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Human Genome and Diseases: Review

Immunology and functional genomics of Behçet's disease

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Abstract. Behçet's disease (BD) is a multisystemic inflammatory disorder. Although the cause and pathogenesis of BD are still unclear, there is evidence for genetic, immunologic and infectious factors at the onset or in the course of BD. This review focusses on the functional genomics and immunology of BD. HLA-B51 is the major disease susceptibility gene locus in BD. An increased number of $\gamma\delta$ T cells in the peripheral blood and in the involved tissues have been reported. However, the T cells at the sites of inflammation appear to be a phenotypically distinct subset. There is also a significant $\gamma\delta$ T cell proliferative response to mycobacterial 65-kDa heat shock protein peptides. Homologous peptides derived from the human 60-kDa heat shock protein were observed in BD patients. There is evidence that natural killer T cells may also play a role in BD.

Key words. Behçet's disease; vasculitis; uveitis; experimental autoimmune uveitis; HLA; streptococcus; herpes simplex virus; heat shock protein; T cells; NK-T-cells; neutrophils; cytokines; endothelial dysfunction; coagulation and fibrinolytic pathway abnormalities.

Introduction

Behçet's disease (BD) is a recurrent systemic inflammatory disorder characterized by four major symptoms consisting of oral aphthous ulcers, ocular lesions, skin lesions, genital ulcerations, and occasionally by inflammation in tissues and organs throughout the body including the vascular system, central nervous system, gastrointestinal tract, lungs, kidneys and joints [1]. This disease is distributed around the world, but a higher prevalence has been found among the Asian and Eurasian populations along the Silk Route stretching to the countries of the Mediterranean region [1]. The prevalence of the disease in Japan is about 15 : 100,000 and reaches the highest level of 370 : 100,000 in Turkey, whereas that in North America and Europe is very low at 1 : 500,000 [2, 3]. There have been no reports of clinical cases of BD among black populations.

Although the cause and pathogenesis of BD are still uncertain, the onset of BD is believed to be triggered by the involvement of some external environmental factors in

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people with a particular genetic background. It has been assumed that infectious agents, immune mechanisms and genetic factors such as HLA-B51 are involved in the onset of BD. The male : female ratio is almost equal in different series, but most of the severe cases with BD are male; hence, it is not clear whether BD should be classified as an autoimmune disease [4]. The mean age at onset is the third decade, children are rarely affected and few neonatal cases have been reported [4]. The main microscopic finding at most sites of active BD is an immune-mediated occlusive vasculitis. At the cellular level, CD4+ T cells were found in the perivascular inflammatory exudates, and Th1 cells responded to various stimuli to produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor β (TNFβ) [5]. Neutrophil hyperactivity, such as increased chemotaxis, active oxygen overproduction and increased endothelial cytotoxicity, is primarily involved in the pathogenesis of BD. In recent studies, an increased number of $\gamma\delta$ T cells in the peripheral blood and involved tissues, and a phenotypically distinct subset of $\gamma\delta$ T cells at the sites of inflammation were reported [6–8]. Furthermore, significant $\gamma\delta$ T cell proliferative responses to mycobacterial 65-kDa heat shock protein (hsp) peptides and their homologous peptides derived from the human 60-kDa hsp were observed in BD patients [9, 10]. Besides T cells, there is evidence that natural killer (NK)-T cells may also play a role in BD.

Therefore, BD is probably not a simple hereditary disease, and exogenous antigen(s) such as bacteria, viruses or other microorganisms might trigger immunological abnormalities which cause the onset of the disease.

Recently, anti-TNF-blocking substances and also IFN-α have been shown to be effective in preventing the severe course of ocular BD.

This review will focus on the functional genomics and immunology of BD.

Functional genomics of BD

There is clear evidence for an association between BD and HLA-B51, one of the split antigens of HLA-B5, and this genetic marker has been identified as the most strongly associated one with the disease [1, 11]. HLA genes are scattered throughout the HLA gene region, which occupies more than 3500 kb on chromosome 6 (6p21.3). This strong association between BD and HLA-B51 has been confirmed in patients of many ethnic groups, particularly those from the Middle to Far East, including Turks, Greeks, Italians, French, English, Germans, Tunisians, Israelis, Jordanians, Iranians, Saudi Arabians, Kuwaitis, Han Chinese, Koreans, Taiwanese and Japanese. One attractive hypothesis is that BD spread through Asian and Eurasian populations from Japan to the Middle East, together with its associated HLA allele, HLA-B51, as a result of the movement of nomadic or Turkish tribes travelling the Silk Route [1]. However, it has not yet been clarified whether the HLA-B51 gene itself is the pathogenic gene related to BD, or if it is another gene in linkage disequilibrium with HLA-B51.

Internal genetic background

In previous studies, the MICA (MHC class I chain-related gene A) gene, located 46 kb centromeric of the HLA-B gene [12, 13], appeared to be a strong candidate as the gene responsible for susceptibility to BD based on its chromosomal localization, its restricted and heat shock-induced expression in epithelial cells [14], its predicted immunological function as a ligand of Vδ1 $\gamma\delta$ T cells [15] and a strong association between a certain transmembrane (TM) microsatellite allele, MICA-A6, and BD [16]. However, the possibility of a primary association of MICA-A6 with BD was lower in the Greek population [17], and lack of an association between BD and the MICA-TM microsatellite polymorphism was reported in the Spanish population [18]. As a result of recent extensive analyses of genetic polymorphism in the extracellular (α1, α2 and α3) domains of MICA, a strong association between MIC-A009 and BD was found in Japanese, Palestinian and Jordanian populations which could be explained in terms of a strong linkage disequilibrium with HLA-B51 [19, 20]. The MICA gene has therefore been suggested not to be directly involved in the pathogenesis of BD, and it is still uncertain whether HLA-B or an unknown gene in linkage disequilibrium is the actual pathogenic gene of this disease.

Recently the genomic sequencing of the entire HLA class I region spanning ~1.8 Mb (1800 kb) from the MICB gene to the HLA-F gene was completed. This region contains the MICA, MICB, HLA-B and HLA-C genes, and 758 microsatellite repeats (STRs: short tandem repeats) have been identified within it [21–23]. A microsatellite generally consists of repetitive sequences of two to five bases, and all eukaryotic DNA contains a family of such repetitive sequences as junk DNA. The number of repetitive sequences varies among individuals, and hence microsatellites can be used as informative polymorphic markers for genetic mapping. The microsatellites identified in this region are densely spread over the BD candidate region and should serve as valuable polymorphic markers for precise mapping of the pathogenic gene. We performed association analysis using HLA-B and eight polymorphic microsatellite markers (C1-2-A, MICA-TM, MIB, C1-4-1, C1-2-5, C1-3-1, C2-4-4 and C3-2-11) covering a region of 1100 kb around the HLA-B gene in order to precisely pinpoint the location of the pathogenic gene responsible for BD (fig. 1) [24, 25]. Since genetic recombination, interchromosomal crossover and genetic

