



# Downregulation of Autophagy-Related Genes in Macrophages From Patients With Behcet's Disease

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## Abstract

**Objective:** Overwhelming inflammatory chemokines and cytokines characterize the immunological profile and inflammatory settings of Behcet disease (BD). The connection between autophagy-related genes (ATGs) and various perspectives of innate and adaptive immunobiology such as antigen presentation, immune tolerance, lymphocyte development and differentiation, cytokine signaling, and inflammation have been implicated. The aim of this study was to evaluate the mRNA expression profile of ATGs in macrophages of patients with BD.

**Materials and Methods:** Whole blood samples were obtained from 10 BD patients and 10 healthy controls. Monocytes were isolated from the blood samples and then differentiated to macrophages using macrophage colony-stimulating factor (M-CSF). After total RNA extraction and cDNA synthesis, quantitative analysis of ATGs including ATG5, ATG7, ATG12, LC3b, mTOR, RAPTOR, and RICTOR was conducted by SYBR Green master mix and real-time polymerase chain reaction (PCR).

**Results:** mRNA expression of all ATGs was downregulated in macrophages of BD patients compared with healthy controls. It is worth to note that the downregulation of ATG12 and LC3b mRNAs in macrophages of BD patients was statistically significant in comparison to that of healthy control group ( $P=0.007$  and  $0.021$ , respectively).

**Conclusion:** Considering the role of autophagy in initiation of immune responses and then clearance of dead cells as well as its participation in the development and differentiation of immune cells, downregulation of ATGs in macrophages of BD patients may be involved in uncontrolled immune response and overproduction of inflammatory cytokines.

**Keywords:** Behcet disease, Autophagy-related genes, Macrophage, Inflammation

## Introduction

Behcet disease (BD) is a multisystemic and chronic inflammatory disorder, mainly characterized by recurrent oral and genital ulcers, ocular involvement, and skin lesions, with periods of remission and relapse (1,2). Epidemiological studies have documented that BD is comparatively frequent in countries located around Mediterranean Sea, the Middle East, China, and Japan, along the ancient Silk Route (3). The exact etiology of BD has not fully explained yet, but the disease occurrence is highly associated with a genetic factor, namely human leukocyte antigen (HLA)-B51. Furthermore, genetic studies have established a strong association of HLA-B with cytokine production including high production of tumor necrosis factor (TNF) and decreased production of interleukin (IL)-10. Gene variations have also been observed in inflammatory cytokines, chemokines, and adhesion molecules, leading to vascular inflammation and consequently vascular damage (4,5).

BD shares characteristics of both autoimmunity and

autoinflammatory. The major features of BD that lead to the classification of the disease as an autoimmune disorder include response to classical immunosuppressive drugs like cyclosporine and azathioprine (6), and involvement of autoantigens such as heat-shock protein 60 (HSP60) in the initiation of the disorder (7). On the other side, specifications of the disease implying the autoinflammatory face of BD comprise high-titer auto-antibodies, a rise in the number of antigen-specific T cells, the role of major histocompatibility complex (MHC) class I molecules, clinical periods of recurrent inflammation by neutrophils mainly (8), and effectiveness of anti-inflammatory agents like colchicine (9). Studies show that macrophages produce macrophage inhibitory protein-1alpha (MIP-1α) chemokine and mediate the activation and recruitment of other leukocytes to the sites of inflammation in BD patients (10).

Autophagy is an intracellular degradation process and plays a variety of physiological and pathological roles through removing aggregated proteins, infectious

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organisms, and damaged organelles by lysosomes. The autophagy proteins are the main executors of the autophagic process in humans (11,12). Autophagy is involved in the normal biology of lymphocytes by facilitating the development of B and T lymphocytes (13). Autophagic pathways and mediators participate in the biology of immune system and inflammatory process. They orchestrate the beneficial and adverse effects of immunity and inflammation, hence may participate in protection against infectious microorganisms, inflammatory conditions, and autoimmune disorders (13). Mounting evidence has disclosed that modifications of the autophagic pathways play a role in the development of several disorders (14). Genetic variants of autophagy-related genes (ATGs) have been associated with the susceptibility to a number of autoimmune diseases, like systemic lupus erythematosus (SLE) (15,16), psoriasis (17), and Crohn disease (18,19). Moreover, ATG5 gene variation was associated with both expression level of the gene and BD risk (20).

