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Differential expression of monocyte/macrophage markers between active and inactive stage of patients with Behçet's disease

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Abstract

Although the exact etiology of Behçet's disease (BD) remains unclear, a complex interaction between T cells and antigen-presenting cells is known to be involved in the immunopathogenesis of BD. This study aimed to identify differentially expressed cell surface markers of peripheral blood mononuclear cells (PBMCs) in active and inactive stage of BD patients. PBMCs were isolated from six healthy controls, eight inactive BD patients and five active BD patients. Different cell phenotypes were analyzed by flow cytometry, serum cytokine levels were detected by ELISA and the morphological structure of polymorphonuclear neutrophils (PMN) was revealed by transmission electron microscope (TEM). The CD11b monocyte marker was slightly decreased in active BD patients (91.5±10.9%) compared with healthy controls (97.5±1.3%), but was not different compared to inactive BD patients (88.8±12.2%). The CD14 monocyte marker was significantly increased in active BD patients (28.9±18.7%, p=0.05) and inactivate BD patients (30.8±21.4%, p=0.08) compared to healthy controls (11.1±3.7%). However, CD16 (FcγRIIIA) was higher in inactive BD patients (93.9±2.4%) than active BD patients (85.3±14%), and CD32 (FcγRII) was down-regulated in active BD patients (26.6±18.1%) compared to inactive BD patients (46.6±30.3%) and healthy controls (71.7±17.4%; p=0.002). Most surprisingly, the mannose receptor marker CD206 was highly expressed with significance in active BD patients (49.7±35.2%) compared to healthy controls (7.4±0.8%) (p=0.02) and inactive BD patients (4.7±3.1%) (p=0.007). In spite of the up-regulation of CD206 in active BD patients, interleukin-10 was markedly increased in the inactive state after improving medication than in the active state. All these findings show that differential surface expression of PBMCs between the inactive and active state of BD patients may influence changes of the disease state following treatment.

Keywords: Behçet's disease, monocyte/macrophage, active and inactive stage, surface markers

Introduction

Behçet's disease (BD) is a rare chronic inflammatory disease characterized by recurrent oral and/or genital aphthous ulcerations, uveitis and skin lesions. Clinical presentation of this disorder is multifaceted with severe chronic inflammation accompanied by articular, central nervous system, gastrointestinal, renal, urogenital, pulmonary and cardiovascular manifestations, all of which are associated with systemic vasculitis, a pivotal pathophysiological feature of BD [1-4]. The exact pathogenesis of BD remains unclear, but autoimmune and autoinflammatory reactions are important [5]. Initially in BD, infiltrated types of cells include $CD4^+$ and $CD8^+$ T cells, macrophages and dendritic cells, followed by neutrophils [6]. Th1/Th2-type immune responses have been investigated in cell-mediated immunity and inflammation in BD [7]. T helper (Th) 1 and Th17 predominant response has been observed in many studies in patients with BD; the response involves the increased production of cytokines including interleukin (IL)-2, IL-6, IL-8, IL-17, IL-12, IL-18, tumor necrosis factor-alpha (TNF-α), and interferon- gamma (IFN-gamma) [8,9].

CD11b is expressed on neutrophils, monocytes, natural killer (NK) cells and a subset of lymphocytes. CD11b has been implicated as having a central role in the migration of leukocytes from peripheral blood to the sites of inflammation [10,11], and is also involved in adhesion, chemotaxis and diapedesis during the process of host defense [12]. A previous study involving the examination of cultured monocytes from BD patients reported the significantly elevated expression of the CD11a, CD11b and CD18 adhesion molecules compared to cells from healthy subjects [13].

CD14 is a co-receptor of innate immunity. BD patients display up-regulated CD14 expression on monocytes and neutrophils and elevated serum soluble CD14 levels [14,15]. Very early activation confirmed by CD69 and CD14 response to heat shock protein 60 (HSP60) on peripheral blood mononuclear cells (PBMCs) of BD patients might be associated with an HSP60-induced innate activation through antigen presenting cells (APCs) [16]. CD16 is a Fc receptor (Fc RIII) that has been directly associated with neutrophil activation. Normally, CD14 and CD16 are found together in secretory vesicles of neutrophils

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Table 1. Clinical and laboratory characteristics of inactive and active BD patients.

Disease condition	Patients	Age	Sex	OU	GU	SL	GI	JI	NEUR	VAS	OL	Pathergy	HLA-B51	ESR	CRP
Active	a	28	M	+	+	+	-	-	-	-	-	-	-	13	<0.01
	b	31	F	+	-	-	-	-	-	-	+	-	+	65	<0.01
	c	29	F	+	+	+	-	+	-	-	-	-	-	41	2.4
	d	19	F	+	+	+	-	+	-	-	-	-	-	20	<1.00
	e	43	F	+	+	+	-	-	-	-	-	-	+	50	2.33
Inactive	a	28	M	--	--	--	--	--	--	--	--	-	-	2	<1.00
	b	31	F	--	--	--	--	--	--	--	--	-	+	10	<1.00
	f	59	F	--	--	--	--	--	--	--	--	--	-	10	<1.00
	g	64	F	--	--	--	--	--	--	--	--	--	+	17	<1.00
	h	53	F	--	--	--	--	--	--	--	--	--	-	19	<1.00
	i	45	M	--	--	--	--	--	--	--	--	--	-	14	<1.00
	j	39	F	--	--	--	--	--	--	--	--	--	-	18	1.54
	k	68	F	--	--	--	--	--	--	--	--	--	-	2	<1.00

M: male, F: female, OU: oral ulcers, GU: genital ulcers, SL: skin lesions, GI: gastrointestinal inflammation, JI: joint involvement, NEUR: neurological involvement, VAS: vasculitis, OL: ocular lesions.

and, when neutrophils are stimulated, CD14 and CD16 co-migrate to the plasma membrane [17]. The intensity of CD16 expression in patients with BD is equivocal [15,18]. In addition, FcγRII, namely CD32, has been detected on T cells, mast cells, monocytes, macrophages, and some epithelial and endothelial cell lineages. The primary function of CD32 appears to be antibody-mediated uptake of antigen and modulation of cellular activation and maturation events [19,20].

Macrophage mannose receptor (MMR), also known as CD206, is a scavenger receptor that is expressed primarily by tissue macrophages and lymphatic and hepatic endothelia in humans and mice [21,22]. MMR's carbohydrate pattern recognition, potent capacity of endocytosis, and role in phagocytosis of microorganisms support a dual role in host defense and homeostasis [23]. In addition, CD206 has been identified in a variety of autoimmune and inflammatory diseases, such as systemic lupus erythematosus, ulcerative colitis, and Crohn's disease [24,25]. However, the role of mannose receptor with other cell surface marker on PBMCs of active and inactive BD patients remain poorly understood in the host defense. In the present study, we investigated the pattern of cell-surface expression of CD11b, CD14, CD16, CD32 and CD206 on PBMCs of active and inactive BD patients before and after medications, respectively. We also attempted to characterize the serum IL-10 levels and cell surface expression during transformation of active to inactive form of BD.

