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Comparison of Expression Levels of miR-29b-3p and miR-326 in T Helper-1 and T Helper-17 Cells Isolated from Responsive and Non-responsive Relapsing-remitting Multiple Sclerosis Patients Treated with Interferon-beta

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ABSTRACT

T helper type 1 (Th1) and Th17 Cells with distinct cytokine profiles including interferon-gamma (IFN- γ) and interleukin 17 (IL-17) have a pivotal role in neuroinflammation and myelin destruction in the central nervous system (CNS) in MS. MicroRNA-29b (MiR-29b) and miR-326 contribute to regulating Th1 and Th17 differentiation and altered expression of the miRNAs could be associated with response to treatment in multiple sclerosis (MS). Therefore, our study aimed to evaluate the percentage of Th1 and Th17 and determining the expression levels of miR-29b-3p and miR-326 in these lymphocyte subpopulations between responsive and non-responsive to interferon beta (IFN- β) therapy in relapsing-remitting multiple sclerosis (RRMS) patients.

The present study was performed on 40 RRMS patients following treatment with IFN- β . The percentage of Th1 cells and Th17 cells were determined by flow cytometry in responsive and non-responsive patients. The expression levels of miR-29b-3p and miR-326 were assessed in Th1 and Th17 cells by quantitative polymerase chain reaction (PCR). Enzyme-linked immunosorbent assay (ELISA) was applied to evaluate the plasma levels of IFN- γ and IL-17A.

No significant difference was observed in the percentage of Th1 and Th17 cells as well as the expression levels of miR-29b-3p and miR-326 (in Th1 and Th17, respectively) in treated patients. Also, we did not find any significant difference in IFN- γ and IL-17A plasma concentration between responsive or non-responsive to IFN- β therapy in patients with RRMS.

IFN- β may regulate other miRNAs in Th1 and Th17 cells than miR29b-3p and miR-326 in MS patients.

Keywords: Interferon-beta; MicroRNA; Relapsing-remitting multiple sclerosis

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INTRODUCTION

Micro RNAs (miRNAs) are single-strand non-coding RNA 19-25 nucleotides in length. They regulate gene expression through 3'untranslated region (UTR) of target mRNAs. The dysregulation of miRNAs expression can induce severe inflammatory responses in some diseases, including different cancers, autoimmune disorders, and neurological disorders.¹

Multiple sclerosis (MS) is shown as the most common disease of the central nervous system (CNS) in young adults. MS is induced by autoimmune chronic inflammation and damage to nerves in the spinal cord and the brain.^{2,3} Relapsing-remitting MS (RRMS) is the most common type of MS, making up approximately 75-85 % of diagnoses.⁴ MS is characterized by distinct cytokine profiles including interferon-gamma (IFN- γ) and interleukin 17 (IL-17) and a high frequency of T helper type 1 (Th1) and Th17 cells.⁵

Previous studies have reported that miR-29b and miR-326 act as regulators of Th1 and Th17 differentiation, respectively. Indeed, miR-29b affects the expression of T-box 21 protein (T-bet) and IFN- γ . IFN- γ establishes a regulatory feedback loop and also increases miR-29b expression.^{6,7} MiR-326 is highly expressed in Th17 cells compared with other subtypes of CD4+ T cells in RRMS patients. Previous studies have reported a significant role for miR-326 in the induction of Th-17 differentiation (by inhibition of ETS-1) and the progression of autoimmune diseases. MiR-326 is known as a potential marker for the diagnosis of relapse and remission phases in RRMS patients,^{8,9} as well as altered expression of miR-326 in T cell-derived exosomes, may be used as a helpful clinical indicator to diagnose and treatment of MS.¹⁰

IFN- β as the first option of treatment of RRMS patients decreases relapse rates, improves clinical signs and symptoms, and slows down the speed of disease progression. But unfortunately, 30–50% of MS patients are non-responsive to IFN- β therapy and this treatment may be worse in neuromyelitis optica (NMO). Therefore, it is important to evaluate the progression of MS disease and the effectiveness of IFN- β therapy in MS patients.