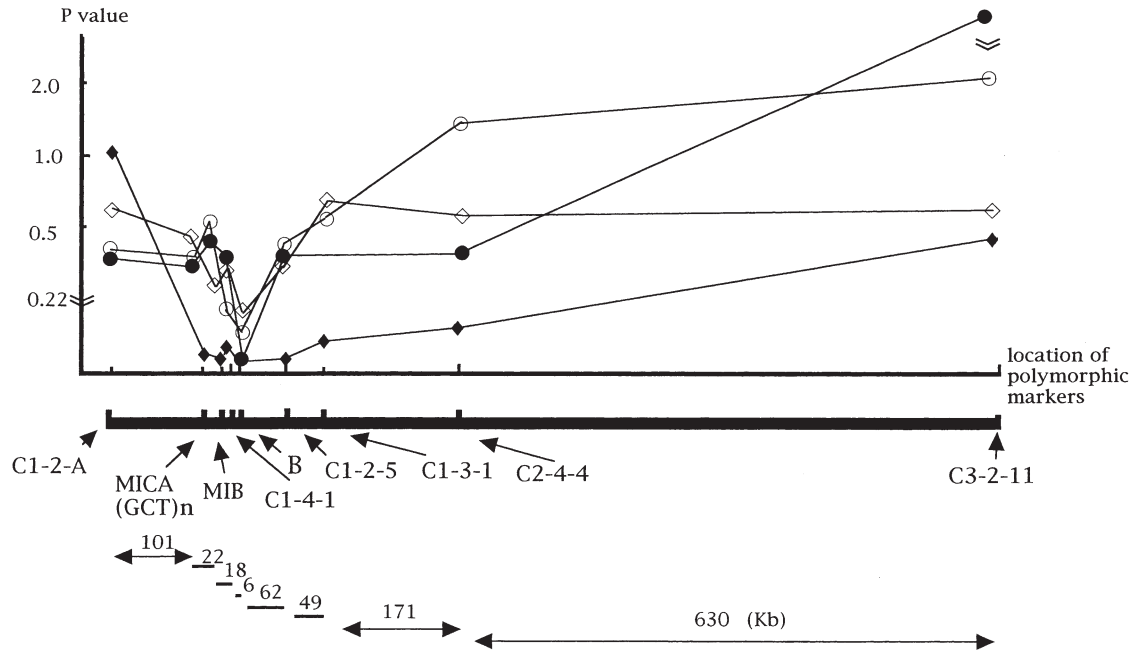

Figure 1. Genotypic differences in the Japanese, Greek, Jordanian and Italian populations with respect to the location of HLA-B and microsatellite loci used for association analysis on Behçet's disease. The location of all markers used in this study are displayed under the map along with the distance (kb) to neighboring markers. The C1-2-A microsatellite is the most centromeric of the markers. Reciprocal of logarithmic *P* values obtained by genotypic differentiation testing are plotted on the vertical axis. The locations of polymorphic markers are plotted on the horizontal axis (◆, Japanese; ○, Greek; ●, Jordanian; ◊, Italian).

exchange may vary in form depending on ethnic origin, four different populations consisting of Japanese, Greeks, Jordanians and Italians were investigated.

Statistically significant differences between patient and control groups were found at one or more alleles of several microsatellite markers. The MICA-A6, MIB-348, C1-4-1-217 and HLA-B51 alleles in particular were commonly increased in frequency with very significant Pc values in patient groups among the four ethnic populations studied. There was no common allele which increased or decreased in frequency at any microsatellite locus centromeric of MICA or telomeric of HLA-B. Accordingly, the MICA-A6 – MIB-348 – C1-4-1-217 – HLA-B51 haplotype appears to have worldwide predominance among groups of BD patients studied. Of these alleles, it must be emphasized that HLA-B51 is by far the one most strongly associated allele with BD (Japanese, Pc = 0.000000000017; Greek, Pc = 0.00000032; Jordanian, Pc = 0.000050; Italian, Pc = 0.0074). Furthermore, in stratification analyses of the confounding effects of MICA-A6, MIB-348 and C1-4-1-217 on HLA-B51 association and vice versa, only the association of B51 was remarkably significant in all four ethnic groups, indicating a primary role in the development of BD. Thus, the significant increase in the frequency of MICA-A6, MIB-348 and C1-4-1-217 in the patient groups can be explained by linkage disequilibrium with HLA-B51.

The genotypic differentiation concerned with the allelic distribution was also analyzed between the patient and control groups (table 1 and fig. 1). Generally, genotypic distribution should be identical within the same ethnic groups. If genotypic differentiation is observed at a locus between patient and control groups, allelic distribution at its locus is supposed to be under the influence of some genetic bias. In the Japanese population, the *P* values were significantly low ($P < 0.01$) at seven loci (MICA-TM, MIB, C1-4-1, HLA-B, C1-2-5, C1-3-1, C2-4-4). Especially, the remarkably low *P* values ($P < 0.0001$) were observed at the MICA-TM, MIB, HLA-B and C1-2-5 loci [26, 27]. Further, in the Greek population, the *P* values were significantly low at the HLA-B locus ($P = 0.00180$) and relatively low at C1-4-1 ($P = 0.00904$), which is located only 6 kb centromeric to HLA-B [27]. In the Jordanian and Italian populations, only the HLA-B locus gave rise to significantly low *P* values (Jordanian, $P = 0.00043$; Italian: P=0.00691) [27, 28].

Association studies with cases and controls are prone to errors mainly arising from population stratification, admixture, genetic drift and so on, and family-based methods such as the transmission disequilibrium test (TDT) are being more frequently used for documenting both association and genetic linkage. Analysis of a small group of multicase BD families has also confirmed the genetic linkage of only the HLA-B locus to BD among the neighboring genes and markers using the TDT [29].

Table 1. Genotypic differentiation between normal and patient groups in Japanese, Greek, Jordanian and Italian populations.

Marker	Japanese (Cont. = 132, BD = 95) *P* value ± s.e.	Greek (Cont. = 52, BD = 55) *P* value ± s.e.	Jordan (Cont. = 50, BD = 49) *P* value ± s.e.	Italian (Cont. = 28, BD = 22) *P* value ± s.e.
C1-2-A	0.38152 ± 0.00065	0.10262 ± 0.00070	0.07004 ± 0.00053	0.22331 ± 0.00076
MICA-TM	0.00001 ± 0.00000	0.09689 ± 0.00121	0.07678 ± 0.00044	0.10818 ± 0.00020
MIB	0.00000 ± 0.00000	0.21318 ± 0.00219	0.06885 ± 0.00055	0.06013 ± 0.00097
C1-4-1	0.00080 ± 0.00004	0.00904 ± 0.00033	0.10165 ± 0.00046	0.07663 ± 0.00017
HLA-B	0.00007 ± 0.00001	0.00180 ± 0.00006	0.00043 ± 0.00003	0.00691 ± 0.00017
C1-2-5	0.00000 ± 0.00000	0.11846 ± 0.00176	0.07020 ± 0.00054	0.05625 ± 0.00080
C1-3-1	0.00036 ± 0.00003	0.16974 ± 0.00064	0.43685 ± 0.00102	0.20040 ± 0.00055
C2-4-4	0.00328 ± 0.00003	0.55011 ± 0.00092	0.07737 ± 0.00052	0.18600 ± 0.00063
C3-2-11	0.12252 ± 0.00078	0.61326 ± 0.00118	0.98143 ± 0.00019	0.22746 ± 0.00082

These results clearly indicate that the real pathogenic gene involved in the development of BD is pinpointed to HLA-B51 itself. However, since HLA-B51 is tightly linked with MICA-A6, and all of the HLA-B51-positive individuals in these populations also possess MICA-A6, a possibility that the MICA-A6 allele is an additional risk factor or further amplifies risk for developing BD cannot be excluded. The presence of HLA-B51-negative BD patients can be explained by the influence of other genetic factor(s) and/or of various external environmental or infectious agent(s).

Primary involvement of HLA-B*51 allele

As described above, it is suggested that the HLA-B gene is a strong candidate locus responsible for the development of BD and that HLA-B51 is the major disease susceptibility gene for BD. Various symptoms of BD have been considered to result from neutrophil hyperactivity. It is interesting that increased neutrophil function has been reported both in the BD patients and healthy individuals who are B51 positive [30, 31]. Further, increased H_2O_2 production by neutrophils after stimulation with formyl-methionine-leucine-phenylalanine (fMLP) has been reported in B51 transgenic mice [31]. Since H_2O_2 production by neutrophils was not increased after stimulation with fMLP in B35 transgenic mice, higher H_2O_2 production by neutrophils in these B51 transgenic mice is not ascribable to the transfection of xenogenic MHC genes. However, this B51 transgenic mouse did not show any characteristic features of BD, possibly because of the low expression level of the B51 molecules. Further analysis is clearly needed by construction of HLA-B51 and human β2 M double transgenic mice.