Materials and methods

Patients and healthy controls

The patient population consisted of 13 patients with BD who presented for the first time or were monitored at the Department of Dermatology, Yonsei University Hospital, Seoul, Korea. According to the International Study Group for BD criteria, the presence of any two of the following symptoms in addition to recurrent oral ulceration is diagnostic: genital ulceration, skin lesions, joint involvement, and ocular lesions. Presently, active BD patients had at least two of the BD symptoms and inactive BD patients who received anti-inflammatory medication were well controlled with no symptomatic states. Two of eight inactive BD patients were transferred from the active BD patient group. The control group consisted of six healthy volunteers (three women and three men; mean age, 29.6±3.5 years), the inactive BD patients consisted of eight (six women and two men; mean age, 48.4±15.0 years) and the active BD patients consisted of five (four women and one man; mean age, 30.0±8.6 years). Detailed clinical characteristics and therapeutic history of these patients are presented in **Tables 1** and **2**. Written informed consent was obtained from all participants prior to enrolling them into this study in accordance with the guidelines of the Declaration of Helsinki Principles.

Cell preparation

PBMCs were isolated from heparinized venous blood by ACK lysing buffer. The cells were washed twice in phosphate-buffered saline (PBS) and then resuspended in PBS. The cell suspensions were finally adjusted to a concentration of 1 x 10^6 cells/ml and were processed further for cellular staining studies.

Flow cytometry

PBMCs were surface-stained with anti-human antibodies CD14 (phycoerythrin (PE)-cy7), CD11b (fluorescein isothiocynate, FITC), CD16 (PE), CD32 (Allophycocyanin, *APC*) (eBiosciences, San Diego, CA, USA) and CD206 (Per-CP) (BD Biosciences

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Table 2. Therapeutic history of inactive BD patients.

Disease condition	Patient	Colchicine	Prednisolone	Azathioprine	Aspirin	Cyclosporine
Inactive	a*	+	+	+	-	-
	b*	+	+	+	-	+
	f	+	-	-	-	-
	g	+	-	-	-	-
	h	+	-	-	+	-
	i	-	+	-	-	-
	j	+	-	-	+	-
	k	+	-	-	-	-

*, These two inactive patients were improved from active patients-a and b.

Pharmingen, San Diego, CA, USA) for 30 min at 4 °C in the dark. Isotype control antibodies were used to estimate the non-specific binding of target primary antibodies. Stained cells were analyzed by flow cytometry using a FACS Canto II (Becton Dickinson, San Jose, CA, USA) with ≥10,000 gated lymphocytes.

Enzyme-linked immunosorbant assay (ELISA)

Serum was obtained from patients and healthy controls and analyzed using commercial ELISA kits for the detection of IL-10 (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The mean and standard deviation were calculated using ELISA values determined for each well. The ELISA reader was Bio-Rad 170-6850 microplate reader (Bio-Rad, Hercules, CA, USA) and samples were read at a wavelength of 450 nm.

Transmission electron microscopy (TEM)

PBMCs were isolated from whole blood of healthy control, inactive and active BD patients and the morphological changes were observed using EM 902A transmission electron microscope (Zeiss, Oberkochen, Germany). In brief, cells were fixed using Karnovsky's fixative solution (2% paraformaldehyde, 2% glutaraldehyde, 0.5% calcium chloride in cacodylate buffer, pH 7.2) for 30 min, washed with cacodylate buffer, dehydrated in a series of graded ethanol and embedded in Epon mixture. After polymerization, ultrathin sections were cut using on Reichert Jung Ultracut S (Leica, Vienna, Austria), mounted on grids, stained with uranyl acetate and lead citrate and analyzed by TEM.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 software (SPSS, Chicago, IL, USA) and analyzed by Kruskal-Wallis Test and Bonferroni correction. A value of $p<0.05$ was considered statistically significant.

Results

Clinical and laboratory features of BD patients

All five active BD patients had severe manifestations consisting of oral ulcers with genital ulcers and skin lesions during the course of the disease. One patient (patient b) did not show genital ulcers and skin lesions, but did have ocular lesions. In addition, two patients (patient c and patient d) had joint complications. However, gastrointestinal infection, neurological involvement and vasculitis were not observed during the study period (**Table 1**). The laboratory tests performed were pathergy test, HLA-B51 detection, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level. Patients with active disease may generate an acute-phase response leading to a significantly raised ESR than inactive disease (37.8±21.39 mm/h and 11.5±6.76 mm/h, $p=0.007$; respectively). Serum level of CRP was varied from <0.01 mg/dL to 2.4 mg/dL in active BD patients, and in inactive BD patients was from <1.0 mg/dL to 1.54 mg/dL. Genetic factor HLA-B51 was positively detected in two active and two inactive BD patients. Finally, a pathergy test was performed; none of the active BD patients showed a positive result (**Table 1**). After blood sampling, active BD patients (Patient a and b) started treatment. Inactive BD patients were treated with colchicine, prednisolone, and azathioprine (n=1); colchicine, prednisolone, azathioprine, and cyclosporine (n=1); colchicine (n=3); colchicine and aspirin (n=2) and prednisolone (n=1). When the active BD patients were changed to inactive stage after medication (**Table 2**), blood was collected for laboratory analysis.

Differential surface expression on PBMCs of active and inactive BD patients

To identify differently expressed cell surface markers between active and inactive BD patients, PBMCs were isolated from healthy volunteers, active and inactive BD patients and labeled with antibodies and analyzed by flow cytometry. The CD11b monocyte marker was slightly decreased in active BD patients (91.5±10.9%) compared to healthy controls (97.5±1.3%), but was not different compared to inactive BD patients (88.8±12.2%). Monocyte marker CD14 was significantly increased in active BD patients (28.9±18.7%, $p=0.05$) and inactivate BD patients (30.8±21.4%, $p=0.08$) compared to healthy control (11.1±3.7%). Fc receptor contributes to the protective role of immune system by binding to pathogens

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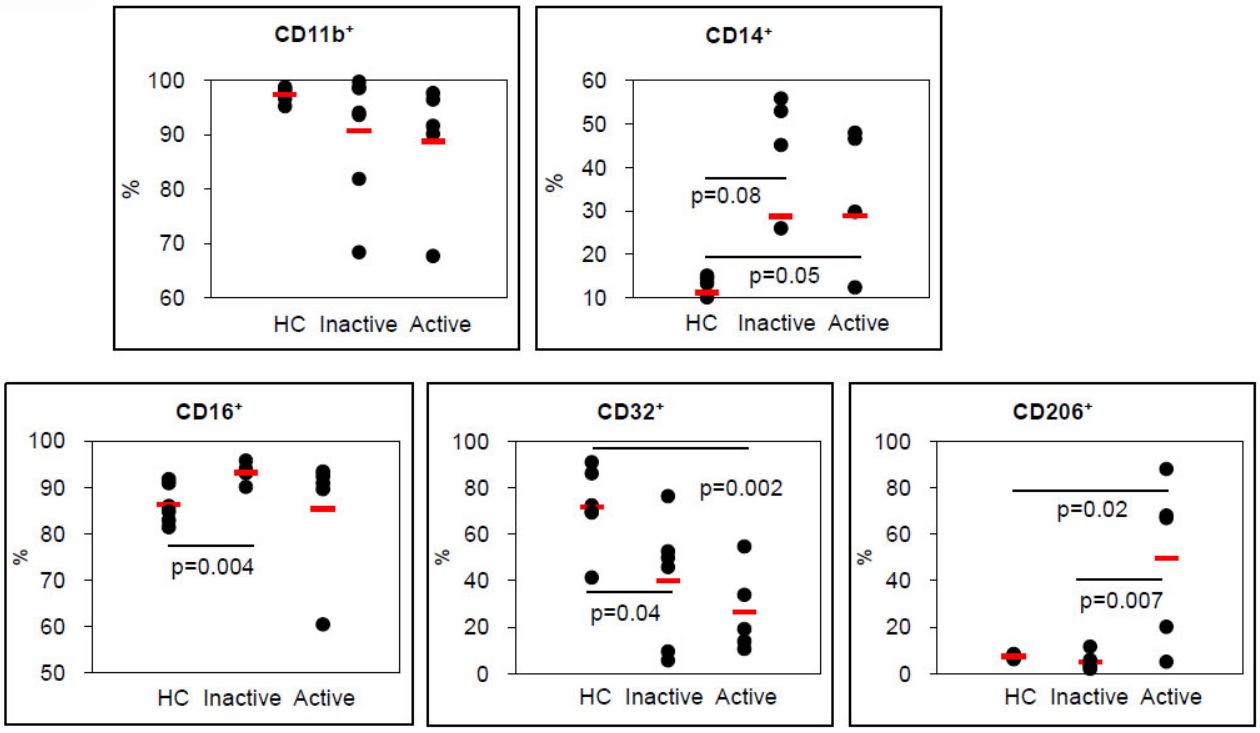

Figure 1. Surface expression on PBMCs of BD patients. PBMCs were isolated from healthy control (n=6), inactive (n=8) and active BD (n=5) patients. The surface expression of CD11b, CD14, CD16, CD32 and CD206 was analyzed by flow cytometry.

