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The Effects of IFN-β 1a on the Expression of Inflammasomes and Apoptosis-Associated Speck-Like Proteins in Multiple Sclerosis Patients

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Abstract This study aims to evaluate the effects of treatment with IFN-β 1α on the expressions of NLRP3, NLRP1, NLRC4, and AIM2, as inflammasomes, and caspase-1, IL-1β, and IL-18, as the downstream molecules of inflammasomes, in a population of Iranian multiple sclerosis (MS) patients. In this study, 30 MS patients (22 women and 8 men) participated. Before receiving any medication and 6Â months after treatment with standard doses of IFN-β 1α 30Â mcg injected intramuscularly once a week, blood samples were taken and then the leukocytes isolated, total RNAs extracted, and complementary DNAs (cDNAs) synthesized. Gene expressions of NLRP3, NLRP1, NLRC4, AIM2, and ASC were evaluated at messenger RNA (mRNA) levels using real-time PCR method; for assessing caspase-1 at protein level, the Western blot method was used. The amounts of IL-1β and IL-18 were measured in plasma using enzyme-linked immunosorbent assay method. Analysis of the results before and after therapy with IFN-β 1α in all patients shows significantly decreased expressions of NLRP3, NLRC4, and AIM2. The plasma levels of IL-1β, after treatment with IFN-β 1α, were significantly decreased in the MS patients. Based on our results, it appears that NLRP3, NLRC4, and AIM2 play critical roles in the progression of MS, probably by mediating Th1 and Th17 responses. It seems that decreased expression of IL-1β is related to decreased production and also functions of inflammasomes.

Keywords Inflammasomes · IFN-β 1α · Multiple sclerosis · Caspase-1 · Cytokine

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system, characterized by demyelination in the brain and spinal cord. Multiple sclerosis is also a chronic disease that develops more likely in the age range of 20–40 years, and its prevalence is higher in women than in men [1]. In the type of relapse-remitting MS (RRMS), women are involved twice compared to men [2]. The spread of MS has been reported in different regions of the world, as in Europe and America from 5 to 6 per 100,000 people suffer from MS [3]. The prevalence of MS in Kerman city in the southeast of Iran is 57.3/100,000 population [4]. In recent years, studies in the Middle East and Iran suggest a relatively high prevalence of the disease in these areas. Although the exact causes of the disease are still unknown, some factors such as inheritance, environmental factors, and even viral infections can be introduced as the underlying causes of the disease [1, 5–7].

The formation of lesions or plaques, areas of inflammation, demyelination, and gliosis in the central nervous system (CNS) white matter are the main reasons for MS symptoms, which can be diagnosed by magnetic resonance imaging (MRI) technique [2]. Plaques in MS contain a variety of cells

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and inflammatory factors. Creation of wounds is due to defects in the regulation of the immune system that leads to increased proliferation, oligodendrocyte myelin, and subsequent T cell activity [8]. When T cells cross the blood–brain barrier and enter the central nervous system, they cause unwanted reactions with the myelin-inflammatory loop that leads to the creation of toxic substances and glial inflammation and results in axonal damage [2]. MS is a chronic disease and disease progression varies from person to person. Significantly more than 85 % of MS patients suffer primarily from RR type [9]. RRMS has repeated periods of flares and part of it is determined as full recovery. Of these patients, about 50–60 %, between 10 and 15 years later, evolve to secondary progressive MS and often have no periods of relapse, but their symptoms are getting worse [10].