Indeed, IFN- β as immune-modulating therapy involved in modulation of the balance among different populations of T cells¹¹⁻¹³ and it seems that IFN- β is effective in RRMS disease driven by Th1, not by Th17. Also, it is suggested that different levels of cytokines

and chemokines among MS phenotypes are related to the heterogeneous response to IFN- β treatment.¹⁴

Thus, it is likely that the Th1/Th17 axis and miRNAs involved in the regulation of Th1 and Th17 differentiation such as miR-29 and miR-326 could be linked to a response to IFN- β therapy as well as may provide a perspective in the prediction of treatment outcome.

Therefore, the present study aimed to assess the percentage of Th1 and Th17 and to determine the expression levels of miR-326 and miR29b-3p in these lymphocyte subgroups between responsive and non-responsive patients with RRMS to interferon-beta therapy.

MATERIALS AND METHODS

Patients

This cross-sectional study was performed on patients with relapsing-remitting multiple sclerosis (RRMS) (n=40) who referred to the MS clinic of Kashani hospital, Isfahan City, during the 2015-2017 years. All participants gave written informed consent and donated samples of blood. Our study was approved by the local ethics committee of the Isfahan University of Medical Sciences, Isfahan, Iran (Code of Ethics: IR.MUI.REC.1394.727). Their demographics and clinical data were collected. All patients were selected by a neurologist based on the McDonald's criteria. Blood samples were obtained from RRMS patients following treatment with recombinant interferon-beta -1-a (IFN- β -1a) and IFN- β -1-b for one year as well as had not been treated with other immune-modulatory drugs during IFN- β therapy. All patients were assessed for expanded disability status scale (EDSS), magnetic resonance imaging (MRI) activity, and relapse rate by the neurologist before (baseline) and after one year of IFN- β therapy to characterize MS patients as IFN- β responders or non-responders. The patients were divided into two groups including responsive patients (n=20) and non-responsive patients (n=20) based on modified Rio score (MRS).¹⁵ MS patients as IFN- β responders were considered when the score of EDSS didn't increase and the MRS score was 0 or 1 after therapy. On the other hand, RRMS patients who fail to respond optimally to IFN- β therapy as determined by clinical examinations, as well as they had MRS scores equal 2 or 3 were diagnosed as non-responders.¹⁶ The patient characteristics are shown in Table 1.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Blood samples were collected in tubes containing ethylene diamine tetraacetic acid (EDTA). Then, Blood samples were diluted with phosphate-buffered saline (PBS, Sigma, Germany). PBMCs were isolated using Ficoll-Paque and then isolated PBMCs were washed twice with PBS. The supernatant was discarded, and the pellet was re-suspended in PBS. The cell viability was determined by trypan blue (Sigma, Germany). The cell suspension was divided into two equal parts, and each part was purified using magnetic-activated cell sorting (MACS).

Purification of Th1 and Th17 Cells by the MACS Method

Isolated PBMCs were purified using the EasySep™ Human Th1/TH17 Cell Isolation Kits (Stemcell™ Technologies, Canada). Purification of Th1 (CD4+CXCR3+) or Th17 cells (CD4+CXCR3-CCR6+) includes two-step, pre-enrichment by negative selection and separation of desired cells by positive selection. According to the statement of the EasySep™ Human Th1/TH17 Cell Isolation Kits, following stimulation with phorbol 12-myristate 13-acetate (PMA)-Ionomycin and intracellular staining, 45-80% and 5-20% of the isolated cells are IFN- γ + and IL-17+ respectively. Thus, the purity of Th1 and Th17 cells were confirmed as 59% and 18% respectively by intracellular flow cytometry using Anti-human IFN- γ PE antibody and Anti-human IL17 PE antibody (eBioscience, USA).