It is now known that the B51 antigen comprises 24 alleles at the amino acid level, B*5101–B*5124 [32]. Recently, a procedure was established involving PCR-SBT (polymerase chain reaction-sequencing-based typing) as well as PCR-SSP as a means of discriminating among all of the HLA-B*51 alleles. HLA-B51 (B*5101–B*5124) allele genotyping was performed among 96 Japanese, 58 Greek, 58 Iranian, 21 Italian and 13 Saudi Arabian BD patients by the PCR-SSP or PCR-SBT method. As a result, HLA-B*5108 as well as HLA-B*5101 were found to be relatively higher in frequency in patient groups from Greek, Iranian, Italian and Saudi Arabian populations [33–36] (table 2). Namely, 13 out of 58 (22.4%) Greek patients, 3 out of 58 (5.2%) Iranian patients, 4 out of 21 (19.0%) Italian patients and 1 out of 13 (7.7%) Saudi Arabian patients carried the B*5108 allele. In contrast, in a Japanese population consisting of 96 BD patients and 132 healthy controls, none carried the HLA-B*5108 allele [37] (table 2). Fifty-six out of 96 (58.3%) Japanese

Table 2. HLA-B*51 allele typing in the Japanese, Greek, Iranian, Italian and Saudi Arabian BD Patients.

HLA	Japanese (*N* = 96)	Greek (*N* = 58)	Iranian (*N* = 58)	Italian (*N* = 21)	Saudi Arabian (*N* = 13)
HLA-B51	57 [59.4%]	44 [75.9%]	36 [62.1%]	15 [71.4%]	10 [76.9%]
HLA-B*5101	56 [58.3%]	34 [58.6%]	33 [56.9%]	11 [52.4%]	9 [69.2%]
HLA-B*5102	1 [1.0%]	0 [0%]	0 [0%]	0 [0%]	0 [0%]
HLA-B*5103	0 [0%]	0 [0%]	0 [0%]	0 [0%]	0 [0%]
HLA-B*5104	0 [0%]	0 [0%]	0 [0%]	0 [0%]	0 [0%]
HLA-B*5105	0 [0%]	0 [0%]	0 [0%]	0 [0%]	0 [0%]
HLA-B*5106	0 [0%]	0 [0%]	0 [0%]	0 [0%]	0 [0%]
HLA-B*5107	0 [0%]	0 [0%]	0 [0%]	0 [0%]	0 [0%]
HLA-B*5108	0 [0%]	13 [22.4%]	3 [5.2%]	4 [19%]	1 [7.7%]
HLA-B*5109 ~ B*5124	0 [0%]	0 [0%]	0 [0%]	0 [0%]	0 [0%]

patients were defined as having B*5101, and one (1.0%) as having B*5102. None of the Japanese patients or healthy controls examined to date have carried the HLA-B*5108 allele.

B*5108 differs from B*5101 by a single nucleotide substitution from adenine (A) to thymine (T) at position 527, resulting in an amino acid change from glutamic acid (Glu) to valine (Val) at codon 152, as well as by three additional contiguous nucleotide substitutions at positions 538–540 (CTG→GAC), resulting in an amino acid change from leucine (Leu) to asparatic acid (Asp) at codon 156 [38]. These amino acids constitute a part of pocket E in the HLA groove [39]. This pocket is not charged and is not primarily related to the specific binding of antigenic peptides. It is unlikely that the two amino acids specific for B*5108, Val at codon 152 and Asp at codon 156, play a primary role in the pathogenesis of BD through specific antigen presentation, although the possibility of some secondary (amplifying or accessory) contribution of the B*5108 allele to the development of BD can not be completely eliminated.

In view of the remarkably different phenotypic frequencies of B*5101 (57.1%) and B*5201 (13.2%) among Japanese patients with BD, which are both distinguished from each other by only two amino acid substitutions at positions 63 and 67, the hypothesis has been presented that having an asparagine (Asn) at position 63 and a phenylalanine (Phe) at position 67 in B*5101 results in a genetic susceptibility to this disease and is primarily responsible for its development [40]. Notably, these two amino acids are known to constitute a part of pocket B in the HLA groove of the class I antigen, which is one of the major pockets and determines amino acid specificity of antigen peptides for binding with HLA molecules (HLA motif) [39, 41]. These two amino acids are shared not only by B*5101 but also by other B*51 alleles (except B*5107), including B*5102 and B*5108. The difference of the allelic distribution between the Japanese population and the Greek, Iranian, Italian and Saudi Arabian populations might be due to differences in the way in which HLA-B*51 alleles were distributed among various ethnic groups. It appears likely that BD spread among Asian and Eurasian populations together with its associated HLA-B*51 allele prior to divergence into suballeles.

Other BD susceptibility genes

HLA-B51 is the strongest genetic predisposition factor for BD defined so far. However, during the TDT analysis of multicase BD families, it has been estimated that the contribution of the HLA-B locus to overall genetic susceptibility to BD is between 12 and 19%, based on the calculations of HLA-B allele sharing in affected siblings [29]. Hence, there should be other genes causing a tendency to BD at different extent. A novel BD susceptibility locus was recently mapped to 6p22-23 using 28 multicase families of Turkish origin [42]. This locus is located more than 15 cM telomeric to the HLA-B, which is far beyond the strong linkage disequilibrium region spanning up to the hemochromatosis (HFE) gene. This telomeric locus is currently being fine-mapped to identify the susceptibility gene.

Role of microbial infections in BD

Microbial infections in BD

Microbial infection has been implicated in the development of BD since the first report of Professor Behçet in 1937. Four principal hypotheses have been suggested: (i) viral, (ii) streptococcal, (iii) heat shock protein (hsp) and (iv) cross-reactive or molecular mimic protein etiologies. Herpes simplex virus (HSV) 1 genome (but not HSV 2) and serum antibodies against the virus have been found in a higher proportion of patients with BD than in controls [43, 44]. Cytotoxic T cells were also found in the circulation of patients with BD. Other viruses such as hepatitis C virus and parvovirus B19 may also have some role for the pathogenesis of BD. There is a consensus that BD is not a result of direct infection by these viruses but possibly a dysfunction of the immune system after infection with these viruses.

Streptococcus sanguis has also been suggested as a causative agent [44–46]. The proportion of *S. sanguis* in the oral flora of patients with BD is significantly increased compared with controls [46]. The isolates from patients with BD were uncommon serotypes and had unique characteristics [47]. Significant antibody responses to these streptococci were observed in patients with BD [48]. Patients show hypersensitivity in skin tests with streptococcal antigens, and symptoms typical of BD are sometimes provoked by injection of the antigen [49]. Recently, this infectious agent was proved to cause BD-like symptoms in experimental gnotobiotic mice [50]. The results of a series of these studies led to the hypothesis that ubiquitous antigens, including hsps of ubiquitous bacteria, may trigger cross-reactive autoimmune responses in patients with BD. Two other species of streptococci, *Streptococcus pyogenes* and *Streptococcus salivalius,* and *Enterococcus fecalis* have been suggested as etiological agents and there may be cross-reactive antigens, including hsps on the surface of the bacteria [51].

Pathogen-derived hsps as targets for the immune response

Both host cells and microbes are confronted with dramatic alterations in their living conditions during infection. With these changing conditions, induction of hsp synthesis is vital for pathogen survival. Subsequently, in-

Table 3. Host responses* to hsp in various diseases.

Disease	hsp family
Autoimmune diseases	
BD	hsp60
Rheumatoid arthritis and reactive arthritis	hsp60
Systemic lupus erythematosus	hsp90
MS	hsp60, hsp70
Psoriasis	hsp60
Atherosclerosis	hsp60
Chronic gastritis	hsp60
Hashimoto's thyroidosis	hsp60
Bacterial infections	
Tuberculosis (*Mycobacterium tuberculosis*)	hsp60, hsp70
Trachoma (*Chlamidia trachomatis*)	hsp60, hsp70
Lyme disease (*Borrelia burgdorferi*)	hsp60, hsp70
Syphilis (*Treponema pallidum*)	hsp60
Gastritis (*Helicobacter pylori*)	hsp60
Fungal infections	
Candidiasis (*Candida albicans*)	hsp90
Histoplasmosis (*Histoplasma capsulatum*)	hsp60, hsp70
Protozoal infections	
Chagas' disease (*Trypanosoma cruzi*)	hsp70,hsp90
Malaria (*Plasmodium falciparum*)	hsp70, hsp90

* Antibodies specific for hsps, T cells specific for hsps, raised expression of self-hsps.

creased pathogen hsp levels in cells lead to rapid degradation of hsps by the host processing machinery. Pathogen-derived determinants may then be efficiently presented by host cells and promote recognition of infected cells by the immune system. It is considered that hsps from the specific bacteria show considerable homology with those of both other bacterial and host hsps, and subsequently enhance host immunity.

