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# MiR-146a promotes oligodendrocyte progenitor cell differentiation and enhances remyelination in a model of experimental autoimmune encephalomyelitis

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ABSTRACT

The death of mature oligodendrocytes (OLs) leads to demyelination in the central nervous system (CNS) and subsequently to functional deficits. Remyelination requires the differentiation of oligodendrocyte progenitor cells (OPCs) into myelinating OLs, which in the CNS with neurodegenerative diseases such as multiple sclerosis (MS), is often inhibited. Among the inhibitors of OPC differentiation are toll-like receptor 2 (TLR2) and interleukin-1 receptor-associated kinase 1 (IRAK1) signaling, and both are negatively regulated by microRNA-146a (miR-146a). Therefore, we hypothesized that increase of miR-146a level in the CNS would foster OPC differentiation and remyelination by inhibiting the TLR2/IRAK1 signaling pathway. Here, we tested this hypothesis using exogenous miR-146a mimics and a mouse model of MS, experimental autoimmune encephalomyelitis (EAE) induced by immunization with myelin proteolipid protein peptide ($PLP_{139–151}$). EAE mice were treated by miR-146a mimics or miR-146a mimic negative controls, respectively, which initiated at day 14 post immunization, once a week for 6 consecutive weeks. Neurological function was monitored daily. Immunofluorescent staining, qRT-PCR and Western blot were used to measure the differentiation of OPCs and myelination, and to investigate the underlying mechanisms of action of miR-146a. Using a fluorescence tracing approach, we found that miR-146a mimics crossed the blood brain barrier and targeted OPCs and microglia/macrophages after systemic administration. MiR-146a mimic treatment substantially improved neurological functional outcome, increased the number of newly generated OLs which may facilitate remyelination in the CNS. The cell number, cytokine level and protein levels of M2 phonotype of microglia/macrophages significantly increased, while cytokine and protein levels of the M1 phenotype significantly decreased after miR-146a mimic treatment. Increased OPC differentiation and remyelination were associated with reduction of TLR2/IRAK1 signaling pathway activity by miR-146a mimic treatment. This study provides insight into the cellular and molecular bases for the therapeutic effects of miR-146a on OPC differentiation and remyelination, and suggests the potential of enhancing miR-146a as a treatment of demyelinating disorders.

## 1. Introduction

Oligodendrocytes (OLs) are the only cells that form myelin to encase axons in the central nervous system (CNS). OLs protect and provide metabolic support to axons and ensure effective transmission of electrical impulses (Nave, 2010; Smith et al., 1979). Damage or death of OLs, caused by, e.g. inflammation, trauma injury, metabolism disturbance and hypoxic–ischemia, lead to demyelination (Love, 2006). Remyelination requires oligodendrocyte progenitor cells (OPCs) which are abundant in the adult CNS to differentiate into mature myelinating

*Abbreviations:* CNPase, 2′,3′ cyclic nucleotide 3′ phosphodiesterase; DAB, 3,3′-Diaminobenzidine; BrdU, bromodeoxyuridine; CNS, central nervous system; CPZ, cuprizone; CY3, cyanine 3; EAE, experimental autoimmune encephalomyelitis; FITC, fluorescein isothiocyanate; IRAK1, interleukin-1 receptor-associated kinase 1; ip, intraperitoneally; miRNA, microRNA; miR-146a, microRNA-146a; MS, multiple sclerosis; MBP, myelin basic protein; PLP, myelin proteolipid protein; NF-H, neurofilament heavy protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OLs, oligodendrocytes; OPC, oligodendrocyte progenitor cell; p.i., post immunization; TRAF6, TNF receptor associated factor 6; TLR2, toll-like receptor 2

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OLs and form new myelin sheaths (Franklin and Ffrench-Constant, 2008; Goldman et al., 2012); however, these distinctive phases are highly orchestrated and strictly controlled by both extrinsic and intrinsic factors (Franklin, 2002; McTigue and Tripathi, 2008). Although spontaneous remyelination occurs after demyelinating CNS damage and provides a partial functional recovery (Chang et al., 2002; Franklin, 2002; Franklin and Ffrench-Constant, 2008; Franklin and Ffrench-Constant, 2017), these OPCs fail to adequately differentiate into mature myelinating OLs (Franklin, 2002; Goldman et al., 2012; Kremer et al., 2011; Kuhlmann et al., 2008), especially during disease progression and ageing (Back et al., 2005; Deshmukh et al., 2013; Franklin and Ffrench-Constant, 2008; Franklin and Ffrench-Constant, 2017; Hanafy and Sloane, 2011; Kremer et al., 2011; Lassmann et al., 2012; Shen et al., 2008; Sloane et al., 2010).

Multiple sclerosis (MS) is the most common demyelinating disease in the CNS. Current therapies targeting the immune system fail to prevent progression of demyelination and axonal degeneration (Najm et al., 2015). Patients with demyelination damage suffer from neurological functional and cognitive deficits resulting in enormous societal financial burden. Thus, therapies for MS aimed to enhance remyelination are urgently required. Although molecular mechanisms underlying inhibition of OPC differentiation into mature OLs are not fully understood, there are intrinsic and extrinsic factors that inhibit OPC differentiation (Back et al., 2005; Franklin, 2002; Franklin and Ffrench-Constant, 2008; Hanafy and Sloane, 2011; Kremer et al., 2011; Kuhlmann et al., 2008; Shen et al., 2008; Sloane et al., 2010). Therapies targeting these inhibitory factors may have great potential to remyelinate axons and consequently to ameliorate MS. Although preclinical studies of mechanisms that regulate remyelination are rapidly growing, and potential therapeutic agents have been screened in early-stage clinical trials (Franklin and Ffrench-Constant, 2017), currently there are no therapies for clinical use to directly promote remyelination (Kotter et al., 2011; Kremer et al., 2011; Najm et al., 2015).

