

Evaluation of IL-1 β and IL-6 Expression following EBNA-1 and BRLF-1 Peptide Treatment in Epstein-Barr Virus-Positive Multiple Sclerosis Patients

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Keywords

Epstein-Barr virus · Multiple sclerosis · Cytokines · IL-1 β · IL-6

Abstract

Introduction: Epstein-Barr virus (EBV/HHV-4) has been implicated in the pathogenesis of multiple sclerosis (MS). This study was conducted to investigate the levels of pro-inflammatory cytokines IL-1 β and IL-6 in healthy EBV carriers and MS patients with prior EBV infection in response to treatment with EBV nuclear antigen 1 (EBNA-1) and replication and transcription activator (BRLF-1/Rta) peptide antigens in whole blood cell culture to assess the cytokine expression across all cells in the peripheral blood. **Methods:** Isolated whole blood cells from the included participants were incubated at a concentration of 10⁶ cells/mL with BRLF-1 or EBNA-1. The amount of IL-1 β and IL-6 transcripts were measured with quantitative RT-PCR at day 3 after incubation. MTT assay was conducted to examine cytotoxicity of the peptides and their effect on cell viability. Changes in cytokine expression and cell viability were analyzed using one-way and two-way ANOVA, respectively. **Results:** Ten MS patients and ten healthy donors were enrolled in the study. Treatment with the peptide antigens resulted in increased cytokines expression in both MS patients and healthy subjects. Furthermore, IL-1 β levels were higher in MS patients

compared to healthy EBV carriers. MTT assay revealed no significant difference in cell viability between the two groups. **Discussion:** The higher levels of IL-1 β in response to EBV antigens in MS patients may reflect the host neuroinflammatory environment and support the notion that immune response against EBV has a role as an aggravating factor in the progression of MS by contributing to the neuroinflammatory cascade.

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Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that is characterized by demyelination of the neuronal cells [1]. Although the precise pathological mechanisms of MS are not elucidated, previous studies have shown that infection with certain viruses such as Epstein-Barr virus (EBV), a member of the *Herpesviridae* family of double-stranded DNA viruses, are associated with a higher risk of MS [1, 2]. Almost all MS patients have been shown to have past EBV infection, and the risk of MS is 2.17 times greater among people with infectious mononucleosis compared to seronegative individuals [3]. In line with this, higher antibody titers for EBV were also observed in MS patients [4, 5]. While cy-

tokine expression is significantly affected by EBV infection, there are no studies comparing this pattern in MS patients with prior EBV infection to healthy EBV-positive individuals. MS is classified as a chronic immune-mediated neurodegenerative disease and, as such, pro-inflammatory cytokines like IL-1 β and IL-6 are among the candidates to be investigated [6].

According to several studies, IL-1 β is secreted in response to autoimmune diseases such as MS, facilitating the migration of active leukocytes toward the CNS [7]. IL-6 is also mainly produced in the acute phase of MS by B and T cells. Expression of IL-1 β and IL-6 were also visualized in MS-associated lesions [8]. According to the previous studies, effective response of CD4 and CD8 T cells and major histocompatibility complex (HLA) class I and class II antigen presentation may be involved in restricted EBV infection and virus control by eliminating proliferating and lytically infected B cells [9]. The results of the previous studies demonstrated that in humanized mice with HLA-DR15-restricted CD4 T-cell clones; there is less efficient detection of EBV-transformed cells, increased CD8 T-cell activation, and recognition of myelin basic protein by these CD4 clones [10]. This may suggest that inefficient viral recognition and infection control may result in a defective CD8 T-cell response directed against the virus that may cross-react with self-antigens that may lead to the autoimmune damage to CNS components observed in MS.

In agreement with the above, a number of genes associated with increased MS risk, which are involved in B and T-cell activation, are also found in association with higher EBV nuclear antigen 1 (EBNA-1) IgG levels in these patients [11, 12]. In addition, certain HLAs that modulate the risk of developing MS have been associated with increased levels of IFNAR2 levels but a reduced response to type I IFN stimulation [13]. As such, cytokine expression may be used as a surrogate marker for the evaluation of the role of antiviral immunity against EBV in the development of MS.