A small percentage of patients with MS (15 %) have more severe degrees of nervous system defects, known as primary progressive MS, who have recurrent episodes of mild defects and are resistant to treatment [2]. Interferon-beta (IFN-β) is an innate immune cytokine from interferon type 1 that acts on multiple metabolic pathways [11]. Studies have shown that MS patients are deficient in the production of IFN-β [12, 13]. Thus, exogenous interferon can cover the defect. These cytokine types also play a crucial role in defending against the virus by interfering with the IgG generation by the plasma, IgG reduction, and adjusted cell function involved in the development of MS, such as natural killer cells [14, 15]. The anti-inflammatory features of interferon make it a significant compound for the treatment of MS. Millward et al. showed that treatment with IFN-β 1a with increasing interleukin (IL)-18 binding protein prevents IL-18 functions, preventing inflammation [16]. Studies also showed that administration of IFN-β levels of IL-18 also reduces MS [17]. The study by Inoue et al. showed that IFN-β can improve inflammation in patients with MS when they are related to the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing (NLRP)3 pathway [18]. In addition, studies have shown that MS patients suffer from increased expression of inflammatory molecules [19]. Inflammasomes may be the most important molecule involved in inflammation. Inflammasomes are a specific class of intracellular receptors which recognize damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) to activate the caspase-1 enzyme [20]. The activated form of caspase-1 provides, in turn, activation of the pre-cytokines IL-1β and IL-18 [20]. Some NOD-like receptors (NLRs), including NLRP1, NLRP3, and NLRPC4, and the absent in melanoma 2 (AIM2) molecules are among the most important families of inflammasomes [20]. It has been documented that IL-1β and IL-18 are the main cytokines involved in the inflammation of MS [21]. Thus, it appears that expression of inflammasomes may be changed during MS. A study on an animal model of MS (experimental allergic encephalomyelitis, EAE) showed that inflammasomes are the most important factors for the activation of caspase-1 and, subsequently, activation of IL-1B and IL-18 to induce MS [22]. Studies also showed that induction of MS in mice without apoptotic speck protein (ASC), the adaptor between inflammasomes and caspase-1, is not possible [23]. However, studies have shown that consuming drugs which mitigate the effects of IL-18 can greatly help MS patients to be treated [24].

Given the role of inflammasomes in the induction of inflammation and the role of IFN-β 1a in reducing inflammation, it has been hypothesized that IFN-β 1a may affect inflammation by altering the expression of inflammasomes. Therefore, this study aims to evaluate the effects of treatment with IFN-β 1a on the expressions of NLRP3, NLRP1, NLRC4, and AIM2, as inflammasomes, and apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), caspase-1, IL-1β, and IL-18, as the downstream molecules of inflammasomes, in a population of Iranian MS patients.

Material and Methods

In this study, 30 MS patients (22 women and 8 men) were examined before receiving any medication. Diagnosis of MS according to the revised McDonald criteria and based on radiology, laboratory, clinical, and neurologist observations was conducted [25]. All patients signed an informed consent form to enter the project. Participants in the study did not suffer from any other inflammatory diseases such as allergy, autoimmune diseases, diabetes types 1 and 2, and heart and kidney diseases. Patients with infectious diseases and under treatment of immunomodulatory medications were excluded from the study. Research permits were obtained from Kerman University of Medical Sciences Ethics Committee, with license number k/92/587. Prior to receiving drug IFN-β 1a (IFN-β pharmaceutical company's CinnaGen composition as the commercial name of Cinnovex, similar to the composition of Avonex), 10 mL of venous blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant and centrifuged for 5 min at 2500 rpm; about 0.5 mL of plasma was separated and then the leukocytes were isolated using Ficoll. Total RNAs were purified and complementary DNAs (cDNAs) were synthesized immediately after leukocyte isolation. The synthesized cDNAs and plasma were stored at –70 °C for further analysis. Six months after treatment with standard doses of IFN-β 1a, blood samples were taken on the basis of previous studies [19]. Just like before, samples were taken for the analysis of plasma, leukocyte isolation, total RNA extraction, and cDNA synthesis and were saved thereafter. Gene expressions of NLRP3, NLRP1, NLRC4, AIM2, and ASC were evaluated at messenger RNA (mRNA) levels using real-time PCR method; for assessing caspase-1 at protein level, the Western blot method

was used. The amounts of IL-1β and IL-18 were measured using enzyme-linked immunosorbent assay (ELISA) method, as described below.

RNA Purification, cDNA Synthesis, and Real-Time PCR Condition

To extract total RNA from patient specimens, a Trizol solution (Bioneer, South Korea) was used. The unity and integrity of RNA was confirmed by agarose gel electrophoresis (1.5 %), and the amount of RNA and the purity and quality of RNA were tested by a photometric and spectrophotometric method at OD260/OD280 nm. Then, using oligo-dT primer and cDNA synthesis kit from the Takara kit (Takara Co., Japan), cDNA was prepared from purified RNA according to the manufacturer's instructions.

