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ORIGINAL ARTICLE

# MicroRNA-92a Drives Th1 Responses in the Experimental Autoimmune Encephalomyelitis

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*Abstract*— Dysregulation of microRNAs (miRNAs) has been linked to the progress of a number of autoimmune diseases including multiple sclerosis (MS), and its animal model, experimental autoimmune encephalomyelitis (EAE). IFN-γ-producing Th1 cells are major players in MS/EAE pathogenesis. It is known that differentiation of T cells towards the Th1 phenotype is influenced by various factors including miRNAs. The miR-92a shows substantial upregulation in MS; however, little is known about its role in the development of autoimmune and inflammatory responses. Herein, we investigated the role of miR-92a in the pathogenesis of MS, focusing on its potential effects on differentiation of Th1 cells. The expression levels of miR-92a were assessed in the spinal cord tissues and splenocytes from mice with EAE using real-time RT-PCR. Next, using transfection with miR-92a mimic sequences, the potential involvement of miR-92a in Th1 polarization was investigated by flow cytometric analysis. Moreover, the expression levels of miR-92a targets were explored in spinal cord tissues of EAE mice. miR-92a expression was enhanced in mouse spinal cord samples at the peak of EAE disease. Overexpression of miR-92a in splenocytes led to increased differentiation of Th1 cells compared with cells transfected with negative control sequences. Enhanced miR-92a expression was accompanied by reduced expression TSC1 or DUSP10, predicted miR-92a targets, in EAE spinal cords. Our data point to a potential role for miR-92a in neuroinflammatory responses in EAE. Our results indicate that miR-92a might affect Th1 differentiation, likely due to downregulation of TSC1 and DUSP10

**KEY WORDS:** multiple sclerosis; experimental autoimmune encephalomyelitis; miR-92a; Th1.

## INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disorder which is characterized by the infiltration of leukocytes into the central nervous system (CNS), loss of myelin, and axonal damage [1]. Experimental autoimmune encephalomyelitis (EAE), in which animals are immunized with myelin antigens, is a commonly used animal model for MS [2]. Numerous studies have examined cellular events occurring in and around CNS lesions in MS and EAE. These studies have led to the widely accepted view that myelin-reactive CD4+ T lymphocytes are crucial in

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disease pathogenesis [1]. In particular, Th1 subtype of T helper cells is considered to be a culprit in promoting autoimmune neuroinflammation in MS/EAE. Production of Th1-type cytokines is increased in CNS lesions during the active phase of disease, and these cytokines have been demonstrated to cause myelin and oligodendrocyte injury [3, 4]. In addition, Th1 cells can activate local microglia and enhance the entry of Th17 cells, another disease-promoting T cell subtype, into the CNS during disease [5].

Despite substantial progress in elucidating cellular events associated with the pathogenesis of MS, molecular mechanisms underlying these cellular responses are only partly understood. MicroRNAs (miRNA) are small endogenous non-coding RNA molecules that serve as post-transcriptional regulators of gene expression. These molecules act by degrading mRNAs or inhibiting their translation to proteins [5]. In recent years, numerous reports have pointed to microRNAs as important players in regulating the activation and differentiation of immune cells [6]. That said, studies have also shown associations between miRNAs and initiation and progression of MS neurological disease. Indeed, there is a growing body of evidence indicating that specific miRNAs (and their respective targets) might have prognostic/diagnostic value in MS [7, 8]. miR-92a belongs to miR-17-92 cluster, also known as oncomiR-1, a cluster of miRNAs that is located within the third intron of C13orf25 locus on the long arm of chromosome 13 (13q31-q32) [9]. Overexpression of miR-17-92 in lymphocytes has been linked to the development of autoimmune diseases, but the underlying mechanisms remain obscure [10]. Of note, miR-17-92 members are dysregulated in peripheral blood specimens from patients with MS [11]. Furthermore, miR-92a overexpression has been detected in the CNS of patients with MS and is likely to contribute to the pathological processes of the disease [12]. Based on the remarkable changes in the CNS of patients with multiple sclerosis, as well as its potential role in immune regulation, in this study, we sought to examine the possible association between miR-92a and immunopathogenesis of MS. Herein, we demonstrate that miR-92a is significantly upregulated in CNS of mice with EAE, and that it contributes to differentiation of Th1 cells likely through targeting TSC1 or DUSP10 genes.

