Inflammation in an Animal Model of Multiple Sclerosis Leads to MicroRNA-25-3p Dysregulation Associated with Inhibition of *Pten* and *Klf4*

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ABSTRACT

Perturbed expression of microRNAs (miRs) has been reported in different diseases including autoimmune and chronic inflammatory disorders. In this study, we investigated the expression of miR-25-3p and its targets in the central nervous system (CNS) tissue from mice with experimental autoimmune encephalomyelitis (EAE). We also analyzed the expression of miR-25 and its targets in activated macrophages and splenocytes.

EAE was induced in 12-week old female C57BL/6 mice; using myelin oligodendrocyte glycoprotein 35-55/complete Freund's adjuvant (MOG35-55/CFA) protocol. The expression of miR-25-3p and its targets, as well as the expression of inflammatory cytokines, were analyzed. We next established primary macrophage cultures as well as splenocyte cultures and evaluated the levels of miR-25-3p and its target genes in these cells following activation with lipopolysaccharide (LPS) and anti-CD3/anti-CD28 antibodies, respectively.

MiR-25-3p expression showed a strong positive correlation with the expression of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1α, and IL-6 pro-inflammatory cytokines. The expression of *phosphatase and tensin homolog* (*Pten)* and *Krüppel-like factor 4* (*Klf4)* was significantly reduced at the peak of the disease. Interestingly, *Pten* and *Klf4* expression showed a significant negative correlation with miR-25-3p. Analysis of miR-25-3p expression in LPS-treated primary macrophages revealed significant upregulation in cells treated with 100ng/ml of LPS. This was associated with suppressed levels of miR-25-3p targets in these cells. However, anti-CD3/anti-CD28-stimulated splenocytes failed to show any alterations in miR-25-3p expression compared with vehicle-treated cells.

Our results indicate that miR-25-3p expression is likely induced by inflammatory mediators during autoimmune neuroinflammation. This upregulation is associated with decreased levels of *Pten and Klf4*, genes with known roles in cell cycle regulation and inflammation.

Keywords: Autoimmune diseases of the nervous system; Encephalomyelitis; MicroRNAs; Multiple sclerosis

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Dysregulation of MiR-25-3p and Its Targets in Experimental Autoimmune Encephalomyelitis

INTRODUCTION

MicroRNAs (miRNAs) are short RNA sequences that target protein-coding mRNAs at 3'UTR conserved sites, influencing their translation and/or degradation.[1] Primary transcript of miRNAs forms hairpin structures that are processed to generate mature single-strand miRNAs. MiRNAs are involved in the regulation of many cell biological processes and their expression levels change in pathological conditions.[2] Numerous studies have shown that miRNAs contribute to the pathogenesis of various diseases including diseases that are associated with chronic inflammation.[3]

Multiple sclerosis (MS) is an autoimmune neuroinflammatory condition that is caused by immune cell infiltration into the central nervous system (CNS) followed by myelin damage, axonal loss, and gliosis. It is not yet clear whether an initial change in the CNS cells/microenvironment leads to neuroinflammation or that the breakdown of immune tolerance in the peripheral immune system is the primary event in the disease process.[4] Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS that simulates many aspects of its pathogenesis. EAE is induced by the immunization of animals with myelin antigens. This leads to the generation of myelin-reactive lymphocytes which in turn cause autoimmune inflammation in the brain and spinal cord.[4] Depending on the animal species/strain and the antigens used for immunization, EAE displays different forms which resemble different clinical aspects of MS. EAE animal model has been successfully used to study MS pathogenesis and to test therapies for this disease.[5] Studies on EAE have revealed that myelin-reactive T cells infiltrate the CNS tissue and release proinflammatory cytokines. Macrophages and microglia are also involved in EAE induction and progress through the production of injurious cytokines as well as phagocytosis of myelin and cell debris.[6]

Expression profiling studies on MS patients' leukocytes and CNS tissue have revealed the dysregulation of various miRNAs compared with healthy controls.[7-11] MiR-25 is one of the miRNAs which has shown altered expression in MS cases in different studies. This miRNA belongs to the conserved miR-106b/25 cluster, which is located on chromosome 7 in the human genome and is transcribed from the intron of MCM7 (minichromosome maintenance complex component 7). MiR-25 precursor generates two mature isoforms, miR-25-5p and miR-25-3p, the latter being the dominant form based on sequencing studies. MiR-25-3p has well-known roles in cycle regulation and cell proliferation, migration, differentiation, apoptosis, and inflammation.[12] A miRNA profiling study from our group showed significant upregulation of miR-25-3p in MS brains, compared with non-MS cases.[13] Other studies have also reported increased levels of miR-25-3p in MS patients' blood.[14] That said, some studies have demonstrated miR-25 downregulation in peripheral Treg cells from MS patients.[15] Dysregulation of miR-106b/25 cluster has also been illustrated during oncogenesis. Indeed, evidence suggests an onco-miR role for miR-25 in different cancers.[16]