RNA Extraction and cDNA Synthesis

The total RNA was extracted from Th1 cells and Th17 cells by the Pico Pure RNA Isolation Kit (Fermentas; Thermo Fisher Scientific Inc, USA) according to manufacturer's instructions. RNA quantity and integrity were assessed using NanoDrop spectrophotometer and agarose gel electrophoresis.

RNA (5 ng/ μ L) was applied for complementary DNA (cDNA) conversion using the miRCURY™ LNA™ miRNA RT Kit (Exiqon, Denmark). The cDNA was made according to the following protocol: 60 min at 42°C followed by heat-inactivation of the reverse transcriptase for 5 min at 95°C.

Real-time qPCR for Quantification of miRNAs

The Real Q Plus Master Mix Green (Amplicon, Denmark) and pre-designed primers (LNA™ Primer mix, Exiqon, Denmark) were used for hsa-miR-29b-3P and hsa-miR-326 quantification by the Step One Software v2.3 (Applied Biosystems, USA). The following thermal cycle conditions were applied: activation/denaturation at 95°C for 15 min followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Expression levels of target genes were normalized using SNORD48 as a reference gene.^{8,17,18} The threshold cycle (CT) was determined automatically by the Step One Software v2.3 software. The relative quantification (Δ CT) method was applied to calculate the fold change expression of microRNAs (Figure 2).

Flow Cytometry Assay

Th1 cells and Th17 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma, Germany) and the appropriate Penicillin-Streptomycin Solution (pen/strep) antibiotic mix. PBMCs were activated by 2 μ L/mL of Cell Stimulation Cocktail 500X of PMA and ionomycin (eBioscience, USA) for 2 hours. One μ L/mL Brefeldin A (BFA) 1000X (eBioscience, USA) as Golgi-plug was added to activated cells and then these cells were incubated at 37°C for 4 hours. Some cells were stimulated with BFA to determine the basal level of intracellular cytokines. As well, other cells as isotype control were stained with mouse IgG2b k ISO Control PerCP Cy5, mouse IgG2a k ISO Control FITC, and mouse IgG1 k ISO Control PE. These cells were stained with anti-human CD3 FITC antibody and anti-human CD4 PerCp-cy5 antibody (all of the antibodies were purchased from eBioscience, USA). Then the cells were fixed using ice-fixation buffer and were incubated for 60 minutes at room temperature in dark. The cell suspension was washed twice with 1X Permeabilization Buffer (eBioscience, USA). The pellet was re-suspended in 100 μ L of Permeabilization Buffer and incubated with anti-IFN- γ PE antibody and anti-IL-17A PE antibody (eBioscience, USA) at room temperature for 60 minutes. Ultimately the stained cells were washed and suspended in PBS. The percentage of Th1 and Th17 cells were analyzed by the BD FACS Calibur System (Becton Dickinson, Co., USA) using Cell Quest Software version 6 (Becton Dickinson, Co., USA) (Figure 1).

ELISA

Plasma samples were collected and stored at -80°C. The concentration of IFN-γ and IL-17A was measured with PeptoTech's Human IFN-γ/IL-17A ELISA development Kit (Pepto Tech, USA) according to the manufacturer's instructions (analytical sensitivity: 2 pg/mL).

Statistical Analysis

We analyzed all data using SPSS software, Version 16 (SPSS Inc. Chicago, Ill, USA). Mann-Whitney U test was applied to compare quantitative expression levels and percentage of cell populations between responsive and non-responsive patients with RRMS. Also, an independent T-test was used to compare cytokine levels between two groups. Data are shown as mean±SD and a *p*<0.05 was considered significant.

RESULTS

Demographic Characteristics of the Patients with MS

The study included RRMS patients (n=40) who were treated with IFN-β for one year. Patients were classified into two groups including responsive patients and non-responsive patients based on MRS. The patient characteristics are shown in Table 1.