MicroRNAs (miRNAs), a class of small non-coding RNAs with ~22 nucleotides, act as master regulator to control post transcriptional gene expression and protein translation (Kosik, 2006). MiRNAs modulate OPC differentiation and myelination during development (Shin et al., 2009; Zhao et al., 2010) and are involved in demyelination diseases (Guerau-de-Arellano et al., 2012; Jr Ode et al., 2012; Li and Yao, 2012). MiR-146a, originally characterized as a regulator of innate and adaptive immune response, by negatively targeting genes of toll-like receptor 2 (TLR2), interleukin-1 receptor–activated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) which trigger activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Gao et al., 2015; Li et al., 2015; Li et al., 2013; Lu et al., 2010; O'Neill et al., 2011; Quinn et al., 2013; Tahamtan et al., 2018; Yang et al., 2012). Emerging studies suggest that the TLR2 pathway substantially activate during MS (Derkow et al., 2013; Hanafy and Sloane, 2011; Marta, 2009; Miranda-Hernandez and Baxter, 2013; Qian and Cao, 2013; Racke and Drew, 2009; Sloane et al., 2010), and contributes to block OPC differentiation into myelinating OLs (Back et al., 2005; Hanafy and Sloane, 2011; Hanafy and Sloane, 2011; Jovicic et al., 2013; Kremer et al., 2011; Lau et al., 2008; Santra et al., 2014; Sloane et al., 2010). IRAK1-deficient mice are resistant to EAE (Deng et al., 2003), and ablation of IRAK1 in OPCs promotes OPC differentiation (Liu et al., 2017a). These data strongly indicate that the TLR/IRAK1 signaling pathway mediates myelination and is clinically relevant to patients with MS. Accordingly, we have investigated the effects of miR-146a on promotion of remyelination in animal models with cuprizone (CPZ) demyelination, stroke, and peripheral neuropathy, and in in vitro primary cultured OPCs (Jia et al., 2016; Liu et al., 2017a; Santra et al., 2014; Wang et al., 2014; Zhang et al., 2017; Zhang et al., 2012). Our vitro study shows that elevation of miR-146a by miR-146a mimics in primary cultured OPCs facilitates OPC differentiation and increased their expression of myelin proteins, whereas attenuation of endogenous miR-146a by siRNA against miR-146a suppressed generation of myelin proteins (Liu et al., 2017a). Moreover, infusion of exogenous miR-146a mimics into the corpus callosum of the mouse after CPZ-induced demyelination robustly promotes OPC differentiation and enhances remyelination (Zhang et al., 2017). In addition, intravenous injection of miR-146a mimics increases axonal myelination in diabetic peripheral neuropathy (Liu et al., 2017b). Underlying mechanism of action studies revealed that miR-146a inversely regulates its target IRAK1 gene in OPCs, and attenuation of IRAK1 in OPCs substantially increases myelin proteins (Liu et al., 2017a). Inhibition of the TLR2 signaling pathway by elevation of miR-146a in the CNS in the CPZ model may mediate remyelination promoted by miR-146a (Zhang et al., 2017). In concert, these in vivo and in vitro studies indicate that miR-146a releases the brakes of remyelination via inhibition of the TLR2/IRAK1/TRAF6/NF-κB signaling pathway to overcome the obstacles of remyelination.

Based on these novel findings of miR-146a on OPC differentiation and remyelination, we thus hypothesize that miR-146a promotes OPC differentiation and remyelination in a mouse model of MS, experimental autoimmune encephalomyelitis (EAE) (Pluchino et al., 2003), and the TLR2 signaling pathway mediates these actions. Thus, here for the first time, we investigate miR-146 as a potential remyelination therapy for MS.

## 2. Material and methods

### 2.1. MiR-146a mimics

MiRIDIAN miR-146a mimics and mimic negative controls were purchased from Dharmacon (Lafayette, CO, USA). They are chemically enginered miRNAs that have been modified to reduce their degradation in vivo, since stability is one of the main challenges for in vivo delivery of miRNA mimics (Trang et al., 2011; van Rooij, 2011). These mimics are double-stranded RNA oligonucleotides and chemical enhancement with the ON-TARGET modification pattern to preferentially program RNA induced silencing complex (RISC) with the active miRNA strand (Liu et al., 2017b; van Rooij and Kauppinen, 2014), and to reduce their degradation in vivo (Bonauer et al., 2009; van Rooij et al., 2008). They have been demonstrated the superior performance and highly effective mimic of endogenous mature microRNA function, preferential programming of RISC with active strand of miRNA and exclusion of passenger strand through proprietary chemical modification pattern in comparison to native double-stranded miRNA(https://dharmacon.horizondiscovery.com/rnai/microrna/miridian-microrna-mimic/). Therefore, these miRNA mimics are highly stable and have been successfully used to effectively elevate endogenous miRNA in vivo (Liu et al., 2017b; Trang et al., 2011). To facilitate efficient delivery of miRNA mimics, mimics were formulated with a novel and nontoxic neutral lipid emulsion system (NeuroAim™ In Vivo Transduction Reagent; BIOO Scientific), followed the vendor's manual.

Moreover, we employed miR-146a mimics fluorescently labeled with CY3 (Dharmacon) to trace the cell targets of miR-146a mimics.

### 2.2. EAE animal model and experimental groups

All animal procedures have been approved by the Institutional Animal Care and Use Committee (IACUC) of the Henry Ford Health System.

Sex plays a significant role in susceptibility to MS, with approximately three times in women than in men (National Multiple Sclerosis Society) (Acharjee et al., 2018). Compare to male SJL mice, female mice exhibit higher incidence and a more consistent relapsing pattern (Miller et al., 2010; Papenfuss et al., 2004); in addition, male SJL mice are more aggressive, the increased stress and production of corticosteroids, which have been shown to negatively affect the induction of EAE (Mason, 1991; Miller et al., 2010). Female SJL mice are preferred in PLP-EAE model induction (Acharjee et al., 2018; Bando et al., 2018; Gerzanich et al., 2017; Karim et al., 2018; Kesidou et al., 2017; Koda

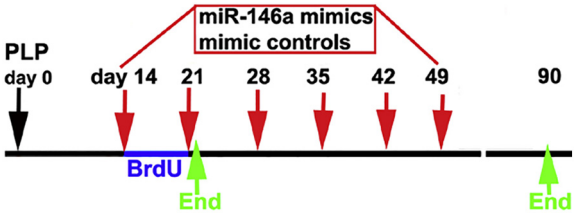


**Fig. 1.** The schematic of experiment design.

et al., 2018; Kornberg et al., 2018; Lecuyer et al., 2017; Stolz et al., 2017; Talebi et al., 2017; Wu et al., 2015).