The expressions of NLRP3, NLRP1, NLRC4, AIM2, and ASC were evaluated using appropriate primers (Table 1) and the master mix containing dye SYBR Green (Takara Co.) in a real-time PCR machine (Corbet, Australia) using the following program: 3 min at 95 °C, followed by 43 cycles of denaturation (10 s), annealing (10 s), and extension. It should be noted that *β.Actin* was used as the housekeeping gene.

At the end of the process, the expressions of these molecules before and after treatment were computed using the $2^{-\Delta\Delta CT}$ formula.

Western Blotting

In this study, the caspase-1 molecule was measured by Western blotting and the data analyzed using ImageJ software. Briefly, protein extraction was performed using the Ripa buffer method and the protein concentration was measured by the bicinchoninic assay (BCA) method. Then, the lysate was equally separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Table 1 Primer sequences of target genes

Genes	Sequences
NLRP1 F	5′-ACTCTCCCTCATTCCCCTAC-3′
NLRP1 R	5′-GCTGTCTCAAAACCCTTCTC-3′
NLRP3 F	5′-GAAGAGGAGTGGATGGGTTT-3′
NLRP3 R	5′-CGTGTGTAGCGTTTGTTGAG-3′
NLRC4 F	5′-TTCGTCTTCTTCCTCCGTCT-3′
NLRC4 R	5′-ATGTCTGCTTCCTGATTGTG-3′
AIM2 F	5′-CAGGAGGAGAAGGAGAAAGTTG-3′
AIM2 R	5′-GTGCAGCACGTTGCTTTG-3′
ASC F	5′-AACCCAAGCAAGATGCG-3′
ASC R	5′-TTAGGGCCTGGAGGAGCAAG-3′
β.Actin F	5′-CTCTTCCAGCCTTCCTTCCT-3′
β.Actin R	5′-GACAGCACTGTGTTGGCGTA-3′

The gel was then transferred to polyvinylidene difluoride. For Western blotting, a primary antibody, anti-caspase-1 (1:400; Santacruz Biotechnology, Santa Cruz, CA), was incubated for 1 h and then rinsed with Tris-buffered saline and Tween 20 (TBST) buffer, incubated with secondary antibody with horseradish peroxidase (1:20,000; Abcam), and finally was incubated for 1 h.

Henceforth, rinsing was performed with TBST three times and then using fluorescence light, produced by enhanced chemiluminescence (BioRAD), and finally determined with the help of a photographic film. The protein β-actin was used as the housekeeping protein.

ELISA Method

To measure the plasma levels of IL-18 (Eastbiopharm, Torrance, CA) and IL-1β (R&D, Minneapolis, MN), the ELISA method was used according to the manufacturer's protocols.

Data Analysis

Data analysis was performed using SPSS-20 software. To determine the effect of gender and time on the expressions of the evaluated molecules, two-way repeated-measures ANOVA was used. However, due to the interaction between sex and time, paired Student's *t* test was used for comparison of the data before and after treatment with IFN-β 1a in all patients and for each sex separately, too. All results are reported as mean ± SEM. $p \leq 0.05$ as the significance level was reported.

Results

Among the 30 patients enrolled, 22 were women (73 %). The results revealed that all of the patients were responders to the IFN-β 1a therapy and have not shown recurrence in MS during treatment with IFN-β 1a.

Analysis of the results before and after therapy with IFN-β 1a in all patients showed decrement in the expressions of the genes of interest (NLRP3, $p = 0.047$; NLRP1, $p = 0.246$; NLRC4, $p = 0.049$; AIM2, $p = 0.052$; and ASC, $p = 0.116$). But only in three cases (NLRP3, NLRC4, and AIM2) was the reduction significant (Fig. 1).