EBV can directly modulate the expression of pro-inflammatory cytokines in monocytes without infecting them [14]. Studies have also demonstrated that the infection of peripheral blood mononuclear cells (PBMCs) with EBV induces IL-1 β and IL-6 expression in these cells [6]. Despite this increasing number of in vitro studies, not much is known concerning the effects of individual EBV antigens on the inflammatory cytokine response of whole blood cells isolated from MS patients, leaving a gap in the understanding of the influence of virus-specific proteins on the inflammatory response of the immune system in MS patients.

Table 1. Subject demographics and clinical characteristics

	HC	RRMS
Number in group	10	10
Female, %	70	70
Age (median)	31 \pm 10.6	34 \pm 4.87
WBC count	6,860 \pm 1,944	7,370 \pm 1,971
EBV seropositivity	10/10	10/10

HC, healthy control; RRMS, relapsing-remitting multiple sclerosis; WBC, white blood cell; EBV, Epstein-Barr virus.

In vitro antigen stimulation of peripheral blood cells in whole blood assays provides a reliable, feasible, and reproducible in vitro method to examine human cellular immune responses in a more physiological environment. Whole blood cultures have been previously demonstrated to reliably report the levels of cytokines in response to mycobacterium tuberculosis antigens and thus may be used as a low-cost proxy-measure alternative to PBMC cultures [15]. Other studies have also mentioned that cytokines production in whole blood assays are more strongly correlated with cytokines expression in monocyte than those analyzed in PBMC cultures. Another downside to PBMC assays is that they may require larger volumes of blood for each sample [16, 17].

We hypothesized that exposing EBV antigens such as replication and transcription activator (BRLF-1/Rta) and EBNA-1 to immune cells in MS patients who are seropositive for EBV would result in a greater release of pro-inflammatory cytokines compared to healthy individuals with prior EBV infection. Therefore, to study the cytokine profile of immune cells, whole blood samples from MS patients and healthy EBV-seropositive controls were treated with BRLF-1 and EBNA-1 and were cultured for 96 h, and gene expression levels of IL-1 β and IL-6 were then measured in peripheral blood cells with an in-house developed real-time RT-PCR. Through analysis of cytokine gene expression, immune response against EBV antigens in MS patients and healthy individuals was assessed to provide further insight into the association between MS and EBV infection.

Materials and Methods

Patient Demographics and Sampling

EDTA-treated peripheral blood samples were drawn from 10 referred MS patients and healthy individuals in Tarbiat Modares University. The participants were screened from individuals who

Table 2. Sequences and characteristics of IL-1 β and IL-6 primers

Primers	Sequence (5' → 3')	T _m , °C	Length, bp	Length of product, bp
Human IL-1 β (forward)	AGGCCGCGTCAGTTGTTGT	62.75	19	194
Human IL-1 β (reverse)	CCGGAGCGTGCAAGTTCAGT	62.87	19	194
Human IL-6 (forward)	AAGCCAGAGCTGTGCAGATGAGTA	63.95	24	177
Human IL-6 (reverse)	AGCTGCGCAGAATGAGATGAGTT	62.80	23	177

T_m, melting temperature; bp, base pair.

were positive for IgG antibodies against EBNA antigen by enzyme-linked immunosorbent assay (Dia.pro, Italy), indicating prior but not primary EBV infection. The participants' demographics and clinical characteristics are listed in Table 1. All patients with MS were in the relapsing-remitting phase and were receiving interferon-beta therapy. All subjects gave informed consent and the study was approved by the Local Research Ethics Committee (Approval No. 96-11-02-52/8432) of Tarbiat Modares University. Written informed consent was obtained from all of the participants.

Stimulation of Whole Blood Cells with EBV Antigens

Whole blood cells were cultured in 24-well plates with Iscove's Modified Dulbecco's Medium (1:4), streptomycin (100 μ g/mL), and penicillin (100 U/mL) antibiotics for 96 h. The blood samples (1,000 μ L/well) were incubated (5% CO₂ at 37°C) with EBNA1 or BRLF1 peptide mixtures (1.6 μ L in each well plate, JPT peptide technologies) or medium alone as control at a concentration of 10⁶ cells/mL. The BRLF-1 and EBNA-1 peptide antigens were selected as representatives of the lytic and latent stages of the EBV infection, respectively.