Previous studies had mainly reported associations between miR-25 dysregulation and MS disease. In this study, we further investigated whether a relationship might exist between miR-25 and inflammatory processes in EAE. Considering the higher abundance as well as the known roles for miR-25-3p isoform in disease, we decided to focus on this isoform. In mice, miR-106b/25cluster is located on chromosome 5 and is transcribed from within the *Mcm7* gene, similar to humans. MiR-25-3p sequence is identical between human and mouse (5' CAUUGCACUUGUCUCGGUCUGA 3'), making murine and human miR-25-3p more likely to bind to similar target transcripts. Experiments were performed to measure miR-25-3p expression in CNS tissue from EAE mice at different time points after disease induction. This was followed by analyzing the levels of the verified targets of miR-25-3p. We then carried out experiments on primary macrophages and splenocytes to examine whether miRNA/target changes observed in CNS might be related to their altered expression levels in activated leukocytes.

MATERIALS AND METHODS

EAE Induction

We used 12 week-old wild-type female C57BL/6 mice for EAE studies. Two weeks before induction, animals were transferred to the laboratory and maintained under a 12/12 h light and dark cycle with free access to water and food. For EAE induction (n=20), mice were immunized with MOG35-55

(Myelin oligodendrocyte glycoprotein 35-55) peptide emulsified in complete Freund's adjuvant (CFA) (EK-2110, Hooke Kit™ MOG35-55/CFA Emulsion PTX, Hooke Laboratories, USA). 0.1 mL of the emulsion was subcutaneously injected to the back, at two points near the base of the tail (200 ng/mouse). On the day of immunization and the next day, pertussis toxin (EK-2110, Hooke Kit™ MOG35-55/CFA Emulsion PTX, Hooke Laboratories, USA) was administered intraperitoneally according to the kit manufacturer's instructions. For the control group (n=10), phosphate buffer saline (PBS) was used instead of MOG emulsion. Mice weight and disability were assessed every day for up to 30 days. After about 14 days, paralysis started in the tail and developed to hind limbs and then, in some animals, to forelimbs in parallel with weight loss. The paralysis status was scored based on a 0-15 scale.[17] EAE mice were assigned to two groups, the first group of mice was sacrificed when they reached the peak score or acute phase of the disease, while the second group was sacrificed when the symptoms had partially subsided (post-peak or chronic phase). Mice were sacrificed using ketamine/xylazine (Alfasan, Iran) overdose and after perfusion with PBS, the CNS was extracted and transferred to liquid nitrogen and then kept in a -80 centigrade freezer. All animal experiments were performed according to institutional guidelines and were approved by the Ethics Committee of Kerman University of Medical Sciences (ethical code: IR.KMU.REC.1397.313).

Splenocyte and Macrophage Cultures

12-week-old C57BL/6 non-immunized mice were put under deep anesthesia and sacrificed by cervical dislocation. Spleens were removed, dissected, and passed through cell strainers with 70 μm pore size (SPL life Sciences, Korea). This was followed by red blood cell lysis, washing, and counting the cells. 2.5×10^6 cells were cultured in 24-well plates (JetBiofil, China) with 1 mL final volume RPMI 1640, 10% FBS, 2% L-Glutamine (BIO-IDEA, Iran). Cells were poly-clonally activated with anti-CD3 (0.5 μg/mL) and anti-CD28 (0.25 μg/ mL) (eBioscience, United States) at the first hour of culture. Splenocytes' total RNA was isolated at three time points; 2, 8, and 24 h after activation.

To establish macrophage cultures, bone marrow cells were isolated from the tibia and femur of C57BL/6 mice, as described before.[18] Cells were differentiated into macrophages in the presence of 50 ng/mL recombinant macrophage colony-stimulating factor (M-CSF) (eBioscience, United States) for one week. Macrophages were then stimulated with 10 and 100 ng/mL Lipopolysaccharide (LPS) (Sigma Aldrich, USA). RNA extraction was performed and gene expression of macrophages was analyzed 24 h after stimulation.