The Frequency of Th1 and Th17 Cells in Responsive and Non-responsive Patients

The percentage of Th1 (CD3+ CD4+ IFN-γ+) and Th17 (CD3+ CD4+ IL17+) cells and also the ratio of Th1/Th17 cells were determined by flow cytometry in responsive and non-responsive patients with RRMS. The percentage of these cells was determined by the Mann-Whitney U test. No significant difference was observed in the percentage of Th1 and Th17 cells between responsive and non-responsive patients with RRMS. Statistical parameters were summarized in Table 2.

The Expression Levels of miR-29b-3p and miR-326 in Th1 and Th17 Cells

Because miR-29b and miR-326 were involved in the regulation of Th1 and Th17 differentiation respectively, the expression levels of miR-29b-3p and miR-326 were evaluated in isolated Th1 and Th17 cells by quantitative PCR. There were any significant differences in the expression levels of miR-29b-3p (*p*=0.38) and miR-326 (*p*=0.19) between the responsive and non-responsive patients with RRMS. The expression ratio of the miR29b/miR326 was not higher in the responsive group.

Table 1. Demographic and clinical characteristics of the study subjects

Characteristic	Responsive Patients	Non-Responsive patients
Number (n)	20	20
Age in years, mean	35	42
Sex, n (%)		
• Female	83	78.57
• Male	17	21.43
EDSS score ^a		
• Baseline	0-6	0-6
• After therapy	0-2	0-5
Relapse rate ^b	0-1	0-1
Activated plaques in the brain		
• Baseline	2	2
• After therapy	2-3	5-6
Cervical plaques		
• baseline	2-3	2-3
• After therapy	2-3	4-6
Lesion load		
• Baseline	2-3	2-3
• After therapy	2-4	7-9

a, Expanded Disability Status Scale; b, Number of MS attacks during a year.

Table 2. Statistical parameters of cell populations

Parameters (mean %±SD)	Responsive	Non-Responsive	<i>p</i>
Lymphocytes	80.35±22.49	79.9±29.04	0.714
T cells (CD3+)	78.36±12.7	62.55±30.78	0.206
T helper cells (CD3+CD4+)	54.17±16.94	28.9±24.46	0.190
Th1 cells (CD3+CD4+IFN γ +))	8.67±7.44	8.55±8.47	0.905
Th17 cells (CD3+CD4+IL17+)	0.79±0.64	0.88±0.68	0.333
Th1/Th17 ratio	9.58±6.38	10.96±8.57	0.805