Immune responses to hsps have been observed in infectious diseases caused by bacteria, protozoa, fungi and helminths, as well as various experimental infections [52, 53] (table 3). Evidently, due to their high conservation among various microbial pathogens, hsps could be major antigens. They are known to induce very strong humoral and cellular immune responses in numerous infections. hsp60 is homologous to the common antigen in various bacteria, including GroEL of *Escherichia coli*. The recent finding that the GroEL complex is involved in bacterial cell wall synthesis suggests ready accessibility of these hsp molecules to antibodies [54]. At least two factors contribute to the fact that hsps represent major antigens in a wide spectrum of infections [52]. These proteins are abundant in the pathogen, especially under stress conditions.

Immunologic memory for cross-reactive determinants of conserved hsps is generated during life based on frequent restimulation by subsequent encounters with various microbes.

Immune response to hsps in human autoimmune diseases including BD

The concept of overexpression of hsps either as proper antigens on the cell surface or as peptides presented by major histocompatibility complex (MHC) products has been central to the hypothesis that hsp-specific antibodies and T cells play a role in the pathogenesis of human autoimmune diseases. Involvement of hsps in autoimmune responses depends on two criteria. First, it is necessary that hsps be expressed by cells of the target organ in a different way from other tissue sites to allow organ-specific recognition by T cells and antibodies. Second, control of natural regulation for organ-specific inflammation must be disturbed. Increased levels of hsp-specific antibodies in serum have been found in BD and other autoimmune diseases (table 3). Immune responses against hsps have been recognized in diseases other than BD, which are suggested to be associated with streptococcal infections such as psoriasis [55] and Kawasaki disease [45].

T cell responses to hsp60 were also found in patients with BD, and four different peptide determinants within hsp60 identified by T-cell-epitope mapping have been suggested to be involved in the pathogenesis of BD (see below) [51, 56]. Oral or nasal administration of hsp peptides without adjuvant induced clinical uveitis in experimental rats [57]. The mucosal route of induction of uveitis is more likely to mimic the clinical situation, because oral streptococci may elicit immune responses which cross-react with the mucosal layer and initiate pathological changes. However, these T-cell-specific responses for hsps may arise in a second response and then be attracted to sites of inflammation. Responses against hsps may reflect a common relation between hsps and other autoantigens in a number of autoimmune disorders.

Induction of tolerance and adjuvant effects by hsps

Since stressful conditions raise hsp synthesis in cells, hsp peptides gain access to the loading compartments of MHC molecules. If hsps were found to serve as target antigens for T cells in the autoimmune process in BD, healthy individuals can control such autoreactive T cells. It has been suggested that T-cell-specific reactions for self-hsps that escaped thymic selection are effectively controlled, and tolerance of T cells may be maintained by permanent encounters of cross-reactive hsps derived from food and commensal organisms [58]. It is well known that hsp65-induced tolerance confers protection against experimental arthritis, such as that induced by streptococcal cell walls and collagen type II. If there is disappearance of tolerance by mucosal bacterial infection (even if the bacterium is a commensal one), introduction of self-antigens via mucosal immunity might cause BD. During oral microbial infection, the protective immune

response can be converted into a pathogenic one. Additionally, foreign or self-hsps have been used successfully as carriers for poorly immunogenic T-cell-independent carbohydrate antigens [59]. Immunization of mice with mycobacterial hsp60 conjugated to peptide or carbohydrate antigens induced hsp-specific antibodies that cross-react only with hsp homologs from other prokaryotes but not with the mammalian hsp60. After mono-infection of *S. sanguis* to germ-free mice, the large inoculum size and heat treatment enhanced immune responses against cell wall and hsp336–351 [50]. Therefore, hsps from oral bacteria such as *S. sanguis* may be important for induction of the self-hsp immune response, especially the specific domain homolog to the bacteria.

Cell surface expression of self-hsps

In a number of autoimmune diseases, hsp expression has been observed in affected cells. Frequently, cell surface expression of hsps is increased in affected tissues in autoimmune diseases, emphasizing a role for hsp determinants in autoimmunity. It has been reported that epidermal expression of hsps is significantly increased in patients with BD [60]. Therefore, overexpression of self-hsps on the target organs allows the hypothesis that hsp-specific T cells may play a role in the pathogenesis.

Host responses to the other bacterial antigens

Fox et al. [61] reported that peptidoglycan provoked chronic inflammation and retinal necrosis similar to that observed in eyes injected with lipopolysaccharides (LPSs). However, due to the crude nature of the cell-wall extracts injected, the specific basis for cell wall-induced inflammation was not determined. Recent evidence suggests that tertiary configuration of peptidoglycan, and its association with the cell wall, or with lipoteichonic acid can directly affect the degree of inflammation. With regard to intraocular inflammogenicity of the cell wall, neither metabolically inactive pathogens nor purified sacculi caused a significant reduction in retinal responsiveness, but they evoked significant inflammation in both the posterior and anterior segments of the eye [62]. It has been demonstrated that lipoteichonic acid from *S. sanguis* has the biological activity to induce intraocular inflammation by intravitreal or intravenous injection [63]. Thus, the cell wall components may also play a role in the pathogenesis of uveitis.

The sequence of *bes-1* encoding a streptococcal antigen has been determined, and reactivity against this protein has been shown in BD patients [64]. The amino acid residues in a portion of *bes-1* show 60% similarity to the human intraocular peptide Brn-3b. Brn-3, a subfamily of POU (*pit-Oct Unc*) domain factors, contains three members, *Brn3a, Brn3b* and *Brn3c*. The POU domain has been found in a class of transcriptional regulators that appear to have an important role in tissue-specific gene regulation. Pit1 plays a critical role in development of the pituitary and regulation of prolactin and growth hormone synthesis; Oct1 is a ubiquitous transcription factor and Oct2 regulates immunoglobulin synthesis in B lymphocytes. Brn-3b is first expressed in migrating, postmitotic ganglion cell precursors in the ventricular zone of developing mouse retinas. However, the role of the protein in the pathogenesis of BD is unknown.

Immunology of BD

Cytokines

BD is characterized by acute inflammatory attacks during a chronic disease course; and cytokines, which are produced by a wide variety of cells, play a crucial role in these inflammatory reactions. Upregulated expression of proinflammatory cytokines and chemokines as well as Th1-type cytokines has been demonstrated in parallel with the clinical activity of BD [65]. However, an enhanced cytokine response can be elicited even in inactive patients following appropriate stimulation [66].

Increased or detectable serum/plasma levels of several cytokines, chemokines and cytokine receptors including TNF-α, IFN-γ, IL-1, IL-8, IL-10 , IL-12, soluble IL-2 receptor (sIL-2R) and 75-kDa TNF receptor (TNFR-75) were detected in BD [67–81]. Serum levels of IL-8, IL-12, sIL-2R and TNFR-75 were reported to be correlated with the clinical activity of BD, whereas lymphocyte-derived IFN-γ was found only in the sera of inactive patients [67, 73–80]. Many investigators reported negative results as well for the detectable levels of various cytokines in the sera of patients with BD, mainly reflecting the problems of measurement of systemic levels of locally active and transiently produced small molecules with a very short half-life, and also the tight regulatory mechanisms of cytokine expression, including soluble receptors and/or receptor antagonists [72, 73, 76, 78, 82, 83].

Mege and colleagues detected spontaneous overproduction of TNF-α, IL-6 and IL-8 in the culture supernatants from monocytes of patients with active BD. When these monocytes were stimulated with LPS, increased production of TNF-α, IL-1, IL-6 and IL-8 was observed in both inactive and active patients [66].

Analysis of intracytoplasmic cytokine expression of individual cells using flow cytometry revealed that the frequency of IL-2 and IFN-γ producing T cells was increased in patients with active BD [80, 84]. Compatible with a strong polarized Th1 immune response in BD, serum levels of IL-12 were also found to be elevated in parallel with the increase in Th1 cells [80]. However, the proliferative response of concanavalin A-activated T cells to exogenous IL-2 was reported to be abnormal in BD [85, 86]. The unresponsiveness of T cells to IL-2 was

explained by a substantial decrease in cells bearing high-affinity IL-2R in patients with active BD, and a decrease in the relative numbers of IL-2R on T cells in inactive disease [85, 86].