RNA Extraction and Reverse Transcription

Total RNA of the upper lumbar cord of EAE models were purified using silica column-based protocol (GA-325-150, Hybrid miRNA GeneAll, GeneAll, Korea), following the manufacturer's instructions. RNA was eluted in nuclease-free water and its concentration was measured by a Nanodrop (ThermoFisher, USA). The Isolated RNA concentration range was between 200-400 ng/μL, and the ratio of absorbance at 260 nm/280 was between 1.8 - 2.2. Complementary DNA synthesis was performed with 500 ng of total RNA using miScript II RT Kit (Qiagen, Germany) for microRNA reverse transcription and TAKARA kit (Takara Bio, USA) for mRNA, according to kit instructions.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA amplification for miR-25-3p was performed using Mm_miR-25_1 miScript Primer Assay (Qiagen, Germany) and MiScript SYBR Green PCR Kit (Qiagen, Germany). Data were normalized based on the amplification results of Hs_SNORD68_11 miScript Primer Assay (Qiagen, Germany). For target genes, real-time RT-PCR was performed using Hot Fire Pol Eva green qPCR (Solic bio dyne, Estonia). Threshold cycles for target genes were normalized using beta-actin threshold cycles. Primer sequences for miR-25-3p targets, cytokines, and beta-actin are provided in Table 1. We used the $2^{-\Delta\Delta Cq}$ method to calculate the relative expression levels. All real-time RT-PCR reactions were performed on a Bio-Rad CFX96 (Bio-Rad, Germany) system.

Immunofluorescence Assay

To evaluate the bone marrow-derived macrophage cells (BMDMs), immunofluorescent staining was performed using antibodies against Iba1 which is a macrophage marker. The cells were cultured on coverslips coated with gelatin (Sigma, Germany), fixed

with 4% paraformaldehyde (Merck, USA) for 20 minutes, permeabilized using 0.2% Triton X-100 (Sigma, Germany) for 30 minutes, incubated with 1% bovine serum albumin (Sigma, Germany) for 1 hour at room temperature. Rabbit anti-Iba-1 antibody was used overnight at 4°C (1:800 diluted in PBS; Wako, Japan), Subsequently, the cells were washed with PBS three times and then incubated with anti-rabbit FITC conjugated antibody (1:1000 diluted in PBS; Abcam) 1 hour at room temperature. Nuclei were stained using nuclear yellow. The immunostained samples were photographed using a fluorescence microscope (Olympus, Japan). In control studies, the primary antibody was replaced with PBS. There was no immunoreactivity in these controls.

Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics version 22. The obtained data were analyzed by nonparametric tests for independent samples, Kruskal-Wallis ANOVA. Null Hypotheses were rejected in cases showing p values less than 0.05. Data are shown as relative fold change (RFC), mean±standard deviation. Plots were prepared using MS Excel 2016.

Table 1. Primer sequences for real-time polymerase chain reaction (PCR) of proinflammatory cytokines and miR-25-3p gene targets

Primer names	Sequence (5'→3')
mmu-beta Actin-F	ATGCTCCCCGGGCTGTAT
mmu-beta Actin-R	CATAGGAGTCCTTCTGACCCATTC
mmu-Bcl2l11-F	GCCAGGCCTTCAACCACTAT
mmu-Bcl2l11-R	TGCAAACACCCTCCTTGTGT
mmu-Klf4-F	GAAAAGAACAGCCACCCACAC
mmu-Klf4-R	GTC CCA GTC ACA GTG GTA AGG
mmu-Pten-F	ATTCCCAGTCAGAGGCGCTA
mmu-Pten-R	TGGCAGACCACAAACTGAGG
mmu-TNFα-F	CCAGTGTGGGAAGCTGTCTT
mmu-TNFα-R	AAGCAAAAGAGGAGGCAACA
mmu-IL-1α-F	GCACCTTACACCTACCAGAGT
mmu-IL-1α-R	AAACTTCTGCCTGACGAGCTT
mmu-IL 6-F	TCCAGTTGCCTTCTTGGGAC
mmu-IL 6-R	GTGTAATTAAGCCTCCGACTTG

RESULTS

MiR-25-3p is Upregulated in the Lumbar Spinal Cord of EAE Mice and Its Expression is Correlated with The Expression of Inflammatory Cytokines

To confirm the induction of neuroinflammation in the EAE groups we first investigated the expression of proinflammatory cytokines in the lumbar spinal cord of EAE mice. Expression levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and IL-6 were examined in tissues extracted in peak (acute) and post-peak (chronic) phases of the disease. As shown in Figure 1a, TNF-α expression was significantly higher in the upper lumbar spinal cord of EAE mice at the acute phase of the disease (Kruskal-Wallis test, *p*<0.001). For IL-1α

(Figure 1b) both acute and chronic phase tissues revealed a significant upregulation compared with control tissues (Kruskal–Wallis test, *p*<0.001, p: 0.026 respectively). IL-6 expression was also significantly induced in the acute phase of disease compared with the control group (Figure 1c, Kruskal–Wallis test, *p*<0.001). These results were consistent with previous reports in EAE.[19] We next examined the expression of miR-25-3p in the RNA reverse-transcribed using the protocol described in the Methods section. As shown in Figure 1d, miR-25-3p expression was significantly induced in tissues extracted at the peak of disease but the expression had returned to near control levels after the peak (Kruskal–Wallis test, *p*<0.001, p: 0.016 respectively). This finding was consistent with miRNA

profiling studies which had shown miR-25-3p upregulation in brain tissues from MS patients. To examine whether miR-25-3p dysregulation could be related to inflammatory cytokines, we performed correlation analyses with TNF-α, IL-1α, and IL-6. As shown in Figure 2a, b and c, these analyses showed highly significant positive correlations between miR-25-3p with all three cytokines. While this does not confirm a causal relation between miR-25-3p and inflammatory cytokines, it does indicate that altered miRNA expression is strongly associated with the inflammatory status in the spinal cord.

