Anti-inflammatory effect of miR-125a-5p on experimental optic neuritis by promoting the differentiation of Treg cells

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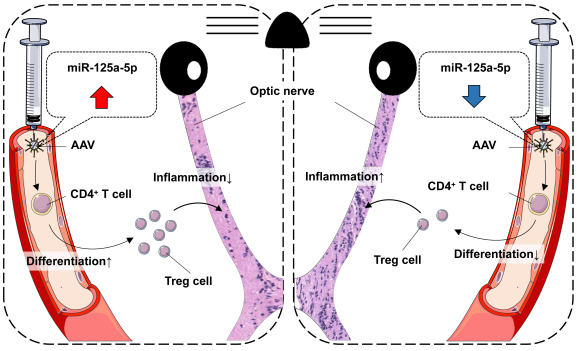
From the Contents

Graphical Abstract

miR-125a-5p affects experimental optic neuritis by promoting the differentiation of Treg cells in mice



Abstract

Methylprednisolone pulse treatment is currently used for optic neuritis. It can speed visual recovery, but does not improve the ultimate visual outcomes. Recent studies have reported that miR-125a-5p has immunomodulatory effects on autoimmune diseases. However, it remains unclear whether miR-125a-5p has effects on optic neuritis. In this study, we used adeno-associated virus to overexpress or silence miR-125a-5p in mice. We found that silencing miR-125a-5p increased the latency of visual evoked potential and aggravated inflammation of the optic nerve. Overexpression of miR-125a-5p suppressed inflammation of the optic nerve, protected retinal ganglion cells, and increased the percentage of Treg cells. Our findings show that miR-125a-5p exhibits anti-inflammatory effects through promoting the differentiation of Treg cells.

Key Words: aquaporin-4; corticosteroids; inflammation; microRNA; neuroprotection; oligodendrocyte; optic neuropathy; regulatory T cells; Th17 cell; visual field defect

Introduction

Optic neuritis refers to inflammatory lesions involving the optic nerve, and it can lead to blindness in young and middle-aged individuals (Chan, 2002; Abel et al., 2019). Inflammatory demyelination is the most common cause of optic neuritis, and environmental and genetic factors may contribute to its pathogenesis (Nuzziello et al., 2018). The clinical features can present as decreased visual acuity, pain during eye movements and visual field defects (Toosy et al., 2014; Luo et al., 2022). The major treatment for optic neuritis in clinical settings is the standard treatment regimen of methylprednisolone pulses. Although this regimen can accelerate the recovery of visual acuity, it does not improve the long-term outcome (Du et al., 2009b; Brownlee and Miller, 2014). Therefore, a novel therapy for optic neuritis patients is needed.

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that function as focused molecular regulators of many genes, including those involved in inflammatory autoimmune diseases and normal immune responses (De Luca et al., 2020; Džafo et al., 2021; Sha et al., 2021; Yu et al., 2021). Although miRNAs account for only approximately 3% of the genome, they regulate up to 90% of genes by suppressing translation or inducing mRNA degradation (Saini et al., 2007). In pathological conditions, miRNAs exhibit aberrant expression, and they can induce disease progression. miR-125a-5p has been demonstrated to be involved in the development of autoimmune-related diseases including rheumatoid arthritis (Rezaeepoor et al., 2020), Hashimoto's thyroiditis (Liu et al., 2020), multiple sclerosis (Nuzziello et al., 2018), chronic spontaneous urticaria and myasthenia gravis (Cron et al., 2018). Exploring the mechanisms of the biological actions of miR-125a-5p is conducive to find new treatment strategies for optic neuritis.

$CD4^+$ T cell implicates in mediating inflammation of autoimmune diseases, including multiple sclerosis, myasthenia gravis (de Faria et al., 2012; Danikowski et al., 2017). While many miRNAs affect autoimmune inflammation by regulate $CD4^+$ T cell differentiation (Li et al., 2010; Murugaiyan et al., 2011). The purpose of the present study was to explore the effect of miR-125a-5p on experimental optic neuritis by using adeno-associated virus (AAV)-assisted *in vivo* overexpression or silencing. We also investigated miR-125a-5p's role in regulation of $CD4^+$ T cells differentiation in experimental optic neuritis.

Methods

This study was approved by the First Affiliated Hospital of Guangxi Medical University Ethical Review Committee (approval No. 2015-KY-GJ-073) on March 6, 2015. Informed consent was obtained from each participant.

Subjects

We conducted a cross-sectional observational study to determine the levels of miR-125a-5p in optic neuritis patients and controls. We recruited optic neuritis patients and healthy individuals from the First Affiliated Hospital of Guangxi Medical University between April 2015 and December 2016. The diagnostic criteria for optic neuritis were based on those used by Feng et al. (2021): (1) acute visual acuity loss, with or without eye pain and papilledema; (2) optic nerve damage-related visual field abnormalities; (3) relative afferent pupillary defect and/or abnormal visual evoked potential (VEP); and (4)

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exclusion of other optic nerve diseases, such as ischemia, compressive, infectious, autoimmunity, trauma, toxicity, nutritional metabolism, and hereditary optic neuropathies. If corticosteroid pulse therapy was administered prior to blood collection, the patients were excluded. During the same period, we recruited healthy participants as controls. The flow chart was shown in **Additional Figure 1**.

Animals

Optic neuritis occurs mainly in women (Quinn et al., 2011), so this study used female animals for the experimental autoimmune encephalomyelitis (EAE) model. Female C57BL/6N wild-type mice (8–10 weeks old, 18–22 g weight) were purchased from the Peking Vital River Laboratory Animal Ltd. (Beijing, China; animal license No. SCXK (Jing) 2016-0006). The mice were maintained under specific pathogen-free conditions in the animal facility of the Laboratory Animal Center of Guangxi Medical University (Nanning, China) and maintained on a 12-hour light/dark cycle with a temperature of approximately 25°C, a relative humidity of 50–60% and ventilation. Mice were randomly assigned to six groups: normal control (n = 12), EAE alone (n = 12), EAE + overexpressed (n = 12), EAE + overexpressed control (n = 12), EAE + silenced (n = 12) and EAE + silenced control groups (n = 12).