In order to measure the expression levels of the cytokines, a quantitative reverse transcriptase PCR assay using a commercial real-time-based absolute quantification kit was performed. To assess the toxicity of EBNA-1 and BRLF-1 peptide antigens, cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction assay (MTT assay).

IL-1 β and IL-6 Expression Measurement

At day 3, RNAs were extracted from a single well for each sample using an RNAsol reagent (ROJE Technologies, Tehran, Iran) according to the manufacturer's instruction. Then, cDNAs were synthesized from the extracted RNAs by a two-step cDNA synthesis kit (Biofact, Daejeon, South Korea).

Primer sequences for human IL-1 β and IL-6 genes were downloaded using the Primer-Blast tool at NCBI website domain (Table 2). The primers were designed and aligned using lasergene7 software (Primer Select, Version 7.0; DNASTAR, Madison, WI, USA). Quantitative RT-PCR (qRT-PCR) was performed by adding 2 μ L from each of the IL-1 β and IL-6 primers and 2 μ L of DNA to real-time PCR master mix (RealQ plus 2X master mix Green; Odense, Denmark). The assay was used to study the temperature gradient and optimization of the primers. The reaction was run at 95°C for 5 min, followed by 40 cycles at 94°C for 30 s and 72°C for 1 min. For each two pairs of primers, the annealing temperature point was 58°C, 60°C, 62°C, and 64°C (ABI, step one plus, USA).

SYBR green qRT-PCR was performed to quantify the number of IL-1 β and IL-6 gene copies. For this purpose, cDNAs were run

with 2 μ L of each primer and 10 μ L of the master mix in the final volume of 20 μ L. The reactions were incubated at 95°C for 1 min followed by 40 cycles at 94°C for 30 s, 62°C for 20 s, and 72°C for 30 s.

Cell Viability and Peptide Antigen Toxicity

Peptide toxicity, cell viability, and cytotoxicity were evaluated in the samples of each group in 24 h, 48 h, 72 h, and 96 h following the start of culture and/or antigen stimulation using an MTT assay kit (Bio idea, Tehran, Iran). The absorbance was measured following administration of MTT solution using a microplate reader at a wavelength of 570 nm.

Data and Statistical Analyses

The EBNA-1 treated group, the BRLF-1 treated group, and the healthy controls were tested for significant differences in cytokine expression and cell viability using GraphPad Prism 7.0 (GraphPad Co., San Diego, CA, USA) software by one-way and two-way ANOVA (and Holm-Sidak's Multiple Comparison), respectively. A *p* value of less than 5% (*p* \leq 0.05) was assumed to be statistically significant.

Results

MTT Assay

Cell viability assessed by MTT assay was reported to be over 70% in all groups at 96 h following antigen stimulation. No significant difference in cell viability and peptide toxicity was observed with two-way ANOVA test for comparison between the groups in 24, 48, 72, and 96 h following culture/peptide treatment. Figure 1 demonstrates the cell viability percentage for all cultures.

qRT-PCR Assay

Limited samples from both groups were examined before conducting the primary study to obtain the optimal time for the differential cytokine expression analysis. Primary analysis of cytokine expression was conducted at 72 h following antigen stimulation, due to the higher levels of gene expression at the aforementioned time point (Fig. 2).

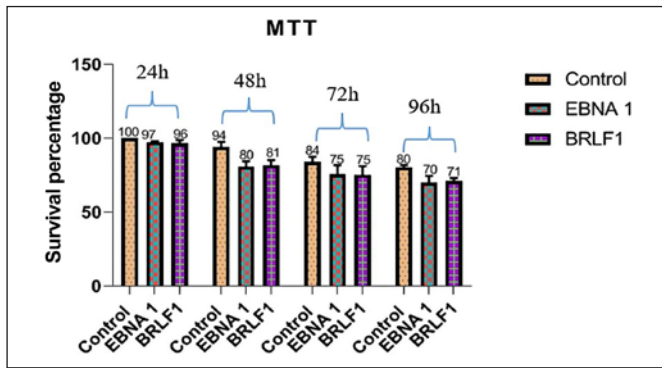


Fig. 1. Toxicity of BRLF-1 and EBNA-1 peptides and cell viability after peptide antigen treatment at 24, 48, 72, and 96 h compared with untreated MS positive controls. No difference in cell viability was observed between the groups.

