CD11c is upregulated in CD8+ T cells of patients with Behçet's disease

Y.J. Park[1], M.J. Park[1], S. Park[2], E.-S. Lee[1]

Department of Dermatology[1] and Microbiology[2], Ajou University School of Medicine, Suwon, South Korea.

Young Joon Park, MD
Mi Jin Park, MS
Sun Park,MD, PhD
Eun-So Lee, MD, PhD

Please address correspondence to:
Eun-So Lee, MD, PhD,
Department of Dermatology,
Ajou University School of Medicine,
164 World Cup-ro,
Yeongtong-gu, Suwon,
443-380 South Korea.
E-mail: esl@ajou.ac.kr

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ABSTRACT

Objective. *Single nucleotide polymorphisms of CD11a and CD11c have been suggested as susceptibility loci in Korean patients with Behçet's disease (BD). As immunoregulatory roles of CD11c+CD8+T cells were previously observed in multiple autoimmune and autoinflammatory diseases, we aimed to investigate CD11a and CD11c in CD4+ and CD8+ subpopulation of BD patients.*

Methods. *Peripheral-blood mononuclear cells were isolated from 21 patients with active BD, 26 patients with inactive BD, 20 patients with recurrent aphthous ulcers (RAU), and 23 healthy controls (HCs). The surface expression of CD11a and CD11c in CD4+ and CD8+ cell populations was analysed by flow cytometry, and CD11a and CD11c mRNA and protein levels from purified CD8(+) T cells were analysed using real-time polymerase chain reaction and western blot.*

Results. *The frequencies of CD11a+ and CD11c+ cells were significantly increased in the CD4+ and CD8+ cell populations of active-BD patients, respectively, than that in the HCs. Additionally, both CD11a and CD11c mRNA and protein levels were significantly elevated in the CD8+ T cells of active-BD patients than that in the HCs.*

Conclusion. *The CD8+ T cells of BD patients exhibited increased CD11c expression levels. Upregulation of CD11c in CD8+ cells may contribute to BD pathogenesis.*

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Introduction

Behçet's disease (BD) is a chronic systemic inflammatory disease clinically characterised by recurrent attacks of oral and genital ulcers, and ocular, skin, and joint lesions. BD etiopathogenesis remains to be fully elucidated, although it is suspected to be an autoinflammatory or autoimmune reaction triggered by an infectious or environmental agent in a genetically susceptible individual (1, 2).

β2 integrins (CD11/CD18) are heterodimeric cell-adhesion molecules expressed on all leukocyte surfaces. These molecules are essential for leukocyte migration and adhesion to inflamed tissue (3) and mediate expression of proinflammatory cytokines during the inflammatory response (4). Increased chemotactic activity of polymorphonuclear cells with increased leukocyte migration has been demonstrated in BD patients, thereby suggesting that these molecules play pivotal roles in BD (5). Four types of β2 integrins have been identified and are classified by an α-unit (*e.g.* CD11a, CD11b, CD11c, and CD11d). Here, we focused on CD11c, given that it is likely to be important in BD pathogenesis for three reasons. First, studies with blocking monoclonal antibodies have suggested a dominant role for CD11c in monocyte adhesion to endothelial-cell monolayers, despite CD11c being expressed at lower levels relative to other β2 integrins (6, 7). As BD lesions display intense perivascular inflammation, CD11c may play an essential role in BD progression. Second, previous studies showed that levels of $CD4^+CD25^{+High}FoxP3^+$ T cells, a typical subset of regulatory T cells, were lower in symptomatic BD patients than asymptomatic patients (8, 9). This finding underscores the possibility that other classes of regulatory/suppressor cells might be involved in BD pathogenesis. Recently, $CD8^+$ T-cell subpopulations co-expressing CD11c were found to constitute an important category of adaptive-immune regulators, with studies showing decreased regulatory roles in *in vivo* rheumatoid arthritis (RA) disease models and expanded roles in the peripheral blood of humans suffering from RA (10). Under appropriate stimulus, $CD11c^{surface-}CD8^+$

Table I. Demographic, clinical, and laboratory features of the BD, RAU, and HC groups.