Induction and evaluation of experimental autoimmune encephalomyelitis

For EAE induction, mice were immunized subcutaneously at four sites on the back with 0.2 mL emulsion containing 4 mg/mL myelin oligodendrocyte glycoprotein peptide (MOG35–55; SBS Genetech, Beijing, China) in phosphate buffered saline and 8 mg/mL heat-killed Mycobacterium tuberculosis (Becton Dickinson, Sparks, MD, USA) in complete Freund's adjuvant (Sigma, St. Louis, MO, USA). Then, the mice were injected intraperitoneally with 200 ng/0.1 mL Bordetella pertussis toxin (Sigma) on the day of immunization and again 48 hours later (Pan et al., 2015; Long et al., 2020). The clinical syndromes were observed in the mice, and an EAE disease score was assessed using a previously described 5-point scale criteria (Quinn et al., 2011), where 1 point and above was considered as the criteria for successful modeling. The EAE stages were defined as follows: disease onset, approximately day 10 after immunization; peak, approximately day 18 after immunization (**Figure 1**).

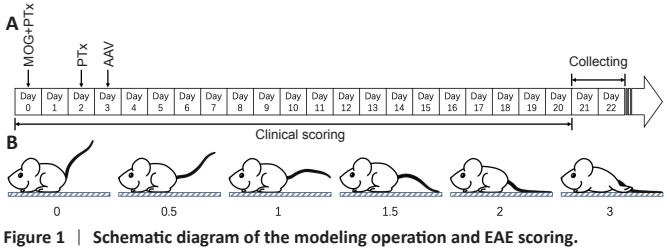

Figure 1 | **Schematic diagram of the modeling operation and EAE scoring.**
(A) Schematic diagram of experimental operation time points. (B) Schematic diagram of tail symptoms for EAE scoring. AAV: Adeno-associated virus; EAE: experimental autoimmune encephalomyelitis; MOG: myelin oligodendrocyte glycoprotein; PTx: pertussis toxin.

AAV transfection

In this study, AAV vectors of serotype 9 carrying a gene-interference sequence were designed and generated by Shanghai GeneChem (Shanghai, China). Four groups of EAE mice were randomly transfected with 0.2 mL of a solution containing 10E+11 units AAV, which was diluted with phosphate buffered saline. miR-125a-5p (EAE + overexpressed group), miR-125a-5p empty vehicle (EAE + overexpressed control group), miR-125a-5p sponge (EAE + silenced group) or miR-125a-5p sponge empty vehicle (EAE + silenced control group) was administered by an intravenous tail injection 3 days after immunization.

Flash VEP

To assess conduction along optic nerves, flash VEPs were performed at the peak of the disease (approximately day 18 after immunization). For recording, mice were anesthetized with 0.1 mL of 1% pentobarbital sodium (Sigma) by intraperitoneal injection. When reflexes were minimal, the mice were fixed on a platform. All VEP experiments were performed unilaterally with the contralateral side covered by a black patch, and were performed in a quiet, dark room. Stimulation was delivered 64 times at a frequency of 1.4 Hz using a Roland Electrophysiological Test Unit (Roland Consult, Wiesbaden, Germany). P2 amplitudes and P2 latencies of flash VEPs were subsequently analyzed.

Quantitative polymerase chain reaction

To assess miR-125a-5p levels in the participants, samples of peripheral venous blood were taken from the median cubital vein of each participant. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation. Total RNA of PBMCs was isolated according to the RNAiso Plus manufacturer's protocol (Takara Bio, Shiga, Japan). cDNA was synthesized using the Mir-XTM miRNA First-Strand Synthesis (Takara Bio) and SYBR® qRT-PCR User Manual protocol (Takara Bio).

Mice were sacrificed by cervical dislocation after anesthesia with 0.1 mL of 1% pentobarbital sodium (Sigma) by intraperitoneal injection at the peak of EAE. Optic nerves obtained from mice were immediately immersed into RNAiso Plus reagent (Takara Bio). Total RNA, containing miRNAs, was extracted according to the manufacturer's protocol. The concentrations of RNA samples were measured with Nanodrop One/OneC (Thermo Fisher Scientific, Waltham, MA, USA).

For cDNA synthesis from RNA, the Mir-XTM miRNA First-Strand Synthesis Kit (Takara Bio) was used according to the manufacturer's instructions. For mRNA reactions, we used 10 μL of RNA and reagents. The reaction condition used was as follows: incubation for 60 minutes at 37°C followed by incubation for 5 minutes at 85°C for reverse transcriptase inactivation. Quantitative polymerase chain reaction (qPCR) was conducted using a thermal cycler (Thermo Fisher Scientific). Then, samples were stored at –80°C immediately until further processing.

We performed qPCR using a SYBR® Premix Ex Taq™ II Perfect Real Time kit (Takara Bio) according to the manufacturer's protocol. miRNA-125a-5p primers were purchased from Takara Bio (Cat# DHM0048). snoRNA U6 was used as an internal standard control (snoRNA U6 forward primer: 5′-GGA ACG ATA CAG AGA AGA TTA GC-3′; snoRNA U6 reverse primer: 5′-TGG AAC GCT TCA CGA ATT TGC G-3′; Takara Bio). The relative expression of miRNAs was calculated by the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008).

Histopathologic evaluation of the optic nerve

To assess pathological changes, isolated optic nerves and eyeballs from mice at the peak of EAE were fixed in 4% paraformaldehyde. Then, they were embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin (H&E). Three sections per optic nerve were cut with a LeicaRM2135 paraffin microtome (Leica Biosystems, Buffalo Grove, IL, USA). Three images of each section (anterior, medial, posterior) were observed with an OlympusBX50 microscope (Olympus, Tokyo, Japan), and scanned with a NanoZoomer S60 pathology section scanner (Hamamatsu, Shizuoka, Japan).

For H&E staining, two researchers blind to group designations independently quantified the inflammatory infiltration using ImageJ 1.52 software (National Institutes of Health, Bethesda, MD, USA) (Schneider et al., 2012). The nuclei number and thickness of the ganglion cell layer (GCL) of the retina sections were quantified. Nuclei were counted on the entire cross-section of the optic nerve and on the longitudinal section under the same scale field.