The patients were divided into two groups, male ($n = 8$) and female ($n = 22$). After treatment with IFN-β 1a, the expressions of all measured genes were decreased in women (NLRP3, $p = 0.050$; NLRP1, $p = 0.127$; NLRC4, $p = 0.055$; AIM2, $p = 0.092$; and ASC, $p = 0.121$). But just about the NLRP3 difference was significant. No significant differences were observed before and after treatment with IFN-β 1a in

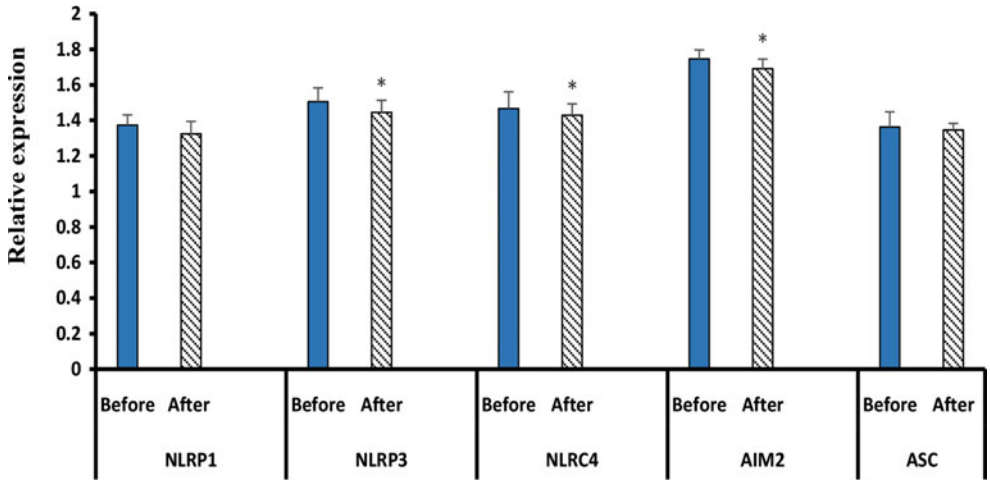

Fig. 1 Bar graph showing the measured changes in gene expression before and after treatment with IFN-β 1a. All the genes were downregulated 6 months after therapy with IFN-β 1a, but only NLRP3 ($p = 0.047$), NLRC4 ($p = 0.049$), and AIM2 ($p = 0.052$) show significant reduction in expression levels. The number of participants was 30 and the data reported as mean ± SEM. $p \leq 0.05$ was considered significant

men: NLRP3, $p = 0.643$; NLRP1, $p = 0.58$; NLRC4, $p = 0.684$; AIM2, $p = 0.573$; and ASC, $p = 0.789$ (Fig. 2).

Protein Results

The data analysis of caspase-1 showed that, 6 months after treatment with IFN-β 1a, the amount of caspase-1 was decreased, but this decline was not significant in all patients ($p = 0.439$), females ($p = 0.55$) and males ($p = 0.64$; Fig. 3).

The plasma levels of IL-1β ($p = 0.035$), but not IL-18 ($p = 0.387$), after treatment with IFN-β 1a were significantly decreased in MS patients, while IL-1β did not differ among female ($p = 0.065$) and male ($p = 0.138$) patients (Figs. 4 and 5). IL-18 levels also were not changed in female ($p = 0.43$) and male ($p = 0.22$) patients (Figs. 4 and 5).

Discussion

Inflammasomes are the main molecules involved in the induction of inflammation via promotion of pro-IL-1β and pro-IL-18 cleavage [26]. There are several investigations which have confirmed the pathologic roles played by inflammasomes and their related downstream molecules, including ASC, caspase-1, IL-1β, and IL-18, in the pathogenesis of MS [27, 28]. Inflammasome activations are associated with neuroinflammation complications such as mitochondrial dysfunction, release of circulating DNA, secretion of reactive oxygen species, and potassium effluxes [29]. Therefore, it can be hypothesized that the molecules and their downstream molecules can be considered as risk factors for MS. Our results showed that IFN-β 1a, as an interferon therapy, can improve the clinical presentation by blocking relapse in treated patients. Therefore, it seems that