[26]. CD16 (FcγRIIIA) is expressed on NK cells, macrophages and neutrophils. Presently, CD16 was higher in inactive BD patients (93.9±2.4%) than active BD patients (85.3±14%). CD32 (FcγRII), which is important in regulating adaptive immunity [27], was down-regulated in active BD patients (26.6±18.1%) compared to inactive BD patients (46.6±30.3%; p=0.04) and healthy controls (71.7±17.4%, p=0.002). Most surprisingly, the mannose receptor marker CD206 was highly expressed with significance in active BD patients (49.7±35.2%) than in healthy controls (7.4±0.8%, p=0.02) and inactive BD patients (4.7±3.1%, p=0.007) (**Figure 1**).

Characterization of CD11b$^+$ subsets in active and inactive BD patients

CD11b is present in neutrophils, NK cells and macrophages, and is overexpressed in chronic obstructive pulmonary disease (COPD) [28]. CD14, the receptor for lipopolysaccharide binding protein, is expressed to a higher degree in blood monocytes than in tissue macrophages [29]. To determine whether the frequencies of co-expressed markers with CD11b$^+$, CD14$^+$, CD16$^+$ or CD32$^+$ cells were analyzed in PBMCs from healthy controls, active and inactive BD patients by flow cytometry. The frequencies of the CD11b$^+$CD14$^+$ cells in inactive and active BD patients were almost similar (27.7±4.1% and 25.9±16.3%, respectively), but were higher than healthy controls (11.0±3.7%) (healthy *vs* inactive, p=0.03; healthy *vs* active, p=0.06). Although the frequencies of the CD11b$^+$CD16$^+$ cells were not significantly different among the groups (healthy 85.5±4.7%, inactive 85.7±9.4% and active 82.2±15.9%), and the frequencies of the CD11b$^+$CD32$^+$ cells were significantly down-regulated in active BD patients (24.8±18%) (p=0.002) and inactive BD patients (47.8±27.8%) (p=0.04), as compared to healthy controls (70.6±18.2%). In addition, active BD patients showed down-regulation of CD11b$^+$CD32$^+$cells compared to inactive BD patients (**Figure 2**).

Up-regulated expression of CD14$^+$CD16$^+$ subsets in active and inactive BD patients

CD14$^+$ cells were increased in both inactive and active groups as compared to healthy controls (**Figure 1**). Similarly, double positive CD14$^+$CD16$^+$ cells were also significantly increased in inactive BD patients (36.1±22.4%, p=0.008) and active BD patient (22.9±17.6%, p=0.02) compared to healthy control (1.7±0.6%). CD14$^+$CD16$^+$ cells were reduced in active BD patients compared to inactive BD patients. Although the difference was not found significant, a similar pattern of surface expression was observed after analysis of CD14$^+$CD32$^+$ cells in inactive BD patients (18.0±13.3%) and active BD patients (10.4±4.4%) and healthy controls (7.9±1.3%) (**Figure 3**).

Increased expression of mannose receptor CD206 in active BD patients

To investigate the expression of mannose receptor in association with monocyte/macrophage subsets, we analyzed

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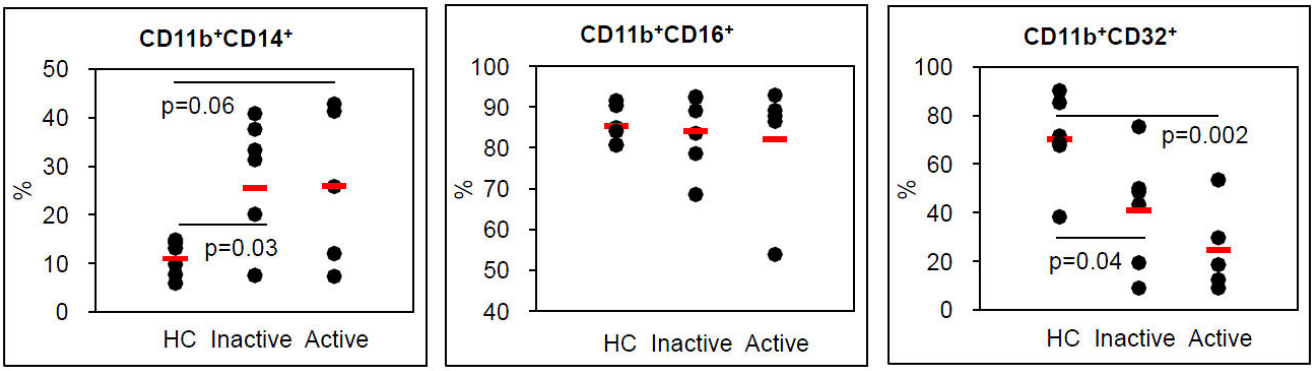

Figure 2. Expression of monocyte/macrophage marker CD11b$^+$ subsets in BD patients. The frequencies of CD11b$^+$CD14$^+$, CD11b$^+$CD16$^+$ and CD11b$^+$CD32$^+$ cells were analyzed by flow cytometry in PBMCs from healthy control (n=6), inactive (n=8) and BD (n=5) patients.

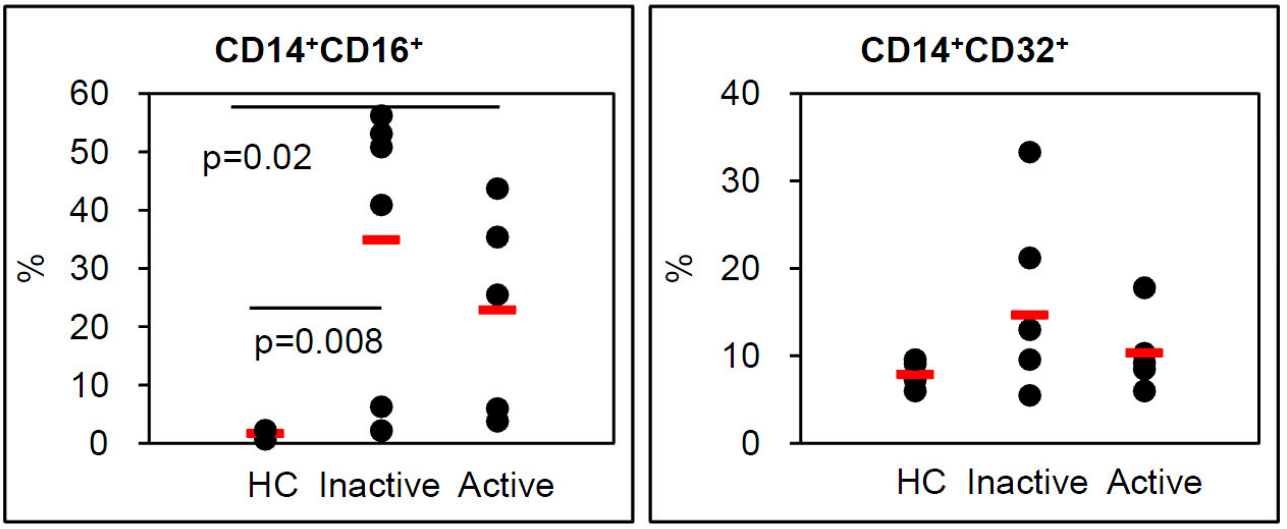

Figure 3. The frequencies of CD14$^+$CD16$^+$ subsets were highly expressed in PBMCs of BD patients. The surface expression of CD14$^+$CD16$^+$ and CD14$^+$CD32$^+$ was analyzed by flow cytometry.