Flow cytometry

To assess the distribution of lymphocyte subsets in EAE mice, lymphocytes in the spleen were analyzed by flow cytometry. Spleens were collected from the mice from each group 18 days after immunization, and the cells isolated from the spleens were hemolyzed with red blood cell lysis buffer (Sigma). Then, the cell suspensions were adjusted to 1×10^7/mL; these were cultured in complete RPMI 1640 medium (Thermo Fisher Scientific, Suzhou, China) and then re-stimulated with a cell stimulation mixture for 4.5 hours before being collected and washed with phosphate buffered saline. Antibodies conjugated to fluorochromes were used to stain the Treg (CD4-PerCP-Cy5.5, Cat#561115; CD25-APC, Cat# 557192; and FoxP3-PE, Cat# 560408), T helper 1 (Th1; CD4-PE, Cat# 557308; CD3-APC, Cat# 553066; and IFN-γ-PerCP-Cy5.5, Cat# 560660), Th2 (CD4-PerCP-Cy5.5, Cat# 561115 and IL-4-APC, Cat# 554436), Th17 (CD4-PerCP-Cy5.5, Cat# 561115 and IL17-PE, Cat# 559502) and B (CD8-APC, Cat# 553035; CD3-PerCP-Cy5.5, Cat# 551163; and CD19-PE, Cat# 557399) cells, by following the manufacturer's instructions. All antibodies were purchased from BD Biosciences (Heidelberg, Germany). Data were acquired from the fluorescence-activated cell sorting Calibur cytometer and the CellQuest software (BD) was used for analysis.

Statistical analysis

No statistical methods were used to predetermine sample sizes; however, our sample sizes were similar to those reported in previous publications (Du et al., 2009a; Chen et al., 2020). The evaluators were blinded to the grouping. Data are presented as mean ± standard errors (SEM) and were analyzed using GraphPad Prism 7.0a for Mac OS X (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and R 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria) (R Core Team, 2016). Data were tested for normality using the Shapiro-Wilk test. For comparisons between two groups, unpaired Student's t-test or Mann-Whitney U test was used, as appropriate. When both eyes of each mouse were used for statistical analysis, generalized estimating equations were used to compare the differences between the two groups. A two-tailed $P < 0.05$ was considered as statistically significant.

Results

miR-125a-5p is downregulated in PBMCs of patients with optic neuritis

Eighteen patients (46.4 ± 3.2 years; 15 female) with acute optic neuritis and 15 healthy controls (39.9 ± 3.4 years; 13 female) took part in this study and all participants were Chinese. All optic neuritis patients underwent blood collection within 3 weeks of onset. Seven patients were positive for serum aquaporin-4 antibodies, six were negative, and five are unavailable.

The expression level of miR-125a-5p was significantly lower in PBMCs of patients with optic neuritis (0.001576 ± 0.0004374) when compared with that of healthy controls (0.004271 ± 0.0009091) (P = 0.0084; **Figure 2**).

miR-125a-5p levels in the optic nerves in EAE mice

The expression level of miR-125a-5p in optic nerve tissue was measured by quantitative real time PCR. Expression of miR-125a-5p in the miR-125a-5p group (0.02648 ± 0.003178) was higher than that in the miR-125a-5p empty vehicle group (0.006109 ± 0.0002749, P = 0.003). The expression of miR-125a-5p in the miR-125a-5p sponge group (0.008207 ± 0.0022) was lower than that in the miR-125a-5p sponge empty vehicle group (0.02049 ± 0.003409, P = 0.0232; **Figure 3**).

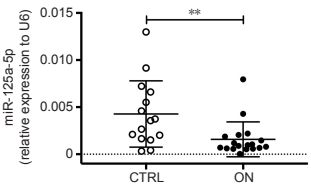

Figure 2 | Expression of miR-125a-5p in the PBMCs of ON and CTRL participants by quantitative polymerase chain reaction.
The expression of miR-125a-5p in ON patients (n = 18) was lower than that in CTRL participants (n = 15). Data are expressed as mean ± SEM. $^{**}P < 0.01$ (unpaired Student's t-test). CTRL: Healthy control; ON: optic neuritis; PBMCs: peripheral blood mononuclear cells.

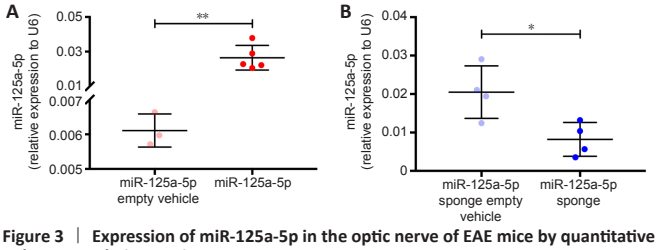

Figure 3 | Expression of miR-125a-5p in the optic nerve of EAE mice by quantitative polymerase chain reaction.
(A) Expression of miR-125a-5p in the miR-125a-5p group (n = 5) was higher than that in the miR-125a-5p empty vehicle group (n = 3). (B) Expression of miR-125a-5p in the miR-125a-5p sponge group (n = 4) was lower than that in the miR-125a-5p sponge empty vehicle group (n = 4). snoRNA U6 RNA was used for the normalization. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ (unpaired Student's t-test). EAE: Experimental autoimmune encephalomyelitis.

miR-125a-5p affects the disease scores of EAE mice

An EAE model was established to determine the function of miR-125a-5p. Then, miR-125a-5p was overexpressed or silenced in the EAE-induced mice. Although no significant difference in EAE prevalence was found between the groups, EAE appeared to be aggravated when miR-125a-5p was silenced (**Figure 4**), according to the parameters for evaluating disease severity (daily and total cumulative scores and maximum clinical score).

miR-125a-5p reduces P2 latency of EAE mice in flash VEP during optic neuritis

Flash VEPs were performed in each group to assess optic nerve function at the peak of the disease. As shown in **Figure 5**, there was no significant difference in P2 latency (138.6 ± 4.964 ms *vs*. 138.7 ± 4.547 ms, P = 0.984) or P2 amplitude (4.255 ± 0.502 μV *vs*. 3.735 ± 0.443 μV, P = 0.442) between the overexpressed and the corresponding control groups. P2 latency in the silenced group (159.8 ± 7.024 ms *vs*. 139.2 ± 5.129 ms, P = 0.023) was prolonged, compared with that in the silenced control group. No significant difference was found in P2 amplitude (5.3 ± 0.612 μV *vs*. 6.26 ± 0.653 μV, P = 0.29) between the silenced and the corresponding control groups (**Figure 5**).

miR-125a-5p affects inflammation of the optic nerve of EAE mice

To assess the histopathological changes, H&E staining was performed at the peak of the disease. In the healthy mice group, the cross-sections of stained optic nerves showed normal cellularity. Compared with those of the healthy control group, the numbers of nuclei in optic nerves of all of the EAE mice groups were significantly increased.