	Active BD (n=21)	Inactive BD (n=26)	RAU (n=20)	HC (n=21)
Age (years old)	48.0 ± 6.6*	47.1 ± 12.18	41.7 ± 11.9	42.8 ± 10.0
Gender (Male:Female)	10:11	12:14	7:13	8:13
Symptoms†				
Oral ulcer	17	6		
Genital ulcer	4	1		
Skin lesion	9	5		
Ocular lesion	2	1		
Arthritis	4	1		
GI involvement	1	0		
HLA-B51 (+)	8/20	15/24		
ESR (mm/h)	17.95 ± 14.74	17.42 ± 13.62		
CRP (mg/L)	0.74 ± 1.58	0.27 ± 0.65		

*Mean ± SD. †Patients may have more than one symptom. Symptoms of inactive BD patients are ones that patients complained of at time of visit. Symptoms were not present at the time of sampling. BD: Behçet's disease; RAU: recurrent aphthous ulcer; HC: healthy control; GI: gastrointestinal; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

T cells developed into CD11c+CD8+ T cells, which showed signature features of regulatory T cells and indicated that CD11c exhibits important suppressive functions. Finally, we identified single nucleotide polymorphisms (SNPs) following genotype and haplotype analysis of both CD11a and CD11c in Korean BD patients (11) and suggested that the SNPs might work as susceptibility loci in Korean populations. Given that genome-wide association studies have identified significant differences in gene function or mRNA expression, and have revealed that these susceptibility loci influence disease pathogenesis, it seemed prudent to check BD-associated CD11a and CD11c expression. In this study, we investigated CD11a and CD11c expression levels in the peripheral blood mononuclear cells (PBMCs) of BD patients. Our results indicate that CD11c expression is elevated on the surface of CD8+ subpopulations, as well as at the mRNA and protein levels of CD8+ T cells. These data may provide an additional framework for evaluating BD susceptibility.

Methods

Subjects

The patient population consisted of 47 BD patients who had presented themselves for the first time or were being actively monitored in the Department of Dermatology, Ajou University Hospital, from March 2012 to February 2013. Diagnosis was made according to the criteria established by the International Study Group (12) and the Japanese research committee (13). Patients were divided into two groups: 1) patients in the active group had at least one BD symptom at the time of sampling, despite treatment; and 2) patients in the inactive group were in a well-controlled disease state without any symptoms or without complaint of symptoms that had lasted >1 week and were not present at the time of sampling