Female SJL/J mice (6–8 weeks old, Jackson Laboratory) were subcutaneously injected with myelin proteolipid protein peptide ($PLP_{139–151}$, HSLGKWLGHPDKF, 100 μg, GeneScript) dissolved in complete Freund's adjuvant (CFA). EAE mice were randomly assigned into a miR-146a mimic group and a miR-146a mimic negative control group. MiR-146a mimics or mimic negative controls (10 mg/kgbw) were intravenously (iv) administered via the tail vein using a syringe needle (27-gauge) under anesthesia with isoflurane, respectively. Treatments were performed once a week for 6 consecutive weeks, initiated at day 14 post immunization (p.i.) (Fig. 1). The dose selection was based on our previous studies (Liu et al., 2017b; Zhang et al., 2017). Moreover, in our lab, we have tested longitudinal miR-146a level in vivo to confirm the stability of miRNA mimics after intravenous administration (Liu et al., 2017b). In this publication, we have shown that a single dose of MiRIDIAN miR-146a mimics (10 mg/kgbw) was intravenously administrated to normal mice, and significantly elevated serum levels of miR-146a over 14 days with a maximum elevation at 1 day after administration compared to the mimic negative control. Based on these published data, we selected 7 days as the interval of treatment in the present study when the miR-146a level is still significantly higher than the mimic control group.

To trace cell targets of mimics in the CNS, an additional group of EAE mice were iv injected with fluorescently labeled miR-146a mimics. To label proliferating cells in vivo, bromodeoxyuridine (BrdU, 100 mg/kgbw) was intraperitoneally (ip) injected daily for 7 consecutive days starting at day 14p.i.. BrdU is a thymidine analog that is incorporated into the DNA of dividing cells during S-phase, therefore, it is used as a marker of cell proliferation. Neurological function was monitored daily up to day 90p.i. ($n$ = 9 per group) by measuring paralysis according to the conventional score 0–5 (Zhang et al., 2005), as follows: 0, healthy; 1, loss of tail tone; 2, ataxia and/or paresis of hind limbs; 3, paralysis of hind limbs and/or paresis of forelimbs; 4, tetra-paralysis; 5, moribund or dead. The higher score, the worse function outcome. The dead mice were scored as 5 until to the end of experiment.

### 2.3. Immunohistochemistry and quantification

EAE mice treated with miR-146a mimics or mimic controls were euthanized at day 22 and 90p.i. ($n$ = 6 per group per time point), respectively. Mice were intracardiac perfused with saline, followed by 4% paraformaldehyde. Spinal cords were removed from vertebral canal, and lumbar spinal cords were obtained. Tissues were prepared for paraffin sections. A series of 6-μm-thick coronal paraffin sections were cut from each block. Every fifth section was used for immunostaining.

Single immunostaining using 3,3′-Diaminobenzidine (DAB) and double immunofluorescent staining with fluorescein isothiocyanate (FITC) and cyanine 3 (CY3) were performed, respectively. The following primary antibodies were used: BrdU (Abcam) as the marker of proliferating cells, adenomatous polyposis coli clone CC1 (APC, GenWay Biotech. Inc) as the marker of the mature OLs, chondroitin sulfate proteoglycan (NG2, Millipore) for OPC marker, ionized calcium binding adaptor molecule 1 (IBA1, Wako) for microglia/macrophage marker, Chitinase 3-like 3 (YM1, R&D) for M2 phenotype of microglia/macrophage marker.

Because mature OLs do not proliferate and OPCs actively proliferate (McTigue and Tripathi, 2008), BrdU injection only labels proliferating OPCs. Thus, when BrdU labeled OPCs differentiate into mature OPCs, this cell population can be identified with double immunofluorescent staining with antibodies against BrdU and APC. To track the cellular targets of miR-146a mimics (pre-labeled with CY3 fluorescence), immunofluorescent stainings with the OPC marker NG2, and the microglia/macrophage marker IBA1 were performed, respectively.

Data were collected by a person blind to the treatment status of each animal. The measured areas were selected in 10 fields within in the white matter of the spinal cord (Zhang et al., 2015). The number of $APC^+$, $BrdU^+$ with $APC^+$ and $YM1^+$ cells were measured under a 40× microscope (Olympus BX40 Axiophots) using a 3-CCDcolor video camera (Sony DXC-970MD) interfaced with Micro Computer Imaging Device (MCID) image analysis system (Imaging Research Inc.). Co-localization of double immunoreactive cells or pre-fluorescently labeled miR-146a mimic signals with phenotype markers of cells was verified along with DAPI positive nuclei by means of 3D two photon microscopy (LSCM, Zeiss LSM 510)(Zhang et al., 2011). The numbers of immunoreactive cells were divided by the measured areas and presented as numbers per $mm^2$. Data are presented as mean ± SE.

### 2.4. Quantitative RT-PCR (qRT-PCR) analysis

EAE mice treated by mimics or mimic controls were euthanized at day 22p.i. The spinal cords were removed and cervical and thoracic spinal cords were obtained. The tissues were homogenized and total RNAs were isolated by miRNeasy Mini Kit (Qiagen). The plasma were obtained as well, total RNAs were isolated by Serum/Plasma miRNeasy Mini Kit (Qiagen).

