Expression of Th2 Cytokines Decreases the Development of and Improves Behçet's Disease–Like Symptoms Induced by Herpes Simplex Virus in Mice

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In the etiology of Behçet's disease (BD), viral infection has long been postulated as a contributing factor, and viral involvement has been demonstrated. However, viral infection alone is not sufficient to explain the pathogenesis of BD, and some evidence suggests that immunologic abnormalities are also important. To study the possible role of immune regulation in the development of BD-like symptoms induced by herpes simplex virus inoculation in ICR mice, macrophages were deleted by use of liposome-encapsulated clodronate (lip-Cl_2MDP). Treatment with lip-Cl_2MDP suppressed the development of BD-like symptoms, and this suppression was correlated with the induction of interleukin-4 expression in mouse spleens. When the Th2 adjuvant ovalbumin (OVA)–alum was injected into mice with BD-like symptoms, their cutaneous symptoms improved. Adoptive transfer with splenocytes from OVA-alum–injected mice also resulted in improvement. These findings suggest that up-regulated Th2 cytokine expression can attenuate the development of and improve some BD-like symptoms.

Behçet's disease (BD) is a chronic multisystemic disorder with mucocutaneous, ocular, arthritic, vascular, gastrointestinal, and central nervous system involvement. The etiology of BD has been linked to viral infection, autoimmune disease, streptococcal-related antigens, specific alleles of the human major histocompatibility complex, and hazardous chemicals [1–4]. Since Hulûsi Behçet first propounded viral etiology in 1937 [5], many studies have suggested a viral involvement in the disease. Sezer [6] isolated a specific virus from patient fluids, and Eglin and Lehner [7] showed herpes simplex virus (HSV) type 1 gene fragments in peripheral blood mononuclear cells of patients with BD by means of in situ DNA-RNA hybridization [7]. By use of DNA-DNA dot blotting and polymerase chain reaction (PCR) analysis, HSV DNA also was detected in leukocytes and saliva from patients with BD [8, 9].

Treatment with acyclovir, which is of proven efficacy in the treatment of HSV infection, did not much alleviate the frequency or severity of orogenital ulceration or other clinical features of BD [10]. Lymphocyte dysfunction has been reported by several research groups. When stimulated by small doses of staphylococcal enterotoxins (superantigen), T lymphocytes from patients with BD had a distinctly lower threshold for interferon (IFN)–γ production than lymphocytes from patients with rheumatoid arthritis or from healthy control subjects [11]. The mucocutaneous lesions of BD were initially infiltrated with CD4 and CD8 cells, macrophages and dendritic cells, followed by neutrophils. Recent attention has focused on Th1 and Th2 cytokines. Persons with active BD have significantly more interleukin (IL)–2–producing CD4 cells than do inactive case subjects and control subjects [12], and IL-12 plasma level and disease activity are correlated [13]. These results suggest that Th1 cells may play an important role in the immunopathogenesis of BD, although others have reported that the cytokine production profile has a mixed Th1/Th2 cell type in active BD [14]. In this study, we attempted to determine whether inactivation of macrophages influences the development of BD and whether related cytokines play a role in the modulation of BD symptoms.

Materials and Methods

Animals and induction of BD symptoms. We used 4–5-week-old male ICR mice (Korean Laboratory Animal Research Center) for this study. By using the method of Hirata et al. [15], we scratched the earlobes of the mice with a needle and then inoculated them with 10^6 pfu/mL of HSV-1 (F strain). Virus inoculation was done 2 times 10 days apart, which was followed by 16 weeks of observation [16]. Mice were bred in temperature- and light-controlled conventional rooms (20°C–22°C; 12-h light cycle starting at 8:00 A.M.). The mice had free access to food and water. During the experimental period, the animals were closely observed and were photographed.

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Animals were handled in accordance with a protocol approved by the Ajou University animal care committee.

