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

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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 (Terry 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 (Luo et al., 2020), multiple sclerosis (Nuzziello et al., 2018), chronic spontaneous uveitis 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; Dankowskik 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-G1-073) 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)
exclusion of other optic nerve diseases, such as ischemia, compressive, infectious, autoimmune, trauma, toxicity, nutritional metabolism, and hereditary optic neuropathies. If corticosteroid pulse therapy was administered and generated from collection, the pupil was 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 Co. (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 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 subcutaneously injected 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 symptoms were recorded 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 was above and 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). For cDNA synthesis from RNA, the Mir-XTM miRNA First-Strand Synthesis Kit (Takara Bio) was used according to the manufacturer’s instructions. For miRNA 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 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# DHM048). miRNA U6 was used as an internal standard control (snORNA U6 forward primer: 5’-GGA AGC ATA CGA AGA AGA TTCG-3’; snORNA U6 reverse primer: 5’-TGG AAC GCT TCA CAG TGC G-3’; Takara Bio). The relative expression of miRNAs was calculated by the 2^−ΔΔCt method (Schmittgen and Livak, 2008).

Histopathologic evaluation of the optic nerve

To obtain histopathological images, the 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 Leica RM2135 paraffin microtome (Leica Biosystems, Buffalo Grove, IL, USA). Three images of each section (anterior, medial, posterior) were observed with an Olympus BX51 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 Image J 1.52 software (National Institutes of Health, Bethesda, Maryland) (Schneider et al., 2012). The 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 × 10^6/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-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 IL-17-PE, Cat#559402) and B cells (CD19-APC, Cat# 556060). Th17 (CD4-PerCP-Cy5.5, Cat# 561115 and IL-17-PE, Cat#559402) and B cells (CD19-PE, Cat# 556060), Th2 (CD4-PerCP-Cy5.5, Cat# 561115 and IL-4-APC, Cat#554436), Th1 (CD4-PerCP-Cy5.5, Cat#551163 and IL17-PE, Cat#559402) and B cells (CD19-PE, Cat# 556060) 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., 2009; Chen et al., 2020). The evaluators were blinded to the grouping. Data were 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). Two-tailed Student’s t-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 in the healthy group (0.0232 ± 0.00491; P = 0.0084; Figure 2). The expression level of miR-125a-5p in the miR-125a-5p sponge group (0.002207 ± 0.000202) was lower than that in the miR-125a-5p sponge control group (0.00249 ± 0.003409; P = 0.0232; Figure 3).

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. Mice were inoculated intracranially with the miR-125a-5p sponge 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 level of miR-125a-5p in the miR-125a-5p sponge group (0.008207 ± 0.00022) was lower than that in the miR-125a-5p sponge control group (0.00249 ± 0.003409, P = 0.0084; Figure 3).

Figure 1 Schematic diagram of experimental operation time points. (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 obtained from Shanghai GeneHeal (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 sponge (EAE + overexpressed control group), miR-125a-5p sponge empty vector (EAE + silenced control group) or miR-125a-5p sponge empty vector (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 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 dark room. Stimulation was delivered 64 times at a frequency of 1.4 Hz using a
Expression of miR-125a-5p in the optic nerve of EAE mice by quantitative polymerase chain reaction.

The expression of miR-125a-5p in ON patients (n = 18) was lower than that in CTRL patients (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.

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

An AEEmodel 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 (159.7 ± 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. 6.2 ± 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 assay 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. There was 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 were disorganized and 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 (82.3 ± 7.851) 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 (9.7 ± 0.378 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).
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 ± 0.1088%) 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.

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., 2019).

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 (Li 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 lumbar sacral spinal cord.
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 neurological scores. This difference could be due to the difference in experimental animals used; miR-125a-deficient mice were used in our study.

CD4+ T cells play a major role in the occurrence and maintenance of a variety of autoimmune diseases, in which Th1 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 TH1 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 the dysregulation 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 (LGN-D or ONH) of patients should 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 (positive or negative) because no commercial test was available at the early time of patient recruitment. This is an open access journal, and all data generated or analyzed during this study are included in this published article and its supplementary information files.

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Author contributions: Study design: YD, JLZ, WY; experiment implementation: JLZ, YLZ, QSS, XL, YLD, GQH; data analysis: JLZ, WY; manuscript writing: JLZ, YD. All authors read and approved the final version of the manuscript.

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Availability of data and materials: All data generated or analyzed during this study is included in this published article and its supplementary information files.

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

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