During the active disease period, the optic nerve tissues show inflammatory cell infiltration and glial cell proliferation, probably due to the inflammatory stimulation. These lead to an increased number of nuclei in tissue sections. In the present study, H&E staining showed moderate scattered foci of increased cellularity and perivascular inflammatory cell infiltration, and the nuclei of the axon fibers appeared to be arranged irregularly in the longitudinal section in all of the EAE mice groups. Compared with the normal control group (214 ± 10.21), the number of nuclei in the optic nerves of mice in the EAE alone group (483.3 ± 14.62, P = 0.0001) was significantly higher. Compared with the overexpressed control group (549.7 ± 19.6), the number of nuclei in the overexpressed group (393 ± 6.245, P = 0.0016) was significantly lower. Compared with the silenced control group (423 ± 13.08), the number of nuclei in the silenced group (627 ± 14.22, P = 0.0005) was significantly higher (**Figure 6**).

miR-125a-5p affects the GCL in the retina of EAE mice

To investigate whether GCL changes were present in the retina, H&E staining was performed at the peak of the disease. The number of nuclei in the EAE group (82.33 ± 7.881) was significantly lower than that in the healthy mice group (220 ± 12.1, P = 0.007). The number of nuclei in the overexpressed group (146.7 ± 5.783) was significantly higher than that in the overexpressed control group (87.67 ± 7.688, P = 0.0036). The numbers in the silenced group (90.67 ± 0.6667) and the silenced control group (83.67 ± 4.177, P = 0.1733) were similar.

The thickness of the GCL in the EAE group (10.48 ± 0.5103 μm) was significantly thinner than that in the normal control group (19.26 ± 2.077 μm, P = 0.0148). The thickness of the GCL in the overexpressed group (20.4 ± 1.898 μm) was significantly thicker than that in the corresponding control group (9.52 ± 0.3781 μm, P = 0.0049). There was no difference in the thickness of the GCL between the silenced (9.473 ± 0.8982 μm) and the corresponding control group (10.52 ± 0.9641 μm, P = 0.4695). These results indicated that MOG35-55 immunization led to the loss of retinal ganglion cells, and that overexpression of miR-125a-5p alleviated the loss. Silencing of miR-125a-5p did not further aggravate the loss (**Figure 7**).

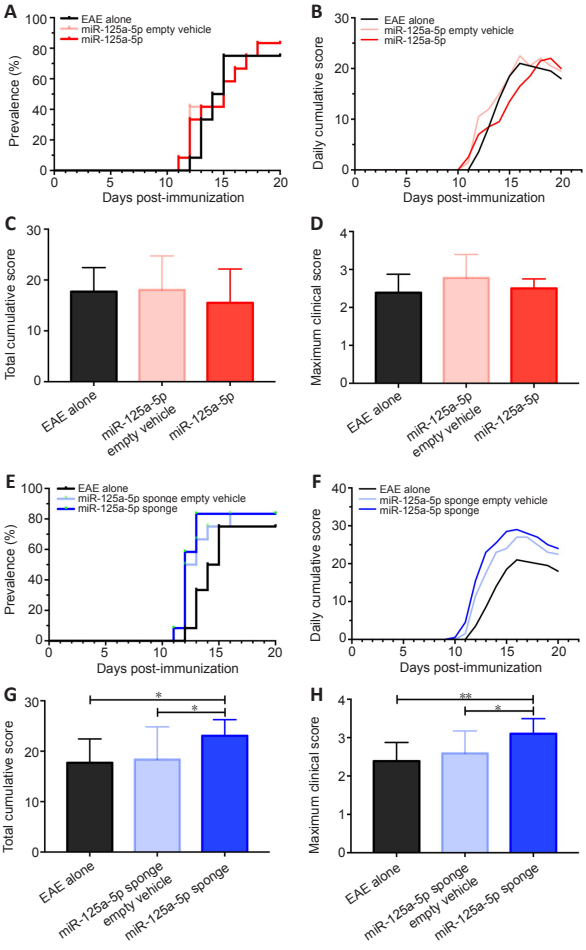

Figure 4 | Prevalence and severity of EAE mice with overexpression (A–D) or silencing (E–H) of miR-125a-5p.
(A–H) Disease prevalence (A, E), daily cumulative scores (B, F), total cumulative scores (C, G) and maximum clinical scores (D, H) were analyzed daily from day 0 to day 20. Higher scores indicate more severe EAE status. Data are expressed as mean ± SEM (EAE alone group: n = 9; miR-125a-5p empty vehicle group: n = 9; miR-125a-5p group: n = 9; miR-125a-5p sponge empty vehicle group: n = 11; miR-125a-5p sponge group: n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$ (Mann-Whitney U test). CFA: Complete Freund's adjuvant; EAE: experimental autoimmune encephalomyelitis; MOG: myelin oligodendrocyte glycoprotein.

