Vitamins A and D Enhance the Expression of Ror-γ-Targeting miRNAs in a Mouse Model of Multiple Sclerosis

Marziyeh Mohammadi-Kordkhayli1,2 · Mohammad Ali Sahraian3 · Samira Ghorbani2 · Fatemeh Mansouri1 · Farideh Talebi4 · Farshid Noorbakhsh5 · Ali Akbar Saboor-Yaraghi1

Received: 5 January 2023 / Accepted: 5 June 2023 / Published online: 23 June 2023
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Abstract

Autoreactive T cells, particularly those characterized by a Th17 phenotype, exert significant influence on the pathogenesis of multiple sclerosis (MS). The present study aimed to elucidate the impact of individual and combined administration of vitamin A and D on neuroinflammation, and microRNAs (miRNAs) involved in T helper (Th)17 development, utilizing a murine model of experimental autoimmune encephalomyelitis (EAE). EAE was induced in C57BL/6 mice, and 3 days prior to immunization, intraperitoneal injections of vitamins A and D or their combination were administered. Th17 cell percentages were determined in splenocytes utilizing intracellular staining and flow cytometry. Furthermore, the expression of Ror γ-t, miR-98-5p and Let-7a-5p, was measured in both splenocytes and spinal cord tissues using RT-PCR. Treatment with vitamin A and D resulted in a reduction in both disease severity in EAE mice. Treated mice showed a decreased frequency of Th17 cells and lower expression levels of IL17 and Ror γ-t in splenocytes and spinal cord. The spinal cord tissues and splenocytes of mice treated with vitamins A, D, and combined A+D showed a significant upregulation of miR-98-5p and Let-7a-5p compared to the EAE group. Statistical analysis indicated a strong negative correlation between miR-98-5p and Let-7a-5p levels in splenocytes and Ror-t expression. Our findings indicate that the administration of vitamins A and D exerts a suppressive effect on neuroinflammation in EAE that is associated with a reduction in the differentiation of T cells into the Th17 phenotype and is mediated by the upregulation of miR-98-5p and Let-7a-5p, which target the Ror γ-t.

Keywords Experimental autoimmune encephalomyelitis · Inflammation · miRNAs · Th17 · Vitamin A · Vitamin D

Introduction

Multiple sclerosis (MS) is an immune-mediated disorder characterized by inflammation and demyelination within the central nervous system (CNS) [1]. Auto-reactive T cells, infiltrating the CNS, are known as crucial components in the neuroinflammatory process [2, 3]. Initially, interferon-γ-producing T helper (Th)1 cells were believed to be the main pathogenic contributors in MS [1], until recent evidence highlighted the significance of Interleukin (IL)-17-producing Th17 cells [4, 5]. Th17 cells and their associated cytokines are present in MS brain lesions and cerebrospinal fluid (CSF), respectively. These cells posses the ability to disrupt blood-brain barrier (BBB), infiltrate the CNS, and recruit additional inflammatory cells to the site of injury [6]. Not only do Th17 cells augment the inflammatory response, but they also directly harm neurons and oligodendrocytes [6, 7]. Several treatments for MS have been shown to reduce the frequency of Th17 cells in the peripheral blood [8–13].
Similar findings have been observed in experimental autoimmune encephalomyelitis (EAE), a widely employed animal model for MS [14–16]. On note, elimination of Th17 cells can suppress the development of EAE and/or decrease the severity of clinical symptoms [6].

Nutritional factors have long been considered potential contributors in the prevention of MS progression [17, 18]. Among micronutrients, vitamin D and vitamin A have garnered substantial attention in relation to the prevention and progression of MS [19–23]. Evidence supporting the role of vitamin D includes epidemiological, clinical, and cell/molecular studies. At the cellular and molecular levels, vitamin D supplementation has demonstrated significant effects in modulating immune cell responses in MS patients and EAE mice [24], such as downregulation of Th17 cell differentiation and cytokine production by CD8+ T cells, while enhancing regulatory T cell (Treg) activity [25]. Similarly, epidemiological and immunological data have linked vitamin A and its analogs to MS pathology. Studies have revealed that vitamin A can reduce immune activation by suppressing the expression of retinoic acid receptor-related orphan receptor gamma) Ror-γ, IL-17, and inflammatory cytokines, while promoting Th2 responses through the upregulation of GATA3 in the CNS of MS patients and EAE mice [26–29]. The etiology of MS and the factors that initiate the damaging neuroinflammation in MS remain unknown. Proposed factors include genetic predisposition, epigenetic alterations such as microRNA (miRNA), and environmental factors [30–33]. MiRNA are a group of non-coding RNA with a length of 19–22 nucleotide that regulate the gene expression post-transcriptionally [34]. They play crucial roles in various physiological and pathological processes, including the regulation of innate and adaptive immune responses [35, 36]. These functions are partially mediated through the regulation of immune cell differentiation [37]. Notably, miRNAs have been implicated in the regulation of T cell differentiation in both healthy and diseased conditions [38–41].

