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Mir223 restrains autophagy and promotes CNS inflammation by targeting ATG16L1

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ABSTRACT

Microglia are innate immune cells in the central nervous system (CNS), that supplies neurons with key factors for executing autophagosomal/lysosomal functions. Macrouautophagy/autophagy is a cellular catabolic process that maintains cell balance in response to stress-related stimulation. Abnormal autophagy occurs with many pathologies, such as cancer, autoimmunity and neurodegenerative diseases. Hence, clarification of the mechanisms of autophagy regulation is of utmost importance. Recently, researchers presented microRNAs (miRNAs) as novel and potent modulators of autophagic activity. Here, we found that Mir223 deficiency significantly ameliorated CNS inflammation, demyelination and the clinical symptoms of experimental autoimmune encephalomyelitis (EAE) and increased resting microglia and autophagy in brain microglial cells. In contrast, the autophagy inhibitor 3-methyladenine (3-MA) aggravated the clinical symptoms of EAE in wild-type (WT) and Mir223-deficient mice. Furthermore, it was confirmed that Mir223 deficiency in mice increased the protein expression of ATG16L1 (autophagy related 16-like 1 [S. cerevisiae]) and LC3-II in bone marrow-derived macrophage mice. Furthermore, it was confirmed that Mir223 deficiency in mice increased the protein expression of ATG16L1 (autophagy related 16-like 1 [S. cerevisiae]) and LC3-II in bone marrow-derived macrophage cells compared with cells from WT mice. Indeed, the cellular level of Atg16l1 was decreased in BV2 cells upon Mir223 overexpression and increased following the introduction of antagonists. We also showed that the 3’ UTR of Atg16l1 contained functional Mir223-responsive sequences and that overexpression of ATG16L1 returned autophagy to normal levels even in the presence of Mir223 mimics. Collectively, these data indicate that Mir223 is a novel and important regulator of autophagy and that Atg16l1 is a Mir223 target in this process, which may have implications for improving our understanding of the neuroinflammatory process of EAE.

Abbreviations: 3-MA: 3-methyladenine; ACTB/β-actin: actin, beta; ATG: autophagy related; ATG16L1: autophagy related 16-like 1 (S. cerevisiae); BECN1: beclin 1, autophagy related; CNR2: cannabinoid receptor 2 (macrophage); CNS: central nervous system; CQ: chloroquine; EAE: experimental autoimmune encephalomyelitis; FOXO3: forkhead box O3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; H&E: hematoxylin and eosin; ITGAM: integrin alpha M; LPS: lipopolysaccharide; MAP1LC3/LC3: microtubule-associated protein 1 light chain 3; miRNAs: microRNAs; MS: multiple sclerosis; PPARγ: peroxisome proliferator-activated receptor gamma; PTPRC: protein tyrosine phosphatase, receptor type, C; RA: rheumatoid arthritis; SQSTM1: sequestosome 1; TB: tuberculosis; TIMM23: translocase of inner mitochondrial membrane 23; TLR: toll-like receptor.

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Introduction

EAE is the classical mouse model of human multiple sclerosis (MS), a chronic inflammatory disease that is characterized by demyelination and degeneration of axonal destruction in the CNS. Although the etiopathogenesis has not been well elucidated, a cascade of studies shows that EAE is triggered by the disruption of the blood-brain-barrier, as well as the activation of microglia and lymphocytes [1–4].

Microglia are the first line of defense in the CNS; they belong to the mononuclear phagocytic family and serve to clear pathogens in the initiation and sustenance of neuroinflammation. The functions of microglia in MS are complex and not well understood, though it has been well established
that microglia contribute significantly to the overall disease severity of MS. In fact, activated microglial nodules are the hallmark of MS lesion formation [5]. Overactive microglia are responsible for detrimental effects leading to profound neurological impairments [6], most of which occur by damage to the mitochondria.

It has been reported recently that enhancement of autophagy could ameliorate the pathogenesis of MS or EAE disease through the limitation of neuro-inflammation [7–9]. Neuro-inflammation and autophagy are 2 critical cellular processes that regulate each other to affect the progression of various diseases in the CNS. The relationship between these 2 processes is complex and includes the suppression of neuro-inflammation by autophagy. However, the signaling mechanisms that relieve this autophagy-mediated inhibition of inflammation to permit a beneficial inflammatory response remain unknown. Recent research found that regulating microglial activation by modulating mitophagy contributes to neuronal survival in Parkinson disease [10], and microglia autophagy plays an important role in the regulation of Parkinson disease [11]. In addition, inhibition of neuro-inflammation by autophagy has great benefits in ischemia [12].

Autophagy is a highly conserved catabolic pathway in the cell that degrades and recycles long-lived proteins and damaged organelles through lysosomal digestion [13]. Autophagy is active at a basal level under normal conditions, but it is rapidly upregulated following exposure to stress factors, including nutrient/hormone deprivation, hypoxic stress, accumulation of misfolded proteins and bacterial invasion [14,15]. Autophagy plays important roles in various organismal processes, such as development and aging, whereas abnormal autophagy leads to pathologies, including autoimmune and neurodegenerative diseases and cancer [16–19]. It has been reported that inducing autophagy could ameliorate several neurodegenerative diseases [20,21], and inhibit inflammation, especially in inflammatory or immune cells such as macrophages and dendritic cells [22,23].