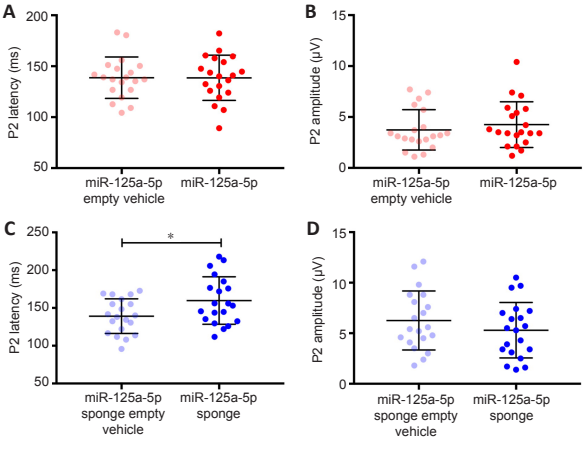

Figure 5 | VEP parameters of EAE mice with overexpression (A, B) or silencing (C, D) of miR-125a-5p.
(A–D) P2 latency (A, C) and P2 amplitude (B, D) were measured at the peak of the disease (approximately day 18 after immunization). P2 latency was shorter in the miR-125a-5p sponge empty vehicle group than in the miR-125a-5p sponge group (C). Shorter P2 latency indicates better optic nerve conduction function. Data are expressed as mean ± SEM (miR-125a-5p group: n = 20 [eyes]; miR-125a-5p empty vehicle group: n = 20 [eyes]; miR-125a-5p sponge group: n = 20 [eyes]; miR-125a-5p sponge empty vehicle group: n = 20 [eyes]). $^{*}P < 0.05$ (generalized estimating equation). EAE: Experimental autoimmune encephalomyelitis; VEP: visual evoked potential.

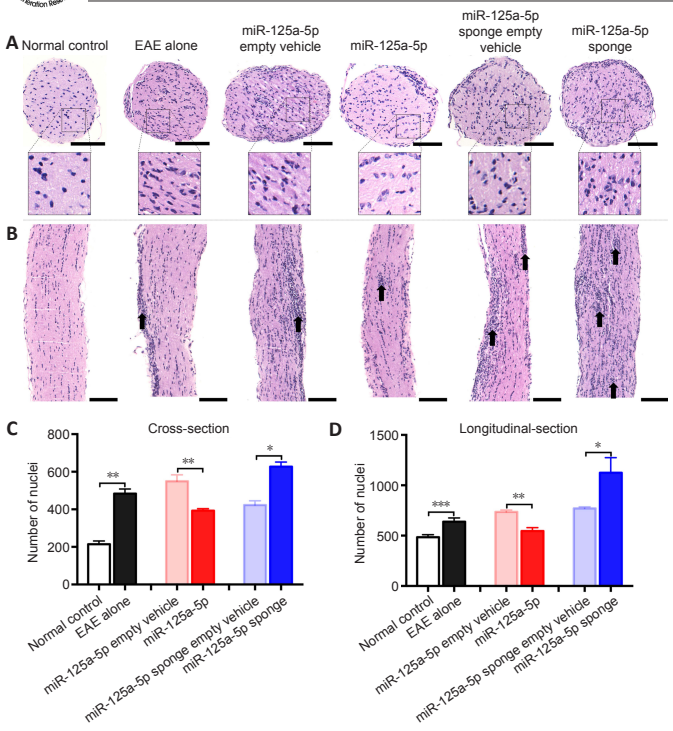

Figure 6 | **Inflammation in the optic nerve of EAE mice.**
The mice optic nerves were collected at the peak of the disease and were stained with hematoxylin and eosin to analyze the degree of inflammation. (A) A cross-section of the optic nerve. (B) The longitudinal section of the optic nerve. Arrows indicate nuclei aggregation. Scale bars: 100 μm. (C) The numbers of nuclei in the optic nerve cross-section and (D) longitudinal section were both higher in the miR-125a-5p group than in the miR-125a-5p empty vehicle group. Data are expressed as mean ± SEM (three sections per optic nerve for three optic nerves of three mice per experiment group). *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Student's t-test). EAE: Experimental autoimmune encephalomyelitis.

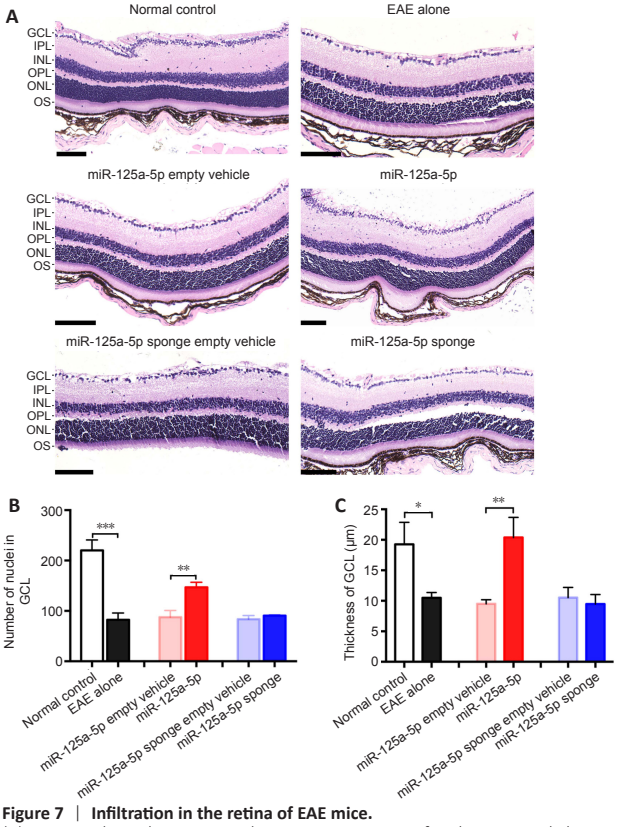

Figure 7 | **Infiltration in the retina of EAE mice.**
(A) Hematoxylin and eosin-stained retina cross-sections of each group. Scale bars: 100 μm. (B) The number of nuclei in the GCL was higher in the miR-125a-5p group than in the miR-125a-5p empty vehicle group. (C) The thickness of the GCL was greater in the miR-125a-5p group than in the miR-125a-5p empty vehicle group. Data are expressed as mean ± SEM (three sections per optic nerve for three optic nerves of three mice per group). *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Student's t-test). EAE: Experimental autoimmune encephalomyelitis; GCL: ganglion cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; ONL: outer nuclear layer; OS: outer segments; OPL: outer plexiform layer.

miR-125a-5p regulates Treg cell differentiation in the spleen of EAE mice
To verify that miR-125a-5p is involved in the activation and regulation of CD4$^+$ T cells *in vivo*, flow cytometry was performed on mice spleens, and then the Th17/Treg ratio was calculated. The percentage of Treg cells was increased significantly in the overexpressed group (1.474 ± 0.1465%) compared with that of the overexpressed control group (0.948 ± 0.1396%, P = 0.0317). The percentage of Treg cells was decreased significantly in silenced group (0.588 ± 0.108%) compared with that of the corresponding control group (1.183 ± 0.2093%, P = 0.031). There was no difference in the percentage of Treg cells between the overexpressed control and the silenced control groups (**Figure 8**). No differences were observed in the numbers of Th1, Th2, Th17 and B cells in the different groups.