Research has shown that several miRNAs are involved in the regulating of Th17 cell differentiation and the balance between Th17 and Treg cells. Studies on miRNAs in different autoimmune diseases have supported the notion that miRNA dysregulation is not only associated with these disorders but also exerts pathogenic effects [42–44]. Investigations into the pathogenesis of MS, using either human samples or EAE mice, have revealed miRNA dysregulation in both the CNS and in peripheral immune system. This dysregulation influences the pathogenic process at various levels [45–50].

While a limited number of studies have indicated a relationship between vitamins A and D and miRNA expression in immune cells [29, 51–53], the molecular mechanisms underlying the effects of these vitamins on miRNA expression in EAE models remain incompletely understood. Furthermore, the association between these vitamins and the expression of the Let-7 family in EAE models has not been reported. Therefore, in the present study, our aim was to investigate the potential therapeutic effects of vitamins A and D, individually and in combination, on the severity of autoimmune neuroinflammation using an EAE animal model. Subsequently, we analyzed the expression of IL17 and the frequency of myelin reactive Th17 cells in splenocyte cultures. Our goal was to elucidate the effect of these vitamins on the differentiation of T cells toward the protective phenotype. We examined miRNAs that could target Ror-γ, a key transcription factor in Th17 cells, and assessed their association with vitamin therapy-induced reduction in Ror-γ expression.

Materials and Methods

Animals

The 6- to 8-week-old, inbred female C57BL/6 wild-type mice were purchased from Pasteur Institute of Iran. The animals were housed under standard controlled conditions, with a 12-h light/dark cycle, a temperature of 20 ± 2 °C, and libitum access to food and water. All experiments and animal care procedures were approved by the Ethics Committee on Animal Experimentation of Tehran University of Medical Sciences (Approval No. IR.TUMS.SP.H.REC.1397.091).

EAE Induction and Neurological Assessment

EAE was induced according to previously established protocols [24]. Briefly, 10-week-old C57BL/6 mice were subcutaneously (SC) injected with 100 μg of myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide emulsified in complete Freund’s adjuvant. In addition, the mice received two intraperitoneal (IP) injections of pertussis toxin in phosphate-buffered saline (PBS) at a dose of 200 ng/mouse/dose (0.1 mL) on the day of immunization and 48 h post immunization, following the manufacturer’s instructions (EK-2110, Hooke Kit™ MOG 35-55/CFA Emulsion PTX). Clinical scores for All groups were assessed daily for up to 30 days after MOG immunization, utilizing a scoring scale ranging from 0 to 15 [54]. Table 1 provides an overview of the EAE scores, which encompass the combined evaluation of the tail and the conditions of all four limbs. Notably, the tail and each hind or forelimbs, are evaluated separately. Thus, a fully paralyzed animal would score 14 and death would be estimated at 15 on the new scale.
**Experimental Design**

The animals were divided into 5 groups, each consisting of seven or eight animals, as outlined below: group I (healthy controls) comprised mice that did not receive MOG injection and were treated with vehicle. The vehicle used was the Solvent of vitamins A and D, which consisted of disodium dihydrogen phosphate, monosodium phosphate monohydrate, ethylenediaminetetraacetic acid (EDTA), sodium ascorbate, sodium chloride, and tween 20 dissolved in distilled water. Group II (EAE group) consisted of EAE mice that were treated with the vehicle; group III (EAE group treated with vitamin A) comprised EAE mice that received 200 μg vitamin A (ATRA, all-trans-retinoic acid) per mouse; group IV (EAE group treated with vitamin D) included EAE mice that received 100 ng calcitriol per mouse; and group V (EAE group treated with combined vitamins A plus D) received a combination of vitamins A plus D, but at half a dose (100 μg of vitamin A and 50 ng of vitamin D). These doses were selected based on the previously conducted research [55–57]. Vitamins A and D were obtained from Kern Pharma (Spain) and Sigma-Aldrich (St. Louis, MO, USA). All treatments were administered IP on a daily basis, starting from 3 days before the immunization to 30 days post-immunization. After day 30, the animals were scarified, and spleen and CNS tissues were aseptically collected. Samples of spinal cord tissue were stored at −80 °C freezers.