To examine the levels of miR-146a, total RNAs were reversely transcribed using the TaqMan® MicroRNA Reverse Transcription (RT) kit (Applied Biosystems). Quantitative RT-PCR (qRT-RCR) reaction was performed using a standard TaqMan® PCR kit protocol (Applied Biosystems) and an ABI 7000 PCR instrument (Applied Biosystems). The mature miR-146a (primer catalog #000468, Applied Biosystems) was normalized against the expression of U6 snRNA as an endogenous normalization control in tissue samples, while the synthetic spike-in control (cel–miR-39 mimic; Qiagen) for internal normalization in plasma samples.

The mRNA levels of iNOS, TNFα and IL-10 were measured by qRT-PCR with SYBR Green PCR mix, 1 μg of RNA from each sample RNA was used to produce cDNA, following the standard protocol supplied with the SuperScript III RTase (Invitrogen). qRT-PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems). Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. The following primers for real-time PCR were designed as follows: glyceraldehyde-3-phosphate dehyrogenase (GAPDH, FWD AGAACATCATCCCTGCATCC, REV CACATTGGGGGTAGGAACAC). iNOS (FWD AGCCCTCACCTACTTCCTG, REV CAATCTCTGCCTATCCGTCTC), TNFa (FWD GGTTC TGTCCCTTTCACTCAC, REV TGCCTCTTCTGCCAGTTCC), IL-10 (FWD, CGGGAAGACAATAACTGCACCC, REV CGGTTAGCAGTATGTTGTCCAGC). The assays were performed in triplicate, and were determined according to the method of $2^{-\Delta\Delta}$CT(Livak and Schmittgen, 2001). Data are presented as fold of mimic control.

### 2.5. Western blot analysis

EAE mice treated with miR-146a mimics or mimic controls were euthanized at day 22p.i. and 90p.i.. The proteins were isolated from the cervical and thoracic spinal cord tissues shared with the RNA analysis. The protein levels of myelin basic protein (MBP, Millipore, a marker for myelinated mature OLs and myelin), YM1 (R&D), iNOS (abcam), TLR2 (Bioss), pNF-κB (cell signaling) and IRAK1 (Santa Cruz Biotechnology)

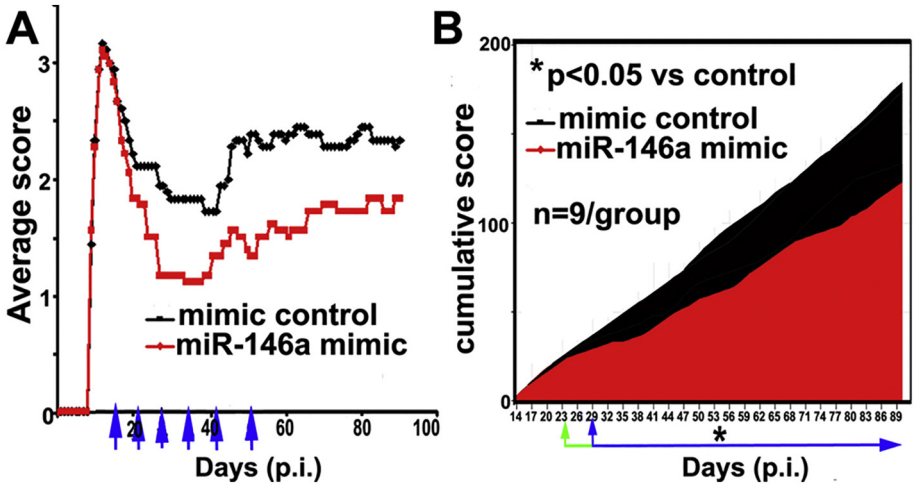


**Fig. 2.** Neurological function was measured daily up to day 90 p.i. with average score (A) and cumulative score (B) in EAE mice treated with miR-146a mimics and mimic controls ($n$ = 9 per group). Blue arrows in panel A indicate the time points of treatment (corresponding to days 14, 21, 28, 35, 42, and 49p.i.). MiR-146a mimic treatment marginally decreased the cumulative score starting at day 24p.i ($p$ = .06), and significantly decreased the cumulative score starting at day 29p.i. and persisted up to day 90p.i., as analyzed by the non-parametric Kolmogorov-Smirnov test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were measured using Western blot. The protein band intensity was quantified based on histogram analysis relative to gel loading internal control β-actin. Measurements were performed in triplicate. Results are presented as fold of mimic control.

### 2.6. Statistical analysis

Data will be evaluated for normality. Since the functional scores are not normally distributed data, we used the non-parametric Kolmogorov-Smirnov test to test the effect of the miR-146a treatment on functional recovery after EAE. Nonparametric Kolmogorov-Smirnov test was also used to test the percentage (mass) of neurological deficits from 2 treatment groups and the area between the two curves (Fig. 2). Analysis was conducted to test the treatment effect up to each follow-up day (exploratory) with a focus on up to 90 days of the follow-up (primary). Data were analyzed as cumulative score.

For morphology, qRT-PCR and Western blot analysis, significance between the two groups was examined using two-way ANOVA analysis. The data are presented as means ± SE. A value of $p < .05$ is considered significant.

## 3. Results

### 3.1. Elevation of miR-146a by exogenous mimics improves neurological functional recovery in EAE mice

We first examined the effect of miR-146a mimic treatment on neurological function outcomes in the PLP-EAE model. After $PLP_{139–151}$ immunization, EAE onset occurred around day 10p.i. MiR-146a mimic treatment was initiated at day 14p.i., the first severity peak of EAE course. This treatment window is clinically relevant and corresponds with disease progression during which demyelination in the CNS develops. Using a 0–5 disease scoring scale (Pluchino et al., 2003), we assessed neurological function daily from $PLP_{139–151}$ immunization day and up to day 90p.i., when at least one remitting-relapsing cycle has occurred ($n$ = 9 per group) (Fig. 2A). Dead mice were scored as 5 until the end of the experiment. When plotted as cumulative disease scores, miR-146a mimic treatment marginally decreased the cumulative score starting at day 24p.i ($p$ = .06), and significantly decreased the cumulative score starting at day 29p.i., which was after the third administration of miR-146a mimics, as analyzed by the non-parametric Kolmogorov-Smirnov test, since the scores were not normally distributed data (Fig. 2B). The functional improvement persisted up to day 90p.i., although the last administration of miR-146a mimics terminated at day 49p.i.. The functional analysis clearly indicates that miR-146a mimics improve neurological outcome.