In consideration of all, it is hypothesized that perturbations of autophagic process in macrophages, as a main source of inflammatory cytokines, might be involved in the inflammatory settings in BD. Hence, we aimed to evaluate the mRNA expression of ATGs, including ATG5, ATG7, ATG12, LC3b, mTOR, RAPTOR, and RICTOR, in macrophages from BD patients and compare them with healthy individuals.

## Materials and Methods

### Study Subjects

In this case-control study, 10 active BD patients ( $38 \pm 11.2$  years old) who were diagnosed with the disease based on the principles of the International Criteria for Behcet's Disease (ICBD) (21) as the case group, and 10 healthy individuals as the control group were included. Clinical specifications of the patients are summarized in Table 1. Healthy subjects ( $34 \pm 6.9$  years old) had no familial history of BD or other autoimmune disorders and were age- and sex-matched with the patient group. The patients were recruited from those who referred to the outpatient clinic of the Rheumatology Research Center, Shariati hospital, Tehran, Iran.

### Isolation of Monocytes and Differentiation of Macrophages

Peripheral blood samples were obtained from BD patients and healthy volunteers and collected into four 9-mL tubes containing EDTA. In order to separate the peripheral blood mononuclear cells (PBMCs), density gradient centrifugation was employed. Samples were processed within 10 hours of collection. Whole blood was diluted 1:2 with PBS (GIBCO, Invitrogen). Lymphocyte separation medium (Lymphodex, Inno-Train) was used for isolation of PBMC buffy coats. We incubated PBMCs with magnetic-activated cell sorting (MACS) columns and CD14 micro-beads to isolate monocytes by positive

**Table 1.** Clinical Characteristics of the BD patients

Characteristic	No. (%)
Male/Female	5 (50)/5 (50)
HLA-B5	5 (50)
HLA-B51	2 (20)
HLA-B27	0 (0)
Oral aphthosis	10 (100)
Genital aphthosis	7 (70)
Uveitis	7 (70)
Retinal vasculitis	0 (0)
Joint manifestation	1 (10)
Pseudo folliculitis	1 (10)
Erythema nodosum	3 (30)
Anterior uveitis	3 (30)
Posterior uveitis	4 (40)
Arthralgia	0 (0)
Monoarthritis	1 (10), Sacroiliitis
Oligoarthritis	0 (0)
Spondyloarthropathy	0 (0)
Gastrointestinal aphthous ulcers	0 (0)
Abdominal vasculitis	0 (0)
Diarrhea	0 (0)
Rectorrhagia	0 (0)
Colitis	0 (0)
Epididymitis	0 (0)
Neurological manifestations	0 (0)
Pathergy	0 (0)
Pulmonary manifestations	0 (0)
Cardiac manifestations	0 (0)
Large vessel involvements	0 (0)
Familial history of BD	0 (0)

selection (all from Miltenyi Biotec, Germany). Flow cytometry confirmed the purity of 90%-94% CD14 positive cells (22). Monocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS; Gibco BRL), 100 µg/mL streptomycin, 100 U/mL penicillin (Sigma), and L-glutamine (Biosera) and differentiated to macrophage by 50 ng/mL of recombinant human macrophage colony-stimulating factor (M-CSF; eBioscience) during 7 days.

### Flow Cytometry Analysis

We performed the staining of monocytes and macrophages by allophycocyanin (APC) conjugated anti-human CD163, fluorescein isothiocyanate (FITC) conjugated anti-human CD206 (both Miltenyi Biotec, Germany), and phycoerythrin-cyanin 5 (PE-Cy5) conjugated anti-human HLA-DR (Biolegend, USA). The simultaneous isotype control staining was performed. The cells ( $2 \times 10^5$ ) were incubated with the mentioned antibodies for 30 minutes at 4°C in darkness. Afterwards, cells were washed and analyzed on a FACS Calibur (Becton Dickinson, USA) flow cytometry system using the FlowJo software (Tree

Star, Ashland, OR, USA). Flow cytometry showed that after 7-day stimulation by M-CSF, the cells were >91% HLA-DR+ and expressed macrophage markers CD163 and CD206 (95% and 91%, respectively) (22).

#### Quantitative Real-time PCR Analysis

High Pure RNA Isolation kit (Roche, Germany) was used in order to isolate total RNA from the macrophages according to the guidelines by the manufacturer. We reverse transcribed total RNA of each sample to cDNA by Transcriptor First Strand Synthesis kit (Roche, Germany) according to the manufacturer's recommendations. The relative expression levels of ATG5, ATG7, ATG12, LC3b, mTOR, RAPTOR, and RICTOR mRNAs were measured using the StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Green (Ampliqon, Denmark) master mix. The values were normalized based on the expression level of endogenous housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers (Table 2) to quantify ATGs and housekeeping gene were designed employing Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). To examine the accuracy and specificity, all primers were checked using the basic local alignment search tool (BLAST) on the USA National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The relative changes in gene expression between patient and healthy control groups were determined using the comparative  $C_T$  method ( $2^{-\Delta CT}$ ) (23).