**Splenocytes Culture and MOG Stimulation**

Splenocyte cultures were prepared from MOG-immunized C57BL/6 mice. The spleen tissues were homogenized, and splenocytes were isolated using Ficoll density gradient centrifugation. To achieve this, 7 mL of Ficoll-Paque was gently added to the homogenized splenocytes using a serological pipette, ensuring a slow and controlled release. This led to a distinct separation between the two layers. Prior to density gradient centrifugation, a single cell suspension formed above the Ficoll medium. Following centrifugation, the splenocytes were located at ed of red blood cells. A total of 2×10⁶ cells were cultured in each well with concentrations of MOG35-55 (MOG in TC Media, 100x, Hooke labs) at 10 μg/mL and 40 μg/mL in RPMI 1640 medium (Gibco) supplemented with 5% FBS (Gibco). The cells were incubated at 37 °C and harvested at 24 and 48 h post-incubation.

**Flow Cytometry**

Splenocyte cultures were exposed to MOG35-55 (40 μg/mL) for 24 h to provide antigen-specific stimulation. For polyclonal activation, phorbol-12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (1 μg/mL) were added for 6 h. The control group consisted of cells that were not stimulated with MOG35-55 or PMA + ionomycin. To begin the analysis, cells were first surface stained with anti-CD4 and anti-CD3 antibodies. Following this, cells were fixed using 1 mL/tube of Bio Legend's Fixation Buffer and incubated at room temperature in the dark for 25 min. Next, permeabilization was carried out using 1 mL of Bio Legend’s Permeabilization Buffer (1x). Following fixation and permeabilization, cells were stained with fluorochrome-conjugated anti-IL-17A antibodies (BioLegend), following the manufacturer’s instructions. The samples were analyzed on a BD FACS Calibur, and the results were analyzed by FlowJo software.

**RNA Isolation and cDNA Synthesis**

Total RNA was extracted from activated splenocyte cultures and lumbar spinal cord tissues using miRNeasy Mini Kit (Qiagen) following the manufacturer’s instructions, and stored at −80 °C. The RNA concentrations were determined using a Nanodrop spectrophotometer system (Thermo Scientific). cDNA was prepared using miScript II RT Kit (Qiagen), while for mRNA analyses, the TAKARA cDNA synthesis kit was used, both following manufacturer’s instructions.

**Real-time RT-PCR**

RT-PCR was employed to evaluate the expression of miRNA and mRNAs in splenocyte cultures and spinal cord tissue. The Step One Plus Real Time PCR system.
Table 2 The primers used for the gene expression of Ror-γt, IL-17, and Let-7 isoforms in the spinal cord and splenocyte

| Gene   | Primer             |
|--------|--------------------|
| Beta-Actin | ATGCTCCCCGCGCTGTAT  |
|         | CATAGGCCAGCTCTGACCCATT |
| Ror-γt | GCTACCAGGAGAAGTCAATGTG  |
|         | CTCCACACCACCGTATTGGC |
| IL-17  | AGCTTTCCATGGTGGTCGCG  |
|         | TCTATCGGTTCTCCTGAC |
| miR-98-5p | TGA GGT AGT AAG TGT TGT T AA |
| Let-7-5p | TGA GGT AGT AAG TGT TGT T AA |

was utilized for the RT-PCRs. The primer sequences utilized are provided in Table 2. The β-actin gene was used as a housekeeping gene for the normalization of the amplified signals of the target genes. For miRNA expression normalization, snord68 and snord72 were used, and the method of relative quantification (2−∆∆Ct) was employed to determine the expression levels of both miRNA and target gene expression.