### 3.2. MiR-146a level increases in the CNS of the EAE mice by exogenous mimic administration

Since stability is one of the main challenges for systemic delivery of miRNA mimics, we first determined whether administration of miR-146a mimics affects miR-146a levels in the blood and CNS of EAE animals by measuring the levels of miR-146a in blood and CNS after administration of exogenous miR-146a mimics.

The EAE mice were treated weekly with miR-146a mimics (10 mg/kg) for 2 consecutive weeks, and blood and CNS tissues were collected 24 h after the last treatment. qRT-PCR analysis revealed that intravenous administration of miR-146a mimics significantly elevated miR-146a level not only in plasma, but also in the spinal cord of the EAE mice compared to the mimic control (Fig. 3A), indicating that miR-146a mimics cross the blood-brain barrier (BBB) after systemic administration.

To further detect the possible neural cell targets of miR-146a mimics, we used miR-146a mimics fluorescently labeled with CY3 (Dharmacon) to trace exogenous miR-146a mimics in the CNS. Using OPC and microglia/macrophage cell markers, immunofluorescent analysis showed that co-localization of CY3 particles within $NG2^+$ OPCs and $IBA1^+$ microglia/macrophages, respectively (Fig. 3B). Collectively, these data indicate that exogenous miR-146a mimics cross the BBB and target OPCs and microglia in the CNS.

### 3.3. MiR-146a mimics target OPCs and promote OPC differentiation and remyelination

Since myelin is important to maintain normal function of axon, we investigated whether these exogenous mimics have an effect on OPCs and OLs after entering the CNS of EAE mice. Using the BrdU chase approach, we examined the effect of miR-146a mimics on OPC differentiation into mature OLs. Double immunofluorescent staining which identified OPC diffrentiation using the cell proliferation marker BrdU and the mature OL marker APC, revealed that the miR-146a mimic treatment significantly increased the number of $BrdU^+/APC^+$ immunoreactive cells in the spinal cords of EAE mice at day 22p.i., while the miR-146a level significantly increased at the same time, and persisted up to day 90p.i. compared to the mimic control group, respectively (Fig. 4A). Accordingly, the numbers of $APC^+$ mature OLs were significantly increased in the miR-146a mimic treatment group at day 22 and 90p.i. compared with the mimic control group, respectively (Fig. 4A). These data indicate that miR-146a increases newly generated mature OLs that were differentiated from proliferating OPCs when BrdU was injected, because mature OLs do not proliferate.

Next, we tested whether the increase of newly generated OLs

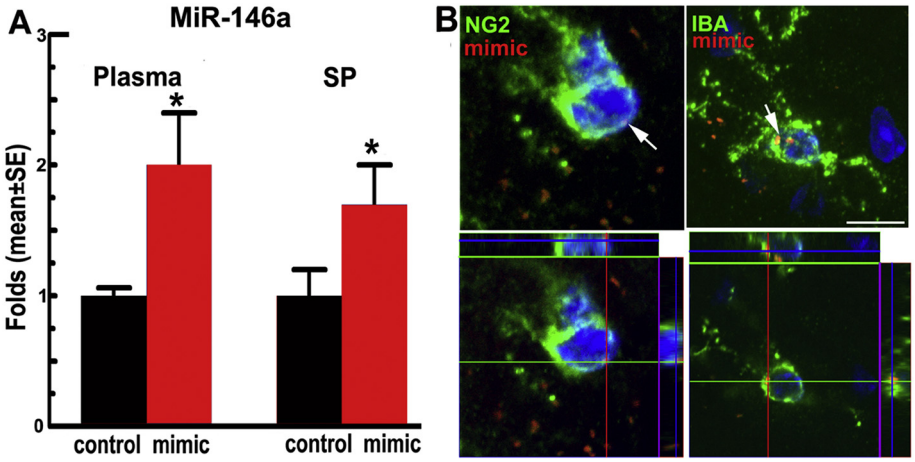


**Fig. 3.** A. RT-PCR results show that miR-146a levels significantly increased in the plasma and spinal cord at day 22p.i in the miR-146a mimic group compared to the mimic control (*$p < .05$). $n = 6$/group. B. (Top panel) Confocal images show mimic-Cy3 (red) colocalized with NG2$^+$ OPCs and IBA1$^+$ microglia (green-cytoplasm; blue-nuclear). (Low panel) 3D confocal images with orthogonal view from X, Y, and Z axis clearly show that red fluorescent signals were localized within cytoplasm of NG2 and IBA immunoreactive cells (green). Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