The decrease in Th1 cells accompanied the improvement in clinical activity of BD without a reciprocal increase in the IL-4 producing Th2 cells [80, 84]. However, the elevated serum level of another Th2 cytokine, IL-10, was reported in BD without any correlation with disease activity [74]. This finding is not contradictory to a Th1-polarized immune response and can be explained by compensatory mechanisms. Upregulated expression of IL-12 receptor β chain and transcription factor Txk in T cells further supports the role of a Th1-polarized immune response in the pathogenesis of BD [87].

Analysis of $\gamma\delta$ T cell populations also revealed that a significantly increased proportion of $\gamma\delta$ T cells produce both IFN-γ and TNF-α and express CD25 (the α chain of IL-2R) on their surface [88, 89]. Yamashita and colleagues reported that CD45RA+ $\gamma\delta$ T cells produce more TNF-α and lymphotoxin-α (LT-α) than CD45RO+ $\gamma\delta$ T cells, while both subsets produce equal amounts of IL-8, but no IL-4 [90].

It was recently demonstrated that neutrophils from patients with BD constitutively express TNF-α messenger RNA (mRNA) and increased amounts of TNF-α with LPS stimulation [91, 92]. BD neutrophils also produce IL-12 and IL-18 spontaneously, which suggests that these activated neutrophils might play a role both in priming themselves and also in the Th1 polarization of the immune response [91, 92].

Infiltration of IFN-γ, IL-6, IL-8 and TNF-α positive cells were observed in a limited number of studies investigating the cytokine expression in mucocutaneous lesions of BD [93, 94]. The enhanced expression of proinflammatory cytokines suggests a defect in the regulatory mechanisms, and it may have a genetic basis. But no strong association could be documented so far between a cytokine gene polymorphism and BD [95, 96].

Although there have been some attempts such as HLA-B51 trangenic mice, hsp-derived peptide-induced uveitis in Lewis rats and herpes simplex virus-induced disease in the ICR mice, there is no established animal model for BD [97–99]. All of these animal models are far from answering questions in BD, especially for understanding the role of cytokines in the pathogenesis.

Very successful and promising results have been obtained using cytokines (such as IFN-α) or cytokine antagonists (anti-TNF-α monoclonal antibodies or TNF receptors) in recent trials for the resistant cases of BD [100–105]. IFN-α has also been shown to reduce the amount of intraocular inflammation in experimental autoimmune uveitis models in rats [105] (after oral and intramuscular administration) and mice (after intramuscular administration) [106, manuscript in preparation]. Okada and colleagues [105] have shown in rats a significant decrease in TNF-α and IFN-γ after IFN-β treatment of interphotorecepter retinoid-binding protein (IRBP)-stimulated splenocytes. Intramuscular administration reduced intraocular IFN-γ and IL-10, suggesting that Th1 and Th2 cells were both suppressed by IFN-α. It seems that the route of administration also influences the cytokine pattern. Analysis of peripheral blood mononuclear cells of BD patients, treated with IFN-α, disclosed significantly decreased levels of NK cells, CD3/TCR$\gamma\delta$+, CD8/TCR$\gamma\delta$+ and CD8/TCR$\alpha\beta$+ T cells. Enhanced expression of HLA-class I antigens in lymphocytes and monocytes was also observed. Before therapy, the levels of CD14+ monocytes, CD3/TCR$\gamma\delta$+, CD8/TCR$\gamma\delta$+ cells, NK cells (CD56/16+) and activated regulatory T cells (CD4/25+, CD8/25+) were elevated in BD patients, compared with healthy control patients [107].

Understanding the characteristics of the cytokine network in BD would help to develop better treatment modalities which may target more specific inflammatory mediators.

T cells

T-cell-mediated immune responses play a central role in the pathogenesis of BD [108]. Enhanced non-specific inflammatory reaction is an important feature of BD. However, an antigen-driven immune response superimposed on this hyperreactivity has been suggested to trigger manifestations of BD [108]. Oligoclonal T-cell expansions correlate with clinical activity of patients with BD, and they show a Th1-type cytokine expression pattern in parallel with activation. T-cell-dominated perivascular infiltrates have mainly been observed in histopathological investigations of the involved tissues [109–113].

Several phenotypical and functional abnormalities were reported in both $\alpha\beta$+ and $\gamma\delta$ T cell subsets in BD. Many investigators detected a reduced CD4+/CD8+ T cell ratio resulting from both a decrease in CD4+ T cells and a concomitant increase in CD8+ T cells [114–116]. A significantly lower frequency of CD4+CD45RA+ (suppressor-inducer) T cells was found in the peripheral blood lymphocytes of patients with active BD compared with healthy controls, despite similar CD4+CD29+ (helper-inducer) T cell frequencies in both groups [117]. Abnormal suppressor activity of T cells was observed at the start of disease exacerbation, which returned to normal when patients became fully active or entered into an inactive phase of BD, suggesting a suppressor regulatory defect in the development of the disease manifestations [114].

The proportions of peripheral blood $\gamma\delta$ T cells and CD8+ $\gamma\delta$ T cells were consistently reported to be elevated in BD [8, 9, 88–90, 118]. A majority of these $\gamma\delta$ T cells were found to be expressing activation markers such as the

α chain of IL-2R (CD25), CD45RA and also producing IFN-γ, TNF-α and IL-8 [88–90].

Analysis of peripheral blood T cell receptor V and J segment gene usages revealed oligoclonal T cell expansions in both CD4+ and CD8+ T cell subsets in BD [8]. T cell expansions were monitored for up to 20 months in eight patients, and at least one of the expansions was reduced and showed a correlation with improved clinical activity in six of them. The restricted V and J gene usages suggested an antigen-driven immune response caused by conventional antigens in the pathogenesis of BD [8, 10]. The authors suggested that involvement of different antigens in the induction of each of the various manifestations of BD might in part explain the observed heterogeneous Vβ gene usages [8]. A recent study also reported oligoclonal T cell infiltration in the anterior chamber of a patient with BD [119].

Several antigens have been reported to stimulate T cells in BD in a hypersensitive manner. T cells can be stimulated by streptococcal antigens to produce IL-6, IFN-γ or neutrophil-potentiating factors [120, 121]. On the other hand, *E. coli*-derived peptides can also stimulate T cells of patients with BD for IFN-γ production [121]. Certain epitopes of microbial hsps were suggested as triggering antigens for cross-reacting inflammatory reactions [56, 119, 122]. Pervin and colleagues identified four peptides from 65-kDa mycobacterial hsps (111–125, 154–172, 219–233, 311–325) and their homologous peptides from human 60-kDa hsps (136–150, 179–197, 224–258, 336–351) as specific epitopes for BD by T-cell-epitope mapping using short-term cell lines from British patients [56]. A similar increased T cell response against these hsp peptides was later documented in Japanese and Turkish patients with BD [10, 122]. Subcutaneous immunization with the hsp peptides induced anterior uveitis in 80% of Lewis rats when they were given in complete Freund adjuvant and accompanying intraperitoneal inoculation of *Bordatella pertussis* [98]. Attempts to induce mucosal tolerance by oral or nasal administration of peptide 336–351 from the human 60-kDa hsps failed, and unexpectedly, up to 90% of rats developed anterior uveitis with mucosal hsp feeding [57]. Treatment using anti-CD4 antibodies reduced the rate of uveitis from 82 to 25% in a dose-dependent manner [57]. However, none of the rats developed posterior uveitis and/or retinal vasculitis similar to the uveitis of BD, and no other finding was detected in the mouth, skin or external genitalia of these animals [57, 98].

