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Aberrant Expression of Three New Members of *IL-1* Family (*IL-36α*, *IL-37* and *IL-38*) in Patients with Multiple Sclerosis

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Abstract

Background & Objective: Multiple sclerosis (MS) is a neurodegenerative disease that is characterized by demyelination and neuronal dysfunction. The study of the expression of cytokine genes seems to be an appropriate option for assessing their potential for the onset and development of the disease. Therefore, in the present study, changes in the expression of three interleukins (*IL-36α*, *IL-37*, and *IL-38*) in MS and their association with clinical factors were investigated.

Material & Methods: In this study, blood samples of 45 MS patients and 45 healthy controls were enrolled. Relative expression of genes was evaluated using the real-time PCR. Finally, the pattern of expression was analyzed by using statistical analyses. In the next step, the relationship between clinical characteristics and *IL-36α*, *IL-37*, and *IL-38* expression was investigated.

Results: The results showed that *IL-36α* and *IL-37* increased in MS patient (1.8 and 3.5 fold respectively, P<0.01). Moreover, a significant reduction in *IL-38* was observed in MS samples (0.21 fold, P<0.01). The dysregulation in *ILs* expression was associated with clinical features.

Conclusion: As a result, the two studied *IL-1* family members (*IL-36α* and *IL-37*) may contribute to the progression of MS, so that increase in their expression is associated with EDSS disability and duration of disease. Another member of *IL-1* family, *IL-38* has a protective effect on MS diseases, and its downregulation is related to severe disability. Further investigation can help to determine accurate functional role of 3 interleukins in MS development.

Keywords: *IL-36α*, *IL-37*, *IL-38*, Multiple sclerosis, gene expression

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Introduction

Interleukins (*ILs*) are a group of cytokines that are expressed by white blood cells (leucocytes) and regulate cell growth and differentiation. *ILs* are classified into different groups depending on the source of their production and their receptor, target cells, and function. One of the most important *IL* families is *IL-1*; the family of *IL-1* cytokines is a set of protein molecules that play a key role in regulating intrinsic immunogenicity activation and are the first line of defense against pathogenic microorganisms (1). *IL-1*, which was initially described as a fever-inducing protein, induces a complex network of inflammatory cytokines and

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regulates inflammatory responses by expressing integrins on leukocytes and endothelial cells (2). The *IL-1* family consists of 11 members that include 7 pro-inflammatory agonists (including *IL-36α*) and 4 distinct antagonists (including *IL-37* and *IL-38*) (3). The genes of all members of the *IL-1* family are positioned as gene cluster on chromosome 2 (4, 5). *IL-36α* is a common mediator of intrinsic and adaptive immune responses. The major function of *IL-36α* is to progress primary inflammatory response to the injured tissue or infection (6). Recently, IL-36 expression has been reported in autonomic dysreflexia lesions, psoriasis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and inflammatory bowel disease (IBD) (7, 8). *IL-37* is often expressed in monocytes, tonsillar plasma cells, and breast carcinoma cells; this type of IL also has anti-inflammatory properties, and its major activity is inhibition of *IL-1*8 activity and intrinsic immunogenicity (9). *IL-38* expression has been reported in the skin, tonsils, thymus gland, liver, and salivary glands (10). *IL-38* plays a role in the pathogenesis of inflammatory diseases and exerting the supportive effect in some autoimmune diseases. *IL-38* binds to the IL-36 receptor, so that both ILs have the same biological role on immune cells and produce anti-inflammatory effects (11). Studies have shown that the *IL-1* family is particularly important in stimulating immune responses such as inflammation, and therefore the immune system's function depends heavily on these types of ILs so that the association between their defect, immunodeficiency, and therefore development of a wide range of diseases, including asthma, AIDS, and autoimmune diseases such as multiple sclerosis (MS), has attracted many researchers' attention (12, 13).