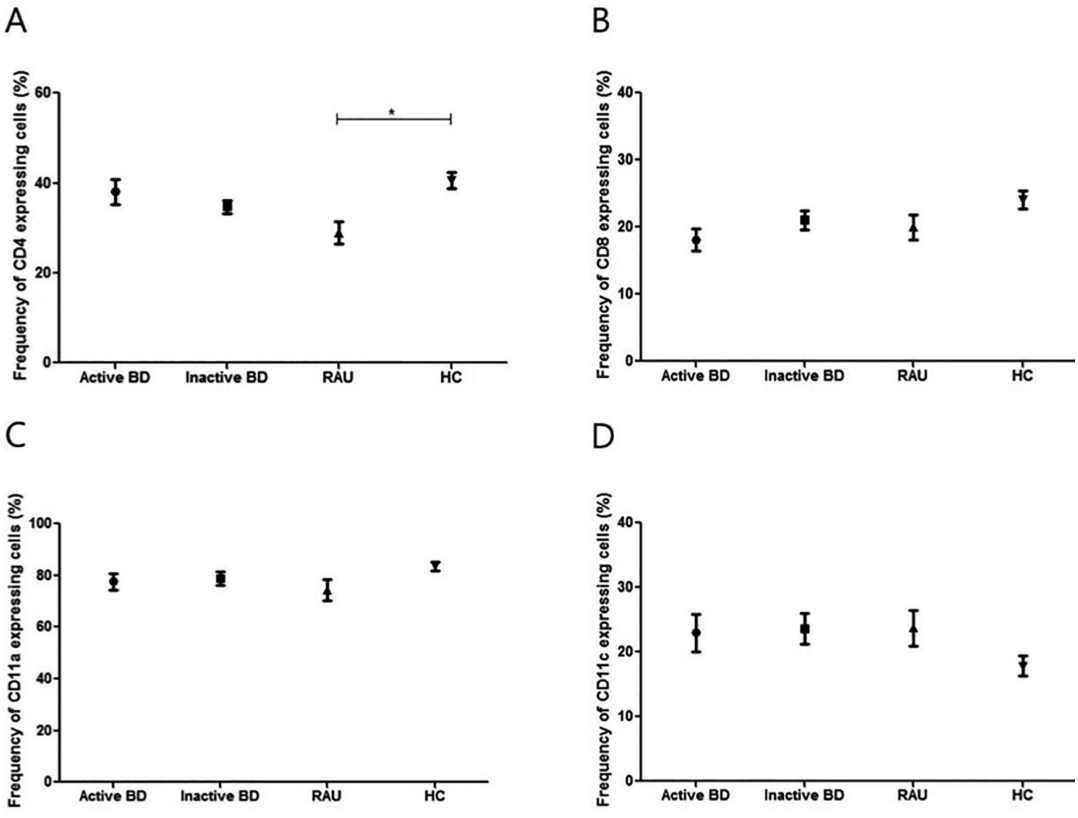

Fig. 1. Frequencies of CD4+ (A), CD8+ (B), CD11a+ (C), and CD11a+ (D) cells in PBMCs from 21 active BD, 26 inactive BD, 20 RAU, and 23 HC subjects. Cells labelled with APC-conjugated anti-CD4, anti-CD8 Ab, FITC-conjugated anti-CD11a, or anti-CD11c Ab were analysed using flow cytometry. There were no significant differences in the frequencies of cells expressing CD4, CD8, CD11a, or CD11c in PBMCs other than decreased frequency of CD4+ cells in RAU patients as compared to that in HC patients. BD: Behçet's disease; RAU: recurrent apthous ulcer; HC: healthy control. Data are shown as mean ± SD. (*p<0.05)

due to administration of anti-inflammatory medication. The disease control and healthy control (HC) groups consisted of 20 age-matched, newly diagnosed patients with recurrent aphthous ulcers (RAUs) without any other evident disease, and 23 healthy volunteers, respectively. Patients fasted for a minimum of 8 hours before having blood drawn (14). Results of laboratory assays including erythrocyte sedimentation rates (ESR) and C-reactive protein (CRP) levels were recorded. HLA-B51 typing by polymerase chain reaction was performed, or when available, previously determined HLA-B51 positivity was recorded. Informed consent was obtained from patients prior to their enrolling in the study. This study was approved by the Institutional Review Board (AJIRB-GEN-13-124).

Cell preparation

PBMCs were prepared from heparinised blood samples by centrifugation over Ficoll Hypaque density gradients (Ficoll Paque Plus, StemCell Technologies, Vancouver, BC, Canada). Cells were washed with phosphate-buffered saline (GIBCO-BRL, Grand Island, NY, USA) with 2% heat-inactivated fetal bovine serum (GIBCO-BRL).

Flow cytometry

To examine cell-surface expression of the indicated molecules, cells were labelled with the following fluorescence conjugated antibodies: APC anti-CD4, APC anti-CD8, FITC-anti-CD11a, and FITC-anti-CD11c (BD Biosciences Pharmingen, San Diego, CA, USA). Cell-surface expression was analysed by multi-colour flow cytometry (BD FACS Canto II, BD Biosciences, San Jose, CA, USA). Analysis of fluores-