Statistical Analysis

Statistical analyses were conducted using SPSS 20 and the graphical representations were generated using GraphPad Prism8. The significance of the results was assessed using two-way analysis of variance (ANOVA) followed by appropriate post hoc tests for multiple comparisons. For two-group comparisons, Student’s t-test was employed. Statistical significance was defined as a P-value of less than 0.05. The data are presented as a mean ± standard error of the mean (SEM).

Results

The Effects of Vitamin A, Vitamin D, and Their Combination on EAE Disease Severity

Studies have demonstrated that vitamin A, vitamin D, and their analogs exert immune-regulatory roles in the context of autoimmune and chronic inflammatory diseases [58, 59]. Previous research conducted by our group and others has demonstrated that the administration of these two vitamins, either individually or in combination, ameliorates the severity of EAE in mice [28, 60–65]. To replicate these previous findings, we treated EAE mice with vitamin A (200 μg/mouse), vitamin D (100 ng/mouse), and a combination of both vitamins at half the dose (100 μg vitamin A + 50 ng vitamin D/mouse). The vitamins were administered intraperitoneally, starting 1 day before immunization. As shown in Fig. 1A, animals treated with the vehicle developed EAE symptoms on day 9 post-immunization. In contrast, animals treated with vitamin A, vitamin D, or the combination of vitamin A plus D displayed disease symptoms between days 13 to 15. There was no difference in the onset of the disease between animals receiving the combination of vitamins and those receiving either vitamin A or D alone. The maximum clinical score (MCS) for vehicle-treated EAE mice was 5.4±1.3, whereas vitamin A–treated EAE mice had an MCS of 1.56 ± 0.6, vitamin D–treated EAE mice had an MCS of 0.78±0.2, and the vitamin A+D–treated EAE group had an MCS of 2.57 ± 0.8 (Fig. 1B). These results confirmed our previous findings regarding the efficacy of vitamins A and D in mitigating the severity of EAE in animals.

![Fig. 1](image_url)

Fig. 1 Clinical scores of experimental autoimmune encephalomyelitis (EAE) disease were recorded daily following immunization with myelin oligodendrocyte glycoprotein (MOG) in various experimental groups (A). The maximum mean clinical score (MMCS) showed a significant reduction in EAE groups treated with vitamin A and vitamin D, as compared to EAE mice (P< 0.001) (B)
The Effects of Vitamins A and D on IL17 Expression in CNS and Spleen and the Frequency of Splenic Th17 Cells

Various mechanisms have been postulated to elucidate the protective effects of vitamins A and D in autoimmune diseases [61, 63–65]. Considering that the pathogenic process of MS and EAE is highly dependent on the differentiation of CD4+ T cells into Th17 phenotype, our investigation aimed to examine the impact of vitamin A and D treatments on T cell differentiation in both the CNS and peripheral immune system.

Firstly, we evaluated the expression of Th17 signature cytokine, IL17, in spinal cord tissues obtained from EAE mice at day 30 post-immunization. Real-time PCR analysis of these tissues showed a significant increase in IL-17 levels in spinal cords tissues of vehicle-treated EAE mice compared to healthy controls (Fig. 2A). However, mice treated with vitamin D showed a significant reduction in IL-17 expression in spinal cord tissue. A non-significant decrease was also observed in mice that received vitamin A or the combination of vitamins A and D (Fig. 2A). Next, we prepared splenocyte cultures from different groups of mice. Splenocytes were re-stimulated with 40 μg/mL of MOG, or with PMA/ionomycin before flow cytometry analysis for surface markers and intracellular IL-17 (Fig. 2B). Our analyses showed that mice treated with vitamin A and vitamin D had lower frequency of Th17 cells in their splenocytes, both in the absence and presence of in vitro MOG re-stimulation (Fig. 2C). Additionally, we analyzed IL-17 mRNA levels in splenocytes 24 and 48 h after stimulation with 10 and 40 μg/mL of MOG antigen. Real-time PCR results showed significant downregulation of IL-17 mRNA in cells stimulated with both concentrations of MOG at 24-h and 48-h time points (Fig. 2D, E).