## MATERIALS AND METHODS

### Mice and EAE Induction

Eight-week-old female C57BL/6 wild-type (WT) mice were obtained from Pasteur Institute of Iran and maintained in the animal facility of Tehran University of Medical Sciences for 4 weeks. Active EAE was induced in 12-week-old C57BL/6 mice by immunization with myelin oligodendrocyte glycoprotein (MOG)-35–55 peptide emulsified in complete Freund's adjuvant [4] (EK-2110, Hooke Kit™ $MOG_{35-55}$/CFA Emulsion PTX, Hooke lab, USA). MOG35-55/CFA emulsion was injected subcutaneously (0.1 ml of emulsion/site) at two sites on the back of each mouse. Mice were also injected intraperitoneally with 200 ng/mouse of pertussis toxin diluted in 0.1 ml PBS on days 0 and 1 after immunization. Mice in the control group were injected subcutaneously with CFA and intraperitoneally with pertussis toxin with the same dose as the EAE mice. Clinical score was recorded daily up to 30 days following immunization on a scale of 0 to 15 [13]. All experiments were performed in accordance with guidelines of the Research Ethics Committee of Tehran University of Medical Sciences. CNS tissue samples were isolated from EAE and control mice at varying times after disease induction (pre-onset, peak of disease, and post- peak phase) and were stored at − 80 °C. The lumbar spinal cord shows the highest degree of inflammation and demyelination in this model of EAE, and was selected for expression analyses [14, 15].

### Isolation of MOG-Primed Splenocytes

Single-cell suspensions were obtained from the spleens of MOG-immunized mice 7 days after EAE induction by dissecting the spleen and passing the tissue fragments through a 100-μm nylon mesh followed by Ficoll centrifugation. Splenocytes were seeded at a density of $2 \times 10^6$ cells in 24-well plates at a final volume of 1 ml RPMI 1640 medium containing 5% FBS (Biosera, France) and re-stimulated with various concentrations of MOG peptide (Hooke labs, USA). Cells were harvested at 12, 24, and 48 h after treatment. Moreover, mouse splenocytes were stimulated with mouse anti-CD3 (0.5 μg/ml) and anti-CD28 (0.2 μg/ ml) (eBioscience) in 24-well plates for different durations (1–72 h) in a humidified 5% CO2 incubator at 37 °C.

### RNA Isolation, cDNA Synthesis, and Real-Time RT-PCR

Total RNA including microRNAs were isolated from EAE lumbar spinal cord tissues and stimulated splenocytes using miRNeasy Mini Kit (Qiagen, Germany). RNA concentrations were measured using a Nanodrop. For analysis of miR-92a expression, 1 μg of total RNA was reverse transcribed into complementary DNA (cDNA) using the

miScript II RT Kit (Qiagen, Germany) followed by real-time RT-PCR using the miScript SYBR Green PCR kit (Qiagen, Germany) on an ABI7500 system (Applied biosystems, USA). Reverse transcription of mRNAs was performed using the TAKARA kit.

MicroRNA threshold cycles were normalized against snord 68 and snord 72 threshold cycles (Qiagen, Germany). Expression of other genes was normalized against β-actin threshold cycles. The sequences of primers used in the study are shown in Table 1.

### Transfection of miR-92a Mimics

To examine whether miR-92a regulates the expression of TSC1 or DUSP10, C57BL/6 splenocytes were seeded at a density of $1 \times 10^6$ cells/well in 24-well plates. Cells were then transfected with miR-92a mimic (Qiagen) or negative control (Mirus) using X-tremeGENE siRNA Transfection Reagent (Roche), according to the manufacturer's instructions. Briefly, oligonucleotides were complexed with X-tremeGENE siRNA Transfection Reagent for 20 min at room temperature and then were added to splenocytes at a final concentration of 50 nM/ml. Four hours after transfection, transfected cells were stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28 (0.2 μg/ml) antibodies (eBioscience) for 48 h in a 37 °C incubator with 5% CO2. Cells were then collected and overexpression of miR-92a in splenocytes was confirmed by the flow cytometry method, 48 h post-transfection with the Label IT® RNAi Delivery Control kit. Finally, total RNA was extracted from transfected cells, and the effect of miR-92a overexpression on target gene levels was evaluated by real-time RT-PCR.

### T Cell Differentiation

To address the question of whether overexpression of miR-92a affects T cell differentiation and cytokine production, intracellular staining and flow cytometry were performed. Cells were isolated from spleens of female C57BL/6 mice at 6–8 weeks of age. $1 \times 10^5$ cells were seeded in each well in 96-well plates and then transfected with 50 nM/ml miR-92a mimic or negative control using X-tremeGENE siRNA Transfection Reagent. Then, cells were activated with anti-CD3 (0.5 μg/ml) and anti-CD28 (0.2 μg/ml) and were differentiated under Th1 or Th17-promoting conditions. To differentiate the cells into Th1, transfected cells were treated with a Th1 cocktail containing IL-2 (20 ng/ml), IL-12 (50 ng/ml), and anti-IL-4 antibody (10 ng/ml) (BioLegend) at 4 h after transfection. Cells were skewed into Th17 phenotype by supplementation with TGF-β (5 ng/ml), IL-6 (100 ng/ml), anti-IFN-γ (10 ng/ml), anti-IL-4 (10 ng/ml), and IL-23 (50 ng/ml) (BioLegend). On day 4, cells were collected and cytokine expression was determined by flow cytometry.