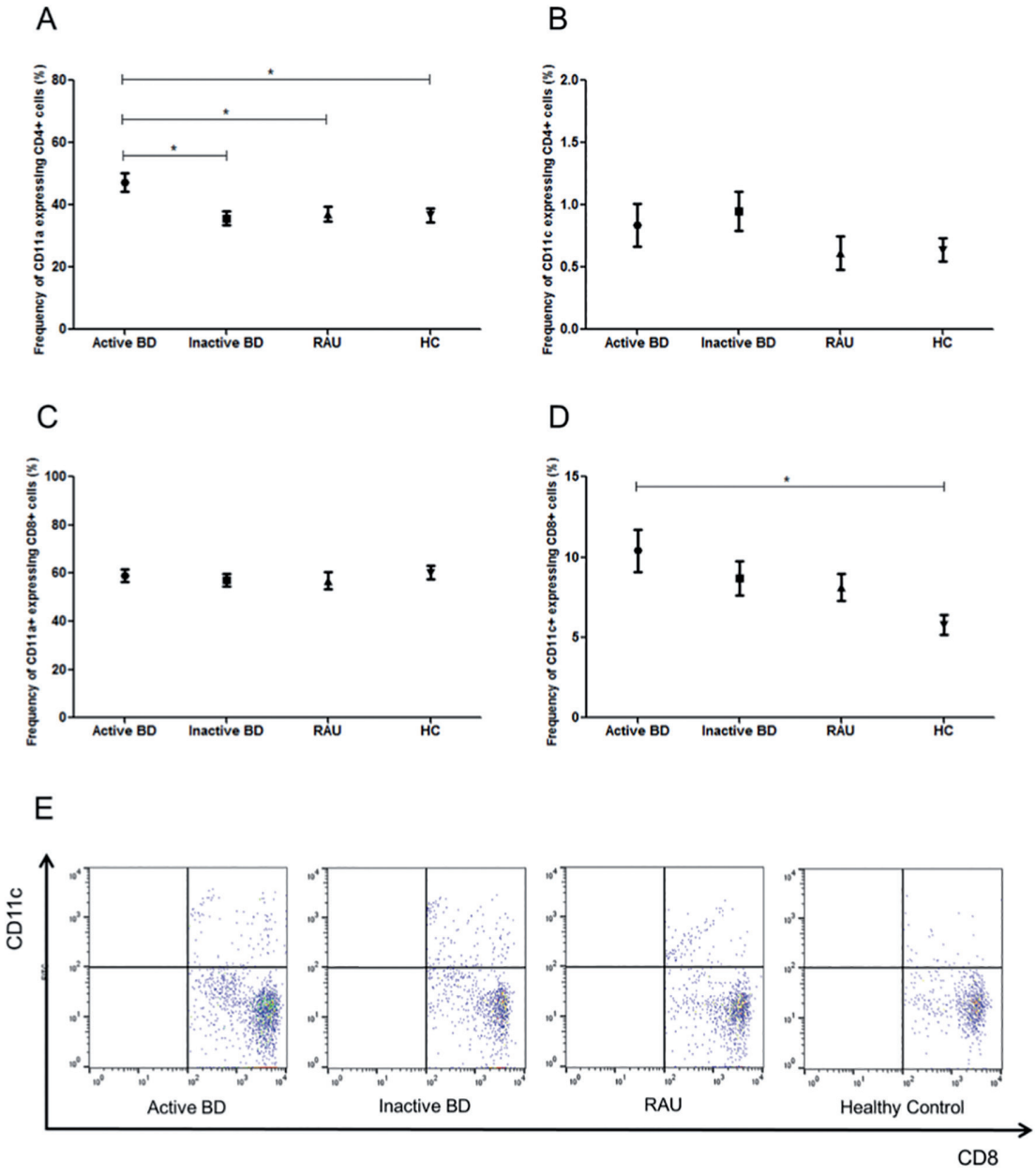

Fig. 2. Frequencies of $CD11a^+$ and $CD11c^+$ cells in $CD4^+$ (A and B, respectively), and CD8+ populations (C and D, respectively), from 21 active BD, 26 inactive BD, 20 RAU, and 23 HC subjects. PBMCs were labelled with APC-conjugated anti-CD4 or anti-CD8 Ab together with FITC-conjugated anti-CD11a or anti-CD11c Ab and analysed using flow cytometry. Increased frequency of $CD11a^+$ cells in $CD4^+$ cell populations (A) and $CD11c^+$ cells in $CD8^+$ cell populations (D) of active BD patients are shown. (E) Representative dot plots of CD11c expression in $CD8^+$ cells. BD: Behçet's disease; RAU: recurrent apthous ulcer; HC: healthy control. Data are shown as mean ± SD. ($^*p<0.05$)