SD, Standard Deviation

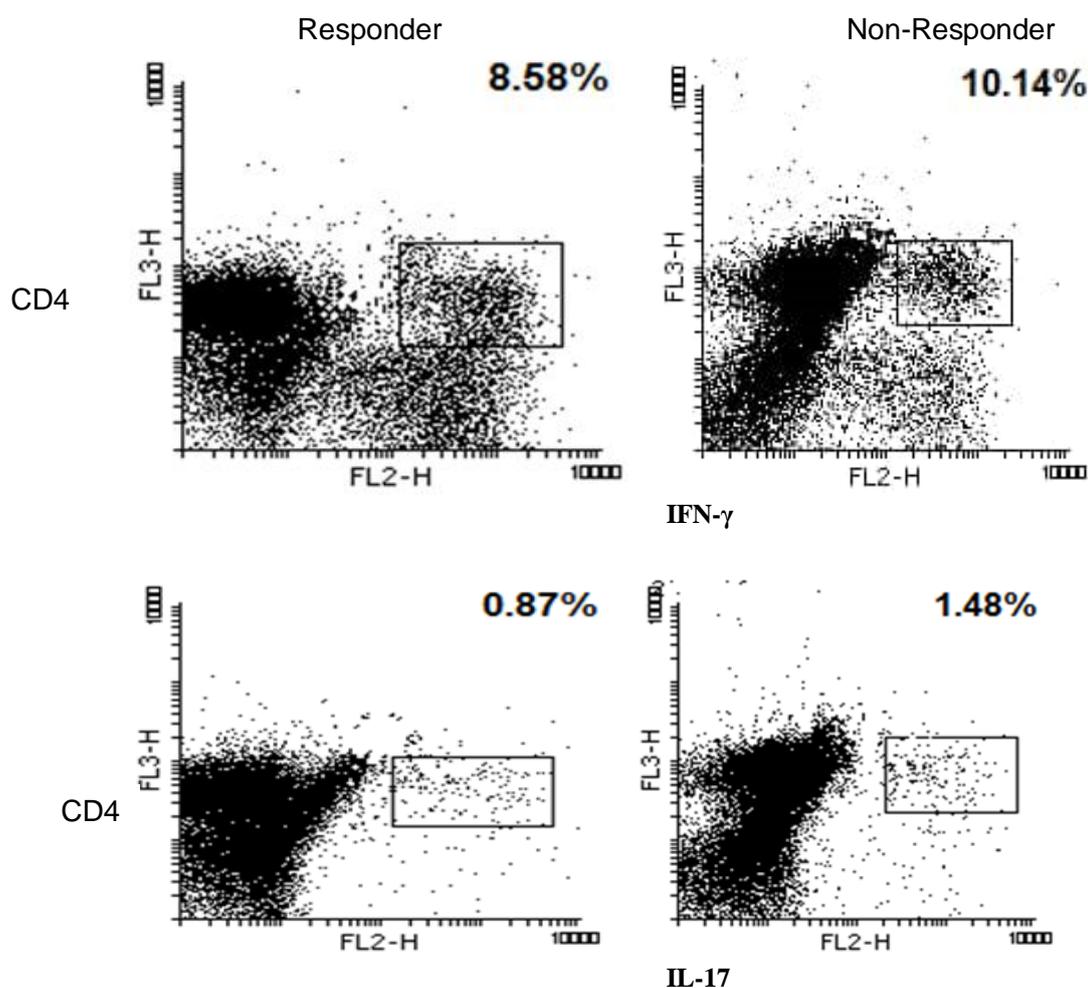


Figure 1. Flow cytometry analysis of the CD3+ CD4 + IFN- γ and CD3+ CD4 + IL-17 expressing cells in responsive and non-responsive Relapsing-remitting multiple sclerosis (RRMS) patients treated with IFN- β . PBMCs activated by stimulation cocktail (PMA+ionomycin), then activated cells stained according to the method described in materials and methods and analyzed using flow cytometry assay. The first lymphocyte population was characterized by FSC/SSC dot plot. Then the lymphocytes were gated on CD3. Next the proportion of CD3+ cells expressing CD4 IFN- γ or CD4 IL-17 (a and b respectively) was evaluated in responsive and non-responsive Relapsing-remitting multiple sclerosis (RRMS) patients. RRMS; Relapsing-remitting multiple sclerosis, IFN- γ ; Interferon-gamma, IL; Interleukin, CD; Cluster of differentiation, PBMCs; Peripheral blood mononuclear cells PMA; Phorbol 12-myristate 13-acetate, FSC; Forward scatter and SSC; side.