CD11b$^+$CD206$^+$ and CD14$^+$CD206$^+$ cells in inactive and active BD patients. These populations were highly expressed in active BD patients with significance compared to healthy control (CD11b$^+$CD206$^+$ cells: 49.4±35.6% in active, 7.4±0.9% in healthy, p=0.02; and CD14$^+$CD206$^+$ cells: 14.6±9.6% in active, 5.6±1.4% in healthy, p=0.05) and inactive BD patients (CD11b$^+$CD206$^+$ cells: 49.4±35.6% in active, 4.4±3.1% in inactive, p=0.007; CD14$^+$CD206$^+$ cells, 14.6±9.6% in active, 4.4±2.9% in inactive, p=0.02) (**Figure 4**).

Change of PBMC surface markers after improvement in two BD patients

Among the five active BD patients, two patients (patient a and b) were followed after treatment with colchicine, prednisolone and azathioprine in the absence or presence of cyclosporine (**Table 2**). When the symptoms changed to the inactive state, the surface expression on PBMCs was analyzed by flow cytometry. After improvement, the frequencies of CD14$^+$ cells were highly increased in both patients at the inactive stage compared to the active stage (patient a from 29.8% to 53.0%; patient b from 7.8% to 44.4%). The frequencies of CD11b$^+$ cells were slightly decreased at the inactive stage (81.9%) compared to the active stage (90.2%) in patient a, and were not different in patient b (inactive 96.4% *vs* active 96.7%). The frequencies of CD14$^+$CD11b$^+$ cells were also higher at the inactive stage (patient a 37.7%, patient b 42.4%) compared to the active stage (patient a 25.9%, patient b 7.4%) (**Figure 5A**). The frequencies of CD16$^+$ cells were slightly increased at the inactive stage (patient a 95.8%, patient b 97.6%) compared to the active stage (patient a 90.9%, patient b 93.4%). The frequencies of CD16$^+$CD11b$^+$ cells were lower at the inactive stage (78.7%) compared to the active stage (86.6%) in patient a, but was similar in patient b (inactive 94.6% vs active 93.0%). In addition, double positive CD16$^+$CD14$^+$ cells were up-regulated at the

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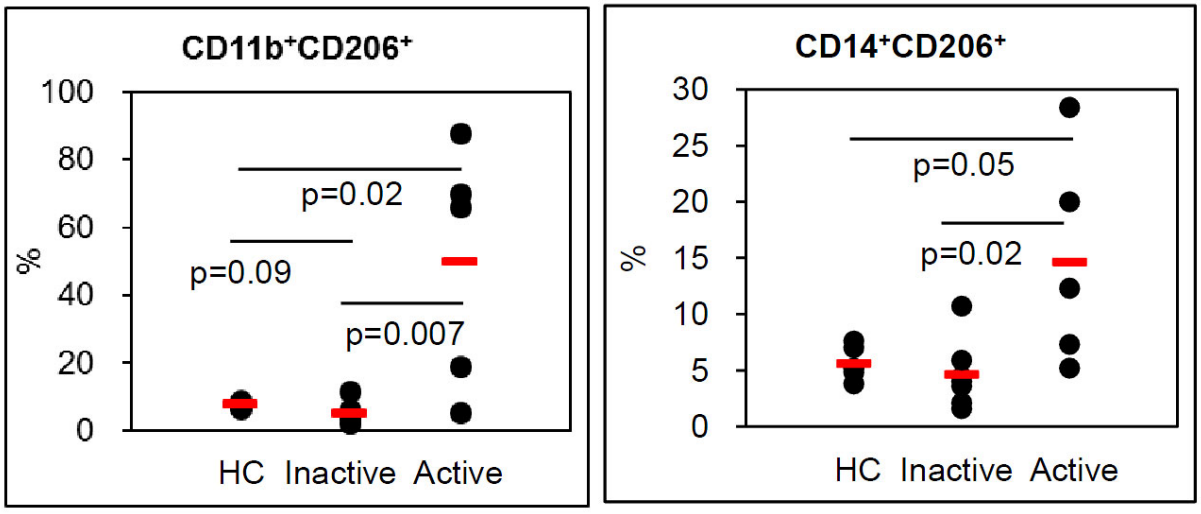

Figure 4. Frequencies of CD206 subsets are highly up-regulated in active BD patients. PBMCs were isolated from healthy control (n=6), inactive (n=8) and BD (n=5) patients. The surface expression of CD11b+CD206+ and CD14+CD206+ was analyzed by flow cytometry.

inactive stage (patient a 53.1%, patient b 43.3%) compared to the active stage (patient a 25.5%, patient b 3.8%) (**Figure 5A**).

In patient a, the frequency of single $CD32^+$ cells was decreased at the inactive stage (active 34.0% *vs* inactive 9.6%), but was increased in patient b (active 54.8% *vs* inactive 85.8%). The frequency of double positive $CD32^+CD11b^+$ cells in patient a was also decreased at the inactive stage (active 29.9% *vs* inactive 9.2%), but in patient b, the frequency of $CD32^+CD11b^+$ cells was increased (active 53.7% *vs* inactive 84.7%). In addition, the frequency of $CD32^+CD14^+$ cells was decreased at the inactive stage (active 17.8% *vs* inactive 9.6%) in patient a, but was increased in patient b (active 6.0% *vs* inactive 38.1%). The frequencies of $CD32^+$, $CD32^+CD11b^+$, and $CD32^+CD14^+$ cells showed opposite pattern of result between patient a and b (**Figure 5B**).

The frequencies of $CD206^+$ cells were highly up-regulated in the active stage (patient a 67%, patient b 68.1%) compared to the inactive stage (patient a 6%, patient b 2.5%). Furthermore, the frequencies of $CD206^+CD11b^+$ and $CD206^+CD14^+$cells were also increased at the active stage (patient a 65.8% and 28.4%, respectively; patient b 67.9% and 7.3%, respectively) compared to the inactive stage (patient a 5.9% and 5.9%, respectively; patient b 2.4% and 2.5%, respectively) (**Figure 5C**).

Variations in the induction of anti-inflammatory cytokine IL-10 after improvement of BD patients

Serum was collected from BD patients a and b in their active and inactive stages. The anti-inflammatory cytokine IL-10 level was evaluated by ELISA. In patient b, the IL-10 level was markedly induced in the inactive stage (33.25 pg/ml) compared to the active stage (5.41 pg/ml). In patient a, IL-10 was slightly increased in the inactive stage (11.39 pg/ml) compared to the active stage (3.36 pg/ml) (**Figure 6**).