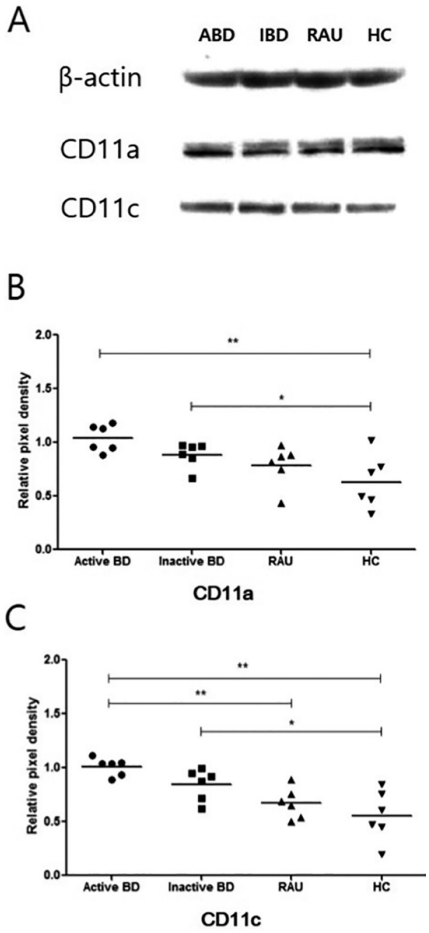

Fig. 3. Increased CD11a and CD11c protein expression in $CD8^+$ T cells of BD. $CD8^+$ T cells isolated from PBMCs were subjected to western blotting. (A) Representative western blot. The band densities of CD11a (B) and CD11c (C), respectively, were normalised against β-actin. Each symbol represents a single subject; bar represents mean value of the corresponding group. BD: Behçet's disease; ABD: Active Behçet's disease; IBD: Inactive Behçet's disease; RAU: recurrent aphthous ulcer; HC: healthy control. ($^*p<0.05$, $^{**}p<0.001$)

cence-activated cell sorting data was performed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

$CD8^+$ T-cell isolation

PBMCs were isolated by Ficoll-Paque density centrifugation, followed by CD8 T-cell purification using the MACS Microbead $CD8^+$ T-Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). This method enabled consistent assessment of $CD8^+$ T-cell purity to >95%.

RNA preparation and real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcription of RNA was performed using an oligo(dT) primer (Invitrogen) and the SuperScript III Kit (Invitrogen). The CD11a and CD11c mRNA levels were analysed by real-time PCR with the ABI Prism 7000 Sequence-detection System (Applied Biosystems, Foster, CA, USA) using the 2–ΔΔCt method. Primers and internal probes for CD11a (Hs00158218_m1), CD11c (Hs00174217_m1), and β-actin (Hs 99999903) were purchased from Applied Biosystems.

Western blot

$CD8^+$ T cells were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor cocktail (Sigma-Aldrich). Fifty micrograms of protein per lane were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose paper. The membranes were blocked overnight at room temperature with 5% skim milk in a TNET buffer containing Tris base, EDTA, NaCl, and 0.2% Tween-20. Blots were incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Anti-CD11a and anti-CD11c antibodies (Abcam, Cambridge, MA, USA) were used. Bands were visualised with enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA). To quantify the expression, the intensities of the bands were measured using Image-Pro Plus Version 4.5 (Media Cybernetics, Inc., Rockville, MD, USA) and expressed as intensities relative to actin.

Statistical analysis

Statistical analysis was performed using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). Differences in the cell frequencies and levels of mRNA and protein between the groups were analysed by analysis of variance (Kruskal-Wallis test) and Mann-Whitney U-test when necessary. A $p<0.05$ was considered statistically significant.

Results

Subject characteristics

A total of 88 subjects entered the study (47 BD patients, 20 RAU patients, and 21 HCs). Baseline demographics and clinical findings for the BD patients are summarised in Table I. There were 22 men and 25 women with a mean age of 47.5 years. Of the 47 patients, 21 were in a clinically active state and 26 were in an inactive state. All subjects in the active group exhibited mucocutaneous symptoms, including oral ulcers, genital ulcers, or skin manifestations. All 47 patients were treated with colchicine. Of the 20 and 24 BD patients with HLA-B51 data from the active group and inactive groups, respectively, 8 from the active group and 15 from the inactive group showed HLA-B51 positivity. Erythrosedimentary ratios (ESR) and C-reactive protein (CRP) levels did not show significant differences between the active and inactive BD groups (15).

Increased frequency of $CD11a^+$ cells in the $CD4^+$ population and $CD11c^+$ cells in the $CD8^+$ population of active-BD patients

We first analysed by flow cytometry the proportions of $CD4^+$, $CD8^+$, $CD11a^+$, and $CD11c^+$ cells in the PBMCs of subjects. Although there were no statistically significant differences in the frequency of cells between the BD groups and HCs (Fig. 1), the frequency of $CD11a^+$ cells in the $CD4^+$ population was significantly higher in active-BD patients relative to inactive-BD patients, RAU patients, and HCs ($p<0.05$; Fig. 2A). There were no statistically significant differences in the frequencies of $CD11c^+CD4^+$ cells and $CD11a^+CD8^+$ cells among all groups (Fig. 2B–C). The frequency of $CD11c^+$ cells in the $CD8^+$ cell population was significantly upregulated in patients with active BD relative to HCs ($p<0.05$; Fig. 2D–E). The frequency of $CD11c^+CD8^+$ cells was slightly higher in HLA-B51 positive patients, but did not reach the threshold for statistical significance (11.19±6.43 *vs*. 7.88±4.94, $p=0.08$). There was no significant difference in the frequency of $CD11a^+CD8^+$ cells between the HLA-B51 positive and negative groups (57.93±11.55 *vs*. 55.41±12.73, respectively, $p=0.64$).