Autophagy involves the formation of double-membrane vesicles in the cytosol called autophagosomes. These autophagosomal membranes eventually fuse with lysosomes to form autolysosomes, where they are degraded and recycled [13,24]. However, the exact assembly platform and membrane source for the generation of these initial structures are still debated. The most likely organelles to provide membranes include specialized phosphatidylinositol-3-phosphate-enriched endoplasmic reticulum domains the Golgi apparatus. There are several important protein complexes that play a key role during autophagosome formation, including the ULK1 (unc-51 like kinase 1) complex, the class III phosphatidylinositol 3-kinase complex, ATG2-WIP1 (WD repeat domain, phosphoinositide-interacting) protein complex, the ATG9 cycling system, and the ATG12–ATG5–ATG16L1 and MAP1LC3/LC3 (microtubule associated protein 1 light chain 3) or GABARAP conjugation systems [25]. Mitophagy, a classic type of cargo-specific autophagy, is the process of catabolism of mitochondria through their encapsulation by compartments termed phagophores (the precursors to autophagosomes) that are coated with the marker LC3 [26]. LC3 protein is covalently conjugated to the lipid phosphatidylethanolamine on the surface of phagophores [27]. LC3 must be cleaved by ATG4 proteins before conjugation with phosphatidylethanolamine [28,29]. While free LC3 (also called LC3-I) is soluble, its lipid-conjugated form (LC3-II) is associated with phagophores and complete autophagosomes. Hence, LC3 conjugation is used as a molecular marker of autophagosome generation and accumulation [30]. Chloroquine (CQ) is considered a lysosomotropic agent that can be trapped in lysosomes to inhibit lysosomal acidification and the activity of the degradative enzymes. Therefore, cells treated with CQ cannot undergo lysosomal digestion and exhibit autophagic vacuole accumulation [31].

The mechanisms regulating mammalian autophagy still require further investigation. Accumulating data have indicated that miRNAs play an important role in autophagy regulation [32,33]. miRNAs control biological events either by triggering the degradation of their target mRNAs through recognition of specific sequences, miRNA response elements (MREs), in the 3’ UTR, and/or through inhibition of their translation. Recent reports have provided evidence that under stress conditions, a number of miRNAs, including Mir144 [34], Mir142, Mir106, Mir93, and Mir20, are capable of modulating autophagy by changing the intracellular levels of key autophagy proteins. Mir223 plays roles in monocytes/macrophages and embryonic stem cell differentiation, osteoclast formation, and bone remodeling. Microarray analyses of miRNA expression found that Mir223 is enriched in microglia [35] and significantly upregulated in MS patients, and therefore the Mir223 expression profile is a promising diagnostic biomarker for MS [36]. mir223 knockout (mir223−/−) mice develop less severe EAE, with increased myeloid-derived suppressor cell numbers in the spleen and spinal cord [37].

However, whether autophagy in microglia plays a role in the mir223−/−–mediated alleviation of neuro-inflammation remains unclear. Here, we discovered that Mir223 deficiency significantly ameliorated CNS inflammation, demyelination and the clinical symptoms of EAE and increased the number of resting microglia and the amount of autophagy in brain microglial cells. In contrast, the autophagy inhibitor 3-MA aggravated the clinical symptoms of EAE in WT and mir223−/− mice. Furthermore, we discovered that Mir223 blocked starvation- and lipopolysaccharide (LPS)-induced autophagy in microglial BV2 cell lines. In light of our results, we provide evidence that a key autophagy protein, ATG16L1, is an important and direct autophagy-related target of Mir223.

**Results**

**Mir223 deficiency suppresses pathogenic CNS inflammation and demyelination during EAE progression in mice**

Previously, several studies demonstrated that certain miRNAs play an important role in the regulation of autoimmunity. One report found that Mir223 regulates EAE though myeloid-derived suppressor cells. We wanted to investigate
whether endogenous Mir223 levels affected the clinical symptoms of EAE in C57BL/6 mice immunized with the MOG[35–55] peptide as well as the mechanism involved. Interestingly, knockout of Mir223 (mir223⁻/⁻) significantly ameliorated the clinical symptoms of EAE compared with wild-type C57BL/6 mice (Figure 1A, B and C). In addition, disease severity, as assessed by the maximal and cumulative clinical score, was significantly decreased in mir223⁻/⁻ mice (Figure 1D and E). These data suggest that endogenous Mir223 levels affect the clinical outcome of EAE.

To characterize disease progression at the level of CNS injury, we performed a histological analysis of the lumbar spinal cord on day 15 post-immunization. At the peak of the acute phase of EAE, hematoxylin and eosin (H&E) and luxol fast blue staining showed that there was less infiltration of mononuclear cells and decreased demyelination in the lumbar spinal cords of mir223⁻/⁻ mice compared with wild-type mice (Figure 1F and G). Thus, Mir223 deficiency improves the pathological and clinical symptoms of EAE.
Mir223 deficiency augments autophagy and resting microglia in EAE mice

Microglial cells play important roles in the regulation of EAE and MS. We assessed whether Mir223 affects microglia in the CNS of EAE mice. Deficiency of Mir223 decreased the infiltration of ITGAM⁺ and PTPRC⁺ cells and especially increased the PTPRC<sup>low</sup> and decreased the PTPRC<sup>hi</sup> cells. Overactive microglia can lead to profound neurological impairment. Here, we found that Mir223 deficiency reduced the number of active microglia and macrophages (ITGAM⁺ PTPRC<sup>hi</sup>) but increased the number of resting microglia (ITGAM⁺ PTPRC<sup>low</sup>). There was also an augmentation of the ratio of the resting microglia vs the active microglia and macrophages in mir223<sup>−/−</sup> mice (Figure 2A and B).

There were considerably higher levels of LC3 protein in the microglia in the brains of mir223<sup>−/−</sup> mice than in wild-type mouse controls in the acute phase of EAE (Figure 2C). In contrast, BCL2 and BECN1/Beclin1 expression did not differ between the WT and mir223<sup>−/−</sup> mice (Figure 2D and E). It is possible that Mir223 may regulate microglia autophagy though pathways other than BCL2 and BECN1, which remain unclear.