MS is an autoimmune, neurodegenerative disease characterized by demyelination and neuronal deficiency in the central nervous system (14). Although MS is one of the most common neurological diseases, its pathogenicity remains to be fully understood. There seems to be a significant heterogeneity in the mechanisms of the disease, but in most cases, at least a strong genetic component is involved (15). Since inflammation plays a crucial role in the development of MS, study of the expression of cytokine genes appears to be a good option to assess the potential for the onset and development of this disease. It has been shown that the *IL-1* family plays a major role in neuritis. During inflammation, the levels of tumor necrosis factor alpha and *IL-1* in the brain increase and their presence may break the blood-brain barrier (16). A study has indicated an increase in serum IL-2 levels in patients with MS, demonstrating the crucial role of IL-2 in MS pathogenesis by activating endothelial cells (17). IL-7 is known as the Pre-B-cell growth factor or lymphopoietin-1. Studies have shown that alpha chain receptor in IL-7 has a significant allelic and functional association with MS (15). IL-2 receptor (IL-2R) and IL-7 receptor (IL-7R) genes are also associated with MS (14). *IL-17*, and induce pro-inflammatory cytokines. Recently, *IL-17* has been confirmed as an important immunological constituent for the nervous system inflammation (18). Therefore, given the importance of ILs in immune responses as well as the role of these proteins in the onset and development of autoimmune diseases, including MS, the changes in the expression of three ILs from the *IL-1* family, including *IL-36α*, *IL-37*, and *IL-38*, in MS were investigated in the present study.

Material and Methods

Blood samples collection and characteristics of participants

In the present study, blood samples were used to detect changes in the expression of three IL genes (*IL-36α*, *IL-37*, and *IL-38*) in MS disease. The participants consisted of 45 patients with relapsing-remitting (RR) MS and 45 health controls. All participants (MS patients and controls) were enrolled from the same geographic area. Diagnosis of MS was made with reference to the McDonald criteria (19). A neurologist also confirmed the diagnosis of MS.

In the present study, controls had no history of neurological, autoimmune, and neurodegenerative diseases. All individuals in this study provided written consent to participate in the study and blood samples were collected after completion of the questionnaires. The questionnaires included the clinical information including age, sex, duration of illness, type of drug, and degree of disability in the patients. To test the gene expression, the experiments were performed in several steps and in accordance with the standard protocols.

Total RNA extraction and cDNA synthesis

In this study, extraction of total RNA was performed on blood samples and MN extraction kit (NucleoSpin RNA Blood, Germany) was used to extract total cell RNA that was then purified according to the instructions of the kit. Nanodrape (Nanodrop2000, Thermo Fisher Scientific, Wilmington, DE, USA) was used to evaluate the quality and quantity of extracted RNAs. Next, the synthesis of complementary DNA (cDNA) was performed by using a specific synthesis kit (Takara, Clontech). The cDNA synthesis was carried out in two steps; first, the addition of random hexamer and oligo-dt primers along with reverse transcriptase (Prime Script RT) and secondly, incubation at 37 ˚C for 15 minutes followed by 5 seconds at 85 ˚C. Finally, the synthesized cDNA was used for real-time PCR.

qPCR assay

The quantitative expression of target genes was performed by qRT-PCR. The sequence of designed primers is shown in Table 1. Rotor-gene 6000 (Qiagen, Hildn, Germany) was used to examine the expression of genes. *GAPDH* was used as internal reference gene. The volume of reaction solution for *IL-36α*, *IL-37*, and *IL-38* and reference gene was 20 µL, consisting of 10 µL of SYBR premix Ex taqII (Takara), 0.2 µL of forward primers (10 pM), 0.2 µLof reverse primers (10 pM), and 2 µL of diluted cDNA. The volume of the resulting solution was eventually increased to 20 µL with addition of nuclease-free water. The PCR temperature protocol in the apparatus was set as follows: Initial denaturation at 95 ˚C for 10 min, 40 cycles of amplification, including 5 s of denaturation at 95 ˚C, 20 s at annealing temperature of the primers (60 ˚C), and 20 s at the extension temperature of the fragments (72˚ C). The melting of products was performed at 70-95 ˚C. Finally, the relative expression of genes was calculated by the $2^{-\Delta\Delta ct}$ method and then the expression pattern was analyzed by statistical analysis.

