Defective autophagy is associated with neuronal injury in a mouse model of multiple sclerosis

Xuedan Feng[1], Huiqing Hou[1], Yueli Zou[1], Li Guo[1,2*]

[1]Department of Neurology, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei, China, [2]Department of Neurology, Key Laboratory of Hebei Neurology, Shijiazhuang, Hebei, China

ABSTRACT

Neurodegeneration, along with inflammatory demyelination, is an important component of multiple sclerosis (MS) pathogenesis. Autophagy is known to play a pivotal role in neuronal homeostasis and is implicated in several neurodegenerative disorders. However, whether autophagy is involved in the mechanisms of neuronal damage during MS remains to be investigated. Experimental autoimmune encephalomyelitis (EAE), an *in vivo* model of MS, was induced in female C57BL/6 mice by immunization with myelin oligodendrocyte glycoprotein p35-55. After that, autophagic flux in the spinal cord of mice was evaluated by detection of LC3-II and Beclin1 protein expressions. EAE mice were then administered with rapamycin and 3-methyladenine (3-MA) for 10 days. Afterward, the changes in LC3-II, Beclin1, and p62 expression, number of infiltrated inflammatory cells, demyelinated lesion area, and neuronal damage, as well as clinical scores, were assessed. Further, apoptotic cell rate and apoptosis-related protein expressions were monitored. We observed an impaired autophagic flux and increased neuronal damage in the spinal cords of EAE mice. We also found that rapamycin, an autophagy inducer, mitigated EAE-induced autophagy decrease, inflammation, demyelination and neuronal injury, as well as the abnormal clinical score. In addition, rapamycin suppressed cell apoptosis, and decreased Bax/Bcl-2 ratio and cleaved caspase-3 expression. Conversely, the effect of autophagy inhibitor 3-MA on EAE mice resulted in completely opposite results. These results indicated that autophagy deficiency, at least in part, contributed to EAE-induced neuronal injury and that pharmacological modulation of autophagy might be a therapeutic strategy for MS.

KEY WORDS: Autophagy; experimental autoimmune encephalomyelitis; multiple sclerosis; neurodegeneration; apoptosis; EAE mice; LC3-II; Beclin1; rapamycin; 3-methyladenine; p62; autophagy deficiency

INTRODUCTION

Multiple sclerosis (MS) is traditionally regarded as a T-cell-mediated autoimmune disorder of the central nervous system that is morphologically characterized by inflammatory demyelination, astrogliosis, and neuroaxonal degeneration [1]. Neurodegeneration occurs during the early stage of MS and is a key indicator of disease progression and irreversible neurological damage in MS patients [2]. The underlying mechanism of neurodegeneration in MS remains to be elucidated. Aside from inflammatory neurotoxicity, neurodegeneration is associated with certain inflammation-independent mechanisms such as oxidative stress, axoplasmic Ca²⁺ accumulation, glutamate excitotoxicity, mitochondrial dysfunction, and protein aggregation and carbonylation.

Autophagy, also known as macroautophagy, is a lysosome-mediated degradation pathway through which cells engulf and transport superfluous or impaired organelles, mis/unfolded proteins, and invading microorganisms to the lysosomes for degradation. In recent years, both *in vivo* and *in vitro* findings have shown that autophagy is a critical mediator of inflammation and immunity [3,4]. Autophagy also plays an essential role in maintaining the health and homeostasis of neurons [5,6]. Impaired autophagy fails to eliminate proteins that are prone to aggregation, thus leading to neuronal cell damage and death [7,8]. A disturbance in autophagy can lead to multiple neurodegenerative disorders, including Alzheimer's disease, Huntington's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Given the established functions of autophagy in inflammation, immunity, and neurodegeneration we hypothesized that autophagy might be responsible for the pathological mechanisms of neurodegeneration in MS. Thus, intervention of autophagy might affect the onset and progress of neurodegeneration in MS.