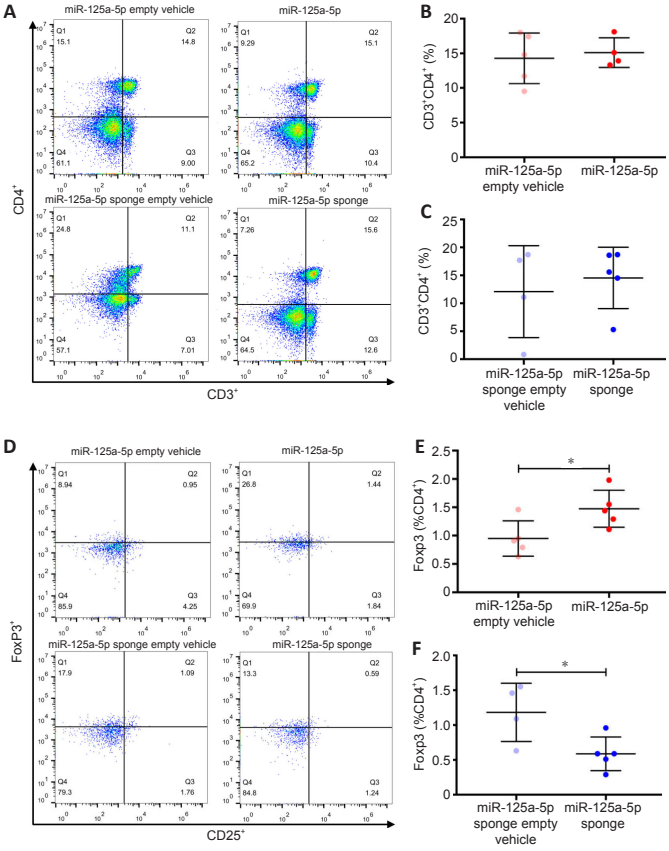

Figure 8 | **Distribution of CD4$^+$ T lymphocytes subsets in EAE mice.**
(A) Flow cytometric analysis of CD4$^+$ T cells. (B) There was no difference in the percentages of CD4$^+$ T lymphocytes between the miR-125a-5p empty vehicle group (n = 5) and miR-125a-5p group (n = 4). (C) There was no difference in the percentages of CD4$^+$ T lymphocytes between the miR-125a-5p sponge empty vehicle group (n = 4) and the miR-125a-5p sponge group (n = 5). (D) Flow cytometric analysis of Treg cells in CD4$^+$ T lymphocytes. (E) The percentage of Treg cells in the miR-125a-5p group (n = 5) was higher than that in the miR-125a-5p empty vehicle group (n = 5). (F) The percentage of Treg cells in CD4$^+$ T lymphocytes in the miR-125a-5p sponge group (n = 4) was lower than that in the miR-125a-5p sponge empty vehicle group (n = 5). Data are expressed as mean ± SEM. *P < 0.05 (unpaired Student's t-test). EAE: Experimental autoimmune encephalomyelitis.

Discussion

miRNAs can post-transcriptionally regulate genes involved in multiple cellular biological functions such as proliferation, differentiation, metabolism and apoptosis (Bartel, 2009). Research into miRNAs has promoted the understanding of diseases and the development of new medications (Issler and Chen, 2015; Chakraborty et al., 2017).

Despite the biochemical mechanisms of miR-125a-5p being largely unknown, several studies have identified dysregulation of this miRNA in neurodegenerative disorders (Herrera-Espejo et al., 2019) and inflammation (Banerjee et al., 2013). One study demonstrated that miR-125a-5p was downregulated in T cells of patients with relapsing-remitting multiple sclerosis (Jernås et al., 2013). Also, it has been reported that the downregulation of miR-125a in T cells of systemic lupus erythematosus patients can contribute to chronic inflammation (Zhao et al., 2010).

miR-125a-5p has been shown to have immunomodulatory effects on autoimmune diseases. Elevated miR-125a-5p expression levels are involved in Th1 cell response in patients with Hashimoto's thyroiditis (Liu et al., 2020). Triptolide-induced miR-125a-5p mediates Treg upregulation and improves symptoms associated with lupus (Zhao et al., 2019). miR-125a-5p affects inflammation infiltration and demyelination in the lumbosacral spinal cord

by regulating the expression of vitamin D receptors in EAE mice (Long et al., 2020), and downregulation of miR-125a in peripheral T cells was identified in patients with multiple sclerosis (Jernås et al., 2013). Our study showed that miR-125a-5p has an anti-inflammatory effect on experimental optic neuritis by promoting Treg cell differentiation.

Reduced VEP amplitudes are believed to reflect a decreased number of optic nerve fibers, and VEP latency prolongation is believed to reflect their demyelination (Raz et al., 2012). In our study, the VEP latency in the silenced group was prolonged compared with that in the corresponding control group, and there was no difference in amplitudes between these two groups. These findings suggest that miR-125a-5p protects the myelin sheath from damage rather than protects the optic nerve fibers. Reduced EAE scores after administration of miRNA-125a were reported by Pan et al. (2015). However, in our study, miRNA-125a-5p did not improve EAE scores. This contradiction may be due to the difference in experimental animals used; miR-125a-deficient mice were used by Pan et al., whereas wild-type mice were used in this study.