Table1. Primer sequences of target and internal control genes in this study and product size

Gene	Primer sequence	Product size (bp)
IL-36α	F: 5′- GAAGGACCGTATGTCTCCAGTC -3′ R: 5′- CACACATCAGGCAGAGATTGAG -3′	125
IL-37	F: 5′- GCAATTGTAATGAGCCTGTTGGG -3′ R: 5′- AGCGAGGAAGGCGTTCAATG -3′	146
IL-38	F: 5′- ACAGCTGGAGGATGTGAACATTG -3′ R: 5′- TGCTCTGGAAGAAGGTGAAGC -3′	77
GAPDH	F: 5′- GTGAAGGTCGGAGTCAAC -3′ R: 5′- GTTGAGGTCAATGAAGGG -3′	116

Assessment of disability level

Diagnosis of MS was made with reference to the McDonald criteria (19) and then confirmed by neurophysiological tests, clinical diagnoses, and magnetic resonance imaging. Degree of disability was determined by Kurtzke Expanded Disability Status Scale (EDSS), which is a method for quantifying disability in MS patients (20). EDSS is based on a neurological test carried out by an expert and quantifies disability in eight functional systems by determining the score of the functional system in each of them. According to this scale, three stages of disability are mild (EDSS = 0-4.0), moderate (EDSS = 4.5-5.5), and severe (EDSS = 6.0-9.5).

Statistical analysis

Data analysis was performed by SPSS (SPSS v.22 Inc., Chicago, IL, USA). T-test and ANOVA were used to determine the significance of gene expression levels. The results were expressed as mean (± SD). $P < 0.05$ was considered significance level. Finally, the graphs were plotted by using the Graph PadPrism7 software (GraphPad Software Inc., San Diego, CA, USA).

Results

In current study, blood samples of 45 MS patients and 45 healthy controls aged between 18 and 45 years of age were studied. Clinical characteristics of the participants are summarized in Table 2. The relative expression of the *IL-36α*, *IL-37*, and *IL-38* genes is illustrated in Figure 1. The relative expression of *IL-36α* was higher in blood samples of patients with MS than in those of controls ($P = 0.005$). Relative expression of *IL-37* in blood samples of patients with MS was significantly greater compared to controls [95% confidence interval (95%CI), $P = 0.02$]. Also, *IL-38* expression significantly decreased in MS patients compared to controls ($P = 0.006$) (Chart 1).

Table2. Demographic and clinical characteristics of patients and controls involved in this study

Index	MS (45)	Control (45)
Mean±SDE age	32.68 ± 1.1	34.24 ± 2.81
Male (%)	11 (24.45)	13 (28.89)
Female (%)	34 (75.56)	32 (71.11)
Disease duration (years)	6.31 ± 1.28	-
EDSS	5.05 ± 2.12	-
Disease subtype	RRMS	-