3-MA-mediated blockade of autophagy attenuates the protective effects of Mir223 on EAE mice

To determine the influence of autophagy on the effects of Mir223 on the progression of MS in vivo, we examined neurobehavioral deficits in 3-MA- and vehicle-treated mice following MOG<sup>[35–55]</sup>-mediated induction of EAE. As shown in Figure 3, the disease course in the EAE mouse model was a chronic progressive-relapsing phenotype. Mir223 deficiency significantly reduced the severity of neurobehavioral deficits, cumulative scores, and maximum neurological disability in EAE mice compared with WT mice. The effect of Mir223 was abolished by 3-MA (10 mg/kg), an autophagy inhibitor (Figure 3A-3F), suggesting that Mir223 plays a role in the progression of EAE at least partly through its effects on autophagy.

Mir223 deficiency promotes autophagy in microphages and BV2 microglial cells stimulated with LPS

Knockout of Mir223 increased autophagy in microglia and resting microglia in the CNS during EAE progression. We differentiated macrophage cells from the bone marrow cells of wild-type C57BL/6 mice or mir223<sup>−/−</sup> mice in vitro to confirm that Mir223 blocks autophagy in microglial cells upon LPS or starvation treatment. LC3 protein expression was increased in bone marrow-derived macrophage cells after stimulation with LPS or starvation, and ATG16L1 protein was mostly increased in mir223<sup>−/−</sup> mouse macrophages compared with those from wild-type mice (Figure 4A). Moreover, a Mir223 inhibitor induced the production of autophagosomes and autolysosomes upon LPS stimulation in microglial BV2 cells (Figure 4B and C). These data indicate that endogenous Mir223 can affect autophagy through the ATG16L1-LC3 pathway during EAE progression.

Mir223 blocks LPS-induced autophagy in BV2 cells

Recent advances have revealed a relationship between autophagy and innate immunity. Autophagy plays a crucial role in sustaining the balance between the beneficial and detrimental effects of immunity and inflammation [38]. It has been reported that LPS induces a non-typical autophagy pathway in BV2 microglia; however, the activation of the typical autophagy pathway using rapamycin inhibits the expression of NOS2 (nitric oxide synthase 2, inducible), IL6 (interleukin 6), and the cell death of microglia exposed to LPS [39]. Autophagy defects, resulting from nutrient deprivation or dysfunction of the autophagy-related proteins, may increase the degree of microglial activation and inflammation.

We then evaluated whether Mir223 overexpression had an effect on LPS-induced autophagy. Past studies have suggested a link between toll-like receptor (TLR) signaling and autophagy in macrophages and other cell types via activation of downstream signaling pathways. TLR4 responds specifically to LPS stimulation. The autophagosome emanates from this signaling platform, and the membrane source for the generation of these initial structures is still debated. The most likely organelles to provide membranes include specialized phosphatidylinositol-3-phosphate-enriched endoplasmic reticulum domains and the Golgi apparatus. Here, we used the mitochondrial proteins TIMM23 and SQSTM1 (sequestosome 1) as a readout for autophagy. LPS-induced GFP-LC3 accumulation (Figure 5A and B), LC3 lipidation (Figure 5C) and TIMM23 degradation (Figure 5D) were decreased following Mir223 overexpression. However, the expression of SQSTM1 did not cause any changes in BV2 cells during LPS stimulation. Cheng et al. found that MIR181A suppresses mitochondrial uncoupler-induced degradation of TIMM23 in SH-SY5Y human neuroblastoma cells, whereas SQSTM1 is not a reliable marker [40], and our results are consistent with these findings. Therefore, we concluded that Mir223 inhibited LPS-induced autophagic activity in BV2 cells.

To confirm that Mir223 inhibited autophagy-induced LC3 turnover and TIMM23 degradation, we performed similar autophagy assays in the presence or absence of the lysosomal inhibitor CQ. Indeed, addition of CQ led to the prominent accumulation of LC3-II and the degradation of TIMM23 in miR-CN-transfected cells upon LPS treatment, indicating the presence of normal autophagic flux under these conditions. However, because Mir223 blocked autophagic vesicle generation, this inhibition-related accumulation of LC3-II and degradation of TIMM23 during LPS treatment was relatively decreased in BV2 cells overexpressing Mir223 compared with mimic-expressing controls (Figure 5E and F).

Mir223 blocked starvation-induced autophagy in BV2 cells

To determine the role of Mir223 in autophagy in microglia, we withdrew nutrition from BV2 cells transfected with Mir223 mimics or mimic controls (Mim-CN) for 4 h to assess changes in autophagy-related genes and proteins. As shown in Fig. S1A and S1B, overexpression of Mir223 significantly blocked starvation-induced GFP-LC3 accumulation. In line with these results, in cell extracts, starvation-activated lipid...
Figure 2. Mir223 deficiency increased autophagy and resting microglia in the brains of EAE mice. (A) Flow cytometric analysis of microglia, demonstrating PTPRC and ITGAM cells isolated from the CNS of EAE mice (n = 6 mice per group), detected on the 15th day after the induction of EAE. The data are shown in a representative plot. (B) The absolute numbers of the cell subpopulations are shown; the black column is for mir223−/− mice. Rest, ITGAM+ PTPRC−; mc, ITGAM+ PTPRChi and low. (C) Autophagy was measured in the brains of the mice. LC3 puncta were visualized by confocal imaging of microglia immunostained for LC3 and AIF1, followed by Alexa Fluor 488/555-conjugated secondary antibodies (green/red); nuclei were stained with DAPI. Representative images are shown. Scale bars: 20 µm. (D) and (E) BCL2 and BECN1 expression were visualized by fluorescence imaging of microglia. Scale bars: 50 µm.
conjugation of free LC3-I to the phagophore membrane to generate LC3-II was attenuated following transfection with Mir223 mimics (Fig. S1C). Furthermore, degradation of the mitochondrial inner membrane protein TIMM23 following starvation was decreased in Mir223-transfected cell extracts (Fig. S1D). These results indicate that Mir223 is a novel miRNA that controls autophagy.