$CD4^+$ T cells play a major role in the occurrence and maintenance of a variety of autoimmune diseases, in which Th17 and Th1 cells are key pathogenic cells. In addition, Treg cells play an important role in inhibiting inflammatory responses and maintaining immune tolerance (Li et al., 2017; Basak and Majsterek, 2021). One study showed that miR-146a-5p affected Sjogren's syndrome by enhancing the differentiation of Th17 cells (Wang et al., 2021a). miR-155 promotes Th17 differentiation and this can have a proinflammatory effect in the pathological process of multiple sclerosis (Basak and Majsterek, 2021). Further, miR-20a affects the symptoms of EAE mice by inhibiting the differentiation of $CD4^+$ T cells into Treg cells (Wang et al., 2021b).

To our knowledge, there has been no previous study showing that miR-125a-5p affects the activation of $CD4^+$ T cells. We found that overexpression of miR-125a-5p promoted the differentiation of $CD4^+$ T cells into Treg cells, and that silencing miR-125a-5p inhibited the differentiation of $CD4^+$ T cells into Treg cells. In addition, miR-125a-5p did not affect the percentages of Th17, Th2, Th1 and B cells. Disturbances in the number or function of Treg cells can trigger or exacerbate autoimmune diseases of the central nervous system (Ronin et al., 2021). These results indicate that miR-125a-5p affects the progression of experimental optic neuritis by regulating the differentiation process of $CD4^+$ T cells into Treg cells.

Two limitations of this study should be noted. First, subtypes of optic neuritis in 28% (5/18) of patients could not be distinguished (e.g., serum aquaporin-4 positive or negative) because no commercial test was available at the early time of patient recruitment. Second, miR-125a-5p levels were not measured in either T cells or the spleen of EAE mice, and should be measured in future studies to provide more evidence for the role of miR-125a-5p in Tregs. Nonetheless, to the best of our knowledge, this is the first study to explore the function of miR-125a-5p in acute optic neuritis.

In conclusion, our study demonstrated that miR-125a-5p exerts anti-inflammatory effects through stimulation of Treg cell differentiation. Silencing of miRNA-125a-5p affects the function and structure of the optic nerve in an EAE mouse model. miR-125a-5p-based therapies may be a potential novel treatment for optic neuritis. Nevertheless, future investigations will be required to confirm its detailed biochemical mechanism.

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Additional file:
Additional Figure 1: *The flow chart of optic neuritis patient study.*