MiR-25-3p Upregulation is Associated with Downregulation of Validated Targets

To examine whether miR-25-3p might have influenced the expression of its target genes with potential roles in inflammatory processes, we searched miRTarBase database to identify miR-25-3p validated targets. A total of 12 targets that were verified by 'reporter assays' were identified in miRTarBase. Of these, we focused on targets that had highly conserved miRNA binding sites among vertebrates, as these miRNA-mRNA interactions are more likely to be critical in biological functions. Target Scanner 7 was used to identify these genes. Among these, we focused on *Bcl2l11 (Bim)*, phosphatase and tensin homolog

(*Pten)*, and *Krüppel-like factor 4 (Klf4)* because of their known roles in inflammation and cell cycle.

Expression analysis of spinal cord tissues revealed no significant changes in *Bcl2l11* transcript levels in EAE groups compared with the control group (Figure 3a). However, *Pten* expression was significantly reduced in both acute and chronic stages of disease (Figure 3b). Likewise, *Klf4* expression was diminished in EAE groups, but this reached statistical significance only in the acute phase (Figure 3c) (Kruskal–Wallis test). When analyzed with relation to miR-25-3p, *Pten,* and *klf4*, but not *Bcl2l11* expression, was significantly negatively correlated with miR-25-3p expression (correlation coefficients/p values were -0.577/0.002 and -0.633/0.0001 for *Pten* and *Klf4*, respectively.

MiR-25-3p Expression in Activated T cells and Macrophages

MS and EAE pathogenesis is associated with the infiltration of different types of leukocytes, including lymphocytes and monocytoid cells. While inside the CNS, these cells get activated either due to exposure to antigens or the inflammatory microenvironment. Considering that miR-25-3p and its targets had shown altered expression in spinal cord tissues, we asked whether any of these changes might parallel similar changes in either T cells or monocytoid cells. We first
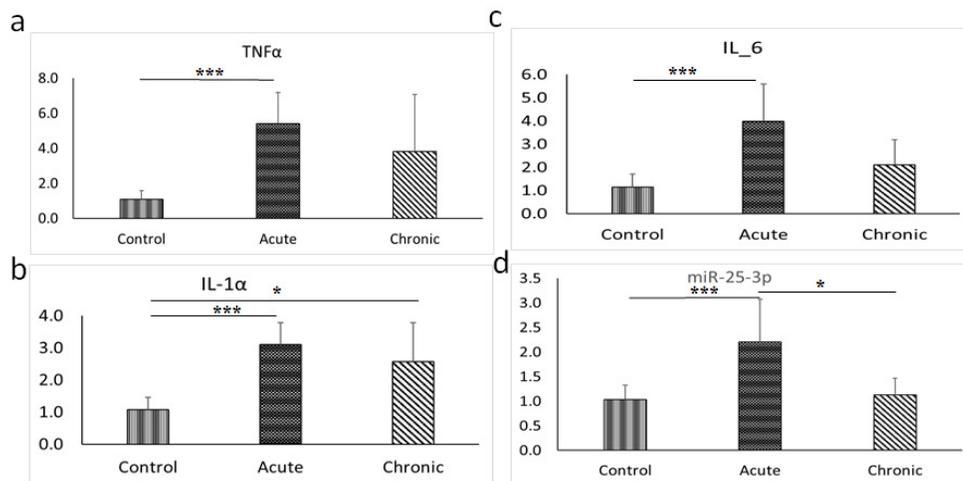