To confirm that Mir223 inhibition of autophagy-induced LC3 turnover and TIMM23 degradation also inhibited autophagic vesicle flux, we performed similar autophagy assays in the presence or absence of CQ. Indeed, addition of CQ led to the prominent accumulation of LC3-II and degradation of TIMM23 in miR-CN-transfected cells, pointing to the presence of normal autophagic flux under these conditions. However, because Mir223 blocked autophagic vesicle generation, inhibition-related accumulation of LC3-II and degradation of TIMM23 during starvation were relatively lower in BV2 cells overexpressing Mir223 compared with mimic controls (Fig. S1E and S1F).

**Inhibition of endogenous Mir223 increases autophagy upon LPS stimulation**

We then tested whether endogenous Mir223 inhibition also had an effect on LPS-induced autophagy. We observed that the formation of LPS-dependent GFP-LC3 puncta was significantly increased (Figure 6A and B). Furthermore LC3-I to LC3-II conversion was stimulated (Figure 6C), and therefore, autophagy was accelerated in cells transfected with Mir223-inhibiting constructs compared with control antagonirs. Moreover, TIMM23 was degraded following the inhibition of endogenous Mir223 upon LPS stimulation compared with controls (Figure 6D). Therefore, inhibition of endogenous
Mir223 using antagomirs led to the further stimulation of autophagic activity during LPS stimulation, indicating that endogenous Mir223 contributes to the limitation of the LPS-activated autophagic response in BV2 cells.

To confirm that inhibition of Mir223 increased autophagy-induced LC3 turnover and TIMM23 degradation, we performed similar autophagy assays in the presence or absence of CQ. Consistent with the above results, accumulation of LC3-II and degradation of TIMM23 during LPS stimulation was relatively higher in BV2 cells transfected with Mir223 inhibitor compared with cells transfected with inhibitor controls (Figure 6E and F). Hence, attenuation of autophagic activity through inhibition of Mir223 led to increased GFP-LC3 accumulation in BV2 cells when endogenous Mir223 was blocked.

**Inhibition of endogenous Mir223 increases autophagy upon starvation**

Antagomirs, or anti-miRNAs, counteract miRNA effects by specifically inhibiting endogenous mature miRNAs. To test the effects of endogenous Mir223 inhibition on autophagy, we transfected cells with Mir223-specific antagomirs (Inhibitor) or control antagomirs (In-CN) and analyzed autophagy under conditions of starvation or no starvation. We observed that the starvation-dependent formation of GFP-LC3 puncta was modestly but significantly increased (Fig. S2A and S2B) and LC3-I to LC3-II conversion was stimulated (Fig. S2C). Hence, autophagy was accelerated in cells transfected with Mir223 inhibitor but not with control antagomirs. Moreover, TIMM23 degradation following starvation was more prominent after inhibition of Mir223 compared with inhibitor controls (Fig. S2D). Therefore, inhibition of endogenous Mir223 using antagomirs led to the further stimulation of autophagic activity during starvation, indicating that endogenous Mir223 contributes to the limitation of stress-activated autophagic responses in microglial cells.

To confirm that inhibition of Mir223 increased autophagy-induced LC3 turnover and TIMM23 degradation, we performed similar autophagy assays in the presence or absence of CQ. However, because inhibition of Mir223 improved autophagic vesicle generation, accumulation of LC3-II and degradation of TIMM23 during starvation was relatively higher in BV2 cells transfected with Mir223 inhibitor compared with those transfected with inhibitor controls (Fig. S2E and S2F). Hence, attenuation of autophagic activity through Mir223 inhibition led to increased GFP-LC3 accumulation in BV2 cells in which endogenous Mir223 was blocked.

**The autophagy-related Atg16l1 gene is a direct target of Mir223**

To determine the mechanism of Mir223-mediated autophagy inhibition, we searched for autophagy-related genes containing potential Mir223 response elements in their 3’ UTRs using the publicly available bioinformatics tools microRNA and miRTarBase. Atg16l1 (GenBank accession number: NM_001205391.1, 138–163) was identified as a Mir223 target by both bioinformatics tools. The predicted interaction between Mir223 and the Atg16l1 3’ UTR is shown in Figure 7A.

To further validate the effect of Mir223 on Atg16l1, the region in the 3’ UTR of the Atg16l1 mRNA containing a potential Mir223 response element was cloned into the 3’ UTR of a luciferase vector. In parallel, we also created a
mutant version of this construct by introducing base changes to the crucial binding residues, primarily in the Mir223 seed sequence-binding region (Figure 7B). Cotransfection of Mir223 mimics together with the wild-type luciferase vector in 293T cells resulted in a significant decrease in luciferase activity compared with control levels (Figure 7C, target). In contrast, Mir223 had no significant effect on the levels of luciferase activity compared to the mutant construct; here, the luciferase activity was similar to control levels (Figure 7C, mutant). These results showed that Mir223 controlled the levels of ATG16L1 by directly targeting the above-described MRE in the 3’ UTR region of the Atg16l1 gene.

The expression of Mir223 was decreased by qPCR in BV2 cells following transfection with Mir223 inhibitor but not with In-CN in the presence or absence of LPS stimulation (Figure 7E). To confirm the bioinformatics-based predictions, we performed immunoblot assays of control- or Mir223 mimic-transfected cell extracts using an ATG16L1-specific antibody. Indeed, ATG16L1 protein levels were decreased in BV2 cells overexpressing Mir223 under starvation conditions (Figure 7F). Conversely, introduction of the Mir223 antagonir, but not control antagonirs, resulted in an increase in ATG16L1 protein levels in BV2 cells during starvation (Figure 7G). Mir223 expression also affected target transcript levels in cells. An increase in Atg16l1 mRNA levels was observed by qPCR in BV2 cells following transfection with Mir223 inhibitor but not with In-CN (Figure 7H).