Vitamin A and D Modulate the Expression of miRNAs Target Ror-γ in Splenocytes

The differentiation of T cells into distinct phenotypes is regulated by a molecular machinery that connects extracellular signals to T cell gene expression program. Non-coding RNAs, specifically miRNAs, play a crucial role as intracellular regulators of gene expression. miRNAs exert their regulatory function by targeting the 3′ untranslated region (UTR) of protein-coding genes, leading to the suppression of translation or degrading of their transcripts [66, 67]. In order to investigate whether the treatment of EAE mice with vitamin A and D influences miRNAs that potentially regulate Th17 cell differentiation, we analyzed the miRNA-target database TargetScan for miRNAs that could target Ror-γ transcripts. Our focus was on miRNA species that were broadly conserved among vertebrates, with conserved binding sites between human and mouse Ror-γ. We identified miR-98-5p and Let-7-5p targeting a single binding site at the Ror-γ 3′UTR (Fig. 3A).

To assess the impact of vitamin A and D treatment on the expression of the Th17-specific transcription factor, Ror-γ (i.e., Rorc), we measured its levels. Our results showed decreased expression of ROR-γ in animals treated with vitamin A and D. However, statistical significance was only observed at the 48-h time point for the 40 μg/mL MOG concentration (Fig. 3B, C). Collectively, these findings suggest that treatment with vitamin A and D, either alone or in combination, can influence the development of Th17 cells by suppressing the transcription factors involved in T cell differentiation. Interestingly, our previous studies have shown that miR-98-5p is downregulated in the brain tissue of patients with MS [68]. Moreover, miR-98-5p and Let-7a-5p have been reported to regulate leukocyte function and inflammatory responses in various tissues [69–71]. We first examined the expression of miR-98-5p in RNA extracted from the spinal cords of control and EAE mice (Fig. 3D). Consistent with previous findings in human tissues, miR-98-5p was downregulated in the spinal cord of vehicle-treated EAE mice compared to healthy controls. However, mice treated with vitamin A and vitamin A+D exhibited a significant upregulation of miR-98-5p, whereas no significant changes were observed in vitamin D–treated mice. A similar expression pattern was also observed for Let-7a-5p (Fig. 3E).

Next, we investigated whether miR-98-5p/Let-7-5p were altered in splenocytes obtained from vehicle or vitamin-treated EAE mice. Analysis of miRNA expression in both control and MOG-stimulated splenocytes revealed a significant increase in miR-98-5p levels at 24- and 48-h time points in vitamin-treated EAE mice (Fig. 4A, B). In the case of the A+D combination, significant upregulation of miR-98-5p was observed only at the 48-h time point in cells stimulated with 40 μg/mL of antigen (Fig. 4B). Increased levels of Let-7-5p were also observed at 24-h for vitamin A–treated animals (10 μg/mL of MOG) and vitamin D–treated animals (40 μg/mL of MOG) (Fig. 4C), as well as at 48-h for both vitamins (40 μg/mL of MOG) (Fig. 4D).

We performed a correlation analysis between miR-98-5p and Let-7-5p and their target -γ in splenocytes derived from different groups of EAE mice. As shown in Fig. 4 E and F, miR-98-5p showed a significant negative correlation with Ror-γ at 24-h time-point. This inverse correlation was even stronger at 48-h time point. Regarding Let-7-5p, no significant correlation with Ror-γ was observed at 24-h (Fig. 4G), but a significant negative correlation was found at 48-h (r = −0.69, P value< 0.01) (Fig. 4H). While a negative correlation between the levels of miRNAs and their target mRNAs does not necessarily indicate a direct physical interaction, it can be considered indirect evidence for the regulation of target mRNA by the miRNA species.
Leukocyte differentiation is a critical step in the immune response against pathogens and the pathophysiology of immune-related disorders. T cells play a key role in the adaptive immune system and their differentiation into various phenotypes is essential for the development of protective or pathogenic immune reactions [72, 73]. Environmental factors, including micronutrients such as vitamins, have been shown to influence the differentiation of immune cells in both normal and disease settings [74–76]. In this study, we investigated the effects of vitamin A and D treatment on the differentiation of Th17 cells and the expression of miRNAs involved in this process in a mouse model of EAE.