Hasan and colleagues reported a significant proliferative response to four mycobacterial hsp-derived peptides in a specifically $\gamma\delta$ T cell subset [9]. The T cell response against mycobacterial hsp peptides showed correlation with the disease activity. The homologous human 60-kDa hsp peptides also stimulated T cells of patients with BD; however, the intensity of stimulation was lower than that of mycobacterial peptides [9]. The authors suggested a regulatory role for these specifically proliferating $\gamma\delta$+ T cells on the $\alpha\beta$+ T cells. On the other hand, Japanese and Turkish studies did not confirm the findings of Hasan and colleagues regarding specific hsp-proliferative response in $\gamma\delta$ T cells [57, 10]. Kaneko and colleagues found an antigen-driven oligoclonal expansion in CD4+ subset of T cells in response to the 336–351 peptide from the human 60-kDa hsps in Japanese patients with BD [117]. The T cell proliferative response to this peptide also showed an association with eye involvement, and proliferating T cells expressed proinflammatory cytokine mRNAs, including IL-8, TNF-α and TNF-β [10].

Hirohata and colleagues showed that this hypersensitivity of BD T cells is not restricted to disease-specific peptides [123]. Staphylococcal enterotoxins could stimulate BD T cells through T cell receptor (TCR) β chain for IFN-γ production at very low concentrations that were not able to stimulate T cells from normal controls or rheumatoid arthritis patients [123]. Following stimulation, there was no selective expansion of Vβ5 and Vβ12 T cells in patients with BD, which were expected to be stimulated by these staphylococcal superantigens. There was also no significant difference in the T cell response to TCR-independent anti-CD3 stimulation between patients with BD and rheumatoid arthritis. Thus, it might be suggested that this hyperresponsiveness results from an intrinsic T cell defect affecting signal transduction through TCR, and is not confined to certain antigens [123, 124].

In addition to the hypothesis of T cell cross-reactivity to the oral mucosal or retinal tissues because of a molecular mimicry between bacterial and self-hsp molecules [45, 125], other observations indicate T cell response against autoantigens which have no similarity to hsps, such as retinal proteins. S-antigen is one of the best-characterized retinal antigens, which can induce experimental autoimmune uveitis in rats. Cellular reactivity against retinal soluble antigen (S-antigen) was reported in BD patients with uveitis [126, 127]. But lymphocyte reactivity was documented against other retinal antigens such as IRBP and S-antigen-derived peptides even in some patients without ocular inflammation [126].

Wildner and colleagues identified a polymorphic HLA-B sequence common in HLA-B27, -B51 and several other HLA-B alleles (B27PD), which shares amino acid homologies with retinal S-antigen [128]. HLA-B-derived peptides can act as autoantigens, and folding abnormalities or aberrant assembly of HLA class I heavy chains as well as enhanced expression due to upregulated immune response increase the possibility of presentation of HLA class I-derived peptides by HLA class II molecules. Although less severe in intensity compared with S-antigen induced inflammation itself, HLA-B-derived B27PD peptide can also induce anterior uveitis in rats [128]. Kurhan-Yavuz and colleagues demonstrated increased T

cell response against retinal S-antigen, retinal S-antigen-derived peptide and B27PD peptide in BD patients with posterior uveitis compared with those with non-Behçet's anterior uveitis or Behçet's patients without eye disease [129]. The role of the autoimmune response against retinal antigens in the initiation phase of uveitis is not clear yet. However, increased frequency of S-antigen-responsive lymphocytes during uveitis attacks suggests that damage of retinal tissue due to recurrent uveitis attacks may uncover sequestered epitopes in immune-privileged regions of the eye and can cause progression of T cell reactivity against self-antigens [127, 129].

Role of NK and NK-T cells

NK and NK-T cells kill target cells, including tumor cells via Fas-mediated or perforin-dependent pathways without prior sensitization [130–132]. In addition, it has been considered that NK and NK-T cells may represent cells involved in cross-talking between innate and acquired immunities, since these cells produce various cytokines, including IFN-γ and IL-4 at a very early stage of inflammation and/or immunological response [133–137]. IFN-γ and IL-4 determine the type of subsequent immunological response (Th1 or Th2). Thus, it seems that NK and NK-T cells play a role in induction and/or regulation of various types of immune reponses, including several autoimmune diseases, through cytotoxicity and cytokine production [130, 135, 138]. On the other hand, it has been reported that certain ocular inflammations, such as BD, result from organ-specific autoimmune responses of the Th1 type [139, 140].

Some investigators reported increased frequencies of both NK (CD16+CD56+) cells and CD56+ T cells in the peripheral blood of BD patients [6, 141]. However, some observed increases only in CD4+CD16+ and CD4+CD56+ T cell subsets, but normal levels of CD16+CD56+ NK cells in BD [142]. Hamzaoui and colleagues even reported decreased frequencies of CD16+ cells in active patients [143]. Activity of NK cells in BD is another controversial issue. Thus far, no concrete evidence shows whether NK or NK-T cells are directly involved in induction or regulation of BD.

NK cells lack receptors specific for a particular antigen. Instead, murine NK cells express Ly 49, a lectin-type receptor (fig. 2). When Ly 49 binds a certain type of MHC class I, an inhibitory signal is transduced intracellularly, and the NK cells stop killing the target. On the other hand, NK cells kill the target when Ly 49 is blocked by antibody (Ab) or those lacking MHC class I. Figure 2 shows a primitive framework for the 'missing-self' hypothesis [144].

In 1987, Fowlkes and colleagues [145] and Ceredig and colleagues [146] reported a unique thymocyte population in CD4/CD8 double-negative (DN) cells. We found a

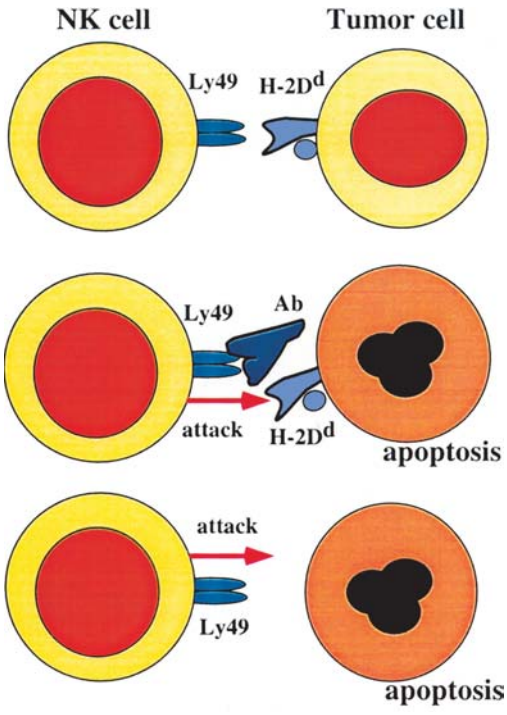

Figure 2. NK cells kill the target missing MHC class I expression.

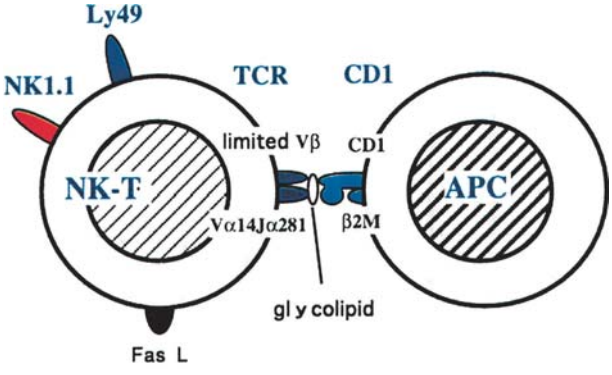

Figure 3. Surface molecules of NK-T cells and their recognition molecules. β2M, β-2 microglobulin.

similar population in CD4+ thymocytes [147]. Now cells are called NK-T cells and constitute more complex subsets [148, 149]. In the thymus, NK-T cells are positively selected in the presence of CD1 or TL molecules expressed on CD4/CD8 double-positive (DP) thymocytes [150, 151]. However, we have demonstrated that thymic medullary epithelial cells are also indispensable for the NK-T generation [152, 153]. NK-T cells express both NK receptors (NK1.1, Ly49) and α/βTCR (fig. 3). These cells phenotypically resemble memory T cells.