#### Statistical Methods

Data analysis was carried out by SPSS software version 22.0 (SPSS, Chicago, IL, USA) and data illustration by graphs was done using Windows-based software of GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). Mann-Whitney U test was applied for

comparing the relative mRNA expression between the patient and control groups. Association analysis ( $\eta^2$ ) was conducted to evaluate relationships between clinical features of the patients and relative mRNA expression of ATGs. Qualitative and quantitative variables were represented as mean  $\pm$  standard deviation (SD) and number (%), respectively.  $P$  value less than 0.05 was set to be statistically significant.

#### Results

Quantitative data indicated that mRNA expression of all ATGs including ATG5, ATG7, ATG12, LC3b, mTOR, RAPTOR, and RICTOR was downregulated in M1 macrophages from BD patients in comparison to healthy subjects (Table 3, Figure 1). Decreased expression level of ATG12 (0.34-fold downregulated,  $P=0.007$ ) and LC3b (0.48-fold downregulated,  $P=0.021$ ) mRNAs was statistically significant.

Association analysis ( $\eta^2$ ) indicated that none of the clinicopathological specifications of the BD patients including gender, HLA-B5 and B51 status, genital aphthosis, uveitis, joint manifestation, pseudo folliculitis, erythema nodosum, anterior uveitis, posterior uveitis, and sacroiliitis were related to mRNA expression of ATGs. More details are shown in Table 4.

#### Discussion

Generally, systemic vasculitis is used to describe 3 major hallmarks of BD. The disease can involve blood vessels of all types and sizes, lungs, joints, nervous system, and gastrointestinal tracts. Immune system abnormalities have long been blamed as the main culprit in the BD pathogenesis, in which immune overresponse occurs possibly due to stimulation through microbial pathogens in genetically-prone individuals. Recent findings have emphasized the importance of inflammation in BD (24). Several cytokines may be released in high levels from

**Table 2.** Primer Sets Used for Quantification of Autophagy-Related Genes by SYBR Green Real-Time PCR

Genes	Forward primer	Reverse primer	Amplicon size (bp)	Tm (°C)
ATG5	5'-AAAGATGTGCTTCGAGATGTGT-3'	5'-CACTTTGTCAGTTACCAACGTC-3'	152	60.54 59.04
ATG7	5'-ATGATCCCTGTAACCTAGCCCA-3'	5'-CACGGAAGCAAACAACCTCAAC-3'	114	58.61 59.15
ATG12	5'-TAGAGCGAACACGAACCATCC-3'	5'-CACTGCCAAAACACTCATAGAGA-3'	153	60.14 58.68
LC3b	5'-GATGTCCGACTTATTCGAGAGC-3'	5'-TTGAGCTGTAAGCGCCTTCTA-3'	167	58.70 59.45
mTOR	5'-GCAGATTGCCAATCTTCGG-3'	5'-CAGCGGTAAGGTGCCCTG-3'	114	60.24 61.21
RAPTOR	5'-ACTGGAACCTACCTTTGGCT-3'	5'-ACTGTCTTCATCCGATCCTTCA-3'	106	59.22 58.90
RICTOR	5'-GCTAGGTGCATTGACATACAACA-3'	5'-AGTGCTAGTTCACAGATAATGGC-3'	200	59.31 58.55
GAPDH	5'-GAGTCAACGGATTGGTCGT-3'	5'-GACAAGCTTCCCCTTCTCAG-3'	185	58.21 58.57

Abbreviations: bp, base pair; Tm, melting temperature.

**Table 3.** Gene Expression Fold Change of Autophagy Gene mRNAs in Macrophages From BD Patients in Comparison to Healthy Controls

Autophagy Gene	Fold Change (BD Patients vs. Controls)	P Value
ATG5	0.52	0.226
ATG7	0.76	0.344
ATG12	0.34	0.007 <sup>a</sup>
LC3b	0.48	0.021 <sup>a</sup>
mTOR	0.55	0.082
RAPTOR	0.58	0.140
RICTOR	0.70	0.427

Abbreviation: BD; Behcet's disease.

<sup>a</sup> Statistically significant at level of 0.05.