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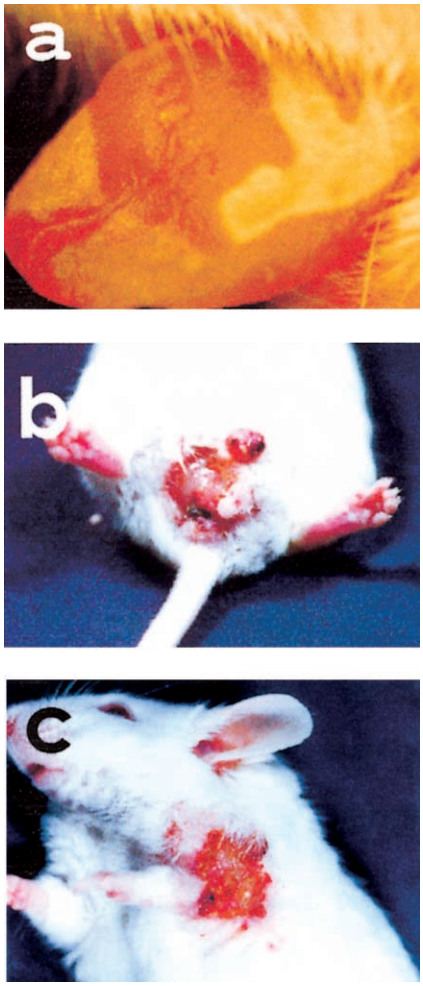

Figure 1. Behçet's disease–like symptoms (oral, genital, and skin: *a–c*, respectively) in herpes simplex virus–induced ICR mice.

Gross observation of BD symptoms. To classify the symptomatic mice as having BD, we followed a revised Japanese classification with minor modifications. Oral, genital, and other skin ulcers (including bulla and crust) and eye symptoms were classified as major symptoms. Arthritis, gastrointestinal ulcers, and neurologic involvement were identified as minor symptoms. Mice with ⩾1 major and 1 minor symptom were classified as having BD. Symptomatic mice were photographed with a Nikon FM2 camera equipped with a 105-mm microlens.

Liposome-encapsulated chlodronate (Lip-Cl_2MDP) preparation. Lip-Cl_2MDP was prepared, as described elsewhere [17]. In brief, 75 mg of phosphatidyl choline and 11 mg of cholesterol (Sigma) were dissolved in chloroform in a round-bottom flask. After low-vacuum rotary evaporation at 37°C, the lipids were dispersed by gentle rotation in 10 mL of PBS, in which 2.5 g of dichloromethylene diphosphonate (Cl_2MDP; Boehringer Mannheim) was dissolved. The resulting liposomes were washed 2 times at 100,000 × *g* for 30 min, to remove free nonencapsulated Cl_2MDP. The liposomes then were resuspended in 4 mL of PBS. Lip-Cl_2MDP was injected intravenously in mice to inactivate their macrophages.

Table 1. ICR mice manifesting symptoms of Behçet's disease (BD) after herpes simplex virus (HSV) inoculation and/or immune cell inactivation.

Treatment	Mice with BD symptoms	Dead mice
Lip-Cl_2MDP	0/50	0/50
Lip-Cl_2MDP + HSV	0/50	9/50 (18.0)
HSV	14/50 (28.0)	10/50 (20.0)

NOTE. Data are no. of mice/total no. of mice (%). Lip-Cl_2MDP, liposome-encapsulated clodronate.

Mice were treated intravenously with 200 μL of lip-Cl_2MDP 2 days before HSV infection.

Antibody preparation. We cultured a hybridoma cell line as follows: ATCC TIB-166 (M3/38.1.2.8 HL2), an anti-mac2 antibody-producing cell line, was cultured in RPMI 1640 medium with 20% fetal bovine serum (FBS). At the time of cell lysis, the supernatant was collected and concentrated (Centriplus-50; Amicon). We measured the concentration of antibody by using a protein assay kit (Bio-Rad). Concentrated antibodies were used for immunohistochemistry.