In the peripheral immune system, it has also been speculated that NK-T cells regulate excess or undesirable immune responses [138, 154, 155]. As shown in figure 4, a self-Ag reactive helper T cell is continuously activated

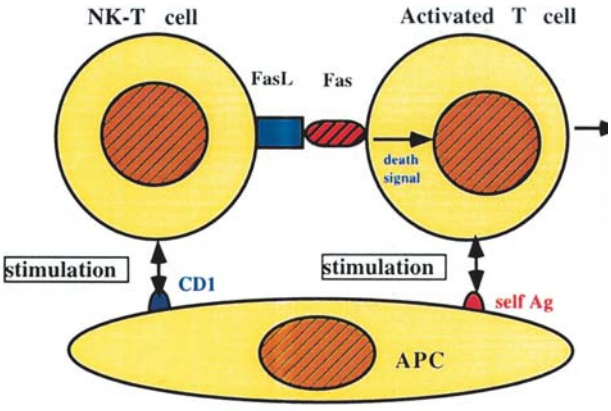

Figure 4. NK-T cell may kill self-reactive T cells via Fas L-Fas interaction.

with self-Ag in vivo and expressing Fas molecules. Under normal conditions, NK-T cells kill this helper T cell, and the autoimmune responses appear to be stamped out before harmful tissue damage takes place. Indeed, it has been reported that the number of NK-T cells decreases in certain types of systemic autoimmune disease in animal models and human cases [156, 157]. Recently, we found that the decrease in NK-T cells was dependent on the pathogenesis of autoimmune-prone mice [158]. At any rate, the decrease in the number of NK-T cells may be related to insufficient regulation of immune responses to self-antigen by NK-T-cells.

Role of NK and/or NK-T cells in experimentally induced EAU, an experimental model of BD

It has been reported that a peptide, K2, deduced from IRBP induces EAU in H-2^k mice (fig. 5) [159, 160]. Using this model, we examined whether NK-T cells play a regulatory role in EAU. H-2^k mice were administrated anti-NK1.1 Ab and immunized with K2 in Freund's complete adjuvant. However, treatment with anti-NK1.1 Ab exerted no influence on T cell response to K2. In addition, anti-NK1.1 Ab treatment rather reduced the clinical severity of EAU [161]. Since this Ab treatment differentially influences NK and NK-T cell populations, it seems difficult to draw a straightforward interpretation from this preliminary experiment. Thus far, our study suggests that NK and/or NK-T cells may enhance local inflammation rather than operate negative regulation. Recently, it was reported that the number of CD56+ T cells increases in patients with BD [107, 162]. Although CD56+ T cells do not necessarily correspond to NK-T cells, this result appears to be consistent with our experiment. In addition, we reported that activated NK-T cells express mRNA of macrophage migration inhibitory factor (MIF) [163]. Thus, it seems that upon stimulation, NK-T cells produce MIF in addition to IL-4 and/or IFN-γ. Administration of anti-MIF Ab inhibited R16, (a peptide deduced from IRBP), induced EAU in a rat system [164]. Indeed, serum MIF levels in patients with BD increased markedly [165]. This series of studies also suggests that NK-T cells enhance ocular inflammation via cytokine production. We have reported that CD8+ NK-T cells produce IFN-γ but not IL-4 [166]. In addition, Miyamoto and colleagues [155] found that OCH, an analog of α-GalCer, induces NK-T cells to produce IL-4, but not IFN-γ. Thus, the role

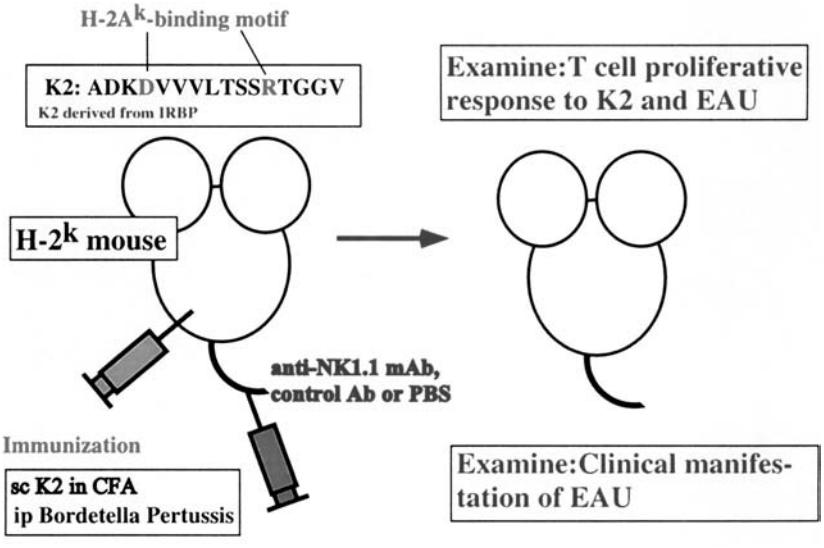

Figure 5. Experimental procedure of K2-induced EAU. To remove NK1.1+ cells mice were injected intravenously with anti-NK1.1 mAb.

of NK-T cells in vivo appears to be determined by NK-T subsets and the nature of stimulatory molecules involved in the response.

Neutrophils

Neutrophils are very important elements of the innate immune response, and they have been postulated as one of the main players of the immunologic dysfunction in BD [167]. An infiltration of neutrophils has been frequently reported in all affected tissues in BD depending upon the timing of the sampling [168–171], and some even called BD a neutrophilic vasculitis.

Most of the phenotypical and functional studies showed findings compatible with in vivo activation of neutrophils [172]. Some cytokines and microbial products such as TNF-α, GM-CSF and formyl peptides stimulate neutrophils to a 'primed' state instead of full activation. Neutrophils can be activated more easily and rapidly with proper signals when they are in the primed state.

Hyperactivity of neutrophils in BD was demonstrated by increased chemotaxis, phagocytosis, superoxide generation and myeloperoxidase levels, as well as higher expression of CD11a, CD10 and CD14 on the cell surface [46, 66, 173–177]. Some of the controversial results, especially in chemotaxis and superoxide generation observed in BD neutrophils under in vitro conditions, can be explained by the differences in the primed state of neutrophils reflecting the clinical activity of BD patients, as well as methodological differences.

The mechanism underlying the hyperfunction of neutrophils in BD remains to be explored. Association of HLA-B51 with enhanced neutrophil function is still a controversial issue (see above) and needs to be confirmed by other methods [31, 172]. Previous studies demonstrated the importance of T cell activation in the stimulation of neutrophils in BD [120]. Supernatants of T cell cultures stimulated either with mitogens or steptococcal antigens caused an increase in neutrophil chemotaxis, phagocytosis and superoxide generation [120]. Enhanced expression of proinflammatory cytokines and chemokines in BD, including TNF-α and IL-8, are suggested to be responsible for the primed state of neutrophils. Incubation of neutrophils from healthy individuals with sera from patients with BD resulted in an increase in adhesion to endothelial cells and also in the expression of CD11a and CD18 [74].

A primary defect in neutrophils specific for BD has not been described so far. However, two-dimensional gel electrophoresis analysis of neutrophil proteins revealed a selective increase of N-terminal truncated actin with a molecular weight of 40-kDa in BD [178]. This truncated form of actin is generated by proteolytic elastase enzyme and may affect the polymerization of actin molecules [179].

Endothelial cells

Vascular involvement is an important feature of BD, and its frequency changes from 9 to 40% in different series of patients [180–184]. Affection of both veins and arteries of all sizes with accompanying thrombotic tendency determines the unique characteristics of BD vasculitis and makes it very difficult to classify among other systemic vasculitides.

Vascular involvement in BD is more frequent and has a more severe course in males. The venous side of the vasculature has predominantly been affected in BD, most commonly as superficial thrombophlebitis and deep vein thrombosis of the lower extremities. However, thrombosis can be seen in any vein, such as in inferior and superior vena cavae, hepatic veins and cerebral venous sinuses. The thrombus seen in BD is defined as sticky to the inflamed vessel wall, and hence pulmonary thromboembolism has been reported very rarely. Arterial involvement is seen less frequently [180, 183]. A true and/or false aneurysm formation is the typical form of arterial disease; however, a thrombotic occlusion can also be seen.