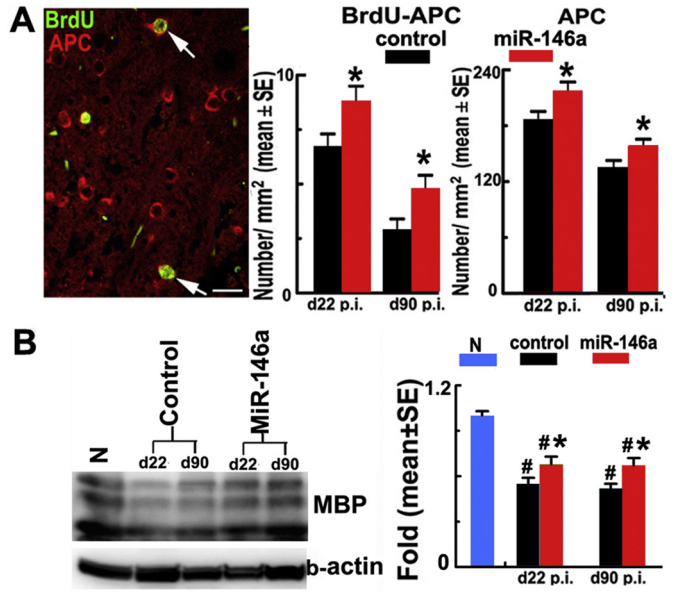


**Fig. 4.** A. Double immunofluorescent images of BrdU$^+$ (green)/APC$^+$ (red) OLs (arrows). Quantitative data show the number of BrdU$^+$/APC$^+$ OLs and APC$^+$ OLs significantly increased in the CNS of the miR-146a mimic group at day 22p.i. and day 90p.i. compared with the control. Scale bar = 25 μm. B. Western blots show that MBP level significantly decreased after EAE, however, the miR-146a mimic treatment significantly increased MBP protein levels at day 22p.i. and 90p.i. compared with the control. #$p < .01$ vs normal, *$p < .05$ vs control, $n = 6$/group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contributes to remyelination. MBP level is an index of the myelin amount. Western blot analysis of the spinal cord tissues showed that MBP protein levels significantly decreased in the EAE mice compared with the normal, however, miR-146a mimic treatment significantly increased MBP protein levels at day 22p.i. and 90p.i. compared to the mimic negative controls (Fig. 4B), which was followed by the significant functional recovery days later. Together, these data suggest that the miR-146a treatment amplifies the differentiation of OPCs into myelinating OLs and remyelination is increased in the demyelination CNS of the EAE animals. These data are consistent with our results from in vivo cuprizone model and from in vitro cell culture studies (Liu et al., 2017a; Zhang et al., 2017), suggesting that miR-146a has a direct effect on OPCs by promoting OPC differentiation into myelinating OLs, leading to enhanced remyelination and improved functional outcome of the EAE model.

### 3.4. MiR-146a mimics promote M2 polarization

In addition to OPCs, we investigated the effects of miR-146a mimics on residential innate immune cells, microglia and infiltrating innate immune cells, macrophages as well, since the tracing approach found that miR-146a mimics targeted microglia/macrophages after crossing the BBB, and the innate immune cells play important role in the processes of myelin damage and repair. The morphology of microglia is closely related to their functional state (Davis et al., 2017). In situations of neuroinflammation, “resting” microglia change their ramified morphology to “active state” with an intermediate and amoeboid forms, which are typically are small and spherical cells, but can also exhibit rod-shape or amoeboid-like (Febinger et al., 2015; Fernandez-Arjona et al., 2017). Using a M2 phenotype marker YM1, analysis of immunostaining revealed that miR-146a mimic treatment significantly increased the number of YM1$^+$ M2 cells in the spinal cord at day 22p.i. compare with the mimic controls (Fig. 5A), and Western blot confirmed that YM1 protein level in the spinal cord significantly increased, while iNOS protein levels significantly decreased at day 22p.i. and 90p.i. in the miR-146a mimic treatment group compare with the mimic controls (Fig. 5B,C). Moreover, qRT-PCR analysis of spinal cord tissues harvested at day 22p.i., further showed that the miR-146a mimic treatment significantly reduced mRNA levels of iNOS and TNFα (from M1 phenotype), but increased mRNA level of interleukin 10 (IL-10) (from M2 phenotype) (Fig. 6). These data suggest that elevation of miR-146a in the CNS increases the cell number and cytokine level of M2 phenotype microglia/macrophages, while decreases the cell number and cytokine levels of M1 phenotype microglia/macrophages under the EAE condition.

### 3.5. MiR-146a mimics reduce TLR2/IRAK1/NF-κB

We investigated the mechanisms underlying the effect of miR-146a mimics on promotion of OPC differentiation with a focus on the TLR2/IRAK1 signaling pathway which has been demonstrated to directly inhibit OPC differentiation by our and other's studies (Back et al., 2005; Liu et al., 2017a; Santra et al., 2014; Sloane et al., 2010; Zhang et al., 2017). Western blot analysis of spinal cord tissues showed that miR-146a mimics considerably reduced protein levels of TLR2, IRAK1, and the downstream target phosphate NF-κB (pNF-κB) at day 22p.i. compared to the mimic control group (Fig. 7), while OPC differentiation and MBP protein level significantly increased at the same time (Fig. 4), suggesting that elevating miR-146a levels in the CNS by administration of miR-146 mimics reduce TLR2/IRAK1 and its signaling pathway and thereby increase OPC differentiation and remyelination.

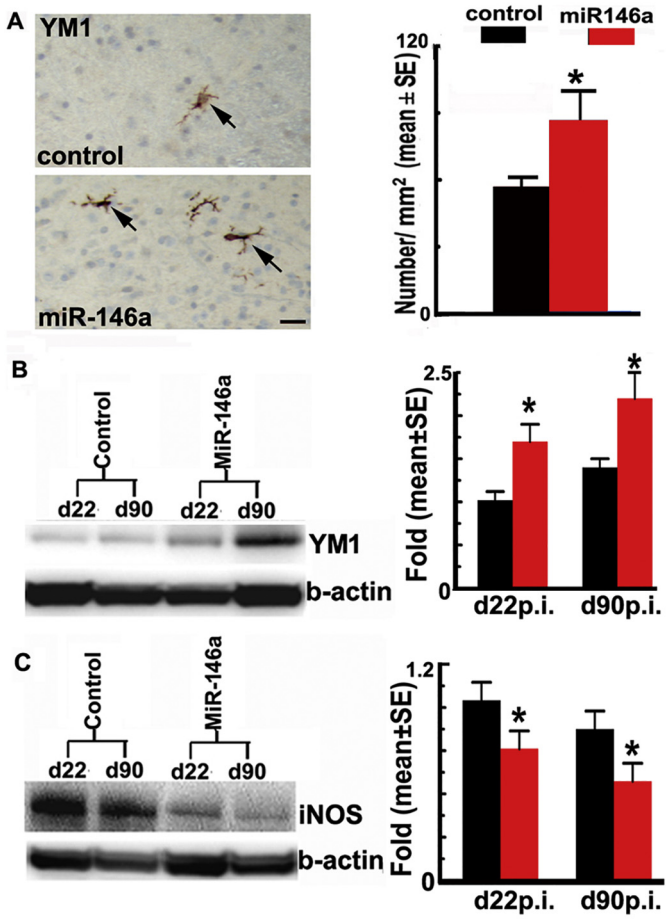


**Fig. 5.** Immunostaining analysis (A) revealed that miR-146a mimic treatment significantly increased the number of YM1$^+$ M2 cells (arrows) in the spinal cord at day 22p.i. compare with the mimic controls. Western blots (B, C) results show protein level of YM1 significantly increased at day 22p.i. and 90p.i., while protein level of iNOS significantly decreased in the spinal cord of EAE mice of the miR-146a mimic group compare with the mimic control group. *p < .05 vs the mimic control. n = 6/group. Scale bar = 25 μm.