Immunohistochemistry. Mouse spleen tissues were embedded in paraffin blocks and were used for histochemistry. Primary antibody (anti-mac2) was produced in our laboratory by using American Type Culture Collection hybridoma cell lines (anti-mac2 antibody, ATCC TIB-166, and M3/38.1.2.8 HL2), since none were commercially available. We used horseradish peroxidase–conjugated rabbit anti–rat immunoglobulin as secondary antibody.

RNA isolation and reverse transcriptase (RT)–PCR. Total RNA was isolated by acid guanidium thiocyanate–phenol-chloroform extraction [18]. Spleen tissues were homogenized in 1 mL of extraction buffer (4 *M* guanidine solution [Aldrich], 25 m*M* sodium citrate [pH 7.0], 0.5% sodium *N*-lauroyl sarcosinate [Fisher], and 0.1 *M* 2-mercaptoethanol [Sigma]). We added a 1:10 volume of chloroform:isoamyl alcohol (49:1) to the samples, which were incubated on ice for 5 min and were centrifuged at 10,000 × *g* for 15 min at 4°C. RNA in the upper aqueous phase was collected, was precipitated with an equal volume of isopropanol, and was washed 2 times in 70% ETOH. RNA pellets were dissolved in distilled water, were quantified at an OD of 260:280 determination, and were visualized in an ethidium bromide–stained agarose gel. We reverse-transcribed 2 μg of RNA with a cDNA kit (Gibco BRL) by using oligo dT primers and avian myeloblastosis virus RT to

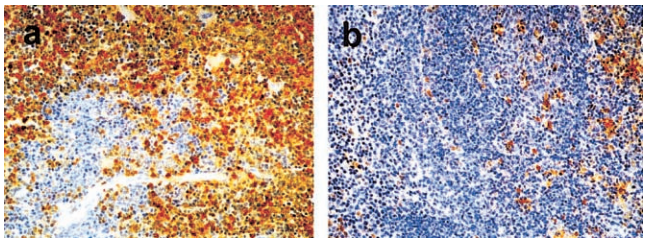

Figure 2. Immunohistochemistry of macrophage-inactivated mice. Spleen tissues of liposome-encapsulated chlodronate–injected mice rarely were stained with anti-mac2 antibody *(b)*, unlike those of PBS-injected mice *(a)*. Figure is 1 of 5 replicate samples from 2 representative experiments.