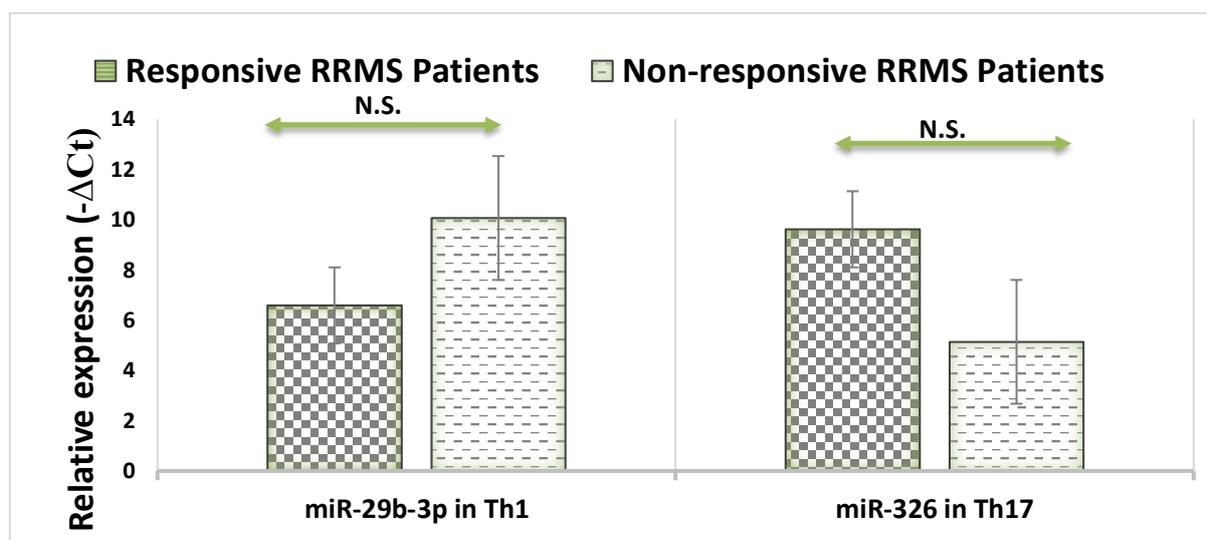


Figure 2. The comparison of expression levels of miR-29b-3p in Th1 and miR-326 in Th17 cells between responsive and non-responsive Relapsing-remitting multiple sclerosis (RRMS) patients treated with IFN- β . The expression levels of these miRNAs were evaluated in RRMS patients following IFN- β therapy for one year by quantitative RT-PCR and the $-\Delta\text{CT}$ method was applied to analyze the results ($p > 0.05$). Expression levels of target genes were normalized using SNORD48 as a reference gene. Bars indicate means \pm Standard Error. RRMS; Relapsing-remitting multiple sclerosis, Mir: MicroRNA, Th; T helper type, IFN- β ; Interferon-beta, RT-PCR; Realtime polymerase chain reaction, CT; Threshold cycle and N.S, non-significant.