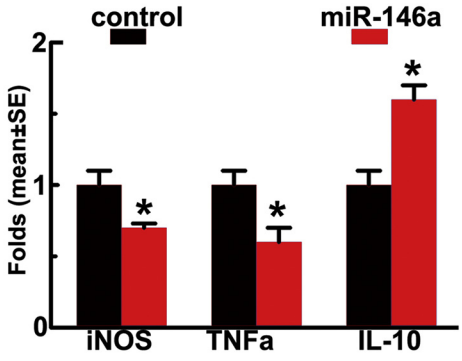


**Fig. 6.** qRT-PCR data show mRNA levels of pro-inflammatory factors iNOS and TNFa significantly decreased, and anti-inflammatory cytokine IL-10 significantly increased in the spinal cord of EAE mice treated with miR-146a mimics compared with those of the mimic controls (*p < .05). n = 6/group.

## 4. Discussion

In our previous studies, we demonstrated the effects of miR-146a on OPC differentiation and remyelination in the Cuprizone toxin-mediated demyelination model (Zhang et al., 2017) and in vitro OPC culture (Liu et al., 2017a), respectively. In the present study, we further demonstrated that miR-146a mimics significantly improve functional recovery in the EAE model. The relapsing-remitting EAE course was induced by $PLP_{139–151}$ which parallels the most common subtype of MS (Miller et al., 2010). To focus the present study on remyelination and to be more clinically relevant, the miR-146a mimic treatment was initiated at the disease peak when demyelination is fully developed in the CNS. We found that robust neurological functional improvement not only occurred during the mimic treatment periods, but also extended to weeks after termination of the mimic treatment.

In the current study, 2 time points (day 22 and 90p.i.) were selected based on functional data in which we found that compared to the control group, miR-146a mimics marginally and significantly improved functional outcomes at day 24p.i, and 29p.i., respectively. We assumed that the morphological and molecular changes precede the functional recovery, i.e., OPC differentiation and myelination which are the key factors of functional recovery; therefore, day 22p.i. was selected, to investigate the effect of miR-146a mimics on OPC differentiation and myelination as well as the potential cellular and molecular mechanisms. To examine the long term effect of miR-146a mimics on myelination, a time point of day 90p.i was selected.

We demonstrated that intravenous administration of the chemically enginered miR-146a mimics cross the BBB, and target the OPC population which is the sole cell resource of remyelination. OPC differentiation identified by newly generated mature OLs (Brdu$^+$/APC$^+$ cells) occurs in the demyelinating spinal cord as early as day 22p.i., followed by functional recovery as indicated by decreased clinical scores; however, OPC differentiation gradually decreased at day 90p.i., with a relapsing event as indicated by increased clinical scores. MiR-146a mimics were shown to further promote OPC differentiation compared with the mimic control group as early as day 22p.i. and up to at least day 90p.i. In the current study, the miR-146a treatment was initiated at the first disease peak (day 14 p.i.) when the disease has fully developed in the CNS, including demyelination damage. We demonstrated that miR-146a mimic treatment increased not only OPC differentiation, but also protein levels of MBP compared with the control group, suggesting that OPC differentiation promoted by miR-146a mimic treatment may increase remyelination. Increase of OPC differentiation and myelination were accompanied by functional recovery from day 27p.i. up to at least day 90p.i., indicating that the effects of miR-146a on OPC differentiation and remyelination contribute to functional recovery after EAE.

The cross-talk between innate immune cells and OPCs are closely related to the remyelination process (Domingues et al., 2016; Miron et al., 2013). Microglia, the resident innate immune cells in the CNS mediate local immune response in the CNS and are robustly activated in MS (Miron et al., 2013; Rawji and Yong, 2013; Zhou et al., 2015). They play an important role in de/remyelination, in conjunction with their polarization from the M1 phenotype with pro-inflammatory property to M2 phenotype with repair property (Domingues et al., 2016; Franklin and Ffrench-Constant, 2017; Liu et al., 2013; Martinez et al., 2008; Mikita et al., 2011; Miron et al., 2013; Miron and Franklin, 2014; Moore et al., 2015; Sun et al., 2017). Systemic regulation of immune cells runs risks of adverse side effects (Gao and Tsirka, 2011), and it is reasonable to target the resident immune cells within the CNS to promote recovery by stimulating endogenous OPCs. Microglia display a remarkable degree of phenotypic plasticity to response the environment, including morphology, molecular markers and cytokines (Davis et al., 2017). The polarization from M1 phenotype to M2 phenotype of microglia/macrophages is critical for OPC differentiation into mature OLs, via removal of inhibitory myelin debris and toxic products, and secretion of growth factors (Kigerl et al., 2009; Kotter et al., 2005; Mikita et al., 2011; Miron et al., 2013; Rawji and Yong, 2013; Zhang et al., 2014). In addition to OPCs, we found that exogenous miR-146a mimics target microglia/macrophages after systematic administration, and significantly promote the M2 phenotype identified by increased YM1$^+$ cell number, YM1$^+$ protein level and the related cytokine (IL-10), associated with the