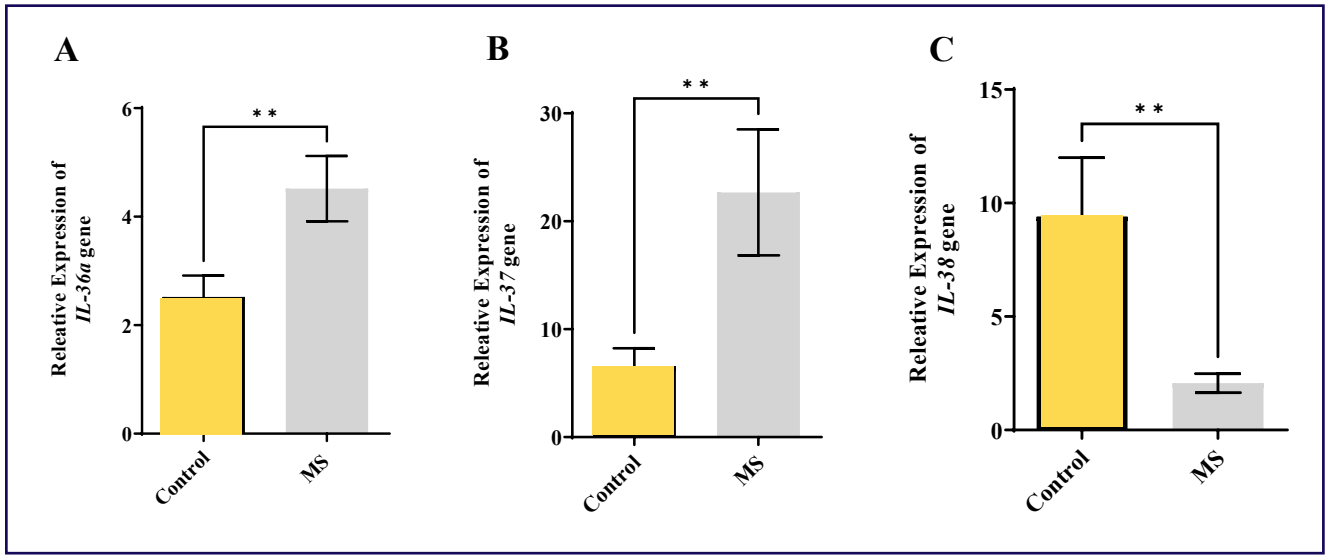


Chart 1. Comparison of the relative expression of the three interleukin genes in relapse-remitting multiple sclerosis patients and healthy controls. A) Relative expression of *IL-36α*, B) *IL-37* and C) *IL-38*

The relationship between the expression of the *IL-36α*, *IL-37*, and *IL-38* genes and age (32 and over 32 years of age), gender, EDSS, and duration of disease (year) was studied separately (Chart 2-4). In the study of the relationship between the expression of the *IL-36α*, *IL-37*, and *IL-38* genes and age (32 and over 32 years of age based on average age in MS samples), it was found that gene expression in participants under 32 and over 32 years of age was not significantly different for all three genes; in other words, there was no statistically significant association between gene expression and age of the participants for all three ILs (Chart 2-4 A). To determine the relationship between gene expression and EDSS, the patients were divided into two groups [mild to moderate disability (EDSS = 0-5.5) and severe disability (EDSS = 6-10)]. The results showed that *IL-36α* gene expression increased in the patients with severe disability (EDSS = 6-10) but there was no statistically significant difference in gene expression of two group (95% CI, P = 0.1) (Chart 2B). To determine the relationship between duration of disease and the expression levels of the studied genes, the patients were divided into 4 groups (1-20 years). The results of the study showed that *IL-36α* gene expression varied in different durations, but there was no statistically significant difference in expression of the gene among different durations of the disease (P > 0.05) (Chart 2C). In examining the relationship between *IL-37* gene expression and disability, it was found that expression of the gene was associated with mild disability (EDSS = 0-5.5) (95% CI, P= 0.02) (Chart 3B). In examining the relationship between *IL-37* gene expression and the duration of the disease, it was found that the expression of the gene increased significantly with increase in the duration of the disease (P = 0.03) (Chart 3C). In the study of relationship between *IL-38* gene expression and EDSS, it was found that the relative expression of the gene was significantly lower in severe EDSS (P = 0.02) (Chart 4B). Regarding the relationship between *IL-38* expression and the duration of the disease, as the duration of the disease increased, the expression of the gene decreased significantly, with minimum gene expression level in the disease duration of 15-20 years (P = 0.0001) (Chart 4C).