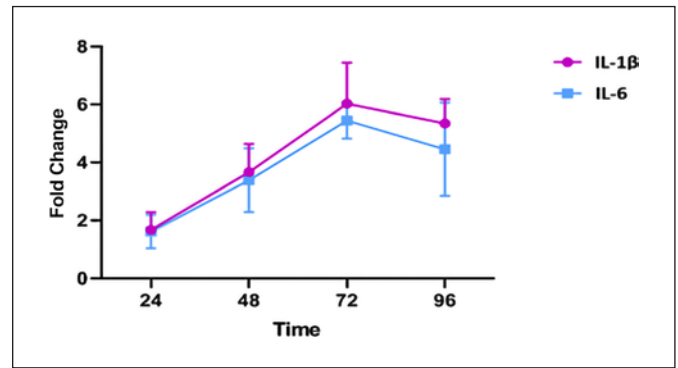


Fig. 2. The limited samples from the groups were treated with EBNA-1 or BRLF-1 and examined for cytokine expression levels at each time point. The proportional increase in cytokine expression is compared to samples without antigen treatment. Seventy-two hour following culture/antigen treatment was selected to reach maximal sensitivity.

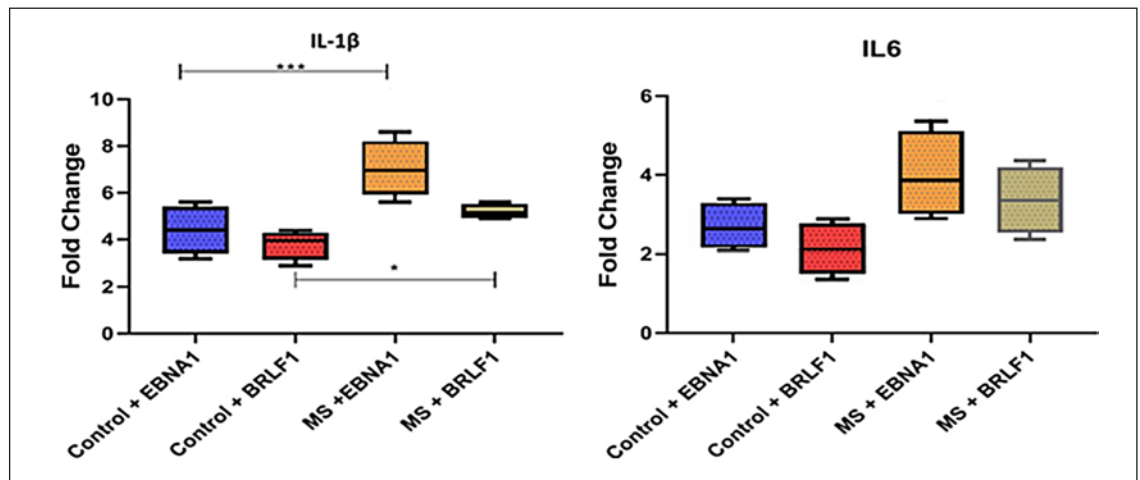


Fig. 3. IL-1 β and IL-6 expression in MS patients and healthy EBV carriers treated with EBNA-1 and BRLF-1 peptides. Error bars represent minimum and maximum data points. Data were reported as proportional change in cytokine expression in samples of each group in comparison to their untreated counterparts ($*p < 0.05$, $**p < 0.001$, $***p < 0.0001$).

SYBR green qRT-PCR assay was performed for both IL-1 β and IL-6 transcripts at 62°C. GAPDH was used as the housekeeping gene to normalize mRNA expression levels. One-way ANOVA statistical test indicated a greater increase in IL-1 β expression in response to EBV peptide antigen treatment of whole blood cells in MS patients compared to healthy individuals (Fig. 3). Furthermore, EBNA-1 triggered a greater cytokine response in whole-blood cells compared to BRLF-1 peptide in both healthy EBV carriers and MS patients.

Discussion

This study investigated the inflammatory response of peripheral blood from whole blood of RRMS patients treated with BRLF-1 and EBNA-1 peptides. The similar levels of cell viability in MTT assay for the study groups also reject the probability that the increased inflammatory response is due to apoptosis or the release of cell contents in the media secondary to cytotoxicity of the peptide antigens. In the present study, BRLF-1 and EBNA-1 pep-

tides were used as surrogates to assess the immune response during the lytic and latent phase of EBV infection and both were reportedly higher in MS patients compared to healthy controls.