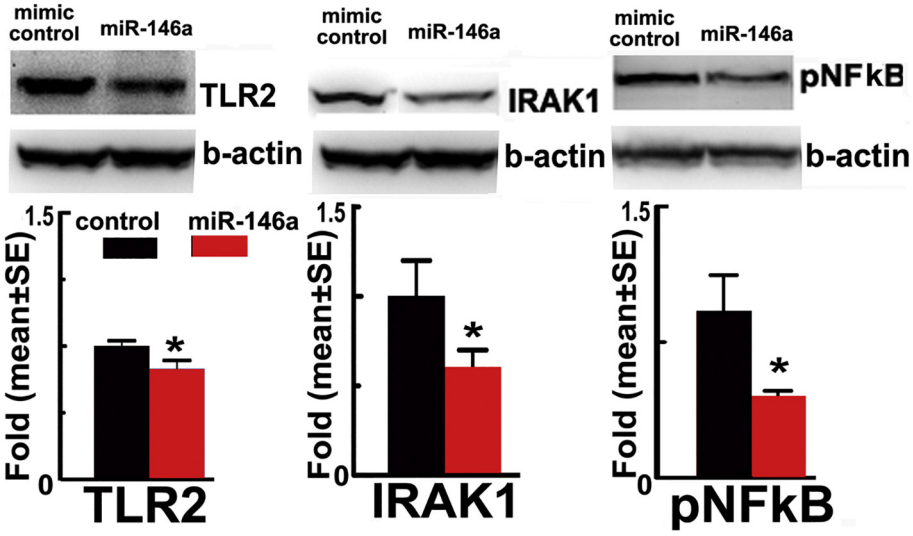


**Fig. 7.** Western blots show protein levels of TLR2, IRAK1 and pNF-κB significantly decreased in the spinal cord at day 22p.i. of miR-146a mimic group compared with the mimic control (*p < .05). n = 6/group.

decreases of pro-inflammatory M1 cytokine level (TNFa), protein and RNA levels of M1 phenotype (iNOS), implying that miR-146a mimics significantly increase M2 phenotype of microglia/macrophages in the CNS and thereby may contribute to remyelination in the EAE model.

In MS/EAE, TLR2 and its key downstream targets IRAK1 and TRAF6 are activated in the lesions, and they have been found to inhibit OPCs from differentiating into mature myelinating OLs and remyelination (Back et al., 2005; Hanafy and Sloane, 2011; Kremer et al., 2011; Liu et al., 2017a; Santra et al., 2014; Sloane et al., 2010). Using gain-off and loss-off function approaches, we and others have demonstrated that IRAK1 is a well known target of miR-146a (Chassin et al., 2012; Halkein et al., 2013; Liu et al., 2017a; Wang et al., 2013; Yang et al., 2012; Zhao et al., 2013), miR-146a may also promote remyelination which is inhibited by the TLR2 signaling. Our in vitro study revealed that elevation of miR-146a facilitates OPC differentiation in primary cultured OPCs by negative control of IRAK1 (Liu et al., 2017a), and our in vivo study further demonstrated that miR-146a mimics promote remyelination in the cuprizone model by inhibiting IRAK1 signaling (Zhang et al., 2017). However, transient elevation of miR-146a by autoimmune response may not be able to adequately modulate remyelination and benefit disease recovery. In the present study, we demonstrated that sustained elevation of miR-146a levels by administering exogenous miR-146a mimics significantly decreased protein levels of TLR2, IRAK1 and pNF-κB, and concurrently the miR-146a mimic treated group exhibited significant increases of mature OLs and MBP protein level, and subsequent improvement of functional recovery. In concert, these data from the present study and complementary data from others indicate that miR-146a promotes OPC differentiation and remyelination via the TLR2/IRAK1 signaling pathway.

We are aware of a possibly contradictory report which employs miR-146a knock-out mice and suggests that miR-146a has an adverse effect on myelination (Martin et al. 2018). This study reported that absence of miR-146a reduced inflammatory responses and demyelination in the demyelination injury model induced by cuprizone in mice in which miR-146a is deleted. We note, however, that in this study by Martin et al., the TLR/IRAK1/TRAF6 signaling pathway activity which is important for immune response was not investigated. Other studies have clearly demonstrated that mice with lack or have deletion of miR-146a are prone to spontaneous induction of hyperinflammatory disease and autoimmune disorder (Boldin et al., 2011; Yang et al., 2012), while overexpression of miR-146a produced the opposite effects (Yang et al., 2012). Thus, it may be problematic to infer from data derived from the miR-146a knock-out mouse that miR-146a has an adverse effect on myelination. Complementary data to ours are also evident in the finding in the cuprizone model that TLR2-deficient mice exhibit reduced microglial activation and oligodendrocyte loss (Esser et al., 2018), and that IRAK1-deficient mice are resistant to EAE (Deng et al., 2003). Therefore, our previous study demonstrating beneficial effects of miR-146a on myelination in the cuprizone white matter toxicity model and our current study in the EAE study are consistent with reports that elevated miR-146a level promotes OPC differentiation, reduces the innate immune response and enhances remyelination via inhibition of TLR2/IRAK1 signaling.

Collectively, this study provides the cellular and molecular bases for the potential therapeutic effects of miR-146a on OPC differentiation and remyelination. We demonstrate that miR-146a mimic treatment targets multiple cell targets, i.e. OPC and microglia/macrophages, to promote OPCs to differentiate into mature OLs, and thereafter, to enhance remyelination by inhibiting the TLR2/IRAK1 signaling pathway in the CNS. The miR-146a mimic is a novel treatment candidate of demyelination diseases, such as MS.

There are caveats in the present study. We will employ the transgenic miR-146a$^{-/-}$ mice to further confirm that miR-146a mimics rescue OPC differentiation and remyelination via the TLR2/IRAK1 signaling. Further investigations are warranted to study the direct links between miR-146a and OPC and microglia using in situ hybridization, and to analyze the TLR2/IRAK1 signaling pathway activity within the specific cell populations using laser capture method in order to harvest the desired cell population. Since MS/EAE involves multiple systems, we do not exclude potential systemic effects after i.v. injection of miR-146a mimics. Future studies to investigate the effects of miR-146a mimics on peripheral immune cells and their secondary effects on OPCs are warranted.

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