Figure 1. Expression of inflammatory cytokines and miR-25-3p in the lumbar spinal cord of control and experimental autoimmune encephalomyelitis mice. Expression levels of TNF-α (a), IL-1α (b), and IL-6 (c) together with miR-25-3p (d) are shown in control as well as acute and chronic EAE groups. Data are shown as relative fold change (RFC) mean ± standard deviation (*p<0.05; ** p<0.01 and ***p<0.001).
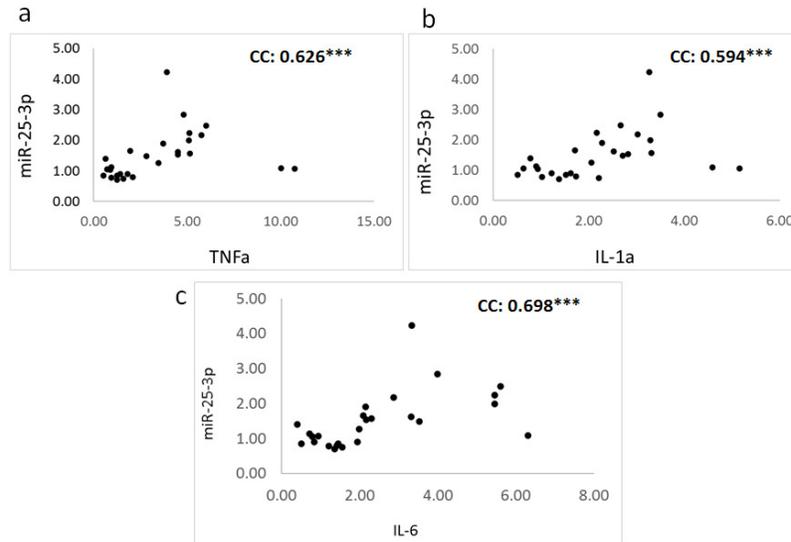
Figure 2. Positive correlation between inflammatory cytokines and miR-25-3p levels. Dot plots show the expression of TNF-α (a), IL-1α (b), and IL-6 (c) versus miR-25-3p in individual mice. Spearman correlation coefficients (CC) and p values are shown on each graph (***p<0.001).
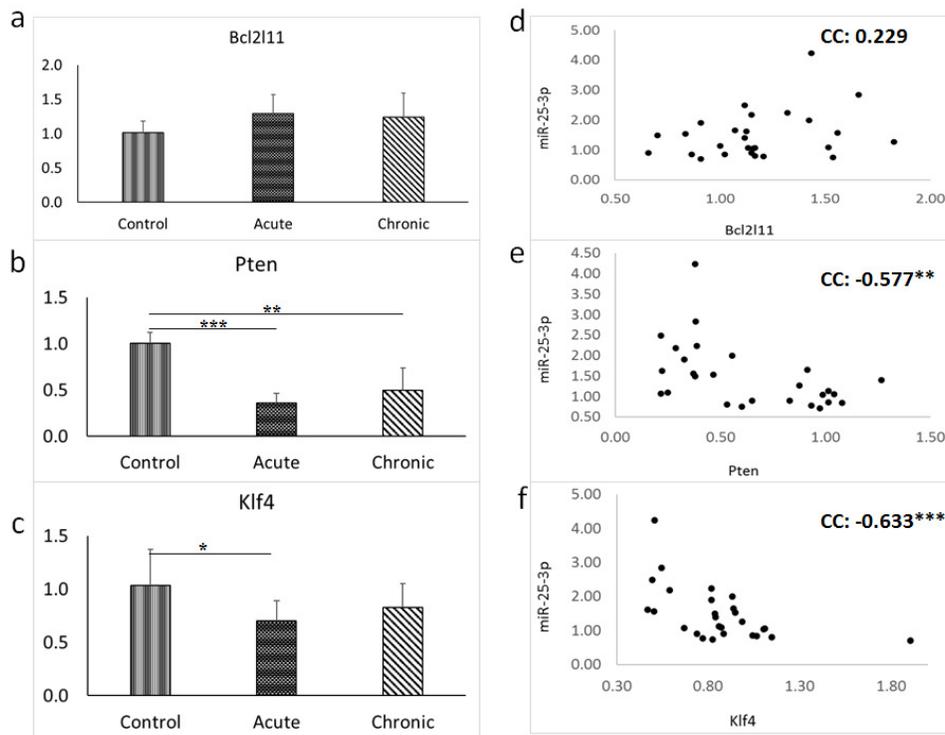

Figure 3. Expression of miR-25-3p target genes in the lumbar spinal cord of control and experimental autoimmune encephalomyelitis mice. Expression of *Bcl2l11* (a), *Pten* (b), and *Klf4* (C) in the spinal cord in different groups of animals are shown. Panels d to f display dot plots and Spearman correlation coefficients (CC) between each of these target genes and miR-25-3p. Data are shown as relative fold change (RFC) mean±standard deviation (* *p*<0.05; **, *p*<0.01, ***, *p*<0.001. Kruskal-Wallis test).

established splenocyte cultures and stimulated them with anti-CD3/anti-CD28 antibodies for different time intervals. Expression of miR-25-3p did not show any significant differences between activated and control splenocytes for any of the studied time intervals. We next prepared primary BMDMs cultures. These cultures were evaluated for the expression of macrophage marker Iba-1 using immunofluorescence assay (Figure 4). BMDMs were then examined for the expression of miR-25-3p in these cells before and following activation with 10 and 100 ng/mL concentrations of LPS. MiR-25-3p showed a significant upregulation in the 100 ng/mL LPS group, compared with unstimulated cells (Figure 5).