Reactivation of the latent virus is associated with altered immune function, particularly with the secretion of cytokines. While the release of cytokines such as IL-1, IL-2, IL-6, IFN- γ , and TNF- α from PBMCs after EBV infection has been examined [6], the current study demonstrated that the cytokine expression may be further increased in individuals suffering from MS. This finding is in line with other reports indicating that both peptides resulted in increased cytokine expression [18–20]. Overall, these findings allude to a possible role of an exaggerated immune response against EBV in development and progression of MS.

Previous literature has already documented the association between MS and cytokines and the reciprocal interaction and communication of T cells using cytokines, which ultimately leads to the infiltration of immune cells to CNS and subsequent demyelination and neurodegeneration [21]. Studies have shown that blocking the IL-1 receptor can lead to successful treatment of a number of autoimmune diseases [22]. Previous reports have also demonstrated that B cells are involved in activating the inflammatory cascade implicated in the development of MS through the production of IL-6 and TNF- α [23]. In addition, there is a direct relationship between the levels of IL-1 β in CSF and the rate of demyelinating lesions in the cortex [24]. While IL-6 may not be completely detrimental in MS, increased levels of IL-6 were observed in active plaques of individuals with MS [8, 25]. IL-6 may be involved in demyelination of neurons through stimulation of antibody synthesis, as it has been well established that IL-6 enhances humoral immunity [26]. It is worth noting that the recent studies using transgenic mice have shown that the production of IL-6 in the CNS alone and without the presence of peripheral IL-6 can promote autoimmune reactions in the host [27]. Consistent with these results, the expression of IL-1 β was significantly increased in MS patients compared to healthy controls.

Given the causative role of pro-inflammatory cytokines in development of inflammation, it seems that BRLF-1 and EBNA-1 peptides can induce inflammation and progression of MS by increasing the expression and production of these cytokines. In this regard, infection of PBMCs with EBV resulted in greater expression of IL-6 compared to seronegative and HSV-1-infected subjects in a previous study [6]. However, the literature is controversial regarding the association of antibodies against

EBV with prognosis or clinical severity of MS. While some epidemiological as well as laboratory studies have suggested an association between EBV and MS [11, 28, 29], a number of other studies have failed to find a correlation between EBV antibodies and disease course or clinical disease activity in MS patients [30–32].

In the context of current conflicting data, the role of EBV in development of MS remains ambiguous. However, the higher levels of antibodies in patients converting to MS from clinically isolated syndrome [33], preliminary evidence regarding the role of antivirals against EBV in MS improvement [34], and the data in this study merit further clinical investigation into the use of nucleoside and pyrophosphate analogues in the experimental treatment of MS. Notably, the role of IL-1 β and IL-6 in inducing autoimmunity by amplification of Th17 CD4 T-cell responses was also demonstrated in previous studies [24, 35, 36].

While the lack of definite proof precludes solid conclusions, increased IL-1 β expression but not IL-6 in response to EBV peptide antigens, coupled by the greater increase in cytokine expression in response to EBNA-1 in this study could be attributed to a CD4⁺ T-cell dominant immune response; as unlike BRLF-1, EBNA-1 is associated with a CD4⁺ immunodominant response [37]. Although cytokine mRNA and protein expression levels have often shown diverging results and poor correlations in several studies with different cell culture systems [38, 39], the relationship between differentially expressed mRNA and mRNA-protein correlations has been shown to be significantly higher than non-differentially expressed mRNAs [40]. Nevertheless, special care should be taken in interpreting the results of this study and inferring cytokine levels from the differential expression of the evaluated cytokine genes.

Statement of Ethics

Written informed consent was obtained from all of the participants. This study and all of its procedures were reviewed and approved by the Local Research Ethics Committee of Tarbiat Modares University, approval number: (96–11–02–52/8432). The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

R.K., M.R., A.S., N.R., and A.M. have made contributions to the conception and design of the study, acquisition of data, analysis, and interpretation of data, drafting the article, and final approval

of the version. M.A.G. has made important contributions to the conception and design of the study and acquisition of data. M.R. supervised the project.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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