Discussion

Fig. 2 The expression level of interleukin-17 (IL-17) mRNA exhibited a significant decrease in EAE mice treated with vitamin D, in comparison to the EAE group treated with the vehicle (A). The flow cytometry gating strategy was employed to identify CD4+T subsets in splenocytes (B). Comparison the effects of vitamin A, vitamin D, and combination A+D on the percentage of phenotypic Th17 cells in MOG and PMA/ionomycin-stimulated splenocyte. The mean fluorescence intensity of IL-17 was measured using by flow cytometry (C). Real-time PCR was used to evaluate the expression levels of IL-17 in MOG-stimulated splenocytes from different study groups. Splenocytes were stimulated with 0 (control), 10, and 40 μg/mL of MOG peptide at 24-h (D) and 48-h (E). The data are presented as the mean ± SEM, n = 8. The values were analyzed using one-way and two-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. The significance levels are denoted as *P<0.05, **P<0.01, ***P<0.001.
upstream signaling converges at the level of transcription factors, miRNAs that target these molecules play a critical role in cell development [78]. Several transcription factors are known to control the differentiation of T helper cells, with Ror-γ being the factor that regulates Th17 differentiation. Ror-γ is encoded by RORC gene in humans and its homolog, Rorc, in mice. RORC generates two RNA isoforms: Ror-γ, which is expressed in multiple tissues, and Ror-γt, which is primarily expressed in immune cells, including Th17 cells. These isoforms differ in their 5’ exons, while their 3’ ends (including the 3’ UTRs) are similar [79]. Several miRNAs target this common 3’ UTR region [80]. In this study, we specifically focused on miR-98-5p and Let-7a-5p miRNAs, which were selected based on our previous miRNA profiling studies that revealed downregulation of miR-98-5p in brain tissues of MS patients [68]. The Let-7 miRNA family comprises multiple miRNAs, including miR-98 and Let-7a subfamilies, which were assessed in this study. The human miR-98 gene (i.e., hsa-mir-98) is located on the short arm of chromosome X and generates two isoforms: hsa-miR-98-5p, the dominant isoform, and miR-98-3p, the minor isoform. A similar situation exists for mouse miR-98. Previous investigations have demonstrated predominantly the anti-inflammatory effects of miR-98-5p in the immune system. Yuan et al. reported the downregulation of miR-98-5p in peripheral blood mononuclear cells (PBMCs) of systemic lupus erythematosus (SLE) patients and its direct targeting of IL-6 mRNA, leading to enhanced production of anti-inflammatory cytokines by human PBMCs [81]. Additionally, Peng et al. demonstrated that miR-98-5p can block the macrophage differentiation into the M2 phenotype by targeting Trib1, and its silencing can reduce disease severity in a colitis model, indicating a pro-inflammatory role of
miR-98-5p [70]. Another study indicated the anti-inflammatory effects of miR-98-5p by targeting vascular cell adhesion molecule 1 (VCAM-1) [70]. Several studies have established a link between miR-98-5p and its protective and anti-inflammatory effects in the context of neuroinflammatory disorders.

Fig. 4 The expression levels of miR-98-5p and Let-7a-5p, which were analyzed using real-time PCR. Splenocytes stimulated with two concentrations of MOG peptide (10, 40 μg/mL) and a control group without MOG restimulation for 24-h (A, C) and 48-h (B, D). The dot plot demonstrates the levels Ror-γt mRNA in relation to miR-98-5p (E, F) and Let-7a-5p (G, H) in splenocytes stimulated for 24- and 48-h. The data are presented as the mean ± SEM, n = 8. The values were analyzed using one and two-way ANOVA with Tukey post hoc test. The correlation analysis was performed using Pearson correlation, with statistical significance indicated as *P < 0.05, **P < 0.01.
Rom et al. illustrated that miR-98-5p decreases inflammation and preserves BBB permeability in neuroinflammatory conditions through its targeting of CCL2 and CCL5 chemokines [82]. Additionally, transgenic mice overexpressing miR-98 exhibited neurological symptom improvements and reduced BBB degradation in a mouse model of middle cerebral artery occlusion (MCAO). [83]. Similarly, miR-98-5p exerts protective effects in animal models of stroke, mediated by the reduction of proinflammatory leukocyte infiltration into the brain and the suppression of microglial differentiation into the M1 phenotype [83]. Studies have also demonstrated the ability of miR-98-5p to diminish neuroinflammation in a peripheral nerve injury model by targeting STAT3 and HMGA2 and reducing the levels of inflammatory cytokines, including TNF-α, IL-1, and IL-6 [84, 85]. Ting et al. investigated the direct involvement of vitamin D in the expression of miR-98-5p, as VDR-D binds to VDRE elements in the miR-98-5p promoter, enhancing its expression. Furthermore, vitamin D indirectly participates in increasing miR-98-5p expression through the inhibition of LIN28A and LIN28, which are miRNA maturation inhibitory proteins [86].