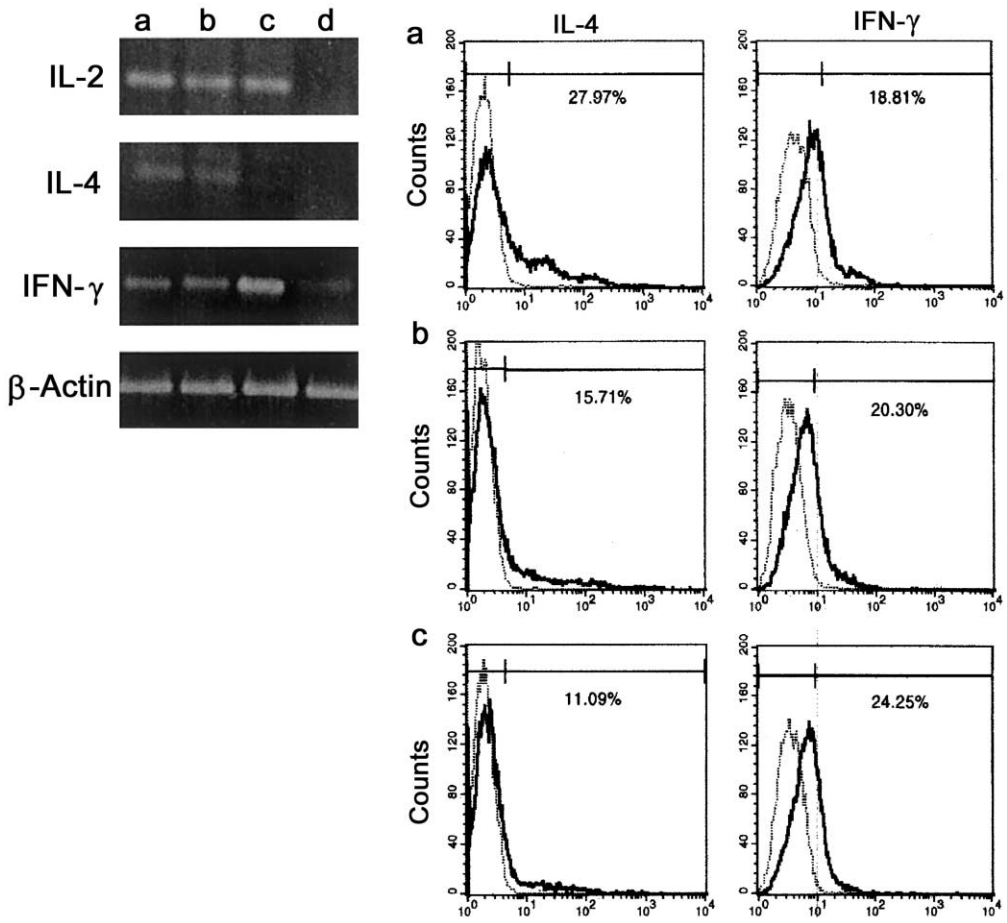

Figure 3. *Left*, Expression of cytokines by reverse transcription–polymerase chain reaction analysis and flow cytometry (FACS) in spleens of mice with Behçet's disease (BD). Groups were injected with liposome-encapsulated chlodronate (lip-Cl_2MDP; *a*), lip-Cl_2MDP and herpes simplex virus (HSV; *b*), and HSV *(c)*. *d,* Normal healthy mice. Data for each group are mean of 3 mice from 2 representative experiments. *Right,* For FACS analysis, 10^7 splenocytes from each group were cultured with Brefeldin A for 5 h and were stained with phycoerythrin- or fluorescein isothiocyanate–labeled anti–mouse IgG. Photographs show 1 of 3 replicate samples from 2 representative experiments. Time schedules of inoculation: *a,* lip-Cl_2MDP (day 0)→lip-Cl_2MDP (day 10)→death (day 21). *b,* lip-Cl_2MDP (day 0)→HSV (day 2)→lip-Cl_2MDP (day 10)→HSV (day 12)→death (day 21). *c,* HSV (day 0)→HSV (day 10)→death (day 21). *d,* Virus culture media (day 0)→culture media (day 10)→death (day 21). IFN, interferon; IL, interleukin.

generate cDNA for use as a template in PCR amplifications. Then we added 2 μL from the RT reaction to 50-μL reaction mixtures containing 50 m*M* KCl (pH 8.4), 20 m*M* Tris-HCl, 2.5 m*M* $MgCl_2$, 200 m*M* dNTPs, 2.5 U of Taq polymerase (Gibco BRL), and 1.2 m*M* primers. Specific primers for cytokines and β-actin were as follows: β-actin [19]; sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, and antisense, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′; IL-2 [20], sense, 5′-TGATGGACCTACAGGAGCTCCTGAG-3′, and antisense, 5′-GAGTCAAATCCAGAACATGCCGCAG-3′; IL-4 [21], sense, 5′-ACGCCATGCACGGAGATGGAT-3′, and antisense, 5′-CAAGCATGGAGTTTTCC-3′; IL-10 [22]; sense, 5′-AGACTTTCTTTCAAACAAAGGACCAGCTGGA-3′, and antisense, 5′-CCTGGAGTCCAGCAGACTCAATACACACTGC-3′; IFN-γ [19]; sense, 5′-AGCGGCTGACTGAACTCAGATTGTAG-3′, and antisense, 5′-GTCACAGTTTTCAGCTGTATAGGG-3′.