### Flow Cytometry

For assessment of intracellular cytokine expression, miR-92a transfected splenocytes were re-stimulated with PMA and ionomycin in the presence of brefeldin A for 4 h to block cytokine secretion. Cells were stained with a PerCP-conjugated anti-mouse CD4 mAb followed by treatment with fixation/permeabilization buffer, and intracellular staining with PE-conjugated anti-mouse IL-17 mAb, and PE-conjugated anti-mouse IFN-gamma mAb according to the manufacturer's recommendations (BioLegend). In brief, following surface staining, cells were resuspended in 1 ml/tube Fixation Buffer and incubated for 20 min at room temperature. After washing, cells were permeabilized with 1 ml Permeabilization Buffer and stained for intracellular markers with antibodies diluted in 100 μl Perm medium. Data were acquired with a BD FACSCalibur flow cytometer and then exported for analysis in the FlowJo software.

### Statistical Analysis

Statistical analyses were done using the SPSS software, Version 21. Comparisons between groups were made using Student's $t$ or Mann–Whitney $U$ tests (two groups) and one-way ANOVA or Kruskal–Wallis tests (three groups). Data are presented as mean ± SEM from three independent experiments and threshold for significance was $p < 0.05$.

**Table 1.** The Reverse and Forward Primers Used in this Study

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *β-actin* | 5′-ATGCTCCCCGGGCTGTAT-3′ | 5′-CATAGGAGTCCTTCTGACCCATTC-3′ |
| *TSC1* | 5′-CTGCTCAGCCAGGTCTCT-3′ | 5′-TCTACTTCCTTGGTGGTGTCA-3′ |
| *DUSP10* | 5′-GGGGACAGACTGAGGTAGCA-3′ | 5′-GCAAAGAACCCCTGGTATTG-3′ |

## RESULTS

### Upregulation of miR-92a in the CNS of Animals with EAE

Analysis of microRNA expression profiles in patients with MS has illustrated miR-92a upregulation in the CNS of MS patients [12]. To examine the possible role of miR-92a in EAE development, we analyzed the expression levels of miR-92a in mice during the course of EAE. Samples from spinal cord tissues were obtained from EAE mice at pre-onset (day 10), peak of disease (day 20), and post-peak phases (day 25). We have previously reported disease severity scores as well as the expression levels of various inflammation-related genes at these disease stages for these mice [16, 17]. When analyzed for miR-92a expression, samples from the pre-onset phase of disease showed no substantial changes when compared with those from control mice. However, expression of miR-92a was substantially higher at the peak of disease, as shown by real-time RT-PCR (Fig. 1). Indeed, tissues isolated from mice at peak of disease displayed miR-92a levels that were almost fivefolds higher than control animals. Also, miR-92a was overexpressed at post-peak of disease, but the levels were not as high as in peak of disease and did not reach statistical significance. These results demonstrated that miR-92a was induced in CNS of mice with EAE and that miR-92a expression levels might correlate with the stage of disease.

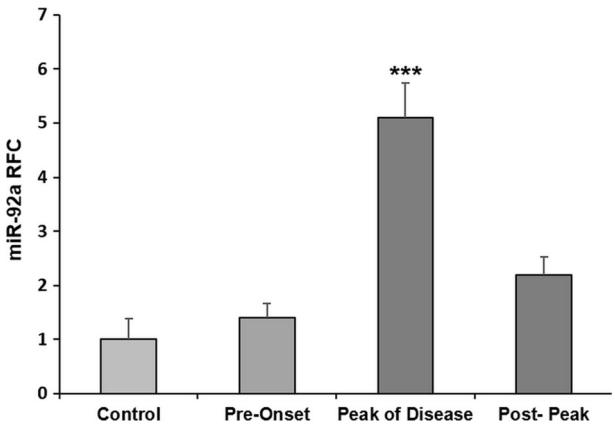


**Fig. 1.** miR-92a expression in the CNS of C57BL/6 mice during EAE. miR-92a expression in spinal cord tissue in different phases of EAE. Expression levels have been normalized against snord68 levels. miRNA levels are shown as relative fold change (RFC). Bar graph shows mean ± SEM for ten mice per group, ***$p < 0.001$, Kruskal–Wallis tests.

### Enhancement of miR-92a Expression Following Splenocyte Activation

In the context of EAE, autoreactive T cells are induced in lymph nodes and the spleen following immunization with myelin antigens. Following activation, these T cells migrate to the CNS, where they are re-activated and initiate an inflammatory response leading to myelin damage [18]. We examined whether immune cell populations in the spleen of mice overexpress miR-92a after EAE induction. To this end, the expression of miR-92a in splenocytes from immunized mice was analyzed following polyclonal or antigen-specific activation at different time points. Following activation of splenocytes with anti-CD3 and anti-CD28 antibodies, miR-92a levels demonstrated no significant changes at 1, 2, 4, 8, 12, and 24 h time points. However, significant upregulation was detected 48 h post-stimulation with further increases at 72 h (Fig. 2). To evaluate whether stimulation of splenocytes with MOG might also affect the expression of miR-92a, splenocytes were stimulated with various concentrations of MOG for 12 or 24 h. Following splenocytes re-stimulation for 12 h, levels of miR-92a showed an upward trend, but it was not significantly different from the controls (Fig. 3a). Moreover, levels of miR-92a following MOG stimulation for 24 h did not reach a statistically significant increase (Fig. 3b).