Overexpression of ATG16L1 abrogated mir223-mediated suppression of autophagy

To further confirm that downregulation of ATG16L1 was responsible for the autophagy-related effects of Mir223, we performed ‘rescue experiments’. In these experiments, Atg16l1 was overexpressed by a plasmid lacking a Mir223 binding site; it was therefore resistant to Mir223-mediated downregulation of Atg16l1. Hence, although Mir223 overexpression decreased endogenous ATG16L1 protein levels significantly, cells cotransfected with the Mir223 mimics and an Atg16l1 plasmid lacking the Mir223 binding site possessed near-physiological levels of total ATG16L1 protein (Figure 8A and B). Under these conditions, the Mir223-mediated suppression of

Figure 5. Overexpression of Mir223 blocks lipopolysaccharide-induced autophagy in BV2 cells. (A) Mir223 repressed the formation of starvation-induced GFP-LC3 puncta in BV2 cells. Stable GFP-LC3-expressing cells were cotransfected with mimics or Mir-CN, and autophagy was evaluated after LPS or control treatment for 4 h. Scale bar: 5 μm. (B) Quantitative analysis of the number of GFP-LC3 puncta per cell in (A). The data are presented as the means± SEM; experiments were performed in triplicate (*p < 0.05, **p < 0.01; NS, not significant). (C) and (D) Western blot analysis of LC3, TIMM23, SQSTM1 and ACTB. (E) and (F) Autophagy was evaluated in the presence or absence of CQ with LPS or no LPS treatment for 4 h. Western blot analysis of LC3, TIMM23, SQSTM1 and ACTB. Densitometric ratios were quantified using ImageJ software. LC3-II:ACTB and TIMM23:ACTB ratios from immunoblots are shown from 3 independent experiments. ACTB was used as the loading control.
autophagy observed during starvation-induced autophagy was reversed upon co-expression of the ATG16L1 protein (Figure 8C and D). In other words, introduction of the ATG16L1 protein was sufficient to return autophagy back to normal levels even in the presence of the Mir223 mimics. These results demonstrated that Atg16l1 was the target of Mir223 for the suppression of autophagy.

Mir223 regulation of autophagy is independent of the BCL2 and PPARG pathways

The antiapoptotic protein BCL2 is involved in autophagy regulation through sequestration of the autophagy-related protein BECN1. In our study, inhibition or overexpression of Mir223 did not significantly affect the levels of BCL2 protein under LPS stimulation conditions in BV2 cells (Fig. S3A and S3B). Therefore, we propose that autophagy regulation by Mir223 did not proceed through BCL2. We also examined whether there are other autophagy-related targets of Mir223. Because Mir223 can regulate IFNG (interferon gamma) by directly targeting PPARG (peroxisome proliferator activated receptor gamma), and PPARG plays an important role in the activation of macrophage cells and in neuronal autophagy during cerebral ischemia-reperfusion injury[41], we wondered whether the PPARG pathway would be affected by Mir223 overexpression. To assess the effect of Mir223 on PPARG, we analyzed the protein levels of PPARG. Although under basal/fed conditions, inhibition or overexpression of Mir223 did not have a differential effect on PPARG expression between cells transfected with Mir223 inhibitor or mimics and control cells, PPARG expression vanished following autophagy activation via LPS stimulation (Fig. S4). Therefore, we concluded that although it is possible that other targets of Mir223 could contribute to its biological effects, ATG16L1 appears to be the major and rate-limiting autophagy-related target of Mir223.

Discussion

In this study, we introduced Mir223 as a novel autophagy regulator. Mir223 deficiency significantly reduced the severity of neurobehavioral deficits, cumulative scores, and maximum
neurological disability in EAE mice compared with WT mice. The effect of Mir223 was abolished by the autophagy inhibitor 3-MA, suggesting that Mir223 plays a role in EAE progression at least partly through autophagy. Here, we found mir223−/− reduced the number of active microglia and macrophages and simultaneously increased the number of resting microglia. Moreover, there were considerably higher levels of LC3 protein in microglia in the brains of mir223−/− mice than in wild-type controls during the acute phase of EAE. Overexpression of Mir223 inhibited GFP-LC3 accumulation, LC3-I to LC3-II conversion and TIMM23 degradation in BV2 cells. These results were validated by stimulation of BV2 cell autophagy upon starvation and LPS treatment. Additionally, antagonist-mediated suppression of endogenous cellular Mir223 accelerated the autophagic response. Together, these data showed that Mir223 is a key player in controlling autophagy. We identified ATG16L1 as a target of Mir223 through miRNA gene blast and luciferase reporter analysis of the MRE found in the 3′ UTR region of the Atg16l1 gene. The results we obtained in this study may be summarized in a mechanistic model as follows (Figure 8E): A certain portion of cellular ATG16L1 protein can form functional ATG12–ATG5–ATG16L1 complexes, followed by LC3 lipidation and autophagy activation. The presence of Mir223 under stimulation leads to the downregulation of ATG16L1 protein levels below the threshold and therefore results in a decrease in LC3 lipidation and inhibition of autophagic activity. Thus, inhibiting Mir223 can regulate autophagy and improve/limit uncontrolled or potentially harmful autophagic activity in cells.

An increasing number of studies have revealed novel knowledge regarding autophagy, and our understanding of autophagy in autoimmune inflammation is rapidly being updated. It is principally thought that autophagy plays an important role in protecting neurocytes from inflammation. BCL2 and BECN1 are important for controlling cellular autophagy in response to environmental stress or treatment with BH3 peptidomimetics. In this study, we found that Mir223 did not affect BCL2 and BECN1 expression during EAE progression or in BV2 cells upon LPS stimulation. These results suggested that Mir223 regulates autophagy through other pathways.