Observation of intracellular morphology of polymorphonuclear neutrophils (PMNs) by TEM

PMNs contain two types of chemically distinct cytoplasmic granules, which appear at different stages of maturation. The larger and dense azurophilic granule (or primary granule) is formed during the promyelocyte stage and contains myeloperoxidase in addition to numerous lysosomal enzymes, neutral proteases, glycosaminoglycans, cationic bactericidal proteins and lysozyme. The specific granule (or secondary granule) is formed during the myelocyte stage. Mature PMNs contain both types of granules: 33% azurophilic and 67% specific granules [30]. To observe the intracellular changes occurred at different stages of BD, we isolated PBMCs from whole blood of healthy controls, and inactive and active BD patients, and observed with TEM. In this study, PBMCs had a normal structural appearance in the healthy control and inactive stage groups. In contrast, huge azurophilic granules were aggregated in the cytoplasm of the neutrophils in active BD patients (**Figure 7**).

Discussion

The exact etiology and immunopathological features of BD are not clear yet. But, immunological properties may play a role in disease sequela. The frequencies of $CD11b^+$ cells in neutrophils are higher in BD patients than in healthy controls [31]. $CD8^+CD11b^+$ cells are reportedly increased in BD patients compared to healthy controls [32], and thalidomide treatment down-regulates $CD8^+CD11b^+$ cells [18]. In another study, CD11b was significantly high in active compared to inactive BD patients or healthy control [33]. However, our data showed lower frequencies of CD11b in BD patients than in healthy controls, although the difference was not significantly different. The prior study reported on BD patients with ocular lesion,

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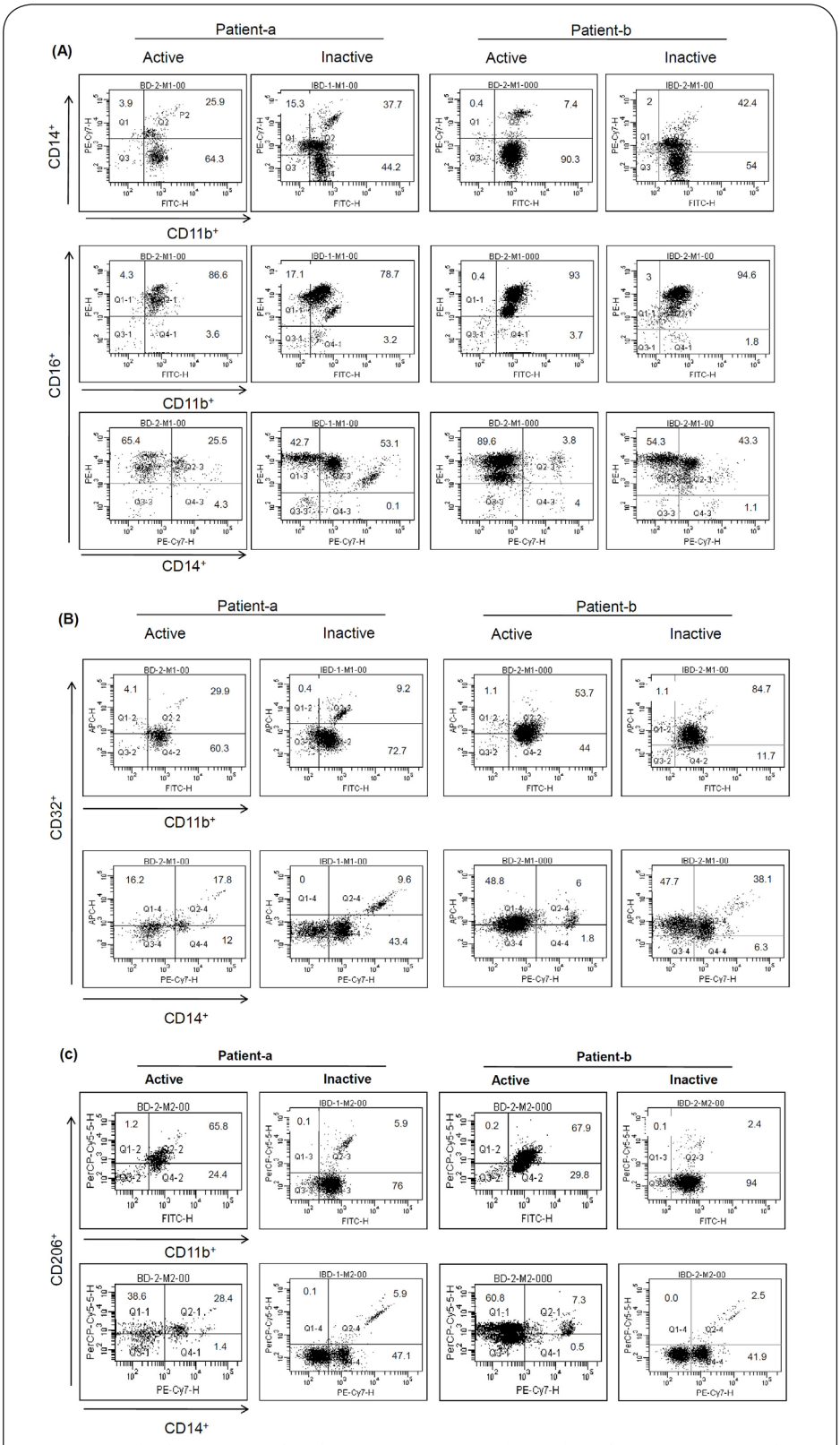

Figure 5. The frequencies of surface expression were changed in after improvement of two BD patients. PBMCs were isolated from two BD patients (patient a and b) before and after treatment with colchicine, prednisolone and azathioprine with or without cyclosporine. A~ C. The surface expression of CD11b, CD14, CD16, CD32, CD206 and their subsets was analyzed by flow cytometry.

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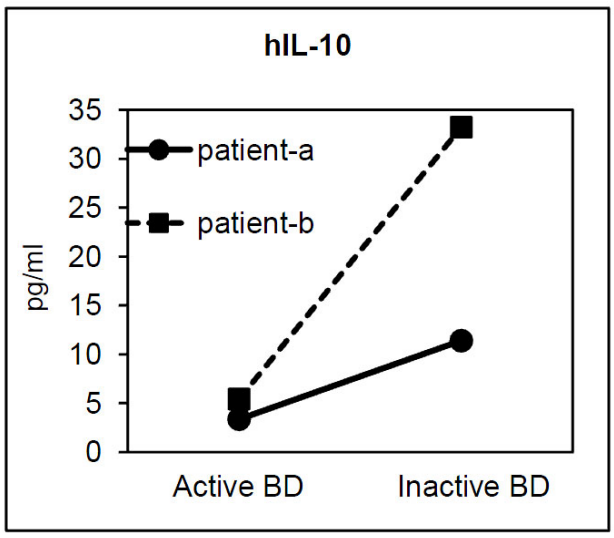

Figure 6. IL-10 serum level is increased after improvement of BD patients. Serum was collected from two BD patients (patient a and b) in their active and inactive stages and evaluated IL-10 level by ELISA.