### Promotion of Th1 Differentiation by miR-92a

To investigate the potential role of miR-92a in development of pathological immune responses in EAE, we

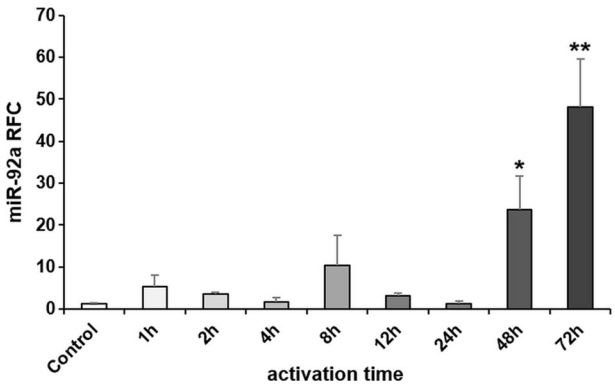


**Fig. 2.** miR-92a expression in polyclonally activated splenocytes. Murine splenocytes were stimulated with anti-CD3 and anti-CD28 for different time points. Quantitative real-time PCR analysis was used to assess miR-92a mRNA levels in splenocytes. Results are presented as the ratio of miRNA levels relative to controls. Data are presented as mean ± SEM, $n = 2$. *$p < 0.05$, **$p < 0.01$, Kruskal–Wallis test.

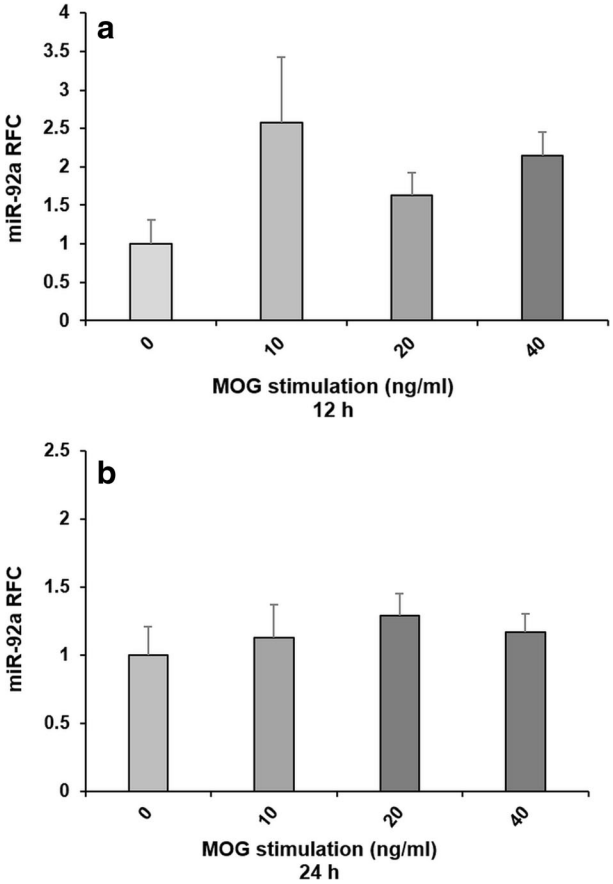


**Fig. 3.** miR-92a expression in splenocytes activated by MOG. Splenocytes from MOG-immunized mice were cultured with different concentrations of MOG and incubated for 12 (**a**) or 24 (**b**) hours. miR-92a expression in MOG-treated splenocytes was determined by real-time RT-PCR. Values are mean ± SEM from two independent experiments. *$p < 0.05$, Kruskal–Wallis test.

examined the effects of miR-92a overexpression in T cell differentiation. Since Th1-secreted IFN-γ and Th17-secreted IL-17 are key mediators involved in EAE pathogenesis, the ability of miR-92a to influence differentiation of these T helper subtypes and stimulate the production of these pro-inflammatory mediators was assessed. Splenocytes from C57BL/6 mice were transfected with miRNA mimic sequences, activated by anti-CD3/CD28, and cultured under Th1 or Th17 polarizing conditions for 4 days. Next, frequencies of IFN-γ- and IL-17A-producing cells were determined by intracellular cytokine staining. As shown in Fig. 4, flow cytometric analyses revealed that overexpression of miR-92a in mouse splenocytes led to a twofold increase in Th1 differentiation compared with cells transfected with negative control sequences, as identified by the increased frequency of IFN-γ-immunopositive cells. A slight but non-significant increase occurred in the frequency of IL-17 immunopositive cells following miR-92a transfection. Increased numbers of IFN-γ-producing cells (but not IL-17-producing cells) following miR-92a overexpression suggest that miR-92a is able to promote the differentiation of Th1 cells, which are able to sustain inflammation and exacerbate clinical signs of EAE.