References

Abel A, McClelland C, Lee MS (2019) Critical review: Typical and atypical optic neuritis. Surv Ophthalmol 64:770-779.
Banerjee S, Cui H, Xie N, Tan Z, Yang S, Icyuz M, Thannickal VJ, Abraham E, Liu G (2013) miR-125a-5p regulates differential activation of macrophages and inflammation. J Biol Chem 288:35428-35436.
Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215-233.
Basak J, Majsterek I (2021) miRNA-dependent CD4(+) T cell differentiation in the pathogenesis of multiple sclerosis. Mult Scler Int 2021:8825588.
Brownlee WJ, Miller DH (2014) Clinically isolated syndromes and the relationship to multiple sclerosis. J Clin Neurosci 21:2065-2071.
Chakraborty C, Sharma AR, Sharma G, Doss CGP, Lee SS (2017) Therapeutic miRNA and siRNA: moving from bench to clinic as next generation medicine. Mol Ther Nucleic Acids 8:132-143.
Chan JW (2002) Optic neuritis in multiple sclerosis. Ocul Immunol Inflamm 10:161-186.
Chen T, Lennon VA, Liu YU, Bosco DB, Li Y, Yi MH, Zhu J, Wei S, Wu LJ (2020) Astrocyte-microglia interaction drives evolving neuromyelitis optica lesion. J Clin Invest 130:4025-4038.
Cron MA, Maillard S, Delisle F, Samson N, Truffault F, Foti M, Fadel E, Guihaire J, Berrih-Aknin S, Le Panse R (2018) Analysis of microRNA expression in the thymus of myasthenia gravis patients opens new research avenues. Autoimmun Rev 17:588-600.
Danikowski KM, Jayaraman S, Prabhakar BS (2017) Regulatory T cells in multiple sclerosis and myasthenia gravis. J Neuroinflammation 14:117.
de Faria O, Jr., Moore CS, Kennedy TE, Antel JP, Bar-Or A, Dhaunchak AS (2012) MicroRNA dysregulation in multiple sclerosis. Front Genet 3:311.
De Luca R, Davis PJ, Lin HY, Gionfra F, Percario ZA, Affabris E, Pedersen JZ, Marchese C, Trivedi P, Anastasiadou E, Negro R, Incerpi S (2020) Thyroid hormones interaction with immune response, inflammation and non-thyroidal illness syndrome. Front Cell Dev Biol 8:614030.
Du C, Liu C, Kang J, Zhao G, Ye Z, Huang S, Li Z, Wu Z, Pei G (2009a) MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. Nat Immunol 10:1252-1259.
Du Y, Ling Y, Zhou CX, Song QX, Wang DM, He JF (2009b) Why we should treat idiopathic optic neuritis with high-dose and long-term corticosteroids in China. Med Hypotheses 72:478-479.
Džafo E, Bianchi N, Monticelli S (2021) Cell-intrinsic mechanisms to restrain inflammatory responses in T lymphocytes. Immunol Rev 300:181-193.
Feng C, Chen Q, Zhao G, Li Z, Chen W, Sha Y, Sun X, Wang M, Tian G (2021) Clinical characteristics of optic neuritis phenotypes in a 3-year follow-up Chinese cohort. Sci Rep 11:14603.
Herrera-Espejo S, Santos-Zorrozua B, Álvarez-González P, Lopez-Lopez E, Garcia-Orad Á (2019) A systematic review of microRNA expression as biomarker of late-onset Alzheimer's disease. Mol Neurobiol 56:8376-8391.
Issler O, Chen A (2015) Determining the role of microRNAs in psychiatric disorders. Nat Rev Neurosci 16:201-212.
Jernås M, Malmeström C, Axelsson M, Nookaew I, Wadenvik H, Lycke J, Olsson B (2013) MicroRNA regulate immune pathways in T-cells in multiple sclerosis (MS). BMC Immunol 14:32.
Li B, Wang X, Choi IY, Wang YC, Liu S, Pham AT, Moon H, Smith DJ, Rao DS, Boldin MP, Yang L (2017) miR-146a modulates autoreactive Th17 cell differentiation and regulates organ-specific autoimmunity. J Clin Invest 127:3702-3716.
Li J, Wan Y, Guo Q, Zou L, Zhang J, Fang Y, Zhang J, Zhang J, Fu X, Liu H, Lu L, Wu Y (2010) Altered microRNA expression profile with miR-146a upregulation in CD4+ T cells from patients with rheumatoid arthritis. Arthritis Res Ther 12:R81.
Liu Y, Ding X, Xiong S, Wang X, Tang X, Wang L, Wang S, Peng H (2020) Circulating microRNA expression profiling identifies miR-125a-5p promoting T helper 1 cells response in the pathogenesis of Hashimoto's thyroiditis. Front Immunol 11:1195.
Long HC, Wu R, Liu CF, Xiong FL, Xu Z, He D, Zhang YF, Shao B, Zhang PA, Xu GY, Chu L (2020) MiR-125a-5p regulates vitamin D receptor expression in a mouse model of experimental autoimmune encephalomyelitis. Neurosci Bull 36:110-120.
Luo W, Deng X, Xu X, Song R, Luo M, Moss HE, Du Y (2022) Development of a prognostic model for predicting multiple sclerosis after optic neuritis: a secondary analysis of data from the optic neuritis treatment trial. J Neuroophthalmol 42:88-96.
Murugaiyan G, Beynon V, Mittal A, Joller N, Weiner HL (2011) Silencing microRNA-155 ameliorates experimental autoimmune encephalomyelitis. J Immunol 187:2213-2221.
Nuzziello N, Vilardo L, Pelucchi P, Consiglio A, Liuni S, Trojano M, Liguori M (2018) Investigating the role of microRNA and transcription factor co-regulatory networks in multiple sclerosis pathogenesis. Int J Mol Sci 19:3652.
Pan W, Zhu S, Dai D, Liu Z, Li D, Li B, Gagliani N, Zheng Y, Tang Y, Weirauch MT, Chen X, Zhu W, Wang Y, Chen B, Qian Y, Chen Y, Fang J, Herbst R, Richman L, Jallal B, et al. (2015) MiR-125a targets effector programs to stabilize Treg-mediated immune homeostasis. Nat Commun 6:7096.
Quinn TA, Dutt M, Shindler KS (2011) Optic neuritis and retinal ganglion cell loss in a chronic murine model of multiple sclerosis. Front Neurol 2:50.
R Core Team (2016) R: A Language and Environment for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org/.
Raz N, Dotan S, Chokron S, Ben-Hur T, Levin N (2012) Demyelination affects temporal aspects of perception: an optic neuritis study. Ann Neurol 71:531-538.
Rezaeepoor M, Pourjafar M, Tahamoli-Roudsari A, Basiri Z, Hajilooi M, Solgi G (2020) Altered expression of microRNAs may predict therapeutic response in rheumatoid arthritis patients. Int Immunopharmacol 83:106404.
Ronin E, Pouchy C, Khosravi M, Hilaire M, Grégoire S, Casrouge A, Kassem S, Sleurs D, Martin GH, Chanson N, Lombardi Y, Lalle G, Wajant H, Auffray C, Lucas B, Marodon G, Grinberg-Bleyer Y, Salomon BL (2021) Tissue-restricted control of established central nervous system autoimmunity by TNF receptor 2-expressing Treg cells. Proc Natl Acad Sci U S A 118:e2014043118.
Saini HK, Griffiths-Jones S, Enright AJ (2007) Genomic analysis of human microRNA transcripts. Proc Natl Acad Sci U S A 104:17719-17724.
Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108.
Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671-675.
Sha N, Wang HW, Sun B, Gong M, Miao P, Jiang XL, Yang XF, Li M, Xu LX, Feng CX, Yang YY, Zhang J, Zhu WJ, Gao YY, Feng X, Ding X (2021) The role of pineal microRNA-325 in regulating circadian rhythms after neonatal hypoxic-ischemic brain damage. Neural Regen Res 16:2071-2077.
Toosy AT, Mason DF, Miller DH (2014) Optic neuritis. Lancet Neurol 13:83-99.
Wang X, Xin S, Wang Y, Ju D, Wu Q, Qiu Y, Niu X, Liu W, Li J, Ji P (2021a) MicroRNA-146a-5p enhances T helper 17 cell differentiation via decreasing a disintegrin and metalloprotease 17 level in primary sjögren's syndrome. Bioengineered 12:310-324.
Wang Y, Xie C, Song Y, Xiang W, Peng J, Han L, Ding J, Guan Y (2021b) miR-20a suppresses Treg differentiation by targeting Map3k9 in experimental autoimmune encephalomyelitis. J Transl Med 19:223.
Yu SJ, Yu MJ, Bu ZQ, He PP, Feng J (2021) MicroRNA-670 aggravates cerebral ischemia/reperfusion injury via the Yap pathway. Neural Regen Res 16:1024-1030.
Zhao X, Tang X, Yan Q, Song H, Li Z, Wang D, Chen H, Sun L (2019) Triptolide ameliorates lupus via the induction of miR-125a-5p mediating Treg upregulation. Int Immunopharmacol 71:14-21.
Zhao X, Tang Y, Qu B, Cui H, Wang S, Wang L, Luo X, Huang X, Li J, Chen S, Shen N (2010) MicroRNA-125a contributes to elevated inflammatory chemokine RANTES levels via targeting KLF13 in systemic lupus erythematosus. Arthritis Rheum 62:3425-3435.

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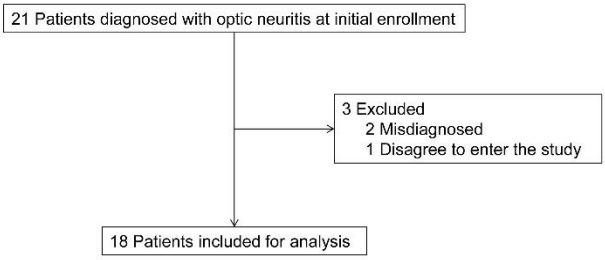

Additional Figure 1 The flow chart of optic neuritis patient study.