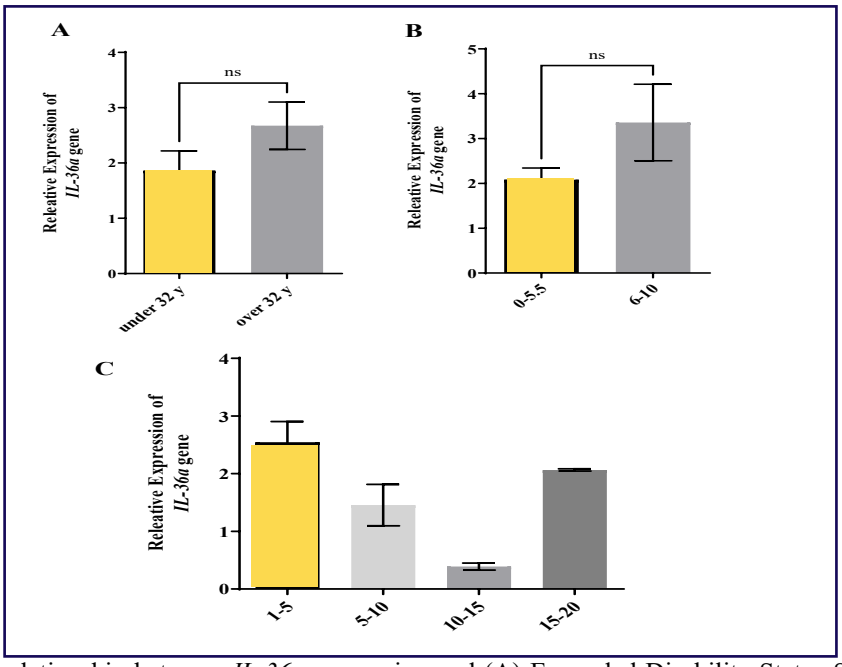


Chart 2. The relationship between *IL-36α* expression and (A) Expanded Disability Status Scale, and (B) duration of disease (year) NS = Not Significant

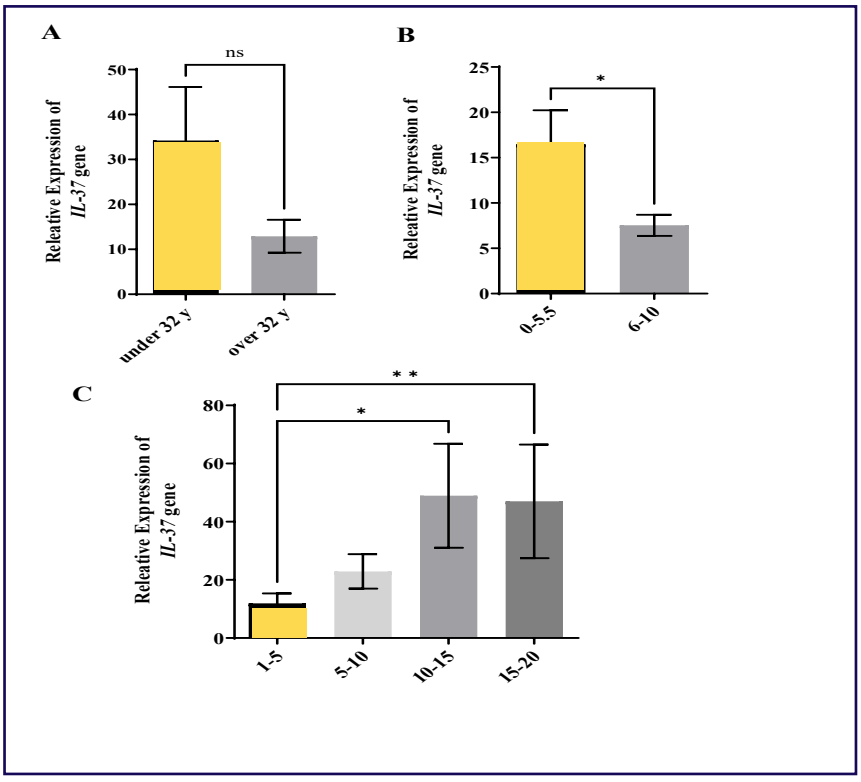


Chart 3. The relationship between *IL-37* expression and (A) Expanded Disability Status Scale, and (B) duration of disease (year)