### miR-92a Downregulates TSC1 and DUSP10 Levels

Considering our findings with regard to miR-92a and its link with Th1 differentiation, we next tried to investigate the mechanisms underlying these effects. miRNAs are involved in negative regulation of gene expression at the post-transcriptional level [19]. To explore how miR-92a is involved in the regulation of T helper fate, we searched for miR-92a target genes which were related to T helper cell differentiation. We searched miRNA target prediction databases TargetScan and miRDB to obtain some information about predicted targets of miR-92a. We focused on targets which had conserved miR-92a binding sites between human and mouse within their 3′ untranslated region. Publications supporting the role of targets in T cell differentiation were considered as another selection criterion. Tuberous sclerosis complex 1 (*TSC1)* and dual specificity phosphatase 10 (*DUSP10)* were selected as targets which met these criteria (Fig. 5a, b). Of note, miRTarBase (http://miRTarBase.mbc.nctu.edu.tw/), a database that includes validated miRNA-mRNA interactions, shows DUSP10 as a validated target for miR-92a [20]. This target validation program also presents *TSC1* as a target for miR-92a (based on NGS evidence). We altered the expression of miR-92a through transfection of splenocytes with miR-92a mimic sequences. Then, the expression of targets following miR-92a overexpression was assessed using RT-PCR. Splenocytes transfected with miR-92a exhibited lower levels of TSC1 and DUSP10 mRNA, as compared with cells transfected with negative control sequences. TSC1 and DUSP10 expression in splenocytes were on average 33 and 44%, respectively, of that observed in negative control cells (Fig. 5c). Thus, it seemed that manipulation of miR-92a in splenocytes could regulate the amount of TSC1 and DUSP10 transcripts. Given that TSC1 and DUSP10 negatively regulate Th1 differentiation [21, 22], this observation suggests that miR-92a has the ability to positively regulate Th1 responses.