We then studied the expression of miR-25-3p target genes in these cells. Expression analyses showed significant downregulation of *Bcl2l11* (Figure 6a), *Pten* (Figure 6b), and *Klf4* (Figure 6c) in stimulated macrophages. Correlation analyses between miR-25-3p and its targets showed a trend towards negative correlation, but this did not reach statistical significance (Figure 6d, e, f). Overall, our data indicated that the observed enhancement in miR-25-3p expression in lumbar spinal cord tissues might be, at least in part, due to increased expression of this miRNA in activated monocytoid cells, but not lymphocytes.
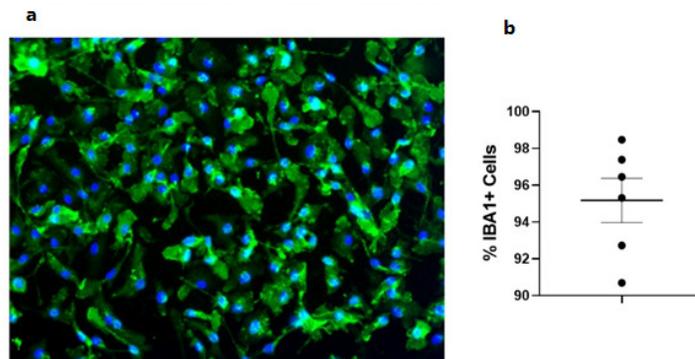

Figure 4. Bone marrow-derived macrophages (BMDMs) stained with anti-Iba-1 antibody (control well). Cultured BMDMs from wild-type mice were stained with anti-Iba-1 antibody and nuclear yellow (NY) (a). Representative images for unstimulated cells are shown. 95% of cells were Iba-1 positive (b). n=6 wells, 12 fields per well.
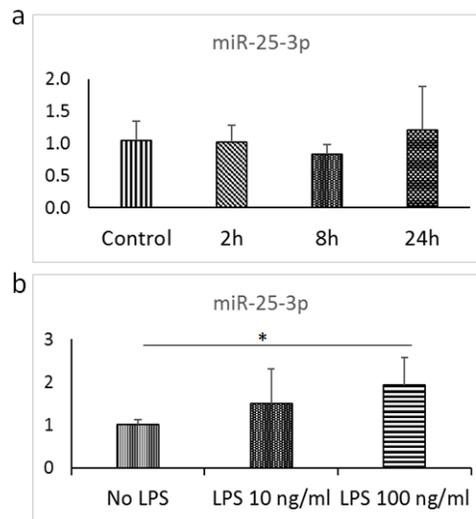

Figure 5. Expression of miR-25-3p in cultured leukocytes. Expression of miR-25-3p in control and anti-CD3/anti-CD28 activated splenocytes (a) and lipopolysaccharide (LPS)-treated bone-marrow-derived macrophages (b) are reported. Data are shown as relative fold change (RFC), mean ± standard deviation (* p < 0.05, one-tailed Kruskal-Wallis test, also confirmed with two-tailed Jonckheere–Terpstra test).
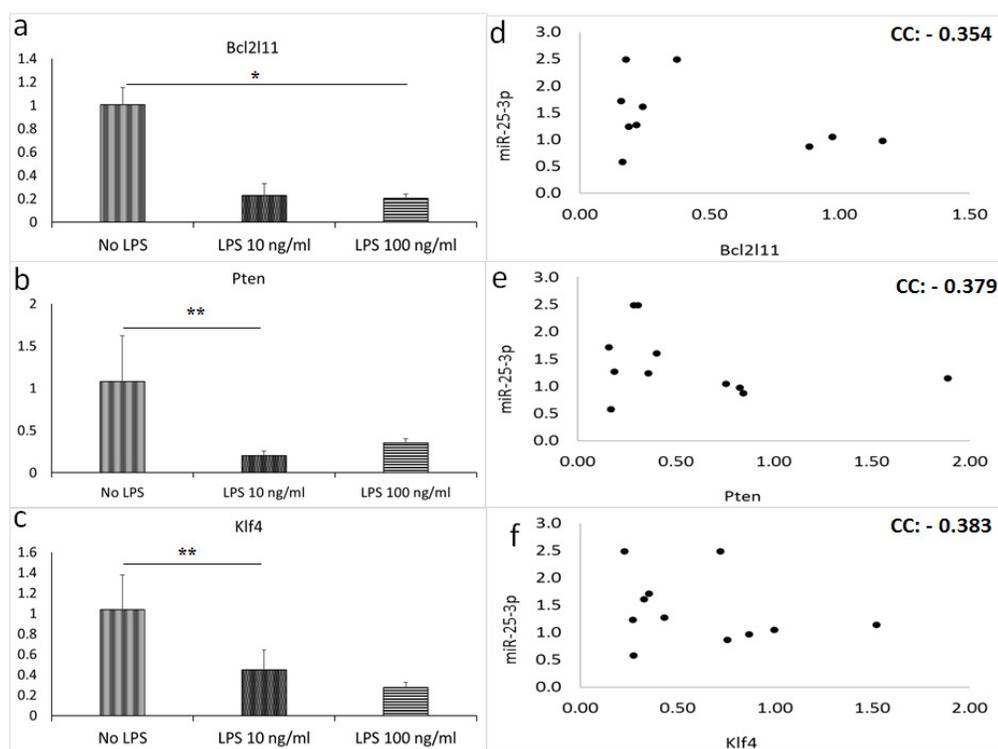
Figure 6. Transcript levels for miR-25-3p target genes in lipopolysaccharide (LPS)-treated macrophages. Expression of *Bcl2l11* (a), *Pten* (b), and *Klf4* (c) in 10 ng/mL and 100 ng/mL LPS-treated macrophages are shown. Panels d to f display dot plots and Spearman correlation coefficients (CC) between each of these target genes and miR-25-3p in macrophages. Data are shown as relative fold change (RFC) mean ± standard deviation (* $p<0.05$; ** $p<0.01$, *** $p<0.001$. Kruskal-Wallis test)