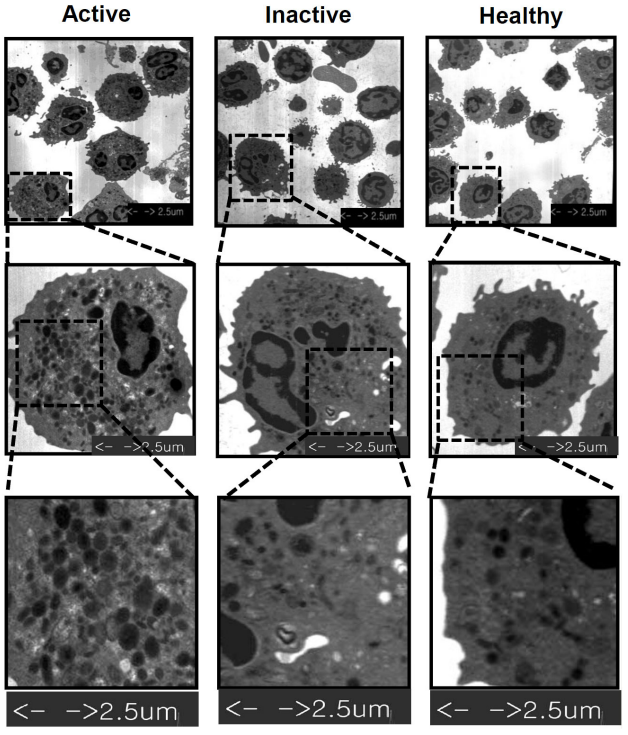

Figure 7. Distinct difference in the intracellular morphology of polymorphonuclear neutrophils of BD patients. PBMCs were isolated from whole blood of healthy control, inactive and active BD patients and the morphological changes were observed using transmission electron microscopy. The inlets of the microphotographs indicate the azurophilic granules in the cytoplasm of the neutrophil. Scale bar indicates the magnification for an individual photograph.

but in our five patients, only one had ocular BD. Therefore, the difference of CD11b expression pattern to the study of Ahn *et al.*, could be explained by different symptom composition.

Presently, the CD11b blood monocyte marker was lower in active BD patients than healthy controls, but the CD14 monocyte marker was highly expressed in inactive and active BD patients compared to healthy controls. In a previous study, Eksioglu-Demiralp *et al.*, reported similar expression pattern of CD14 in BD [15]. Houman *et al.*, reported no significant differences in the proportion of $CD11b^+CD14^+$ cells between the active and inactive stages, but $CD11b^+CD14^+$ cells were highly expressed compared to healthy controls [34]. Our data also demonstrated a similar expression pattern as reported by Houman. $CD11b^+CD32^+$ cells were significantly down-regulated in active and inactive BD patients compared to healthy controls, whereas no significant differences were evident in the proportion of $CD11b^+CD16^+$ cells between healthy controls and both active and inactive BD patients. These results indicate that among the $CD11b^+$ subsets, $CD11b^+CD14^+$ and $CD11b^+CD32^+$ cells are important in the induction of BD, but are unrelated with symptoms. The $CD11b^+CD14^+$ and $CD11b^+CD32^+$ subsets were not significantly different between active and inactive BD patients. The $CD14^+$ subsets and $CD32^+$ subsets in CD11b+ population displayed a reciprocal negative regulation according to the inhibition of $CD32^+$ macrophage activation from $CD14^+$ monocytes. CD14 is a membrane-bound protein that is expressed in monocytes, macrophages, polymorphonuclear neutrophils [35] and dendritic cells [36]. CD32 is a marker of indication in monocyte activation [37] and one of the Fc-IgG receptors [38]. Fc-IgG receptors contribute to the pathogenesis of immune complex- and auto-antibody mediated diseases such as vasculitis, rheumatoid arthritis or autoimmune neutropenia [38,39]. CD32 also plays an essential role in the removal of antigen-antibody complexes from the circulation and cell-to-cell interactions mediating antibody-dependent cell-mediated cytotoxicity. $CD14^+CD16^+$ monocytes are reportedly increased in sepsis patients with severe infection [40] and efficiently produce the pro-inflammatory cytokine TNF-α, while they produce no or little of the anti-inflammatory cytokine IL-10 [41]. Our results also showed significantly up-regulated frequencies of $CD14^+CD16^+$ subsets in BD patients (**Figure 3**) and markedly elevated IL-10 levels in improved inactive BD patients compared to active BD patients (**Figure 6**). In BD patients, the frequencies of $CD14^+CD16^+$ cells were higher in the inactive stage than in the active stage, although the difference was not statistically significant. In addition, a similar pattern was also observed in $CD14^+CD32^+$ cells.

A recent study reported that anti-TNF-α monoclonal antibodies (Infliximab and Adulimumab) are effective in treating patients with Crohn's disease (CD), which might contribute to the resolution of inflammation [42,43]. In addition, antibody against TNF-α induced the formation of a new population of macrophages in a Fc region-dependent

manner; these macrophages had an immunosuppressive phenotype because they inhibit the proliferation of activated T cells, produce anti-inflammatory cytokines, and express the macrophage marker CD206 [44]. However, in this study, a clear and significant increase was observed in the frequencies of $CD206^+$, $CD11b^+CD206^+$ cells and $CD14^+CD206^+$ cells in active BD patients compared to the inactive stage. Significant down-regulation was also observed in each individual inactive BD patient, which was recovered after receiving a combined therapy. Although patients with inflammatory bowel disease responding to infliximab displayed increased numbers of $CD206^+$ cells [45], our data show that combination therapy with colchicine, prednisolone and azathioprine with or without cyclosporine improved BD symptoms with the reduction of CD206 macrophages. Although there have been several reports on mannose binding lectin polymorphism in BD [46-48], until now there was no report on mannose receptor related one. Here, we report that mannose receptor CD206 is related to BD, and specifically the presence of symptoms in active BD patients.

Although no differences were evident in the frequencies of $CD11b^+CD14^+$ cells between the active and inactive stages (**Figure 2**), after improvement with the combination therapy the frequencies of $CD11b^+CD14^+$ cells were markedly increased in the improved stage in both patient groups.

In a previous study, the frequencies of $CD14^+CD16^+$ monocytes were lower in rheumatoid arthritis patients compared to normal subjects [49]. In this study, the frequencies of $CD14^+CD16^+$ were higher in BD patients compared to normal subjects (**Figure 3**), and treatment up-regulated $CD14^+CD16^+$ cells in both patients. No effect of cyclosporine related to the frequencies of $CD16^+CD14^+$ cells after improvement was evident (**Figure 5A**). An opposite pattern of expression was observed in $CD32^+$ subsets between both patients after improvement, in which the frequencies of $CD32^+CD11b^+$ and $CD32^+CD14^+$ cells were lower in the inactive stage of patient a, but was higher in patient b. These data show that a complicated immune response regulated the improvement of BD patients, which may vary with their choice of drugs or may be related to the ocular involvement.

PMNs are the most abundant white blood cells in the peripheral blood of humans, and are associated with the host defense. They are known as the "first line defense", particularly against bacterial infections [50]. Because of their cytotoxic and proteolytic potential, PMNs can also attack and damage the surrounding tissue, and thus can contribute to destructive inflammatory processes [51]. There is increasing evidence that PMNs are not only effector cells of the acute inflammatory reaction, but that they also participate in chronic inflammatory diseases, such as rheumatoid arthritis, primary vasculitis and inflammatory bowel disease [52,53]. In this study, we found a distinct morphological difference in PMNs of active BD patients, in which huge azurophilic granules were aggregated in the cytoplasm of the neutrophil in active BD patients. In contrast, in inactive BD patients, the granules had disappeared or were decreased. Koga *et al*., also reported that the quantity of intracytoplasmic granules in blood monocytes and macrophages were correlated with disease severity in Kawasaki disease [54]. These intracytoplasmic granules store inflammatory mediators and are considered structural markers of inflammation [55], therefore, having a role in pathology of inflammatory diseases.