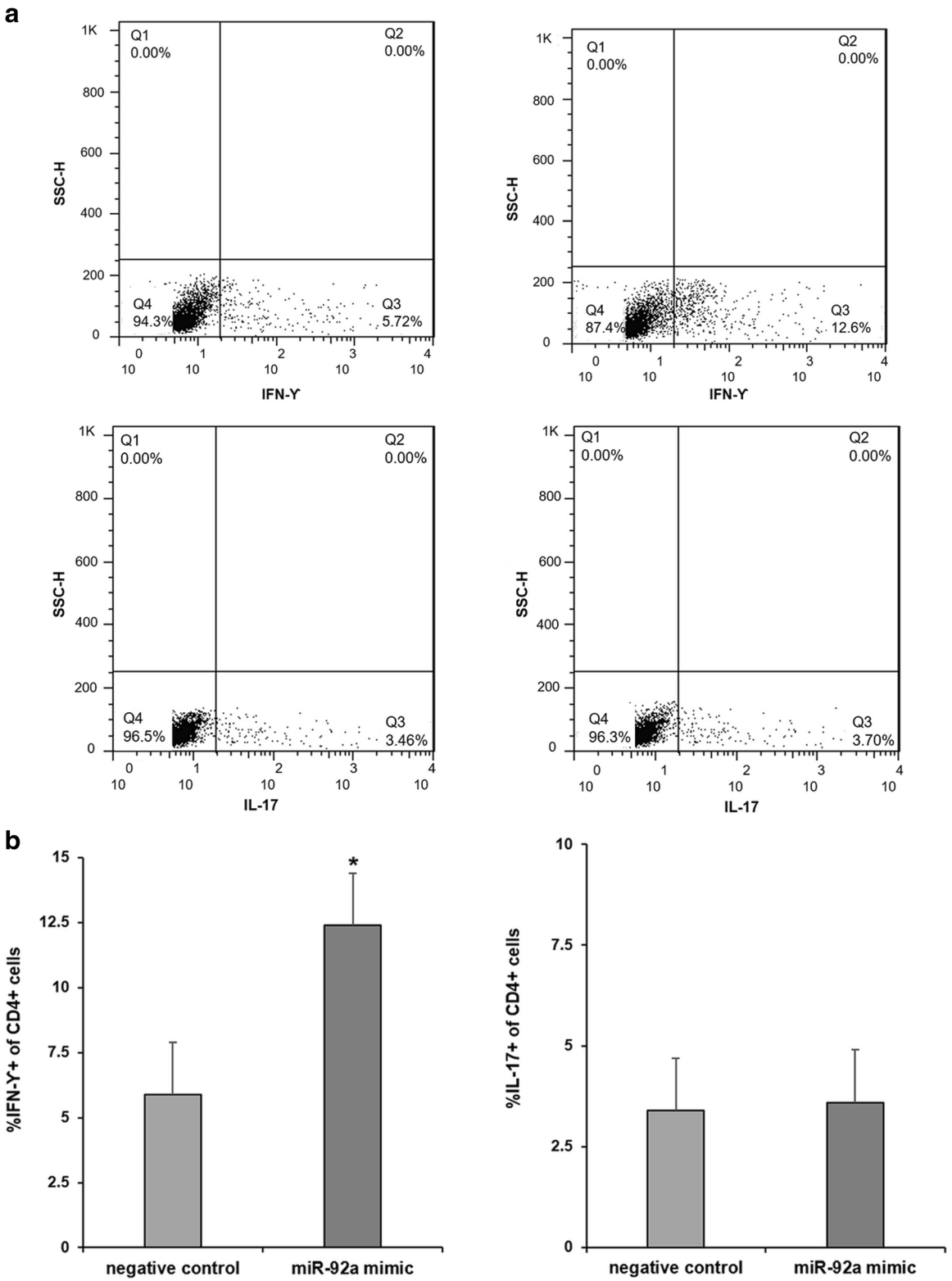


**Fig. 4.** miR-92a overexpression shifts the differentiation of Th cells in murine splenocytes. **a** Representative flow cytometry dot-plots. Numbers in quadrants indicate the percentage of the cells producing IFN-γ or IL-17 in the CD4+ gate. **b** Graphs summarize flow cytometry findings. Results are presented as mean ± SEM from three independent experiments. *$p < 0.05$, Mann–Whitney *U* test.

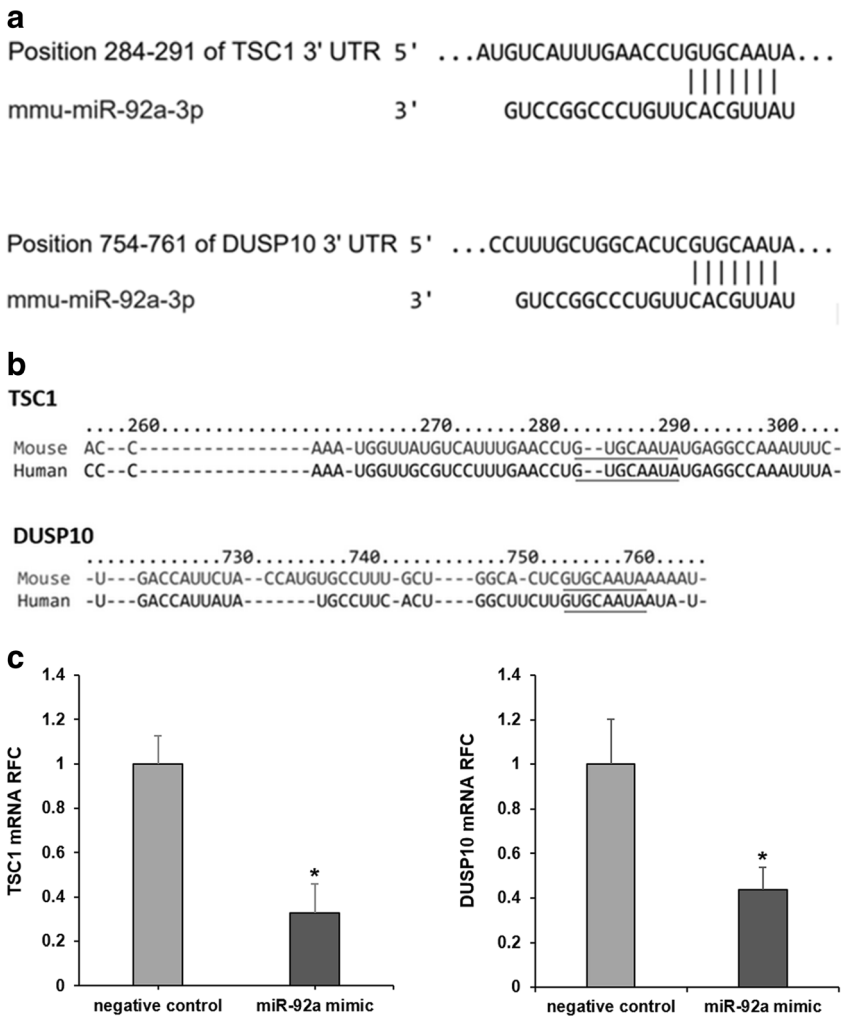


**Fig. 5.** Analysis of miR-92a targets which contribute to Th1 differentiation. **a** Alignment of predicted miR-92a binding sites with TSC1 and DUSP10 3′UTR, downloaded from TargetScan. **b** The 3′UTR of TSC1 and DUSP10 mRNAs contains one binding site for miR-92a homology between TSC1 and DUSP10 miRNA binding sites between human and mouse. **c** Real-time RT-PCR analysis of the expression levels of TSC1and DUSP10 RNA in splenocytes after transfection with miR-92a mimics or negative control sequences. Bar graph summarizes means ± SEM from three independent experiments. (*$p < 0.05$).