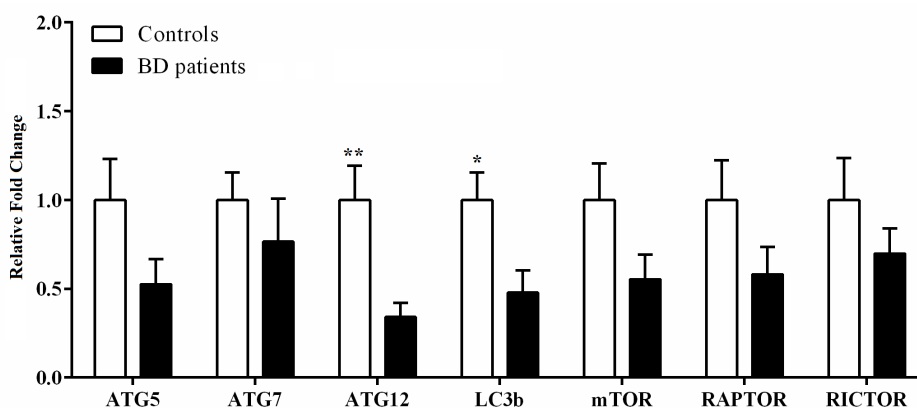
lymphocytes and monocytes of BD patients. As a main pathogenetic pathway in BD, overactivation of neutrophils with enhanced chemotaxis and cytokine production as well as upregulation of adhesion molecule have been implicated (25). T cell mediated immune responses, on the other hand, are the core players in the immune overactivation and cytokine overproduction in BD (26). Studies demonstrated that perturbations of the autophagic pathways might take part in the pathogenesis of infectious diseases and autoimmune disorders (27,28).

The hypothesis whether modifications in ATGs and impaired autophagy function may culminate in inflammatory autoimmune disease led us to evaluate the mRNA expression of such genes in BD patients (13). Several single nucleotide polymorphisms (SNPs) in ATG5 has been associated with predisposition to SLE (15,29,30). In SLE, there are autoimmune responses against autoantigens originated from self-dying cells. ATG5 genetic variants have also been linked to BD susceptibility (20). Despite an unknown mechanism of influence of such SNPs on ATG5 expression and function, impaired ATG5-dependent negative thymic selection results in multiorgan inflammation and autoimmunity in mice (31). There are also other effects because of

loss of ATG5 function such as modulation of interferon (IFN) and release of pro-inflammatory cytokines (32,33), clearance of dying cells (34) and antigen presentation by dendritic cells (35). These effects might also contribute to the autoimmunity and inflammation. As a result, a link between dysregulated expression of ATGs and autoimmune pathogenesis is biologically plausible. In this study, we observed insignificant downregulation of ATG5, ATG7, mTOR, RAPTOR, and RICTOR, while statistically significant downregulation of ATG12 and LC3b in macrophages from BD patients in comparison to healthy control group was disclosed.

ATG5, which is the key regulator of autophagosome-precursor synthesis, interacts with Fas-associated protein with death domain (FADD), and mediates the IFN- $\gamma$ -related cell death. ATG5 is activated by ATG7 and the consequent participation of ATG5 and ATG12 acts as an E1-like activating enzyme in a ubiquitin-like manner, which affects several intracellular processes such as appearance of autophagic vesicles and repression of innate and adaptive immune system (36). ATG5 is in conjugation with ATG7 and ATG12, which facilitates expansion of isolation membranes (phagophores) (37). Microtubule-associated protein 1 light chain-3b (LC3b) affects the fusion of autophagosome with lysosome and the expansion of autophagic membrane by its conversion from cytosolic form to phosphatidylethanolamine -conjugated form (38).

One of the explanations for impaired expression of ATGs in BD patients may be fulfilled in consideration of putative role of infectious agents in BD etiopathogenesis (39). Given the role of autophagy in the defense mechanisms against infectious pathogens, ATG impairment might provide an explanation about the relation between bacterial infection and BD development. The most generally agreed hypothesis for the role of infection in BD pathogenesis is that these antigens have high homology with human molecules such as heat shock protein 65 (HSP65). Mycobacterium derived HSP56, which has high homology with human HSP60 protein, causes the

**Figure 1.** Relative Fold Change of ATGs in Macrophages From BD Patients in Comparison With Healthy Individuals. Downregulation of ATG12 and LC3b was statistically significant (\*\*  $P = 0.007$ , \* $P = 0.021$ ).