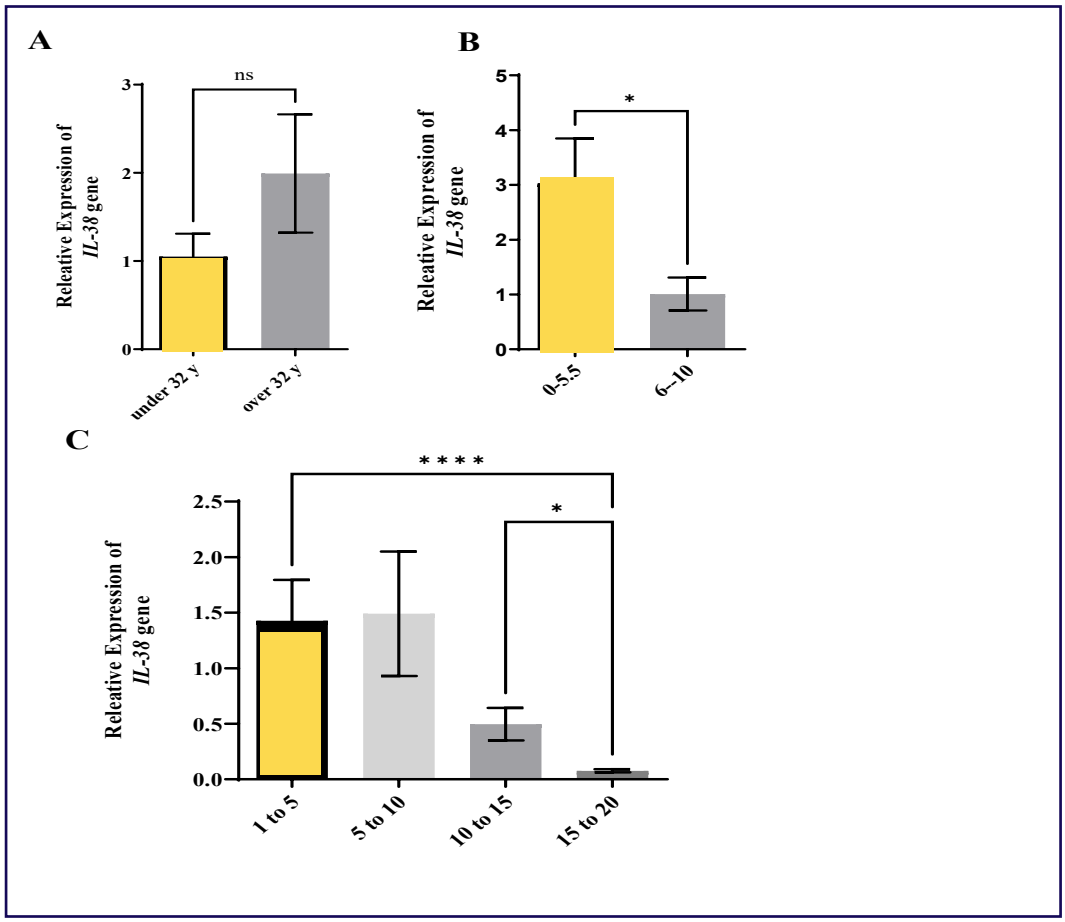


Chart 4. The relationship between *IL-38* expression and (A) Expanded Disability Status Scale, and (B) duration of disease (year)