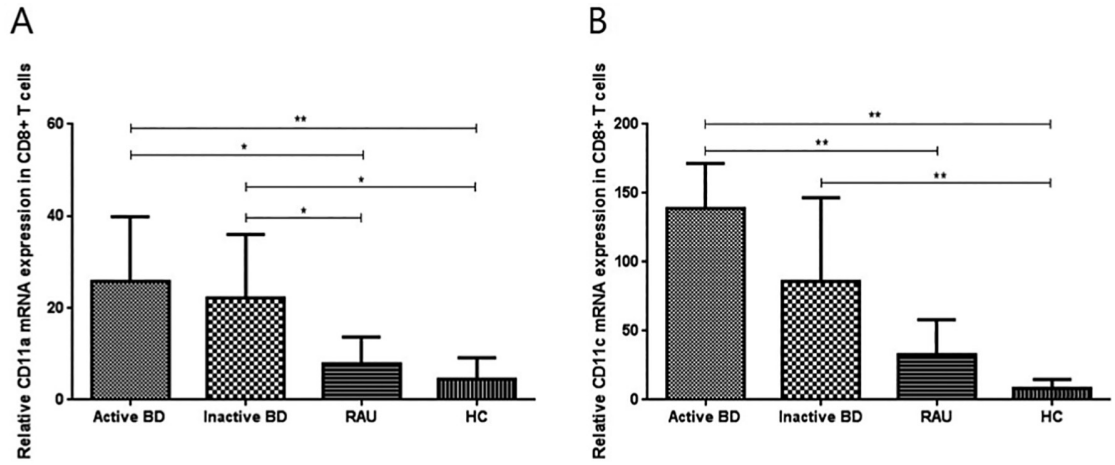

Fig. 4. Increased relative mRNA expression levels of CD11a and CD11c in $CD8^+$ T cells of BD. mRNA levels of CD11a (A) and CD11c (B) in isolated $CD8^+$ T cells from five subjects from each group were determined by real-time RT-PCR using β-actin as a reference gene. BD: Behçet's disease; RAU: recurrent aphthous ulcer; HC: healthy control. Data are shown as mean ± SD. ($^*p<0.05$, $^{**}p<0.001$)

Increased expression of CD11a and CD11c in the $CD8^+$ T cells of BD patients

To determine whether the expression of CD11a and CD11c was increased in the $CD8^+$ T cells of BD patients, $CD8^+$ T cells were isolated from six active-BD, six inactive-BD, six RAU, and six HC subjects for western blot analysis. CD11a protein expression was significantly elevated in both active- and inactive-BD patients relative to HCs ($p<0.001$ and $p<0.05$, respectively; Fig. 3A and C). CD11c levels were significantly increased in the $CD8^+$ T cells of active-BD patients as compared with both RAU patients and HCs ($p<0.001$; Fig. 3B–C). In order to verify differences in CD11a and CD11c expression levels observed between BD patients and the control groups, relative mRNA levels in purified $CD8^+$ T cells were quantified. CD11a mRNA expression levels in $CD8^+$ T cells were significantly higher in active- and inactive-BD patients as compared with RAU patients and HCs (Fig. 4A). CD11c mRNA levels were also significantly higher in active-BD patients as compared with RAU patients and HCs, and in inactive-BD patients relative to HCs ($p<0.05$; Fig. 4B). These results showed increased CD11a and CD11c expression levels in the $CD8^+$ T cells of BD patients.