DISCUSSION

In this study, we explored the potential role of miR-25-3p in the pathogenesis of EAE, an animal model of MS. Previous studies on this model of EAE have revealed that the highest level of inflammation occurs in the lumbar spinal cord.[20] Hence in this study, we focused on lumbar spinal cord tissues. Expression analyses revealed significant induction of this miRNA in the acute but not the chronic phase of EAE, and this was associated with suppressed levels of two of its important target transcripts, *Pten* and *Klf4*. Examining the miRNA levels in activated lymphocytes did not show any changes. However, LPS-activated macrophages displayed alterations similar to those in EAE spinal cords.

Several groups have investigated miR-25-3p levels in MS patients, mostly using peripheral blood leukocytes. De Santis et al have shown that miR-25 is

downregulated in peripheral Treg cells in MS patients.[15] Sievers et al also found decreased expression levels for members of miR-106b-25 and miR-17-92 clusters in B cells from untreated MS cases.[21] However, in a study by Nuzziello et al, researchers examined the expression of various miRNAs in blood samples from adult-onset MS and found that miR-25-3p was among the upregulated miRNAs.[14] In another study by the same group, high-throughput next-generation sequencing on pediatric MS cases demonstrated increased levels of miR-25-3p in MS compared with controls.[22] Studies on miR-25-3p expression in the CNS tissue in MS/EAE are limited. In a miRNA profiling study performed by our group, miR-25-3p showed significant upregulation in the brains of MS patients compared with non-MS controls.[13] Whether these alterations in miR-25-3p can influence the disease process is not well-known. Of note, a study by Finardi et al has shown that mice lacking miR-106b-

25 and miR-17-92 clusters together have diminished EAE disease severity.[23]

In this work, we also investigated the levels of several miR-25-3p targets that had been previously validated by reporter assays. *Bcl2l11 (Bim)* is one of the miR-25-3p targets which is involved in regulating apoptosis. In apoptosis-related signaling, *Bcl2l11* is phosphorylated by MEK/ERK pathway, translocated to mitochondria, and initiates programmed cell death by inducing BAX-like proteins.[24] Studies indicate that Bim-dependent apoptosis is regulated by TGFβ signaling through SMAD3 and mitogen-activated protein kinase (MAPK) phosphatase MKP2.[16,24] Increased miR-25-3p has been reported in multiple cancers where it inhibits *Bcl2l11* and thereby blocks TGFβ-dependent apoptosis.[16,25] In our study, we could not find any significant change in *Bcl2l11* expression between EAE and the control group.

Pten is a protein that plays essential roles in cell polarity, migration, and metabolism.[26] *Pten* is considered to be a tumor suppressor and negatively regulates the PI3K pathway. Enhanced expression of miR-25 inhibits *Pten*, resulting in over-activation of PI3K/Akt signaling which is tightly linked with cell proliferation.[26,27] In the context of inflammation, *Pten* acts mostly as a regulator of inflammatory processes. It is known to decrease airway inflammation in bronchial asthma.[28, 29] *Pten* is also involved in T cell differentiation, where it acts as a key regulator in Th17 cell development, cells that play pathogenic roles in EAE.[30] Deleting *Pten* in Th17 cells blocks their development and ameliorates EAE disease.[30] These roles for *Pten* seem contradictory, and they might point to the cell-specific behavior of the molecule. In our study, we detected decreased levels of *Pten* in EAE lumbar spinal cords, and there was a negative association with miR-25-3p levels.