Discussion

MS is an autoimmune inflammatory disease of the central nervous system; however, the exact cause of the disease is unknown (21). The disease occurs in two stages, inflammatory and neurodegenerative. The inflammation process in MS is known to entail inflammatory cytokines. On the other hand, the association of *IL-1* family cytokines and MS disease has been demonstrated (14). Therefore, in the present study, the relationship of the *IL-36α*, *IL-37*, and *IL-38* expression with MS was investigated. According to the findings of the present study, there was a significant difference in *IL-36α* expression between patients with MS and controls. Previous studies have shown that *IL-36α* expression increases in rheumatoid arthritis and psoriatic arthritis. However, regarding IL-36R, it has been determined that this cytokine does not significantly affect joint inflammation in animal model (22). Studies have shown that agonistic cytokines are involved in the development of intrinsic and adaptive immunogenicity, while antagonists restrict uncontrolled immune and inflammatory responses. Therefore, *IL-36α* is likely to be involved in the development of intrinsic immunogenicity and induce the production of pro-inflammatory cytokines (22, 23). However, the biology of IL-36 cytokine, particularly its potential different functions, or the expression patterns of various types of IL-36 have been poorly understood. The activation and expression of IL-36 appear to be regulated separately, but its relative expression in different organs and tissues has

regarding the expression of this IL-36 has yet been offered and data on this topic are limited. The results showed that the relative expression of *IL-37* was significantly higher in blood samples of patients with MS than in those of healthy controls. Comparison of the results of our study and other studies showed that our observations are in agreement with other studies. Imaeda et al. (2013) reported in detail that *IL-37* was identified in inflamed mucosal tissue of patients with inflammatory bowel disease (IBD), but it was not seen in controls (24). Yanqun Li et al. (2014) reported that *IL-37* expression levels were significantly higher in patients with Graves' disease (GD) than in the healthy people. Yanqun Li et al. demonstrated that *IL-37* expression was directly correlated with GD activity. The results of that study indicated that inflammatory responses in GD might stimulate the production of *IL-37* in peripheral blood mononuclear cells (PBMCs), especially as the serum levels of *IL-37* and expression of *IL-37* in PBMCs are directly correlated with the levels of pro-inflammatory cytokines TNF-α, IL-6, and *IL-1*7 in GD patients (25). It should be noted that investigations by Nold et al. (2010) have confirmed this, in which pro-inflammatory cytokines TNF-α, *IL-1*b, *IL-1*8, and *IL-1*2 induced expression of *IL-37* in vitro in PBMCs (5). However, Ye et al. (2014) examined another aspect of this mechanism, and reported that *IL-37* could suppress PBMCs in patients with systemic lupus erythematosus (SLE). A feedback mechanism is activated in patients with SLE by up-regulation of *IL-37* to prevent the production of pro-inflammatory cytokines and the incidence of widespread and uncontrolled immune responses resulting from inflammatory activities in autoimmune diseases. Ye et al. also showed that expression of *IL-37* was directly correlated with SLE activity (26). The study of Yanmei Li et al. (2014) showed that *IL-37* expression significantly increased in patients with ulcerative colitis (UC), a chronic inflammatory disorder, compared to the healthy people (27). In vivo studies have also shown that *IL-37* expression is increased by inflammatory activity and pro-inflammatory cytokines in autoimmune diseases such as rheumatoid arthritis and SLE, especially their active phase (26). Research findings have shown that *IL-37* can reduce the symptoms of dextran sulfate sodium (DSS)-induced colitis and the inflammatory processes in psoriasis by inhibiting the expression of inflammatory cytokines in the disease (28, 29), and can cause inflammatory liver injuries through producing effects on hepatocytes and non-parenchymal cells (30). In other words, *IL-37* prevents the spread of symptoms and the development of the diseases mentioned above by inhibiting the uncontrolled production of inflammatory cytokines. It has recently been proven that *IL-37* is a natural inhibitor of intrinsic immunogenicity and inflammatory responses. However, studies have suggested that the expression of *IL-37* increases in inflammatory tissues, which inhibits additional inflammatory responses (31). *IL-37* has been shown to be induced by several toll-like receptor (TLR) ligands and pro-inflammatory cytokines such as *IL-1*b, TNF-a, and IFN-c (31-33). In the present study, increased expression of *IL-37* in MS patients can confirm the high rate of inflammation in the disease. According to available evidence, despite the inhibitory effects of *IL-37* on inflammation, its role in specific immune responses, such as the functions of dendritic cells, has not yet been fully elucidated. In other words, intrinsic immunogenicity in inflammatory conditions increases the production of cytokines (including *IL-37*) and chemokines, and, on the other hand, changes the activity and function of dendritic cells (34). Extensive evidence suggests that *IL-37* inhibits inflammatory responses that are generally related to the activity of autoimmune diseases (31, 35). The results clearly showed that high expression of *IL-37* was associated with severe degrees of disability due to MS. It is clear that MS is a neurological disease and this autoimmune disease is commonly associated with severe and periodic damage to myelin