In our previous studies, we reported that the combination of vitamins A and D as well as vitamin A or D alone reduced the expression level of Ror-γt in splenocyte of EAE mice [55]. As previously mentioned, we observed decreased levels of miR-98-5p in brain tissues from MS patients [68]. In the present study, we have made a novel discovery: miR-98-5p levels were diminished in the spinal cords of EAE mice compared with healthy controls. However, EAE mice treated with vitamins A and A+D showed a significant increase in miR-98-5p expression compared to untreated EAE mice. Additionally, miR-98-5p levels were decreased in splenocyte of EAE mice compared to healthy controls. Nevertheless, EAE mice treated with vitamins A or vitamin D exhibited a significant increase in miR-98-5p expression level after 24 and 48 h of restimulation of lymphocytes with 10 and 40 μg/mL of MOG, respectively, compared to EAE mice. These increases were negatively correlated with Ror-γt levels.

Notably, a half dose of combination treated of vitamins A+D demonstrated a more pronounced effect on miRNA expression levels in the spinal cord of EAE mice compared to a single full dose of vitamin D alone. These findings, combined with previous research conducted by other groups, suggest that miR-98-p plays an anti-inflammatory and protective role in MS/EAE. The protective effects are induced by vitamins A and D, which target the Th17-specific Ror-γ transcription factor. This mechanism may partially explain how vitamins A and D improve neurological symptoms by reducing the onset and severity of EAE, protecting BBB integrity by decreasing pro-inflammatory leukocyte infiltration and IL-17 cytokine levels in the brain, and decreasing the frequency and differentiation of Th17 cells. Th17 cells are known to play a pivotal role in the EAE model and pathogenesis of MS, as they are associated with elevated levels of IL-17 in the blood and active lesions of MS patients [15].

Human Let-7a miRNAs are transcribed from three orthologous genes: Let-7a1, Let-7a2, and Let-7a3. These genes are located on the long arms of chromosomes 9, 11, and 22, respectively. All three genes produce the same mature Let-7-5p sequence. Let-7 miRNAs exhibit widespread expression in various tissues. Within the immune system, Let-7 miRNAs are expressed in lymphocytes and macrophages, exerting influence on the differentiation of these cells [87–89]. Notably, Let-7 miRNAs are essential for T cell survival [89].

The expression of Let-7 is altered in the context of neuroinflammation and neurodegenerative diseases such as MS. This altered expression negatively regulates the differentiation of naive CD4+ T cells into pathogenic Th17 cells and the development of EAE [90]. This regulatory effect is achieved through the reduction in the frequency of IL-17A+ and GM-CSF+ cells, as well as the downregulation of cytokine receptor genes Il1r1 and Il23r. It is known that IL-1R1 and IL-23R signaling pathways play critical roles in Th17 cell differentiation. Mice lacking either receptor or ligand are completely resistant to EAE development [91–93]. Let-7 miRNAs play an important role in downregulating the migration of pathogenic cells, particularly Th17 cells, within the CNS [90]. This regulatory effect is achieved through the modulation of proliferation and migration processes that are mediated by chemokine receptors, such as CCR2 and CCR5. Experimental studies utilizing MS receptor-deficient mice have provided evidence implicating CCR2 and CCR5 in the transportation of pathogenic CD4+ T cells into the CNS [94, 95]. In the present study, we observed a downregulation of Let-7a-5p expression following the induction of EAE. However, we found that treatment with vitamins A and A+D led to an increase in Let-7a-5p levels in the spinal cord. Furthermore, Let-7a-5p levels were decreased in splenocyte of EAE mice compared to mice treated with vitamin A and D, and this decrease was negatively correlated with Ror-γt levels. Notably, vitamin D has been shown to enhance in let-7a-2 through its interaction with VDRE located in the promoter region of the pre-let-7a-2 gene [96]. These findings, in conjunction with previous studies conducted by other research groups, provide evidence that vitamins A and D possess the capability to reduce the presence of pathogenic Th17 cells in the CNS. This reduction is achieved through the modulation of cell differentiation, decreased frequency of IL-17 production, and reduced migration of these cells into the CNS and spleen. The mechanism underlying these effects involves the
upregulation of Let-7 levels, which target Ror-γt, a key transcription factor involved in Th17 cell function. As a result, these effects may contribute to a reduction in inflammatory activity and the development of EAE.