The amplification was processed in a thermal cycler (model 900; Perkin Elmer) with an initial 5-min denaturation at 94°C, followed by 35 cycles of the profile: 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The products were subjected to electrophoresis on a 1.8% agarose gel and were visualized under UV light.

Flow cytometric analysis of intracellular cytokines. Before intracellular cytokine staining, splenocytes were freshly isolated. Brefeldin A (5 g/mL; Sigma) was added for the last 4 h of incubation, to accumulate cytokines in the Golgi complex. Cells were harvested, were washed in culture medium containing brefeldin A, and were fixed with 4% formaldehyde in 1% FBS containing PBS for 20 min at room temperature. Then cells were permeabilized with 0.1% saponin in PBS containing 1% FBS and 0.1% sodium azide (saponin buffer) for 10 min at room temperature. Cell suspensions then were treated with fluorescein isothiocyanate– or phycoerythrin-conjugated anti–IL-4, IFN-γ antibody (CalTag) suspended in permeabilized buf-

fer. Samples were analyzed by means of flow cytometer (FACS Vantage; Becton Dickinson) with ⩾20,000 gated lymphocytes.

Induction of Th2 cytokine. Immunization consisted of 2 mg of ovalbumin (OVA) adsorbed onto 2 mg of $Al(OH)_3$ adjuvant given by intraperitoneal injection on day 0. Spleens were removed 7 days later, and splenocyte suspensions were cultured for 2 days with or without 0.15-μg/mL OVA in Dulbecco's MEM. For the control experiment, OVA and complete Freund's adjuvant (CFA) were injected instead of OVA-alum. OVA-CFA (2 μg of OVA and 100 μL of CFA) was mixed through a double-hubbed emulsifying needle before injection. Cytokine production was assessed by RT-PCR analysis, to determine whether alum could induce Th2 cytokines. Mice that developed symptoms of BD were injected with OVA, either in alum or emulsified in CFA. Treatment occurred on day 0 and on day 14 after BD symptoms developed.

Adoptive transfer. Splenocytes (5×10^6) isolated from the spleens of mice treated with OVA in alum or emulsified in CFA were transferred intravenously to mice exhibiting symptoms of BD. Mice were observed for 7 days.

Results

Macrophage inactivation could decrease the incidence of BD-like symptoms. To determine whether macrophages play a role in the development of HSV-induced BD-like symptoms, ICR mice were treated with lip-Cl_2MDP to inactivate macrophages. Symptoms appeared in the inoculated animals. As reported elsewhere [9], these symptoms included bulla and crust in the face region, erythema on the scratched earlobe, eye symptoms (e.g., hypopyon, iridoretinitis, retinal degeneration, and uveitis), and skin ulcerations of the earlobes, scruff, genitalia, and other regions (figure 1). Of interest, 14 (28%) of 50 HSV-inoculated mice without lip-Cl_2MDP treatment developed BD-like symptoms; however, no mice treated with lip-Cl_2MDP before HSV inoculation had BD-like symptoms. The control group, mice treated with lip-Cl_2MDP alone, did not exhibit any BD-like symptoms. Mortality was similar between the HSV-inoculated mice with and without lip-Cl_2MDP (18% vs. 20%; table 1).

Spleen macrophages are nearly depleted by Lip-Cl_2MDP treatment (as shown by staining with anti-mac2 antibody). To permit in vivo studies of macrophage function and immunologic process, Van Rooijen [17] developed a lip-Cl_2MDP–mediated macrophage suicide technique. Lip-Cl_2MDP–engulfed macrophages were killed by apoptosis after phospholipase-mediated disruption of the liposomal bilayers and release of the Cl_2MDP [17]. To confirm that the treatment with lip-Cl_2MDP depletes the macrophages of ICR mice, their spleens were immunohistochemically analyzed. Lip-Cl_2MDP was intravenously injected 2 times 10 days apart in ICR mice. The spleen tissues of lip-Cl_2MDP–injected mice rarely were stained with anti-mac2 antibody (figure 2*B*), unlike those of PBS-injected mice (figure 2*A*).