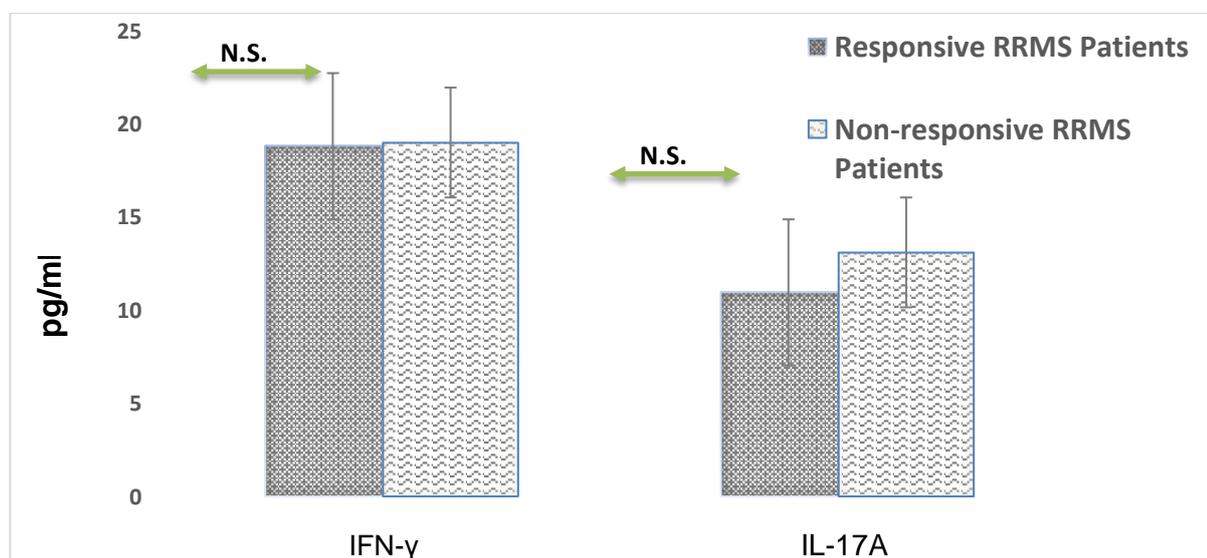


Figure 3. The plasma levels of IFN- γ and IL-17A in responsive and non-responsive Relapsing-remitting multiple sclerosis (RRMS) patients. The plasma levels of IFN- γ and IL-17A were measured by ELISA in responsive and non-responsive RRMS patients ($p > 0.05$). Data are displayed as means \pm Standard Error. RRMS; IFN- γ ; Interferon-gamma, IL; Interleukin, ELISA; Enzyme-linked

immunosorbent assay Pg/ml; Picogram/milliliter and N.S, non-significant

Plasma Levels of IFN- γ and IL-17A

The plasma concentrations of IFN- γ and IL-17A were measured by ELISA. There were no significant differences in levels of IFN- γ and IL-17A between responsive and non-responsive patients ($p=0.360$ and $p=0.907$) respectively.

As well, we did not observe any significant difference in the ratio of IFN- γ /IL-17 between responsive and non-responsive patients ($p=0.959$).

DISCUSSION

RRMS is a complex disease with an unpredictable clinical course and variable pathological patterns.¹² Th1 and Th17 cells with distinct cytokine profiles, including IFN- γ and IL-17 respectively, have important roles in the pathology of MS.⁵ The therapeutic role of IFN- β as immune-modulating therapy is related to modulation of the balance among different populations of T cell.¹³ Based on the context of the specific pathology, type I Interferons (type I IFNs) have Inflammatory and anti-inflammatory functions and thus there is the heterogeneity of IFN- β response in MS patients.¹⁹

In the current study, the percentage of Th1 and Th17 cells and cytokine profile (IFN- γ and IL-17A) were investigated and compared between responsive and non-responsive patients with RRMS. However, we observed no significant difference in the percentage of Th1 cells and Th17 cells between responsive and non-responsive patients with RRMS. Several studies have demonstrated that IFN- β worsens Th17-mediated inflammatory diseases such as RRMS, NMO, psoriasis, rheumatoid arthritis, systemic lupus erythematosus and ulcerative colitis.²⁰⁻²⁵ As well as, Axtell et al have shown that IFN- β could contribute to improving Th1 cells-induced EAE and worse Th17 cells-induced disease.^{14,21} Whilst some findings indicate that IFN- β prevents Th17 cell differentiation and triggers the apoptosis in Th17 cells.^{26,27} Our results may have become non-significant because our sample size was too small, which makes it impossible to classify the diseases to drive by Th1 or Th17 and so it's not conceivable to compare our results with another study.

Furthermore, we evaluated and compared the expression levels of miR29b-3p and miR-326 in Th1 and Th17 cells between responsive and non-responsive patients with RRMS. There was not any significant

difference in the expression levels of miR-29b-3p in Th1 cells and miR-326 in Th17 cells between the responsive and non-responsive patients with RRMS. Hecker et al reported decreased expression of miR-29b-5p in PBMCs from responsive patients with MS. Moreover, they observed the down-regulation of the mir-29 family one month after the start of IFN- β therapy.²⁸ However, Fattahi et al reported that miR-29b-5p and miR-29b-3p were down-regulated in isolated PBMCs from non-responsive patients with RRMS compared to responsive patients with RRMS following IFN- β treatment for more than one year.²⁹ It seems IFN- β affects miR29b-3p in other immune cells and not Th1 cells in RRMS patients.