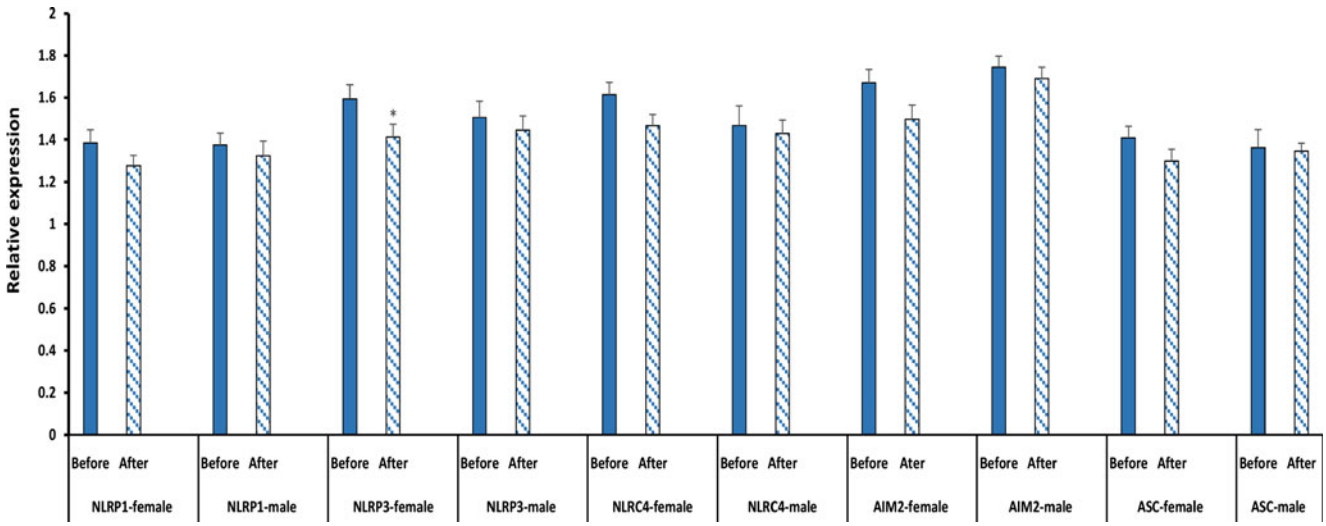

Fig. 2 Bar graph showing the measured changes in gene expression before and after treatment with IFN-β 1a in males ($n = 8$) and females ($n = 22$). All the genes were downregulated after drug treatment, but only in females was the expression level of NLPR3 decreased significantly ($p = 0.05$). The data were reported as mean ± SEM. $p \leq 0.05$ was considered significant

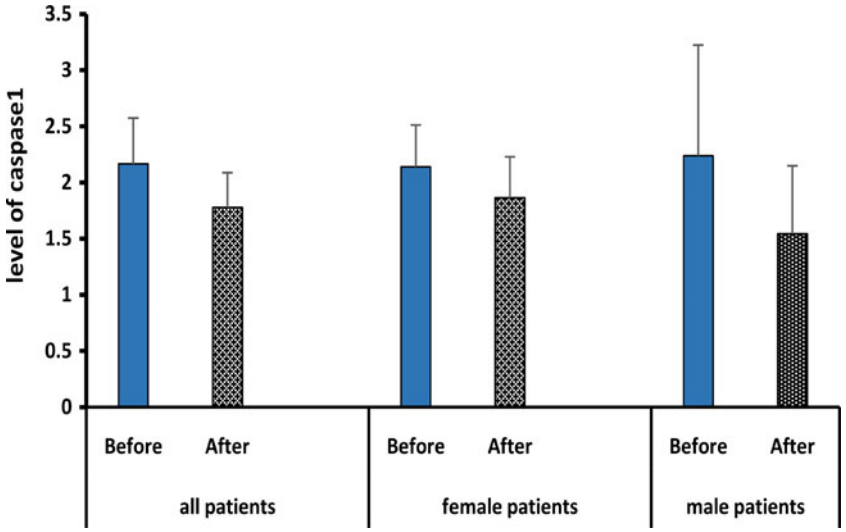

Fig. 3 Caspase-1 was measured by Western blotting in patients before and after treatment with IFN-β 1a. The number of patients was 30 and data in three groups—all patients, female group (22 patients), and male group (8 patients)—were observed. Caspase-1 expression was decreased after treatment in MS patients ($p = 0.439$), female group ($p = 0.55$), and male group ($p = 0.64$), but the differences were not significant. Data were reported as mean ± SEM. $p \leq 0.05$ was considered significant. β-Actin was used as the control protein