sheaths and demyelination, local inflammation, and axonal degeneration. During the early stages of relapsing remitting MS (RRMS), myelin can be regenerated. However, with the progression of the disease, remyelination does not suffice and leads to the destruction of axons, nerve damage, and exacerbation of the symptoms (36). Therefore, it can be concluded that with the exacerbation and development of MS, the disability in MS patients increases, thus increasing the expression of *IL-37* to deal with uncontrolled inflammatory responses and suppress them.

The results of the present study on the expression of *IL-38* revealed that *IL-38* expression was significantly reduced in patients with MS compared to healthy participants. The effects of *IL-38* may be similar to those of IL-36-Rα, because it binds to the IL-36 receptor, and particularly inhibits the Th17 response (37). Van et al. (2012) showed that *IL-38* bound to IL-36 Rα. *IL-38* and IL-36Rα reduce *IL-17* and IL-22 production (38). Yuan et al. (2015) showed that *IL-38* was involved in regulating inflammation and immune response. It was also found that Th17 plays an important role in many autoimmune diseases, including MS, and has an independent regulatory mechanism for the differentiation and development of the disease, so that mature Th17 cells secrete cytokines such as IL-22 and *IL-17*, but *IL-38* inhibits the production of these cytokines (39). Yuan et al. (2016) in a study on *IL-38*, found that *IL-38* expression significantly reduced the serum levels of TNFα, IFγγ, IL-6, *IL-17*, and IL-22. These results indicate that *IL-38* expression in mice produces protective effects against liver damage by inhibiting inflammatory cytokines (40). Mora et al. (2016) used *IL-38* to restrict the response of inflammatory macrophages. They found that the decrease in *IL-38* in apoptotic cells increased IL-6 and IL-8, as well as activation of AP1 in early macrophages. Consequently, by regulating the *IL-38*-dependent immune mechanism, *IL-38* may help eliminate inflammation, autoimmune responses, and cancer (41).

Conclusion

According to the present study, *IL-36α* expression in MS patients increased. Regarding *IL-37*, interlukin is generally a natural inhibitor of intrinsic immunogenicity and inflammatory responses. However, studies have shown that antagonistic cytokines such as *IL-37* limit the uncontrolled immune and inflammatory responses. Hence, it is expected that in MS, the expression of this IL will increase in response to uncontrolled immune responses resulting from autoimmune responses. The results of our study and other studies have clearly confirmed this. It has also been shown that *IL-38* plays an important role in the pathogenesis of inflammatory diseases and has a protective effect in some autoimmune diseases. In this condition, with the development of MS disease, it seems that the immune system undergoes certain changes, so that with the decrease of *IL-38* expression, the production of pro-inflammatory cytokines increases, and therefore decreased expression of *IL-38* gene in patients with MS, compared to healthy people, can lead to an increase in the concentration of this group of inflammatory cytokines in the blood. However, further studies are needed to offer an accurate functional role for *IL-38* in the stages of neuritis due to MS.

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Conflict of Interest

We certify that there is no conflict of interest with any financial organization.

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