There was no significant difference in miR-326 expression in Th17 cells between responsive and non-responsive patients with RRMS. Similarly, Fattahi et al observed no significant difference in miR-326 expression in PBMCs between responsive and non-responsive patients with RRMS.¹⁸ Waschbisch et al presented that miR-326 and miR-155 are involved in Th17 differentiation. As well as, they did not observe any significant change in the expression level of the miR-326 in MS patients treated with IFN- β versus untreated MS patients for at least three months.³⁰ It's likely that IFN- β therapy does not restore the expression of deregulated miR-326 and may regulate other involved miRNAs in Th17 development other than miR-326.

Moreover, we observed no significant differences in the levels of IFN- γ and IL17A between responsive and non-responsive patients with RRMS. Also, Fattahi et al reported there is no significant difference in IFN- γ level following treatment with IFN- β between responsive and non-responsive patients with RRMS.²⁹ Taheri and colleagues have compared serum levels of IL-4, IL-6, IL-10, IL-17A and IFN- γ in RRMS patients' responder and non-responder to Cinnovex (IFN- β -1a), they showed that significantly IFN- γ was higher in responders compared with non-responders patients; conversely, IL-6 and IL-17A were lower in responders between two groups.³¹ Criteria used for the classification of IFN- β responders and non-responders in our study are different from their study, besides, the fewer number of patients evaluated in the present study ($n=40$) than the mentioned study ($n=231$) is one of the probable reasons for such contradiction. On the other hand, we used various forms

of IFN-β including IFN-β1a and IFN-β1b in treated RRMS patients, but they only evaluated the effects of Cinnovex in the patients. Also, the follow-up period is not the same in the two studies.

Different genetic profiles of patients in assessed populations (Isfahan compared to Hamadan) may result in heterogeneous responses to IFN-β treatment in both studies.

Similar to our findings, some studies do not get any significant differences in IL-17 and IL-23 values in patients with RRMS.³² Whereas some findings show decreased levels of IL-17 and IL-23 in responsive patients with MS to IFN-β therapy.³³ As well as, MS patients with levels of IL-17F higher than 200 pg/mL were correlated with poor response to treatment.³⁴ Bushnell et al do not find any correlation between IL-17F serum values and response to IFN-β therapy.³⁵ In this study similar to our study, IL-17 levels were lower than 50pg/mL another major difference between us and other studies is that we measured IL-17A plasma values instead of IL-17F. Therefore, IL-17F and IL-17A as single factors could not predict treatment outcome.³⁶ Measurement of a combination of other cytokines in concert with MS pathogenesis and recognition of the synergistic interactions between potent mediators on immune regulation might be helpful to predict response to treatment in patients with RRMS. Besides, cohort studies could be an efficient way to identify immune-based biomarkers of response to treatment and a more correct conclusion; we suggest measurement of baseline levels of these cytokines in MS patients.

There was some limitation in the current study. We investigated IL-17A and IFN-γ levels in the plasma. Diagnostic analysis of cytokine levels in serum or plasma might be influenced by the period between blood collection and arrival in the laboratory. As well as, it is necessary to monitor cytokine at different time points. Also, the volume of blood samples obtained from patients was insufficient, thus it was not possible to perform a complete blood count (CBC) test and to evaluate the absolute number of cells. Furthermore, our sample size was too small. Since a small sample size decreases statistical power. It may be one of the reasons that many of our results have become non-significant.

In conclusion, we observed no significant differences in the percentage of Th1 and Th17 and expression levels of miR29b-3p and miR-326. Therefore, this result may be due to our sample size is small or a cohort study may be needed. Furthermore,

IFN-β may affect other miRNAs in Th1 and Th17 cells rather than miR29b-3p, and miR-326 in MS patients or IFN-β may affect miR29b-3p and miR-326 in other immune cells such as monocytes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest concerning this study.

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