Lip-Cl_2MDP treatment up-regulated IL-4 expression at mRNA and protein levels. To address the possible role of cytokines affected by macrophage knockout in the development of BD-like symptoms, we used RT-PCR analysis to analyze the mRNA expression of cytokines in the spleens of mice treated with lip-Cl_2MDP, lip-Cl_2MD and HSV, or HSV alone. In all 3 experimental groups, IL-2 and IFN-γ were expressed. However IL-4 mRNA was expressed only in the spleens of mice treated with lip-Cl_2MDP or lip-Cl_2MDP combined with HSV (figure 3, *top*).

To confirm the protein expression of intracellular cytokines, we analyzed the expression of IL-4 and IFN-γ by means of flow cytometry. We found that IL-4 expression in splenocytes was significantly up-regulated in mice treated with lip-Cl_2MDP or lip-Cl_2MDP combined with HSV, compared with that in HSV-inoculated mice (figure 3, *bottom*). IFN-γ expression was increased slightly in mice inoculated with HSV alone.

Th2 adjuvant and alum hydroxide mixed with OVA induced Th2 cytokines with accompanying improvement of skin symptoms. To determine whether the Th2 adjuvant could systemically induce Th2 cytokines (e.g., IL-4 and IL-10), especially in the spleen, OVA-alum was injected into normal mice. In splenocytes cultured from the OVA-alum–injected group, Th2 cytokine IL-4 and IL-10 and Th1 cytokine IFN-γ mRNA expression were demonstrated by RT-PCR analysis. In the OVA-CFA–injected group, only IFN-γ was expressed (figure 4). To test whether Th2 cytokines could attenuate the BD-like symptoms, mice with BD-like symptoms were injected with OVA-alum. Improvement was noted in 6 of 9 mice (figure 5). The symptoms that improved the most were cutaneous problems, such as skin ulcers and crust on the face, earlobes, scruff, and back (table 2). The remaining 3 mice had unchanged symptoms (e.g., ocular and neurologic involvement). In OVA-FCA–injected BD-like mice, none of 6 animals showed any improvement.

Symptom improvement after transfer of splenocytes from OVA-alum–injected mice to BD-like mice. Adoptive transfer with

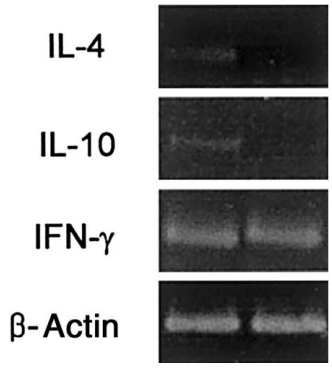

Figure 4. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of splenocyte cultures from ovalbumin (OVA)–alum– or OVA–complete Freund's adjuvant (CFA)–injected mice. Aseptically isolated splenocytes from OVA-alum– or OVA-CFA–injected mice were cultured for 2 days in Dulbecco's MEM with 0.15 μg/mL of OVA. Collected splenocytes underwent RT-PCR analysis. Splenocytes were cultured from OVA-alum– or OVA-CFA–injected mice (*left* and *right lanes,* respectively). IFN, interferon; IL, interleukin.

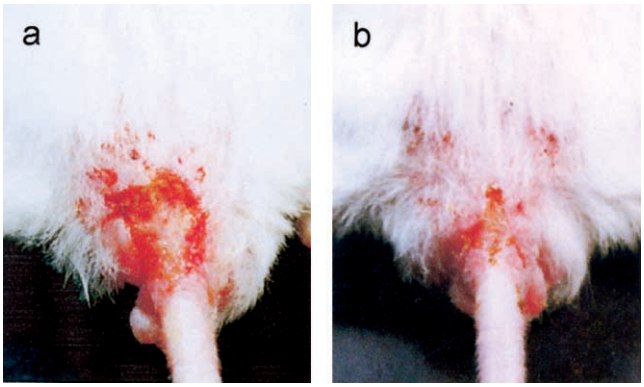

Figure 5. Photographs of mice before *(a)* and after *(b)* ovalbumin (OVA)–alum injection. OVA-alum was injected intraperitoneally 2 times 2 weeks apart into mice with Behçet's disease with skin ulcerations on their backs.