the Iranian version of IFN-β 1a is suitable for the treatment of Iranian RRMM. The results also revealed that IFN-β 1a modulates the expression of some inflammasomes, including NLRP3, NLRC4, and AIM2 (but not NLRP1), at mRNA levels. The results also demonstrated that the plasma levels of IL-1β were significantly decreased in IFN-β 1a-treated patients. In accordance with the fact that IL-1β is secreted from immune and non-immune cells after cleavage (activation), hence, it may be concluded that decreased expression of IL-1β is related to the decreased production and also functions of inflammasomes. Based on our results, it may be hypothesized that IFN-β 1a improves the clinical presentation of MS via decreasing the plasma levels of IL-1β in a NLRP3-, NLRC4-, and AIM2-dependent manner. Previous studies revealed that NLRC4, NLRP3, and AIM2 recognize PAMPs and DAMPs, while NLRP1 almost recognizes narrow ranges of PAMPs [30]. Thus, it is plausible that IFN-β 1a targets inflammasomes which are activated by various ranges of PAMPs and DAMPs, the ligands released in autoimmune diseases, including MS. Accordingly, NLRP3 can be activated not only by microbial PAMPs but also by various ranges of endogenous DAMPs, including uric acid crystals, alum, reactive oxygen species, intercellular ATP, silica, and amyloidal-β [31–33]. AIM2 is also able to recognize self-DNA from apoptotic cells and activate caspase-1 via ASC [34]. Collectively, due to the results presented here, it appears that IFN-β 1a targets the pathways which lead to the downregulation of the most important inflammasomes involved in MS. A study by Malhotra and colleagues revealed that, although the mRNA levels of NLRP3 and IL-1β after 24 months of treatment with IFN-β were increased in responder MS patients, their expressions were lower in comparison to non-responder MS patients [35]. Interestingly, their results also demonstrated that IFN-β therapy has no effect on the expressions of NLRP1 and IL-18 [35]. Additionally, their results showed that IFN-β therapy has not altered the expression of NLRC4 [35]. Based on our results which revealed that the expressions of NLRP3 and IL-1β were decreased after therapy with IFN-β 1a, the results of Malhotra were in contrast with ours. Due to the fact that our results revealed sex-dependent effects of IFN-β 1a on the expression

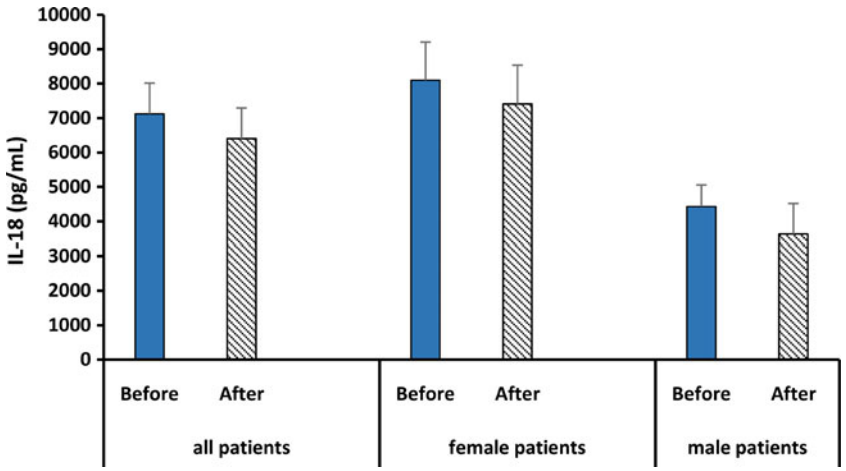

Fig. 4 IL-18 was tested by ELISA in plasma before and after treatment with IFN-β 1a. IL-18 level reduced after treatment in MS patients, but not significantly ($p = 0.387$). The plasma levels of IL-18 were not changed after treatment in males ($p = 0.22$) and females ($p = 0.43$). Data are reported as mean ± SEM. $p < 0.05$ was considered significant