## Dysregulation of miR-92a Targets Transcript in EAE CNS Tissues

As shown in Fig. 1, the expression levels of miR-92a were significantly higher in the CNS at the peak of EAE disease compared with those from pre-onset or control groups. To gain further insight about the effects of miR-92a in gene regulation during EAE, the levels of the target genes were analyzed in EAE tissues. The abundance of *TSC1* transcripts was markedly diminished in the CNS at pre-onset and also at the peak of the disease compared with control samples. *TSC1* levels were slightly reduced at the post-peak phase, although this was not statistically significant (Fig. 6a).

When we analyzed DUSP10 levels, the expression displayed a marked reduction at the peak of disease compared with control or pre-onset. DUSP10 transcripts showed higher expression levels at pre-onset compared with other phases or compared with the control group (Fig. 6b). These changes do not necessarily indicate a direct effect by miR-92a; nonetheless, these results display an inverse correlation between the abundance of target genes and miR-92a during EAE disease.

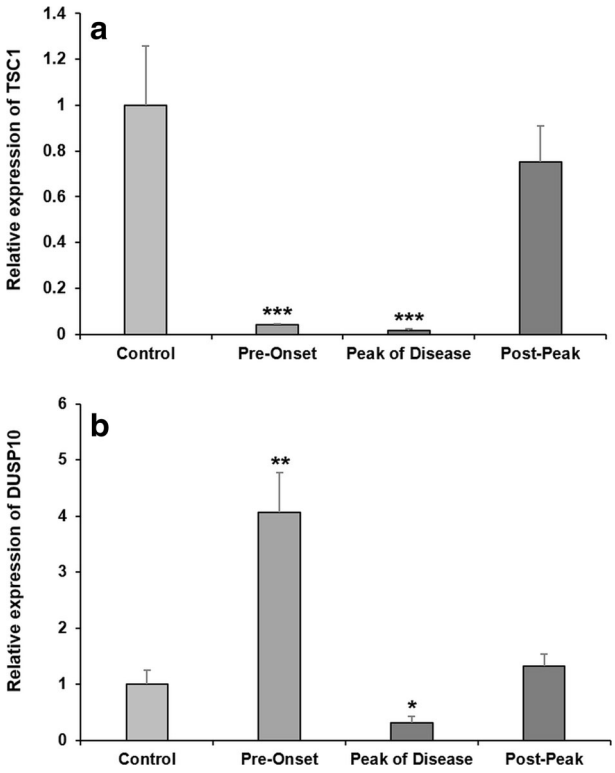


**Fig. 6.** Expression of miR-92a targets in EAE tissues. miR-92a target transcripts were assessed using real-time RT-PCR analysis on RNA extracted from EAE and control tissues. **a** TSC1 was significantly decreased in peak of disease and Pre-Onset phases of EAE in comparison with control. **b** DUSP10 was significantly decreased in peak of disease phase of EAE in comparison with control or Pre-Onset phase. Data are presented as mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, one-way ANOVA.

## DISCUSSION

Elucidating molecular mechanisms that affect T cell differentiation is essential for understanding the pathogenesis of T cell-mediated autoimmune disorders and for identification of potential targets for therapeutic intervention. While T cell differentiation is chiefly regulated by specific sets of cytokines and transcription factors, epigenetic elements including microRNAs are known to "fine-tune" the process in physiological and pathological settings.

In the current study, we explored the role of miR-92a in EAE, as well as in the T cell differentiation process. This miRNA was selected based on two sets of previous findings: miRNA expression profiling in brain white matter tissue from MS patients and reported roles for miR-92a (and miR-17-92 cluster) in regulating inflammation and leukocyte function [12, 23–25]. Indeed, miR-92a is known to play potentially important roles in several categories of human disease, including neoplastic- and inflammation-related conditions. Of note, cancer biology studies have reported altered expression as well as pathogenic roles for miR-92a in various cancers including brain, breast, gastric, lung, and colorectal cancers [26–30]. These roles are consistent with miR-92a effects in regulating apoptosis and cell cycle [26, 31, 32]. miR-92a is also known to be involved in inflammatory conditions, including SLE and scleroderma [33–35]. In the context of cardiovascular disease, induction of miR-92a is involved in endothelial dysfunction caused by oxidative stress and it can contribute to development of atherosclerotic lesions [36, 37]. In the current study, analysis of miR-92a expression in spinal cord tissues from EAE mice showed a substantial increase in miR-92a levels almost 20 days after disease induction, when clinical signs of disease were most severe. This finding was consistent with MS brain white matter profiling data, which had also shown induced miR-92a levels in MS [12]. However, it should be noted that, unlike the MS profiling study, in the current study, we looked at miRNAs extracted from total spinal cord tissue which included both white matter and gray matter. Hence, the altered expression was the average of miRNA expression in both white and gray matter components. We next explored the role of miR-92a in T cell biology after polyclonal or antigen-specific activation of cells. Polyclonal activation of T cells using anti-CD3/CD28 antibodies led to upregulation of miR-92a at 48 and 72 h time points, a finding that was consistent with increased miR-92a levels in EAE tissues. However, when splenocytes from MOG-immunized animals were stimulated with the antigenic peptide they showed only a non-significant upward trend for miR-92a. Whether this is due to low frequency of MOG-specific T cells in splenocyte cultures, or different kinetics of T cell activation, is unclear and requires further experiments. It should also be noted that activated T cells are one of several cellular players in inflamed EAE tissues, and enhanced miR-92a expression might be a consequence of altered expression of the miRNA in monocytoid or neural/glial cells. We next asked whether altered miR-92a levels might affect EAE-related pathogenic pathways including T cell differentiation. As mentioned above, several studies have pointed to the role of miR-17-92 cluster in promoting T cell responses. Jiang et al. have shown that miRNAs encoded by miR-17-92 cluster are key players in Th1 responses, where they promote T cell proliferation and IFN-γ production and decrease post-activation cell death and Treg differentiation [24]. Another study by Wu et al. has shown that miR-17-92 expression is required for virus-specific Th1 responses as well as expansion of T follicular