Multiple genetic studies have implicated the autophagy-related gene Atg16l1 in this regulatory process, and luciferase reporter assays have indicated that MIR106B and MIR93 target ATG16L1 mRNA [42]. Zhai et al. reported miRNA-mediated regulation of ATG16L1 in colonic epithelial cells as well as Jurkat T cells. Dual-luciferase reporter assays found that MIR142 is a key miRNA that regulates ATG16L1 by targeting the 3′ UTR. The study revealed that MIR142 is a new autophagy-regulating small molecule that acts by targeting ATG16L1, indicating a role of MIR142 in intestinal inflammation and Crohn disease[43]. Other researchers have found that ATG16L1 is also a target gene regulated by Mir20a [44,45]. Bacillus Calmette-Guérin infection of macrophages
results in enhanced expression of Mir20a, which decreases the expression levels of LC3-II and the number of LC3 puncta in macrophages. In addition, Mir20a promotes Bacillus Calmette-Guérin survival in macrophages, whereas transfection with a Mir20a inhibitor has the opposite effect. Moreover, transfection with a Mir20a mimic leads to a significant reduction in the number of autophagosomes per cellular cross-section, but transfection with a Mir20a inhibitor increases the number of autophagosomes per cellular cross-section [46]. In ischemic kidney injury and HK-2 cells under hypoxic conditions, the expression of punctate LC3 and ATG16L1 is increased and Mir20a expression is decreased. The 3′ UTR luciferase reporter assays indicate that Mir20a targets Atg16l1 mRNA. Overexpression of Mir20a and antagoMir20a also confirm these results [44]. In osteoclasts, a dual-luciferase reporter assay revealed that Mir20a directly targets Atg16l1 by binding to its 3′ UTR [45].

We identified ATG16L1 as a target of Mir223 through miRNA gene blast and luciferase reporter analysis of the MRE found in the 3′ UTR region of the Atg16l1 gene, and we found that introduction of mutations to this sequence abolished the effect of Mir223. We showed that Mir223 overexpression attenuated ATG16L1 protein and mRNA levels, and more importantly, reintroduction of ATG16L1 protein in the presence of Mir223 mimics blocked autophagy. Therefore, our results established ATG16L1 as an important target of Mir223 during autophagy regulation. Whether autophagy inhibits or enhances inflammatory cell infiltration depends on the related signaling pathways targeted by different stimulators and cells. Moreover, further studies are needed to reveal the role of autophagy during autoimmune inflammatory reactions.

Mir223 was previously shown to be involved in various biological processes, such as development, differentiation, hematopoiesis, and immune modulation. In fact, autophagy has also been described as an important regulator of similar physiological events [47–50]. Study of the effects of autophagy on these processes could provide valuable information about the importance of Mir223. Mir223 was upregulated in the blood and lung parenchyma of tuberculosis (TB) patients and in TB-infected mice. Deletion of Mir223 renders TB-resistant mice highly susceptible to acute lung infection [51]. The apoptosis rate of peripheral blood macrophages is decreased in active TB patients compared with healthy controls. Transfection of human macrophages (TDMs and MDMs) with MIR223 inhibits macrophage apoptosis. Moreover, researchers found that MIR223 directly suppresses FOXO3 (forkhead box O3), and FOXO3 plays a critical role as a mediator of the biological effects of MIR223 in macrophage apoptosis [52]. In macrophages, MIR223 may regulate apoptosis by directly targeting FOXO3. Activation of CNR2 (cannabinoid receptor 2, [macrophage]) can ameliorate the pathogenesis of EAE, whereas deleting Cnr2 decreases LC3-II/LC3-I and BECN1 expression and increases CASP1 (caspase 1) activation and IL1B production in EAE mice. In contrast activation of CNR2 with the specific agonist HU-308 induces inverse effects. At the same time, one study reported that HU-
expression was increased in the spleen and CNS during EAE progression (unpublished data). At the same time, autophagy was repressed in brain microglial cells, whereas Mir223 deficiency increased LC3 expression and ameliorated EAE pathogenesis.

Some researchers have identified an increase in autophagy during the progression of autoimmune disease. Systemic lupus erythematosus is an autoimmune disease, and overexpression of TLR7 has been found to be associated with this disease. We ablated autophagy in Tlr7-transgenic (Tg) mice, and, in the absence of autophagy, these mice were cured of lupus. This was also supported by the survival of autophagy-deficient mice compared to the Tg(Tlr7)1Boll/Tlr7.1 Tg mice [54]. Rheumatoid arthritis (RA) is a kind of autoimmune disease that involves dysregulation of CD4+ T cells. One report demonstrated that autophagy is significantly increased in CD4+ T cells from RA patients and that increased autophagy is also observed in activated CD4+ T cells. Increased resistance to apoptosis is also observed in CD4+ T cells from RA patients. These results provide novel insight into the connection between the pathogenesis of RA and autophagy [55].

Autophagy deficiency is associated with many diseases. Therefore, autophagy has been proposed as a cell-protective mechanism. Similarly, we observed that inhibition of Mir223 can ameliorate the pathogenesis of EAE by increasing autophagy in brain microglial cells. Further research also showed that intraperitoneal injection of 3-MA in mice can aggravate the pathogenesis of EAE. Therefore, changes in the Mir223 level might modulate autophagy and affect the clinical pathogenesis of EAE. Further studies are required to explore the contribution of Mir223 expression to other autoimmune diseases regulated by macrophages.