Conclusion

The identification of environmental factors that can influence the development of autoimmune diseases is crucial for the development of preventive and therapeutic strategies. Furthermore, understanding the mechanisms through which these factors exert their effects is valuable for both pathogenesis and therapeutic perspectives. Long-term use of anti-inflammatory drugs may have negative effects, making it important to explore alternative treatment options that shift the balance toward protective elements with potentially fewer side effects, such as the use of nutrients.

In this study, we present a novel mechanism by which vitamins A and D modulate miRNA expression in EAE models. Our data indicate that these vitamins may exert their protective effects in EAE development by increasing the expression of miR-98 and Let-7. These miRNAs target a Th17-specific transcription factor, leading to a reduction in the frequency and expression of IL-17 cytokines that play a role in the pathogenesis of MS. Moreover, micronutrients, including vitamins, can impact various disease-related cellular and molecular mechanisms both within the immune system and the repair processes within CNS. Further investigations, including immune profiling studies in human subjects, will be necessary to gain a deeper understanding of the underlying mechanisms involved in the protective effects of these compounds.

Acknowledgements The authors express their sincere gratitude to all the faculty members at Department of Immunology, School of Public Health, Tehran University of Medical Sciences, for their valuable discussions and contributions to this work.

Author Contribution Marziyeh Mohammadi and Fatemeh provided reagents and prepared the materials. Farideh and Samira developed new software and analyzed the bioinformatics data. Marziyeh wrote the manuscript, and Dr. Farshid made revisions to the manuscript. All authors have read and approved the final manuscript.

All authors contributed to the study conception and design. Dr Ali akbar Saboor-Yaraghi and Dr Farshid Noorbakhsh supervised the study and Dr Mohammadali Sahraian served as adviser. Dr Ali akbar Saboor-Yaraghi, Dr Farshid Noorbakhsh, and Marziyeh Mohammadi kordkhayli designed experiments. Marziyeh Mohammadi kordkhayli performed experiments and data collection and analyzed and provided new tools. Fatemeh Mansouri provided reagents and prepared the material. Farideh Talebi and Samira Ghorbani developed new software and bioinformatics data. Marziyeh Mohammadi kordkhayli wrote the manuscript and Dr Ali akbar Saboor-Yaraghi and Dr Farshid Noorbakhsh made manuscript revisions. All authors read and approved the final manuscript.

Funding This work was supported by research grants from the National Institute for Medical Research Development (NIMAD, grant No. 977636) and Tehran University of Medical Sciences (TUMS, grant No. 97-02-27 38998) and Iran National Science Foundation (INSF, grant No. 97005464). Dr Ali akbar Saboor-Yaraghi has received research support from all these centers. The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data Availability The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing Interests Marziyeh Mohammadi, as a first author and PhD student, has received financial from Tehran University of Medical Sciences (TUMS) for this project, which represents her PhD project. Samira Ghorbani and Farideh Talebi have no financial interests associated with this work. Fatemeh Mansouri, as laboratory expert, has also received financial from TUMS. Dr. Ali akbar Saboor Yaraghi, Dr. Farshid Noorbakhsh, and Dr Mohammadali Sahraian as supervisors and adviser have received research funding from TUMS.

Ethics Approval All experiments conducted in this study, “Vitamins A and D enhance the expression of Ror-γ-targeting miRNAs in a mouse model of multiple sclerosis”, as well as the animal care methods were approved by the Ethics Committee on Animal Experimentation of Tehran University of Medical Sciences The approval for this study was granted on July 18, 2018, with the approval reference number IR.TUMS.SPH.REC.1397.091.

Consent for Publication The authors state that no human samples were utilized in this study. All samples utilized in this research were obtained from animals’ sources, specifically mice.

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