**Table 4.** Association of Disease Manifestations of BD Patients With Relative mRNA Expression of ATGs

Group	ATG5		ATG7		ATG12		LC3b		mTOR		RAPTOR		PICTOR	
	$\eta^2$	<i>P</i>	$\eta^2$	<i>P</i>	$\eta^2$	<i>P</i>	$\eta^2$	<i>P</i>	$\eta^2$	<i>P</i>	$\eta^2$	<i>P</i>	$\eta^2$	<i>P</i>
Gender	0.016	0.683	0.180	0.999	0.165	0.999	0.069	0.999	0.344	0.999	0.338	0.999	0.447	0.999
HLA-B5	0.049	0.999	0.224	0.999	0.123	0.999	0.000	0.999	0.162	0.999	0.222	0.999	0.261	0.999
HLA-B51	0.117	0.378	0.168	0.999	0.136	0.999	0.029	0.644	0.043	0.999	0.268	0.999	0.027	0.999
Genital aphthosis	0.084	0.267	0.005	0.999	0.034	0.999	0.136	0.533	0.082	0.999	0.205	0.999	0.94	0.999
Uveitis	0.222	0.800	0.299	0.999	0.336	0.999	0.345	0.533	0.194	0.999	0.344	0.999	0.039	0.999
Joint manifestation	0.316	0.600	0.296	0.999	0.369	0.999	0.291	0.800	0.361	0.999	0.359	0.999	0.418	0.999
Pseudo folliculitis	0.277	0.999	0.264	0.999	0.327	0.999	0.317	0.800	0.218	0.999	0.230	0.999	0.429	0.999
Erythema nodosum	0.284	0.800	0.299	0.999	0.293	0.999	0.160	0.533	0.484	0.999	0.219	0.999	0.588	0.999
Anterior uveitis	0.049	0.999	0.065	0.999	0.136	0.999	0.003	0.999	0.260	0.999	0.111	0.999	0.13	0.999
Posterior uveitis	0.096	0.206	0.054	0.999	0.123	0.999	0.191	0.444	0.076	0.999	0.183	0.999	0.084	0.999
Sacroiliitis	0.316	0.600	0.296	0.999	0.369	0.999	0.291	0.800	0.361	0.999	0.359	0.999	0.418	0.999

activation of  $\gamma\delta$ T cells in cross-reactive manner in BD patients (1,40,41). Additionally, in vivo study indicated that ATG5 played a role in LPS-induced inflammatory response of macrophage polarization from mice and suggested that ATG5 mediated a negative regulatory feedback mechanism to suppress the inflammatory response (42). Therefore, downregulation of ATGs in macrophages of BD patients might be the possible cause of increased inflammatory responses, which needs to be explored for possible mechanisms.

A variety of immune and nonimmune cells seem to be responsible for overwhelming inflammatory setting in BD by overproduction of the pro-inflammatory cytokines. Moreover, high levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  have been attributed to pro-inflammatory macrophages, suggesting the role of macrophage-dependent inflammation in the pathogenesis of BD (43). On the other side, high level of IFN- $\gamma$  activates macrophages and monocytes to produce reactive oxygen and nitrogen species as well as several monokines, and causes efficient self-antigen presentation by MHC class II molecules to self-recognizing T helper 1 cells (44-46). To prematurely conclude, decreased expression of ATGs in macrophages from BD patients may be involved in the inflammatory manifestations of the patients.

The ATG5, which is required for antigen presentation, plays a role in enhanced viral clearance (47). The autophagy machinery impairments have been related to increased viral replication (48). The presence of herpes simplex virus (HSV) type 1 genome was indicated within the peripheral blood lymphocytes of BD patients (49,50). A 289 base pair genomic segment of HSV type 1 was also amplified by PCR from the saliva fluid, genital and gastrointestinal ulcers of patients with BD (51,52). It should be noted that aberrant expression of ATGs may be related to the role of viral infection in BD pathogenesis.

In conclusion, this study demonstrated the downregulation of ATGs in M1 macrophages from BD patients in comparison to healthy control group. Lower expression of ATGs can be involved in BD pathogenesis

in a number of putative approaches of pro-inflammatory cytokines overproduction, aberrant self-antigen presentation, and bacterial and viral infections, among others. However, limitations of this study, such as small sample size and evaluation of a number of ATGs, should be noted and improved in further studies to shed light on the role of ATGs in BD pathogenesis.

#### Conflict of Interests

The authors declare that they have no conflict of interests.

#### Ethical Issues

Informed consent forms were obtained from all subjects participated in the study. The Ethics Committee of Tehran University of Medical Sciences concurred with the study protocol (Ethics Code: IR.TUMS.REC.1394.1941).

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