may play a detrimental role in the development of skin lesions in patients with BD.

## 6. Learning from HSV-Infected Mice as a Model of Behçet's Disease

Most animal models of disease are not perfectly matched to their corresponding human disease, but they are often the most appropriate research tool. Furthermore, the complexity of BD makes it difficult to develop a single experimental animal model to understand the mechanisms responsible for all BD symptoms. Currently, the HSV-induced BD mouse model is the model that most closely mimics the symptoms, histological characteristics, immunological abnormalities, and responses to therapeutic modalities of patients with BD. This mouse model has been a useful tool for the study of BD pathogenesis and pharmacotherapy. Since the successful development of an HSV-induced BD mouse model was first reported in 1998 [11], over 20 papers using this model have been published. These papers have involved such topics as immunological abnormalities, including major histocompatibility (MHC) relevance [34, 35], Th1/Th2 balance [36], and $CD4^+CD25^+Foxp3^+$ regulatory T cells [38]; therapeutic targets, such as TNF$\alpha$ [39], IL-6 [40], and IL-17 [40]; and conventional and potential therapies involving colchicines [41, 42], thalidomide [29], famciclovir [34], gemcitabine (2′,2′-difluorodeoxycytidine) [43], IL-4 DNA vector [44], and vitamin D3 [45].

## 7. Innate Immunity and Tripartite Motif-Containing Proteins in Viral Infection

Tripartite motif-containing (TRIM) proteins, a family of RING domain-containing E3 ligases, play diverse functions in cell biology and immunity [46]. Several TRIM proteins exhibit direct antiviral activity, by restricting various stages of the viral replication cycle [47]. Some TRIM proteins also regulate signalling of immune pathways mediated by pattern recognition receptors [48]. With recent elucidation of the role of TRIM proteins as viral restriction factors or regulators of the viral RNA/DNA sensing pathway and the inflammasome, the role of TRIM proteins as key molecules in antiviral immunity has become increasingly apparent [47, 48]. TRIM proteins contribute to the regulation of immune responses, including the production of type I interferons and proinflammatory cytokines such as interleukin-1$\beta$, and they have been reported to have pathogenic roles in numerous autoimmune diseases, including systemic lupus erythematosus, gout, type 2 diabetes, rheumatoid arthritis, and Crohn's disease [47, 49, 50].

Because of the role of TRIM proteins in antiviral immunity, pattern recognition receptor signalling, inflammasome activation, and autoimmunity, it has recently been suggested that these proteins may contribute to the pathogenesis of BD. A single nucleotide polymorphisms study published in 2010 demonstrated that TRIM39 was independently associated with BD, suggesting that it may contribute to the pathogenesis of the disease [51]. TRIM39R (although not TRIM39B) was subsequently reported to be related to the regulation of the type I interferon response [52].

TRIM19, which is also known as the promyelocytic leukaemia (PML) protein, organizes PML nuclear bodies, which function in antiviral immunity [53]. The genomes of HSV-1 and human cytomegalovirus are both subject to transcriptional repression by mechanisms involving PML and nuclear body components [54]; these are important innate

antiviral defence mechanisms. TRIM19 enhances interferon-$\gamma$-mediated antiviral gene expression [55], and its depletion has led to increased replication of HSV-1 in previous studies [53, 54]. As HSV infection appears to play an important role in the pathogenesis of BD and TRIM19 functions in innate defence mechanisms against HSV, this suggests that TRIM19 may contribute to the development of BD.

Several other TRIM proteins also possess antiviral properties and are being investigated as potential mediators of autoimmune or chronic inflammatory disorders [46–48]. However, studies regarding the relationship between TRIM proteins in BD are rare. Since TRIM proteins have the potential to function as a potent cellular detection mechanism and can allow cells to stimulate broad-spectrum immunity [56], they may be candidate molecules for connecting the innate immune system to the pathogenesis of BD. Therefore, further investigations to reveal the role of TRIM proteins in BD might aid in further elucidating the pathogenesis of this disease.

## 8. Therapeutic Efficacy of HSV-Targeted Treatments in BD Patients and the BD Mouse Model

There is currently no single curative therapeutic agent for BD. Present treatment of BD is empirical and focuses on symptom relief, not a complete cure. The treatment generally depends on the clinical manifestations of each patient and is thus primarily patient-specific [57–60]. Although various therapeutic agents have been used for BD, the treatment results vary widely between agents and individuals, and the results are far from optimal [60].

As HSV has been substantially implicated in the pathogenesis of BD [7–11], most strategies have been centred on treating the HSV infection itself or blocking the HSV-related immunologic pathways. Although treatment with acyclovir failed to reduce the frequency and severity of orogenital ulceration or other clinical manifestations in BD patients [61], treatment with famciclovir, a prodrug form of penciclovir that inhibits viral DNA polymerase, was effective in improving BD-like symptoms and preventing relapse in the mouse model of BD [34].

TNF-$\alpha$, a representative proinflammatory cytokine, is primarily produced by T cells, polymorphonuclear cells, dendritic cells, and macrophages [62]. In macrophages, production of TNF-$\alpha$ can be induced by physical, chemical, and biologic stimuli, including ischemia, trauma, irradiation, tumour cells, complement, and various other cytokines [39]. The TNF-$\alpha$ signalling pathway can also be induced by bacterial and viral infections. As viral infection appears to be a triggering factor for BD, studies focusing on reducing or blocking TNF-$\alpha$ signalling have been performed. Injecting TNF-$\alpha$ small interfering RNA or anti-TNF-$\alpha$ blockades (infliximab or etanercept) inhibited TNF-$\alpha$ gene expression and improved symptoms in a BD mouse model [39]. Synthesized pyridine compound derivatives (SK94 and SK126) are also potential therapeutic candidates for BD, as they have been shown to downregulate cell adhesion molecules and TNF-$\alpha$, and ameliorate symptoms in the BD mouse model [32].

Investigations continue to be conducted to more fully elucidate the pathogenesis of BD and to develop novel therapeutic agents. Peptides such as Hsp-65/60 are now being considered as possible new therapeutic targets for patients with BD [63]. Further investigations, including clinical trials in humans, are required to validate the effectiveness and safety of candidate agents. Additionally, more in-depth studies are required to investigate the role of immune reactions in BD, including not only adaptive responses but also innate immune reactions, such as those related to TRIM proteins.

## 9. Conclusion

The exact pathogenesis of BD remains elusive, although research continues to increase our understanding of the complex pathogenetic mechanisms of the disease. Infectious agents, such as HSV and *Streptococcus sanguinis*, have long been postulated as possible environmental triggers of BD, and the role of HSV continues to be a main focus of BD research. Clinical observations and the detection of HSV DNA in saliva and genital ulcers of BD patients prompted us to investigate the effects of HSV in BD *in vitro* and *in vivo*. Establishing a BD mouse model using HSV inoculation facilitated our investigations of the pathogenesis of BD and, more importantly, the therapeutic efficacy of emerging medicines. As current treatment options for BD are limited and often unsatisfactory, it is hoped that recent research findings will lead to the development of successful therapeutic strategies for BD in the near future.

## Conflict of Interests

The authors have no conflict of interests to declare.

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