helper cells [23]. In a relatively different set of findings, and of direct relevance to the current study, De Kouchkovsky et al. have reported that miR-17-92 is required for accumulation of Tregs and that Treg-specific loss of the miRNA cluster results in exacerbated autoimmune responses in EAE [11]. In contrast, Liu et al. have reported that miR-17-92 cluster promotes Th17 responses and that T cell-specific loss of the cluster leads to amelioration of EAE disease [25]. Considering all these previous reports, we tried to delineate the role of miR-92a in T cell differentiation fate, and examine the impact of its expression in driving Th1 or Th17 responses. Our results indicated that increased miR-92a levels supported a Th1 response but not a Th17 response. Our finding was consistent with findings of the Wu et al. and Jiang et al. studies, which had shown a reduction in Th1 responses in the absence of miR-17-92 cluster [23, 24].

In the last part of this study, we tried to gain some insight into the molecular players which could link miR-92a with T cell polarization. Each miRNA species is capable of targeting and regulating numerous downstream genes, and a thorough screening for a miRNA's downstream players in the context of a particular cell biological phenomenon would likely require a high-throughput transcriptomic/proteomic study. Herein, we used a limited but more feasible approach to identify potential downstream molecules. We used miRNA target prediction databases combined with literature review and came up with two miR-92a targets which could affect T cell differentiation: TSC1 and DUSP10, both being negative regulators of Th1 differentiation. Tuberous sclerosis complex 1 (TSC1) is a tumor-suppressor gene which acts as a negative regulator of mTORC1, itself a major regulator of cell growth [38]. In the context of immune signaling, mTORC1 plays a central role in T cell fate determination [39]. Studies have revealed that T cell-specific deletion of the TSC1 gene results in augmented Th1 differentiation [21]. Chornoguz et al. have reported that mTORC1 modulates Th1 differentiation through the promotion of T-bet phosphorylation not only in mouse CD4+ T cells but in human CD4+ T cells as well [40]. In addition, mice which harbor T cell-specific deletions of Rheb, an mTOR-related GTPase, fail to secrete IFN-γ and are resistant to the development of EAE [39]. In our study, transfection of splenocytes with miR-92a mimic sequences led to significant suppression of TSC1 levels, indicating that T helper fate might be determined, at least in part, through the miR-92a/TSC1/mTORC1 pathway.

The dual specificity phosphatase 10 (DUSP10), also known as mitogen-activated protein kinase phosphatase 5 (MKP5), negatively regulates the activation of MAP kinases [41]. This MKP has distinct functions in both innate and adaptive immunity. DUSP10 has been shown to inhibit the JNK signaling pathway after TCR ligation and reduce the activity of activator protein-1 (AP-1) transcription factor. Increased JNK activity in the absence of DUSP10 gives rise to the increased AP-1-dependent production of T cell cytokines [22]. Both Th1 and Th2 cells lacking DUSP10 have significantly enhanced levels of JNK activity [42]. In the current study, overexpression of miR-92a led to significant downregulation of DUSP10 levels in splenocytes, a finding which supports, but does not confirm, a role for miR-92a-DUPS10 axis in T cell fate determination.

Overall, our findings support the hypothesis that miR-92a is involved in the pathogenesis of EAE, perhaps by tipping the balance of T cell differentiation towards pathogenic Th1 cells. miR-92a might function as a positive regulator of Th1 differentiation, and this regulation is likely to occur, at least in part, through attenuating the expression of negative regulators of Th1 differentiation, DUSP10 and TSC1. Regulation of miR-92a expression or inhibiting its interaction with its downstream players through miRNA binding site blockers might represent a potential therapeutic strategy in autoimmune demyelination.

## COMPLIANCE WITH ETHICAL STANDARDS

All experiments were performed in accordance with guidelines of the Research Ethics Committee of Isfahan University of Medical Sciences.

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

**Ethical Approval.** All experiments were performed in accordance with guidelines of the Research Ethics Committee of Isfahan University of Medical Sciences (Code of Ethics: IR.MUI.REC.1394.3.134).

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