Discussion

In this study, we demonstrated that $CD11c^+CD8^+$ cell frequency and CD11c expression in $CD8^+$ T cells were increased in active-BD patients relative to HCs. A recently identified subset of $CD8^+$ T cells showed increased CD11c expression in several *in vivo* animal models of autoimmune and autoinflammatory diseases, including collagen type II-induced arthritis, experimental autoimmune uveoretinitis, and hapten-induced colitis (16). The $CD11c^+CD8^+$ T cells in these models were regulatory T cells (16). A T-cell costimulatory molecule, 4-1-BB, induced expansion of $CD11c^+CD8^+$ T cells that suppressed development of RA in mice (17). Inhibition of RA development was assumed to be the result of suppression of antigen-specific $CD4^+$ T cells in both number and function (17), however, $CD11c^+CD8^+$ T cells were also capable of working as immune effectors. The 4-1BB T cells enhanced herpes simplex virus type-1 (HSV-1)-specific $CD8^+$ T-cell responses by induction of $CD11c^+CD8^+$ T cells producing IFN-γ (18). The increased IFN-γ level supported the anti-viral action of conventional cytotoxic T cells (18). It should be noted that not only is HSV-1-induced BD a commonly used animal model of BD, but significant increases in the HSV-1 genome in PBMCs and saliva have also been reported (19, 20). Thus, our results showing increased CD11c expression in the $CD8^+$ T cells of BD patients raises the question as to whether $CD11c^+CD8^+$ T cells can suppress BD development, or rather play a protective role against HSV-1, a potential environmental trigger of BD.

We also showed an increased frequency of $CD11a^+$ cells in the $CD4^+$ cell population from active-BD patients as compared with inactive-BD and RAU patients and HCs. Similar upregulation of CD11a in $CD4^+$ T cells was previously observed in Chinese systemic lupus erythematous patients, suggesting that microRNAs might be involved, particularly microRNA-126 (21). Although global microRNA profiling of PBMCs in Sardinian BD patients did not detect differential expression of microRNA-126 (22), other BD patient populations may display different results, so we cannot rule out the possibility of microRNA being a missing link between our gene susceptibility study and the present study (11). Although the frequency of $CD11a^+$ cells in the $CD8^+$ cell population of BD patients was similar to HCs, we observed elevated CD11a mRNA and protein levels in the $CD8^+$ T cells of BD patients relative to HCs. The CD11a protein is an α-unit of heterodimeric β2 integrin [CD11a/CD18 (also called lymphocyte function-associated antigen)] and constitutes the most abundant protein involved in leukocyte adhesion. It is presumed that CD11a expression increases during the progression of inflammatory disorders. $CD11a^+CD4^+$ and $CD11a^+CD8^+$ T-cell counts were elevated in chronic psoriasis patients (23). However, this is not always the case, as seen in inflammatory bowel disease, where the frequency of $CD11a^+$ T cells decreased relative to HCs (24).

In a recent study by our group, we were able to determine that the major CD11a genotype, rs11574944 CC, and the haplotype rs11574944C-rs2230433G-rs8058823A both play a role in lower-

ing BD susceptibility, while two major CD11c genotypes, rs2230429CC and rs2929GG, and the haplotype rs2230429C-rs2929G are involved in increasing BD susceptibility in Korean patients (11). The results presented here indicate that it is plausible that the functional basis of the loci association involves aberrant CD11a and CD11c expression in $CD4^+$ and/or $CD8^+$ T cells. In contrast, the frequencies of $CD11c^+CD8^+$ cells and $CD11a^+CD8^+$ cells did not show significant correlations to HLA-B51 in our study, despite the fact that the relationship of HLA-B51 to BD has been widely identified in diverse populations (15). The pathogenic role of HLA-B51 in BD has been considered to act in connection with natural killer (NK) cells, rather than $CD8^+$ cells (15); hence, $CD11c^+CD8^+$ T cells may act in a regulatory capacity irrespective of NK cells or increase IFN-γ level in addition to production thereof by NK cells (25).

The limitations of this study include possible selection bias due to the experimental setting, a dermatology clinic, in which most of patients exhibited mucocutaneous symptoms without further complications. Therefore, our findings might be limited to mucocutaneous BD. Additionally, specific information about the patients' medication regimens was not included and could have affected the surface expression levels of CD11 proteins (26). Despite these limitations, our study is the first to demonstrate increased expression of $CD11c^+CD8^+$ T cells in active BD patients. Our results suggest that this group of cells is likely to be associated with BD pathogenesis in mucocutaneous BD patients and could extend to other BD patients, as well. Further study is required to identify the exact functions of these cells in BD.

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