In conclusion, a comparative analysis of different expression of monocyte/ macrophage markers revealed a considerable variation in phenotypes between active and inactive stage of BD patients. The CD14 monocyte marker was highly expressed in active and inactive BD patients compared to healthy controls, but another monocyte marker, CD11b, was decreased in active BD patients compared to healthy control. Moreover, their subsets were also expressed differently in active BD patients compared to healthy control. Mannose receptor (CD206) and its subset were consistently highly expressed in active BD patients compared to inactive BD patients. Furthermore, the recovery state of the BD patients showed down-regulated frequencies of CD206 in both patients with or without ocular symptoms after treatment with colchicine, prednisolone and azathioprine with or without cyclosporine. In accordance with CD206, anti-inflammatory cytokine IL-10 was highly up-regulated in improved state with combined drugs with or without cyclosporine. Taken together, the data reveal that the peripheral inflammatory environment during active stage of BD might be dominated by monocytes, which depend on the expression of surface markers representing polarized phenotypes. In the future, we shall perform the large scale study to overcome this limited cases.

Competing interests
The authors declare that they have no competing interests.

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References
1. Hatemi G, Silman A, Bang D, Bodaghi B, Chamberlain AM, Gul A, Houman MH, Kotter I, Olivieri I, Salvarani C, Sfikakis PP, Siva A, Stanford MR, Stubiger N, Yurdakul S and Yazici H: **EULAR recommendations for the management of Behcet disease**. *Ann Rheum Dis* 2008, **67**:1656-62. | Article | PubMed
2. Yurdakul S and Yazici H: **Behcet's syndrome**. *Best Pract Res Clin Rheumatol* 2008, **22**:793-809. | Article | PubMed
3. Calamia KT and Kaklamanis PG: **Behcet's disease: recent advances in early diagnosis and effective treatment**. *Curr Rheumatol Rep* 2008, **10**:349-55. | Article | PubMed
4. Kontogiannis V and Powell RJ: **Behcet's disease**. *Postgrad Med J* 2000, **76**:629-37. | Article | PubMed Abstract | PubMed Full Text

5. Direskeneli H: **Autoimmunity vs autoinflammation in Behcet's disease: do we oversimplify a complex disorder?** *Rheumatology (Oxford)* 2006, **45**:1461-5. | Article | PubMed
6. Cho S, Kim J, Cho SB, Zheng Z, Choi MJ, Kim DY and Bang D: **Immunopathogenic characterization of cutaneous inflammation in Behcet's disease**. *J Eur Acad Dermatol Venereol* 2012. | Article | PubMed
7. Raziuddin S, al-Dalaan A, Bahabri S, Siraj AK and al-Sedairy S: **Divergent cytokine production profile in Behcet's disease. Altered Th1/Th2 cell cytokine pattern**. *J Rheumatol* 1998, **25**:329-33. | Article | PubMed
8. Kim J, Park JA, Lee EY, Lee YJ, Song YW and Lee EB: **Imbalance of Th17 to Th1 cells in Behcet's disease**. *Clin Exp Rheumatol* 2010, **28**:S16-9. | Article | PubMed
9. Bardak Y and Aridogan BC: **The demonstration of serum interleukin 6-8, tumor necrosis factor-alpha, complement, and immunoglobulin levels in Behcet's disease with ocular involvement**. *Ocul Immunol Inflamm* 2004, **12**:53-8. | Article | PubMed
10. Springer TA: **Adhesion receptors of the immune system**. *Nature* 1990, **346**:425-34. | Article | PubMed
11. Nielsen HV, Christensen JP, Andersson EC, Marker O and Thomsen AR: **Expression of type 3 complement receptor on activated CD8+ T cells facilitates homing to inflammatory sites**. *J Immunol* 1994, **153**:2021-8. | Article | PubMed
12. Butcher EC: **Warner-Lambert/Parke-Davis Award lecture. Cellular and molecular mechanisms that direct leukocyte traffic**. *Am J Pathol* 1990, **136**:3-11. | PubMed Abstract | PubMed Full Text
13. Onder M and Gurer MA: **The multiple faces of Behcet's disease and its aetiological factors**. *J Eur Acad Dermatol Venereol* 2001, **15**:126-36. | Article | PubMed
14. Sahin S, Lawrence R, Direskeneli H, Hamuryudan V, Yazici H and Akoglu T: **Monocyte activity in Behcet's disease**. *Br J Rheumatol* 1996, **35**:424-9. | Article | PubMed
15. Eksioglu-Demiralp E, Direskeneli H, Kibaroglu A, Yavuz S, Ergun T and Akoglu T: **Neutrophil activation in Behcet's disease**. *Clin Exp Rheumatol* 2001, **19**:S19-24. | Pdf | PubMed
16. Direskeneli H and Saruhan-Direskeneli G: **The role of heat shock proteins in Behcet's disease**. *Clin Exp Rheumatol* 2003, **21**:S44-8. | Pdf | PubMed
17. Detmers PA, Zhou D, Powell D, Lichenstein H, Kelley M and Pironkova R: **Endotoxin receptors (CD14) are found with CD16 (Fc gamma RIII) in an intracellular compartment of neutrophils that contains alkaline phosphatase**. *J Immunol* 1995, **155**:2085-95. | Article | PubMed
18. Direskeneli H, Ergun T, Yavuz S, Hamuryudan V and Eksioglu-Demiralp E: **Thalidomide has both anti-inflammatory and regulatory effects in Behcet's disease**. *Clin Rheumatol* 2008, **27**:373-5. | Article | PubMed
19. Parren PW, Warmerdam PA, Boeije LC, Arts J, Westerdaal NA, Vlug A, Capel PJ, Aarden LA and van de Winkel JG: **On the interaction of IgG subclasses with the low affinity Fc gamma RIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2**. *J Clin Invest* 1992, **90**:1537-46. | Article | PubMed Abstract | PubMed Full Text
20. Fanger NA, Wardwell K, Shen L, Tedder TF and Guyre PM: **Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells**. *J Immunol* 1996, **157**:541-8. | Article | PubMed
21. Stahl PD: **The mannose receptor and other macrophage lectins**. *Curr Opin Immunol* 1992, **4**:49-52. | Article | PubMed
22. Taylor ME: **Structure and function of the macrophage mannose receptor**. *Results Probl Cell Differ* 2001, **33**:105-21. | Article | PubMed
23. McKenzie EJ, Su YP and Martinez-Pomares L: **The mannose receptor, a bi-functional lectin with roles in homeostasis and immunity**. T*rends Glycosci Glycotechnol* 2002, **14**:273-279.
24. Zhang Z, Maurer K, Perin JC, Song L and Sullivan KE: **Cytokine-induced monocyte characteristics in SLE**. *J Biomed Biotechnol* 2010, **2010**:507475. | Article | PubMed Abstract | PubMed Full Text
25. Radwan P, Radwan-Kwiatek K, Tabarkiewicz J, Radej S and Rolinski J: **Enhanced phenotypic and functional maturation of monocyte-derived dendritic cells from patients with active Crohn's disease and ulcerative colitis**. *J Physiol Pharmacol* 2010, **61**:695-703. | Pdf | PubMed
26. Igietseme JU, Eko FO, He Q and Black CM: **Antibody regulation of Tcell immunity: implications for vaccine strategies against intracellular pathogens**. *Expert Rev Vaccines* 2004, **3**:23-34. | Article | PubMed
27. van de Velde NC, Mottram PL and Hogarth PM: **FcgammaRII and multi-system autoimmune disease**. *Springer Semin Immunopathol* 2006, **28**:329-38. | Article | PubMed
28. Stockley RA: **Neutrophils and the pathogenesis of COPD**. *Chest* 2002, **121**:151S-155S. | Pdf | PubMed
29. Wahlstrom J, Berlin M, Skold CM, Wigzell H, Eklund A and Grunewald J: **Phenotypic analysis of lymphocytes and monocytes/macrophages in peripheral blood and bronchoalveolar lavage fluid from patients with pulmonary sarcoidosis**. *Thorax* 1999, **54**:339-46. | Article | PubMed Abstract | PubMed Full Text
30. Bainton DF: **Selective abnormalities of azurophil and specific granules of human neutrophilic leukocytes**. *Fed Proc* 1981, **40**:1443-50. | PubMed
31. Macey M, Hagi-Pavli E, Stewart J, Wallace GR, Stanford M, Shirlaw P and Fortune F: **Age, gender and disease-related platelet and neutrophil activation ex vivo in whole blood samples from patients with Behcet's disease**. *Rheumatology (Oxford)* 2011, **50**:1849-59. | Article | PubMed
32. Kibaroglu A, Eksioglu-Demiralp E, Akoglu T and Direskeneli H: **T and NK cell subset changes with microbial extracts and human HSP60-derived peptides in Behcet's disease**. *Clin Exp Rheumatol* 2004, **22**:S59-63. | Article | PubMed
33. Ahn JK, Chung H, Lee DS, Yu YS and Yu HG: **CD8brightCD56+ T cells are cytotoxic effectors in patients with active Behcet's uveitis**. *J Immunol* 2005, **175**:6133-42. | Article | PubMed
34. Houman H, Hamzaoui A, Ben Ghorbal I, Khanfir MS, Feki M and Hamzaoui K: **Tc1/Tc2 ratio in the inflammatory process in patients with Behcet's disease**. *Mediators Inflamm* 2004, **13**:247-53. | Article | PubMed Abstract | PubMed Full Text
35. Tobias PS and Ulevitch RJ: **Lipopolysaccharide binding protein and CD14 in LPS dependent macrophage activation**. *Immunobiology* 1993, **187**:227-32. | Article | PubMed
36. Verhasselt V, Buelens C, Willems F, De Groote D, Haeffner-Cavaillon N and Goldman M: **Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway**. *J Immunol* 1997, **158**:2919-25. | Article | PubMed
37. Durbin AP, Vargas MJ, Wanionek K, Hammond SN, Gordon A, Rocha C, Balmaseda A and Harris E: **Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever**. *Virology* 2008, **376**:429-35. | Article | PubMed Abstract | PubMed Full Text
38. Ravetch JV and Kinet JP: **Fc receptors**. *Annu Rev Immunol* 1991, **9**:457-92. | Article | PubMed
39. Unkeless JC, Scigliano E and Freedman VH: **Structure and function of human and murine receptors for IgG**. *Annu Rev Immunol* 1988, **6**:251-81. | Article | PubMed
40. Blumenstein M, Boekstegers P, Fraunberger P, Andreesen R, Ziegler-Heitbrock HW and Fingerle-Rowson G: **Cytokine production precedes the expansion of CD14+CD16+ monocytes in human sepsis: a case report of a patient with self-induced septicemia**. *Shock* 1997, **8**:73-5. | Article | PubMed
41. West SD, Goldberg D, Ziegler A, Krencicki M, Du Clos TW and Mold C: **Transforming growth factor-beta, macrophage colony-stimulating factor and C-reactive protein levels correlate with CD14(high)CD16+ monocyte induction and activation in trauma patients**. *PLoS One* 2012, **7**:e52406. | Article | PubMed Abstract | PubMed Full Text
42. Sinitsky DM, Lemberg DA, Leach ST, Bohane TD, Jackson R and Day AS**: Infliximab improves inflammation and anthropometric measures in pediatric Crohn's disease.** *J Gastroenterol Hepatol* 2010**, 25**:810-6. | Article | PubMed
43. Papadakis KA: **Adalimumab for the treatment of Crohn's disease.** *Expert Rev Clin Immunol* 2006, **2**:11-5. | Article | PubMed
44. Vos AC, Wildenberg ME, Duijvestein M, Verhaar AP, van den Brink GR and Hommes DW: **Anti-tumor necrosis factor-alpha antibodies induce regulatory macrophages in an Fc region-dependent manner**. *Gastroenterology* 2011, **140**:221-30. | Article | PubMed
45. Vos AC, Wildenberg ME, Arijs I, Duijvestein M, Verhaar AP, de Hertogh G, Vermeire S, Rutgeerts P, van den Brink GR and Hommes DW: **Regulatory macrophages induced by infliximab are involved in healing *in vivo* and *in vitro***. *Inflamm Bowel Dis* 2012, **18**:401-8. | Article | PubMed
46. Kim J, Im CH, Kang EH, Lee EY, Lee YJ, Park KS and Song YW: **Mannose-binding lectin gene-2 polymorphisms and serum mannose-binding lectin levels in Behcet's disease**. *Clin Exp Rheumatol* 2009, **27**:S13-7. | Article | PubMed
47. Park KS, Min K, Nam JH, Bang D, Lee ES and Lee S: **Association of HYPA haplotype in the mannose-binding lectin gene-2 with Behcet's disease**.