Materials and methods

Animal experiments

Female C57BL/6 mice aged 7–8 weeks were purchased from the Academy of Military Medical Science (Beijing, China). mir223+/− mice were kindly provided by Fei Gao’s lab (Institute of Zoology, Chinese Academy of Sciences, Beijing), which is a global knockout. The Mir223 gene is located on the X chromosome. The animals were housed and fed in a specific pathogen-free animal facility at the Experimental Animal Center of Tianjin Medical University (Tianjin, China). The experiments were performed in accordance with the guidelines for animal care and were approved by the Animal Ethics Committee of Tianjin Medical University (Tianjin, China).

For EAE induction, the C57BL/6 female mice were immunized (subcutaneously) with 200 µg of myelin oligodendrocyte glycoprotein (MOG residues 35–55). The peptide sequence was Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-ArgVal-Val-His-Leu-Tyr-Arg- Asn-Gly-Lys, and the purity was >95% (CL Bio-Scientific Co., Ltd.). The immunization was performed by mixing the MOG[35–55] peptide with complete Freund’s adjuvant containing 500 µg of heat-killed H37Ra, a Mycobacterium tuberculosis strain (Difco Laboratories, 231,141). Pertussis toxin (200 ng) (List Biological Laboratories, 180) in PBS (BBI Life Sciences, A610100-0001) was administered intraperitoneally on the day of immunization and again after 48 h. For inhibition of autophagy, mice were treated with 3-MA (10 mg/kg, Sigma, M9281) daily. The mice were weighed and examined daily for disease symptoms, which were assessed using the following standard score system: 0, no obvious changes in motor functions; 1.0, limp tail; 2.0, limp tail and wobbly gait; 3.0, bilateral hind limb paralysis; 4.0, complete hind limb and partial forelimb paralysis; and 5.0, death [56].

Histopathology and immunohistochemistry

The lumbar spinal cords from the female C57BL/6 mice and mir223+/- mice (n = 6) were perfused transcardially with 4% (weight:volume) paraformaldehyde and were then dissected and post-fixed for 48 h. The spinal cord paraffin sections (7 µm) were stained with H&E to detect inflammatory cell infiltration and with luxol fast blue (Alfa Aesar, 1328–51-4) to assess demyelination.

The brains from the wild-type C57BL/6 mice and mir223+/- mice (n = 6) were perfused transcardially with 4% (weight:volume) paraformaldehyde and then were dissected and post-fixed for 48 h. The brain paraffin sections (7 µm) were stained with DAPI (Thermo Fisher Scientific, 00–4959–52), LC3A/B (Cell Signaling Technology, 12,741), BCL2 (Abcam, ab32124), BECN1 (Cell Signaling Technology, 3495), AIF1/IBA1 (allograft inflammatory factor 1) (Abcam, ab107159) and Alexa Fluor 488 (Proteintech, SA00006-2) and Alexa Fluor 546 (Thermo Fisher Scientific, A11056) secondary antibody conjugates (green and red, respectively).

Intracellular cytokine staining

The single-cell suspensions were isolated from the brain and spinal cords of WT or mir223+/- EAE mice by grinding and filtering the tissue through a cell strainer. Then, the macrophages and microglial cells were separated by Percoll (GE Healthcare Bio-Sciences AB, 17–0891–09) gradient centrifugation at 630g for 25 min. The cell surface markers were assessed with APC-conjugated anti-mouse PTPRC antibody (eBioscience, 17–0451–82) and PE-conjugated anti-mouse ITGAM (eBioscience, 12–0112–82). Nonspecific staining was monitored with isotype antibody controls PE IgG2b (eBioscience, 12–4031–82) and APC IgG2b (Sungene biotech, R20022-11A). The cells were analyzed by BD FACS Canto II (BD Biosciences, USA), and the acquired data were analyzed using FlowJo software.

Separation of macrophages from bone marrow

 Femurs and tibias, removed from wild-type C57BL/6 mice and mir223+/- mice immediately after they were sacrificed, were cleaned of muscle tissues, placed in a petri dish with 70% ethanol for 2–3 min and washed twice in sterile culture medium. Bone ends were cut, and the marrow was flushed out into a new petri dish with 10 ml of RPMI (Gibco, 31,800–022). Cells
were then washed and resuspended in RPMI supplemented with 10% fetal bovine serum (FBS; Gibco, 10,099,141) and 10 ng/ml CSF1/M-CSF (Peprotech, 315–02). After an adhesion step of 5 days on a plastic dish at 37°C and 5% CO₂, the cells were stimulated with LPS (Sigma, L4516) or starvation for 4 h.

**Cell culture and treatment**

The mouse microglial cell line BV2 was obtained from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). The cell line was grown in Dulbecco's modified Eagle's medium (Gibco, 12,800–017), supplemented with 10% FBS, and 10 U/ml penicillin-streptomycin (Gibco, 15,140–122). The cells were cultured in a 5% CO₂ humidified incubator at 37°C. LPS and hydroxychloroquine (CQ; Sigma, H0915) were dissolved in DMEM and dimethyl sulfoxide (Solarbio, D8370), separately. To induce autophagy, cells were treated with starvation or LPS for 4 h.

**Transmission electron microscopy**

The cells were fixed with ice cold 2.5% glutaraldehyde (pH 7.4). After being washed 3 times with 0.1 M phosphate buffer, pH 7.4, the cells were fixed with 1% osmium tetroxide for 1 h. The samples were dehydrated in a graded series of ethanol concentrations and embedded in Spurr resin (TED Pella, 18,300–4221). The ultrathin sections were cut to a thickness of 70 nm and then double-stained with uranyl acetate and lead citrate. The number of autophagic vacuoles was determined for a minimum of 100 cells. The samples were examined and photographed with a JEM-1230 transmission electron microscope (JEOL, Japan) by FCEN Company (Shanghai Fucheng Biological Technology Co., Ltd., China).

**Target prediction for miRNA**

miRNA targets were identified using the publicly available bioinformatics tools microRNA (http://www.microrna.org/microrna/home.do) and miRTarBase (http://mirtarbase.mbc.ntcu.edu.tw/).