splenocytes from OVA-alum–treated mice also resulted in the improvement of BD-like symptoms (e.g., cutaneous ulcers of the face, earlobe, and scruff and genital inflammation). In the control experiment, splenocytes isolated from OVA-FCA–treated mice were transferred to BD-like mice. These resulted in the deterioration of skin ulcers of the BD-like mice or generation of other symptoms, such as ocular and cutaneous involvement (figure 6).

Discussion

There is a hypothesis about the relationship between the pathophysiology of BD and the autoimmune responses seen in the disease. Antigens, such as bacteria, virus, and heat-shock proteins, stimulate macrophages, and stimulated macrophages activate T cells and neutrophils or induce tissue damage directly [23]. Therefore, to determine whether elimination of macrophages can affect the development of BD-like symptoms, we used lip-Cl_2MDP to inactivate macrophages in a mouse model. Animals treated with HSV combined with lip-Cl_2MDP had a lower incidence of BD-like symptoms than did those treated with HSV alone. These results suggest that macrophages may play an important role in the development of this disease. Macrophage deletion did not seem to affect mortality in the 2 groups: HSV infection might be macrophage independent, whereas the development of BD-like symptoms requires macrophage activity. The primary defense after HSV infection is mediated predominantly by polymorphonuclear cells rather than by macrophages, which therefore resulted in fewer BD-like symptoms. From this, we suggest that the incidence of BD differs, depending on macrophage depletion, but that mortality after HSV infection does not [24].

A possible polarization of T lymphocytes toward the Th1 type in BD has been suggested in experimental uveoretinitis and in humans [25]. Plasma levels of IL-12, tumor necrosis factor receptor–75, and soluble IL-2 receptor correlate with disease activity in BD [26, 27]. Activated macrophages induce cellular immunity by activating Th1 cell responses and by suppressing Th2 cell responses [28]. IFN-γ represents the key cytokine produced by Th1 cells. IFN-γ is a potent immunoregulatory factor that plays an important role in the activation of macrophages by regulating antigen presentation. Antigen-presenting cells exposed to virus secrete the Th1 cytokine, IFN-γ. IL-2 may contribute to the host defense by inducing the clonal expansion of cytokine-producing T cells and by augmenting the production of IFN-γ [29].

As shown in figure 4, inoculation with HSV alone induced IL-2 and IFN-γ expression. Lip-Cl_2MDP treatment also induced IFN-γ but to a lesser extent. IL-4 expression was seen only in the lip-Cl_2MDP–treated groups. IL-4 drives the differentiation of naive CD4-positive cells in a Th2 direction [30]. Up-regulated IL-4 mRNA levels could be influential in decreasing the onset of recurrent BD-like ulceration in response to HSV by altering the Th1/Th2 balance in the direction of Th2 cells.

Macrophages induce cellular immunity by activating Th1 cell responses and by suppressing Th2 responses [31], and macrophage depletion in mice shifts the Th1 response to a Th2 re-

Table 2. Symptoms of mice with Behçet's disease (BD) and response to ovalbumin (OVA)–alum treatment.

Mouse, symptom(s)	Improvement observed
1	
Skin ulcer	Yes
Arthritis	No
2	
Facial skin crust and periocular crust	Yes
Earlobe edema	Yes
3	
Facial skin crust and ulcer	Yes
Genital inflammation	Yes
4	
Skin ulcer	Yes
Eye involvement	Yes
5	
Skin ulcer	Yes
6	
Earlobe edema	Yes
Facial skin crust and ulcer	Yes
7	
Earlobe edema	Yes
Skin ulcer	Yes
8	
Eye involvement	No
9	
Skin ulcer	Yes
Neural involvement	No

NOTE. OVA-alum was injected 2 times intraperitoneally at 2-week intervals after BD symptoms occurred. BD symptoms improved in 6 of 9 mice after OVA-alum immunization. None of 6 mice injected with OVA–complete Freund's adjuvant had any improvement.