Tissue Antigens 2005, **65**:260-5. | Article | PubMed
48. Wang H, Nakamura K, Inoue T, Yanagihori H, Kawakami Y, Hashimoto S, Oyama N, Kaneko F, Fujita T, Nishida T and Mizuki N: **Mannose-binding lectin polymorphisms in patients with Behcet's disease**. *J Dermatol Sci* 2004, **36**:115-7. | Article | PubMed
49. Cairns AP, Crockard AD and Bell AL: **The CD14+ CD16+ monocyte subset in rheumatoid arthritis and systemic lupus erythematosus**. *Rheumatol Int* 2002, **21**:189-92. | Article | PubMed
50. Kobayashi SD and DeLeo FR: **Role of neutrophils in innate immunity: a systems biology-level approach**. *Wiley Interdiscip Rev Syst Biol Med* 2009, **1**:309-33. | Article | PubMed Abstract | PubMed Full Text
51. Mahmudi-Azer S and van Eeden SF: **Neutrophil 'connectivity': key to neutrophil-mediated tissue injury?** *Crit Care* 2003, **7**:285-7. | Article | PubMed Abstract | PubMed Full Text
52. Nikolaus S, Bauditz J, Gionchetti P, Witt C, Lochs H and Schreiber S: **Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin 10 during intestinal inflammation**. *Gut* 1998, **42**:470-6. | Article | PubMed Abstract | PubMed Full Text
53. Iking-Konert C, Ostendorf B, Sander O, Jost M, Wagner C, Joosten L, Schneider M and Hansch GM: **Transdifferentiation of polymorphonuclear neutrophils to dendritic-like cells at the site of inflammation in rheumatoid arthritis: evidence for activation by T cells**. *Ann Rheum Dis* 2005, **64**:1436-42. | Article | PubMed Abstract | PubMed Full Text
54. Koga M, Ishihara T, Takahashi M, Umezawa Y and Furukawa S: **Activation of peripheral blood monocytes and macrophages in Kawasaki disease: ultrastructural and immunocytochemical investigation**. *Pathol Int* 1998, **48**:512-7. | Article | PubMed
55. Melo RC and Dvorak AM: **Lipid body-phagosome interaction in macrophages during infectious diseases: host defense or pathogen survival strategy?** *PLoS Pathog* 2012, **8**:e1002729. | Article | PubMed Abstract | PubMed Full Text

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