Klf4 is an essential transcription factor, which is involved in various cell functions. An extracellular signal-regulated kinase (ERK) phosphorylates *Klf4* and regulates its cellular level.[31] Similar to *Pten*, *Klf4* suppresses PI3K and hence, it negatively affects cell proliferation.[32] Studies on inflammation have reported contradictory roles for *Klf4*. Hartmann et al have reported that miR-103-mediated *Klf4* suppression promotes vascular inflammation.[33] Another work by Li et al has also indicated an anti-inflammatory role for *Klf4* in LPS-induced inflammation.[34] In the context of neuroinflammation, *Klf4* is known to decrease vascular

endothelial inflammation.[35] Nonetheless, other studies have pointed to pro-inflammatory roles for *Klf4*.[36,37] An immunohistochemical study has reported *Klf4* upregulation in EAE in rats, where it has been mostly detected in astrocytes.[38] *Klf4* is also required for T cell development but its levels decrease after T cell maturation.[39] *Klf4* deficiency leads to diminished Th17 differentiation and resistance to EAE induction in mice.[40] In this study, we observed a significant decrease in *Klf4* levels in the acute phase of EAE which was negatively correlated with miR-25-3p levels. It is conceivable that miR-25-3p-mediated suppression of *Klf4* might exert either a proinflammatory role at the vascular level or an immunomodulatory effect at the level of lymphocytes.

In MS/EAE, macrophages and lymphocytes are recruited to the CNS.[6] This is associated with morphological changes and activation of resident microglia. Infiltrated macrophages as well as locally activated microglial act as antigen-presenting cells which lead to activation of autoreactive T cells.[41] These cells also produce copious amounts of proinflammatory cytokines which have direct myelotoxic effects.[42] Leukocyte infiltration together with microglial cell activation contributes to altered gene expression observed in CNS during MS/EAE. Moreover, altered expression of genes in other neural cells, i.e. neurons and astrocytes, might cause further changes in CNS transcripts. In this study we tried to gain some insight into the first mechanism; i.e. whether activation of monocytoid cells and/or lymphocytes might underlie miR-25-3p alterations in the CNS during EAE. To this end, we used in vitro cultures of primary lymphocytes and macrophages and measured miR-25-3p and its targets' expression under resting and stimulated conditions. While this *in vitro* system is an oversimplified representation of what happens *in vivo*, studies have shown that it could still provide some useful information.[18,43] As illustrated in the results, we failed to detect any significant miR-25-3p alterations in polyclonally activated lymphocytes. There are two potential explanations for this observation. One possibility is that miR-25-3p expression in T cells is simply not a function of T cell activation during inflammation and/or immune response. The other possibility is that polyclonal T cell activation by the antibodies does not mirror *in vivo* T cell activation. In the context of autoimmune inflammation, T cells and other leukocytes experience a complex

microenvironment that includes various cytokines and inflammatory mediators. T cells do likely alter their miR-25-3p (and perhaps other ncRNAs) transcription while in the presence of these molecules, and mere TCR signaling is not sufficient for the alteration of miR-25-3p expression. Unlike T cells, we observed miR-25-3p upregulation in macrophages activated with LPS. This finding was associated with decreased levels of miR-25-3p targets, *Pten* and *Klf4* in macrophages. Considering that *Pten* and *Klf4* mostly act as regulators of inflammation, it is conceivable that miR-25-3p-mediated downregulation of these molecules might promote the inflammatory processes mediated by these cells. These findings are in corroboration with other studies which show that exosome-derived miR-25-3p stimulates the secretion of pro-inflammatory cytokines from tumor-associated macrophages.[44] Another possibility is that miR-25-3p release by macrophage exosomes and subsequent uptake of these exosomes by other cells might lead to further suppression of immune regulatory molecules in a paracrine fashion. Enhanced expression of miR-25-3p in activated monocytoid cells might partly explain its increased levels in CNS. Nonetheless, as mentioned above, it is likely that increased expression of miR-25-3p in neurons and glial cells also contributes to this upregulation.

Results of this study suggest that miR-25-3p is upregulated in the spinal cord of animals with EAE, where it shows a positive correlation with the levels of inflammatory cytokines. The increased miR-25-3p expression also shows a significant negative correlation with *Pten* and *Klf4* expression in CNS. Interestingly we could find the same pattern of miR-25-3p, *Pten,* and *Klf4* expression in activated macrophages. Activation of macrophages, which is a crucial part of MS/EAE pathogenesis, might lead to increased levels of miR-25-3p and suppression of protective genes such as *Pten* and *Klf4* in CNS. We believe that the relation between miR-25-3p and neuroinflammation is bidirectional; inflammation (including macrophage activation) leads to miR-25-3p upregulation, and enhanced miRNA levels promote inflammation by suppressing immune regulatory molecules.

Overall, our data point to potential roles for miR-25-3p in the pathogenesis of MS/EAE. In this study, we did not explore the effects of *in vivo* manipulation of miR-25-3p expression, which would be required to have a more conclusive interpretation of the role of this miRNA in autoimmune neuroinflammation.

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