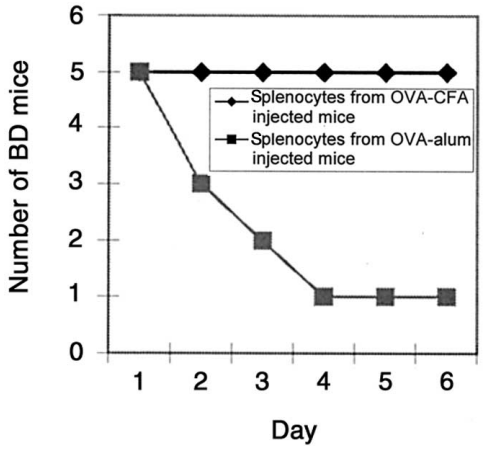

Figure 6. Transfer of splenocytes from ovalbumin (OVA)–alum–treated mice attenuates symptoms of mice with Behçet's disease (BD). Aseptically isolated 5×10^6 splenocytes from OVA-alum– or OVA–complete Freund's adjuvant (CFA)–injected mice were transferred intravenously into mice with BD. Injection was OVA (2 μg) mixed in 2 mg of alum hydroxide or emulsified in 100 μL of CFA. OVA-alum or OVA-CFA was injected 2 times 2 weeks apart. Two days after the final injection, mice were killed, and splenocytes were isolated. $n = 5$ mice per group.

sponse [32]. Our studies also show that HSV inoculation induces the expression of Th1 cytokines alone, whereas lip-Cl_2MDP or lip-Cl_2MDP combined with HSV treatment induces the expressions of both Th1 and Th2 cytokines. Thus, lip-Cl_2MDP knocks out macrophages that regulate the expression of cytokines secreted by macrophages. The degree of macrophage inactivation is correlated with the incidence of BD-like symptoms. In HSV-inoculated mice with macrophages inactivated by anti-mac1 antibody, 20% developed BD-like symptoms versus 10% of mice with macrophages inactivated by anti-mac1 and anti-mac2 antibodies [33]. IL-4 mRNA, even with weak expression, could help the improvement of symptoms in ICR mice. Thus, Th2 cytokine expression might be critical in inhibiting BD-like symptoms induced by HSV.

The use of CFA as an adjuvant in immunocompetent mice produces a predominantly Th1 type response, whereas alum produces a polarized Th2 type response [34]. The effect of alum is due to the suppression of Th1 development via an IL-4–dependent mechanism [35]. Six of 9 BD-like mice improved when injected with OVA-alum, although 4 regressed 2–3 weeks after the initial improvement. The 3 mice that did not improve had ocular and neurologic symptoms. In clinical experience, the pathogenesis of BD can be divided into several subtypes by the type of disease expression. For example, ocular symptoms reflect more humoral immunity balance, whereas mucocutaneous symptoms are related to cell-mediated immunity. Bang [36] suggested that therapeutic plans are divided into 2 categories by humoral or cell-mediated immunity involvement. Another possible explanation could come from experiments with the Lewis rat. Many researchers induced recurrent autoimmune uveitis similar to ocular symptoms of BD in the Lewis rat but no other BD symptoms (e.g., orogenital ulcerations) [37]. We assume that the different responses to OVA-alum treatment can be explained with different pathogenic mechanisms between orogenital and skin involvement and ocular and neural involvement.

In conclusion, we demonstrated that macrophage inactivation decreases the incidence of HSV-induced BD in mice. This result was associated with the induction of IL-4 expression. Both Th2 adjuvant treatment and adoptive transfer could attenuate some of the disease symptoms. Therefore, Th2 cytokine expression induced by macrophage inactivation may be closely related to the development, deterioration, and improvement of BD induced by HSV.

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