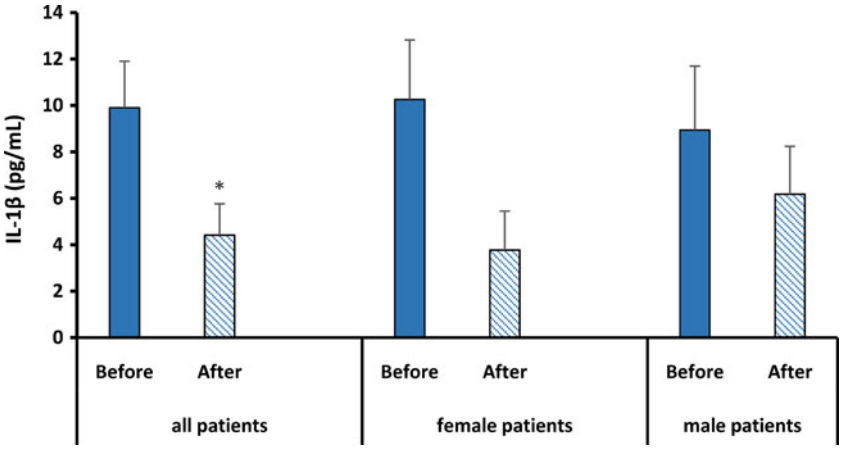

Fig. 5 IL-1β was measured by ELISA in the plasma of patients before and after treatment with IFN-β 1a. IL-1β, after drug treatment in a total of 30 MS patients, dropped significantly ($p = 0.035$). But in terms of gender, significant differences were not observed before and after treatment with IFN-β 1a (females, $n = 22$, $p = 0.065$; males, $n = 8$, $p = 0.138$). Data were reported as the mean ± SEM. $p \leq 0.05$ was considered significant

of NLRP3, hence, it may be proposed that the differences between participants regarding sex or other ethnic variations may be the reasons for the discrepancy. However, their results approved the effects of IFN-β 1a therapy on the expressions of NLRP3 and IL-1β in responders, which were lower than in non-responders. Another study on an animal model of MS (EAE) showed that IFN-β 1a suppresses NLRP3 activation and ameliorates EAE [36]. Inoue and colleagues reported that IFN-β 1a therapy in an animal EAE model is effective only when the development of EAE depends on NLRP3 [37]. In parallel with our results, Guarda et al. demonstrated that IFN-β 1a inhibits IL-1β production and activation [38]. Collectively, based on our results and the mentioned studies, it appears that NLRP3, NLRC4, and AIM2, as inflammasomes, play critical roles in the progression of MS, probably by mediating Th1 and Th17 responses [26].

Our results also showed that the mRNA levels of ASC and the protein levels of caspase-1 were not altered following treatment with IFN-β 1a. Based on the fact that ASC and caspase-1 play as the adaptor and enzyme proteins, respectively, and that they exist in the cytoplasm and ready to be activated by inflammations, so, it seems that IFN-β 1a decreases their functions, rather than expressions, by decreasing the expressions of NLRP3, NLRC4, and AIM2. The results were also confirmed by previous investigations [35]. Additionally, due to the results, NLRP1 did not change after treatment with IFN-β 1a; therefore, it may be hypothesized that ASC and caspase-1 may be used by NLRP1, hence their unchanged expressions after IFN-β 1a therapy. It seems that more studies on the effects of IFN-β therapy on NLRP1 and also its related molecules (ASC and caspase-1) can shed light to improve our knowledge.

The results also demonstrated that the expression of NLRP3 has been decreased in female patients, but not in males, following 6 months of IFN-β 1a therapy. Due to the fact that the expression of NLRP3 in all participants was significantly decreased following IFN-β 1a therapy, it seems that the decline is sex dependent and that women were more sensitive than men to IFN-β 1a. In contrast, although the serum levels of IL-1β and the mRNA levels of NLRC4 and AIM2 were significantly decreased after 6 months treatment with IFN-β 1a, their values in females and males were not significant. So, it appears that the decreased expressions of the molecules following IFN-β 1a therapy were sex independent. Accordingly, the authors suggested that more studies need to be performed to explore the roles of gender on the expression of NLRP3 in MS.

Conclusion

Based on our results, it seems that IFN-β 1a decreases inflammation via NLRP3, NLRC4, and AIM2 expressions, which can lead to decreased cleavage of IL-1β and IL-18, which play key roles in the pathogenesis of multiple sclerosis. The main pathway used by IFN-β 1a for decreasing the expressions of NLRP3, NLRC4, and AIM2 has yet to be clarified, but it may be hypothesized that it downregulates the inflammasomes directly, via affecting the responsible transcription factors, and indirectly, via affecting the positive related pathways.

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Compliance with Ethical Standadrs

Conflict of Interest The authors declare that they have no conflict of interest.

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