**MicroRNA, siRNA, plasmid and transfection**

*Mir*223 mimics, control mimics (Mim-CN), *Mir*223 inhibitor (a *Mir*223-specific antagonist), and control inhibitor (In-CN) were synthesized by Ribobio (Guangzhou, China). For the dual-luciferase assay, the MRE found in the 3’UTR region of the luciferase 1-AP), anti-ACTB/β-actin (Vazyme, Ab101-01), anti-BCL2 (Abcam, ab32124) and anti-PPARG (Proteintech, 22,061-00) were dissolved in DMEM and dimethyl sulfoxide (Solarbio, D8370), separately. To induce autophagy, cells were treated with starvation or LPS for 4 h.

**GFP-LC3 analyses**

Forty-eight h after cotransfection of *Mir*223 mimics, Mim-CN, inhibitor, In-CN and GFP-LC3 (HANBio, China), BV2 cells were incubated for 4 h in EBSS medium or in DMEM-FBS containing 500 ng/ml LPS for 4 h. Cells were fixed in 4% formaldehyde for 10 min, washed with PBS, mounted and inspected under 100 × magnification using a BX60 fluorescence microscope (Olympus, Germany), followed by image analysis with ImageJ software (National Institutes of Health, Bethesda, MD, USA) after quantifying pixel (200 pixels) intensities of both cellular compartments and subtracting background fluorescence. At least 100 GFP-positive cells per condition were counted, and the graphs were plotted as the number of GFP-LC3 puncta per cell.

**Western blot analyses and antibodies**

Protein extraction was performed with RIPA buffer supplemented with complete protease inhibitor cocktail (Roche, 04–693–131–001) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, P7626). The lysates were clarified by centrifugation at 3600 g for 20 min at 4°C, and the protein concentration was quantified using the BCA protein assay kit (Applygen Technologies Inc., P1511). Cell extracts (5–10 μg) were denatured for 5 min in 5 × SDS-PAGE loading buffer (CWbio, cw0027), separated on 10% SDS-PAGE, and transferred onto PVDF membranes (Roche, 03010040001). Following blocking in 5% nonfat milk in TBST (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20 [SbaseBio, A336], pH 7.4) for 1 h at room temperature, membranes were incubated in 5% milk-TBST solutions containing the following primary antibodies: anti-ATG16L1 (Cell Signaling Technology, 8089), anti-LC3 (Cell Signaling Technology, 12,741), anti-SQSTM1/p62 (Cell Signaling Technology, 5114), anti-TIMM23 (Proteintech, 11,123–1-AP), anti-ACTB/β-actin (Vazyme, Ab101-01), anti-BCL2 (Abcam, ab32124) and anti-PPARG (Proteintech, 22,061–1-AP) at 4°C overnight. Then, secondary anti-mouse or anti-rabbit antibodies coupled to horse-radish peroxidase (anti-mouse Tianjin Sunge Biotech Co., Ltd., LK2002; anti-rabbit Tianjin Sunge Biotech Co., Ltd., LK2001) were applied in 5% milk-TBST for 1 h at room temperature, and protein bands were revealed with chemiluminescence HRP substrate (Millipore, WBKLS0500). Protein loading volume and strip exposure time for every antibody were detected before the formal experiment. Band intensities were quantified using ImageJ software.

**RNA isolation and RT-PCR analyses**

Total RNA was extracted using TRIzol reagent (Invitrogen, 15,596–026) according to the manufacturer’s instructions. cDNA was reverse transcribed from total RNA (DNase
treated) using M-MLV reverse transcriptase (Invitrogen, 28,025–013) and random hexamers (Invitrogen, 48,190–011).

Real-time-PCR for Mir223 and Atg16l1 mRNA quantification

A SYBR Green Quantitative RT-PCR kit (Roche, 04–913,914-001) was used for single-step qRT-PCR reactions. To activate the SYBR green, an initial cycle of 95°C for 10 min was performed, followed by PCR reactions with 40 cycles of 95°C for 15 sec and 60°C for 1 min. Then, a thermal denaturation protocol was used to generate the dissociation curves to verify the amplification specificity (a single cycle of 95°C for 60 sec, 55°C for 60 sec and 80 cycles of 55°C for 10 sec). Changes in mRNA levels were quantified using the 2-ΔΔCT method with Gapdh (glyceraldehyde-3-phosphate dehydrogenase) mRNA as a control. The following primers were used: Atg16l1 primers, 5′-CAGAGCAGCTAATAGCGCT-3′ and 5′-AAAAAGGGGAGATTGCGACAGA-3′; Gapdh primers, 5′-AGCCACATCGCTGCAACAC-3′ and 5′-GCCA AATACGACCAAATCC-3′; Rnu6 primers, 5′-CTCGCTTC GCCAGACA-3′ and 5′-AACGCTTCAGAATTTGCGT; Mir223 RT primers, 5′-GTCGTAATCCAGTGAGGGTTC GAGGTATCCGACTGCTGAGGTA-3′ and Mir223 primers, 5′-GCCCGRCCAGUUUGCAGAAUA-3′ and 5′-GTCGAGGGTCCGAGGT-3′. Reactions were performed in duplicates and the number of independent experiments (n = 3) was indicated. The reactions were performed with a 7500 Fast Real-Time PCR System (Applied Biosystems, USA).

Statistical analyses

Statistical analyses were performed using Student’s two-tailed t-test or ANOVA. Data were analyzed using Microsoft Office Excel 2010 or SPSS Statistics 15 and presented as the mean ± SEM using the GraphPad Prism statistical program. Values of p < 0.05 were considered significant. No potential conflicts of interest were disclosed.

Disclosure statement

The authors have no financial conflict of interests.

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