Thrombotic tendency is the unique feature of BD amongs other systemic vasculitides, and understanding the exact mechanism underlying this 'procoagulant state' is an elusive task [185, 186]. Perivascular mixed cellular infiltration is a common finding in all of the manifestations; and endothelial dysfunction, which results from immune-mediated inflammatory infiltrate, is believed to be the basis of the observed thrombotic tendency in BD.

Endothelial dysfunction

Endothelial cells have pleiotropic functions which maintain the integrity of the vessel lumen to keep the blood flow intact through procoagulant, anticoagulant and fibrinolytic activities. Several studies have revealed increased blood levels of endothelium-derived von Willebrand factor, thrombomodulin and E-selectin, indicating endothelial activation and/or injury in BD [187–201]. Elevated plasma von Willebrand factor levels were detected even in patients in complete remission [187]. Basal thrombomodulin levels were found to be elevated, and no more increase could be observed following desmopressin infusion [188, 191]. Some investigators report decreased levels of soluble thrombomodulin levels in patients carrying factor V Leiden mutation [192].

Endothelium-dependent flow-mediated dilation of arteries was found to be reduced in BD compared with control subjects, using brachial artery Doppler investigation [193]. Flow-mediated dilation of arteries is dependent on the function of constitutive endothelial nitric oxide synthase (eNOS), which produces nitric oxide from L-arginine. Recently, a single nucleotide polymorphism in the

eNOS gene (Glu298Asp) was found to be associated with BD [194]. Despite indications of impaired function, both decreased and elevated plasma levels of nitric oxide metabolites were reported in active patients [195–197]. In inactive patients, increased activity of the superoxide dismutase enzyme, which has a scavenger function for superoxides, also suggests the involvement of nitric oxide metabolites in the pathogenesis of BD [198].

Impaired synthesis of endothelial cell-derived prostaglandins was previously reported in active BD patients [199]. On the other hand, others reported increased levels of both endothelium-derived antiaggregant $PGF_1\ \alpha$ and platelet-derived proaggregant TXB_2 levels in patients with BD [200].

Antibodies against endothelial cells have been detected in up to 50% of patients with BD, especially when microvascular endothelial cells are used as antigen source [187, 201–203]. Western blot analysis revealed a 44-kDa endothelial cell surface antigen reacting with immunoglobulin (IgM) anti-endothelial cell antibodies (AECAs) in the sera of patients with BD [203]. Most studies show a correlation between the presence of AECAs and clinical activity of the patients. However, no complement-mediated cytotoxic activity of these antibodies on the endothelial cells could be documented, and the functional importance of AECAs other than reflecting endothelial injury is yet to be shown [201, 204].

Coagulation and fibrinolytic pathway abnormalities

No specific defect in the coagulation cascade has so far been demonstrated in BD [185, 186, 189, 205]. However, several findings indicate activation of both coagulation and fibrinolytic pathways in BD patients with or without thrombosis. Increased levels of thrombin-antithrombin III complex (TAT) and prothrombin fragment 1+2 support intravascular thrombin generation in these patients as a result of activation of the coagulation cascade [189, 191]. All of the known inherited or acquired procoagulant conditions associated with increased risk of thrombosis, such as deficiencies of protein C, protein S and antithrombin III, or factor V Leiden and prothrombin 20210A mutations, or hyperhomocysteinemia, also contribute to the prothrombotic state of BD to some extent depending on their prevalence in the population. There are reports of BD patients with extensive thrombosis due to coexistence of protein C, protein S deficiencies or factor V Leiden and prothrombin gene 20210A mutations [206–209]. Severalfold increases in the risk of thrombosis have been described in carriers of factor V Leiden and prothrombin gene mutations in patients with BD [210–213]. An association between hyperhomocysteinemia and thrombosis has also been described in BD [214]. Some studies report the presence of anticardiolipin antibodies in up to 25% of patients with BD. However, a recent reassessment did not point out a primary role for anticardiolipin antibodies in the thrombotic tendency of BD [215].

Increased levels of plasmin-α_2-antiplasmin complex (PAP) in BD patients possibly indicate fibrinolytic activity compensatory to excessive thrombin generation [191]. On the other hand, some studies revealed defective fibrinolysis following venous occlusion or after desmopressin infusion, or impaired plasminogen activator binding kinetics [216–218]. Tissue plasminogen activator (t-PA) activity was found to be decreased, normal or increased in different studies [205, 218, 219]. The concentrations of free t-PA, u-PA and free plasminogen activator inhibitor-1 (PAI-1) were elevated in BD, but the antigen (free and PAI-1 bound) levels of t-PA and u-PA were normal, suggesting defective t-PA/PAI-1 complex formation [218].

Conflicting results in different studies may have arisen from various reasons: differences in disease activity of patients, measurement of circulating levels of hemostatic parameters, which are generally active locally, and varying prevalence of procoagulant mutations in different populations. Some of these studies also lack power for conclusive results, mainly because of the low number of patients.

All of the hemostatic studies in BD support an imbalance towards a prothrombotic state at different levels. Endothelial dysfunction resulting from immune-mediated vasculitis is the main determinant in this thrombotic tendency, and no other disease-specific defect has been defined so far. None of the findings of these prothrombotic markers differs in patients with or without clinically overt thrombosis. Any condition which contributes further to the prothrombotic side of this dysequilibrium, such as the presence of common procoagulant polymorphisms, an inflammatory insult on endothelial cells due to an increase in disease activity or trauma, can be the critical factor determining the development of a thrombotic event.

Conclusions

There is evidence that various factors play important roles in the etiology of BD. Besides hereditary factors, immunological dysfunctions have been observed, probably triggered by exogenous antigen(s) found on bacteria, viruses or other microorganisms. The real pathogenic gene involved in the development of BD is the HLA-B gene itself, and the major disease susceptibility allele is HLA-B51 (HLA-B*51 including HLA-B*5101, B*5102 and B*5108 at the DNA allele level). The presence of HLA-B51-negative BD patients can be explained by the influence of other genetic factor(s) and/or of various external environmental or infectious agent(s). In relation to

this aspect, some of us (N. M., S. O.) are planning to conduct genome-wide mapping of BD pathogenic gene(s) with association analysis using more than 30,000 polymorphic microsatellite markers which we have recently collected from the human genome at 100-kb (which corresponds to the average length of the linkage disequilibrium of microsatellite markers) intervals.

Whether the initiating event is a microbial infection or inflammation at a site of manifestation is important for the presentation of unusual, generally nondisplayed antigenic determinants to immune systems. In BD, various conditions must exist to enable a self-reactive response. Hsp336-351 is a uveitogenic antigen, and the immune response against this hsp can occur in BD. Specific bacterial infection such as *S. sanguis* on the mucosal surface can bring self-reactive responses and a Th1 cytokine array that will lead to enhanced MHC display and hightened levels of antigen processing. If regulation is not impaired, these events are usually brought under control.

BD seems to be associated with multiple dysfunctions of T cells (e. g. strong Th1 response, $\gamma\delta$-T-cells) and consequently expression of high amounts of IL-2 and IFN-γ. Even inactive patients could disclose enhanced cytokine responses following appropriate stimulation of their lymphocytes. TNF-α could be a key cytokine for this disorder, because reduction of TNF-α using blocking substances or IFN-α results in amelioration of the disease. Several antigens have been reported to stimulate T cells in BD, such as streptococcal antigens and mycobacterial hsps.

Involvement of NK and NK-T cells in the pathogenesis of BD is still a controversial issue. It appears that NK1.1+ cells rather enhance ocular inflammatory responses. However, our study (K. O.) is just beginning, and experimentation in which specific elimination of NK-T cell populations in vivo is performed appears to be needed. We (K. O.) are now carrying out EAU experiments in NK-T knockout mice to elucidate the precise role of NK-T cells. Besides lymphocytes, neutrophils also seem to play a major role in BD. Hyperactivity of neutrophils in BD has been found, probably induced by TNF-α and IL-8.

Finally, endothelial dysfunction as well as abnormalities of coagulation and fibrinolytic pathways are documented in BD patients. Hemostatic studies indicate an imbalance towards a prothrombotic state in BD patients which probably contributes to disease pathogenesis by causing occlusive vasculopathy.

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