*Corresponding author: Li Guo, Department of Neurology, The Second Hospital of Hebei Medical University, No. 215 Heping West Road, Shijiazhuang 050000, Hebei, China. E-mail: guoli12321@126.com

To test this hypothesis, we assessed the effects of pharmacological induction and inhibition of autophagy on neuronal cell injury in experimental autoimmune encephalomyelitis (EAE), a well-established animal model of MS suitable for studying neurodegeneration. Moreover, we sought to characterize alterations in autophagic activity and to determine the possible molecular mechanisms of autophagy during neurodegeneration in EAE mice. To date, anti-inflammatory and immune modulating therapies are the major forms of MS treatment. It is necessary to fully understand the mechanisms of neurodegeneration in MS so that more efficient therapeutic strategies can be employed.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (aged 6-8 weeks, weighted 18-20 g) were purchased from Vital River Laboratories, Beijing, China. Mice were maintained under specific pathogen-free conditions at room temperature (24 ± 2°C), with 12 hours light/dark alternate cycle, and were given free food and drink. All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Hebei Medical University and the local Experimental Ethics Committee.

EAE induction and evaluation

Mice were randomly distributed to control group and age-matched EAE group. Each group was divided into three subgroups: Premorbid (13 days), fastigium (20 days), and paracmastic (30 days). EAE was induced in mice with subcutaneous injection of 250 μg myelin oligodendrocyte glycoprotein (MOG) p35-55 peptide (Lysine Bio-system, Xian, China), as previously described [9]. All peptides were dissolved in complete Freund's adjuvant (CFA; Sigma, MO, USA) containing 4 mg/mL of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI, USA). On the day of immunization and 2 days later, mice received an intraperitoneal (IP) injection of 500 ng pertussis toxin (Alexis, San Diego, CA, USA). Clinical scores were measured daily as follows: 0 - No paralysis; 1 - Loss of tail tone; 2 - Hindlimb weakness; 3 - Hindlimb paralysis; 4 - Severe hindlimb and forelimb paralysis; 5 - Moribund or death.

For the analysis of autophagic flux in the spinal cord of EAE mice, phosphate buffered saline (PBS) alone (vehicle) or PBS combined with 60 mg/kg chloroquine (CQ; Sigma-Aldrich) was injected IP once a day for 7 days, starting at EAE onset (clinical score = 1.0). Autophagic flux was determined by the difference in LC3-II protein levels in the absence or presence of CQ.

Rapamycin and 3-methyladenine (3-MA) treatment

3-MA, an autophagy inhibitor, and rapamycin, an autophagy inducer, were used in this study to suppress and induce autophagy, respectively. Mice were separated randomly into a control group (mice immunized only with CFA; n = 10), EAE-Veh group (EAE mice treated with vehicle; n = 15), EAE-Rapa group (EAE mice treated with rapamycin; n = 15), and EAE-3-MA group (EAE mice treated with 3-MA; n = 15). Rapamycin (2 mg/kg; Sigma) or 3-MA (1.5 mg/kg; Sigma) IP injections were given once daily for 10 days started from the 10th day of EAE induction. EAE-Veh mice received a daily IP injection of the same volume of vehicle (distilled water).

Histological evaluation

The intact spinal cords were removed from the sacrificed mice in each group 20 days post-immunization. Following fixation of the lumbosacral enlargements with 4% (w/v) paraformaldehyde (Sigma-Aldrich), some of the tissues were processed for paraffin embedding. The spinal cords were sliced into 5 μm-thick axial sections. These sections were stained with Luxol fast blue (LFB), hematoxylin-eosin (H&E), and Cresylviolet to assess demyelination, inflammatory lesions, and changes in the number of Nissl bodies, respectively. Semi-quantitative evaluation of inflammation and demyelination was assessed and scored as previously described [10]. For Nissl staining, motor neurons were counted according to the reported criteria [11].

Apoptosis assay

Apoptosis was measured by terminal-deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL) assay (Roche, Mannheim, Germany). Spinal cord tissue slides were stained with TUNEL reagents, Alexa fluor 568-conjugated-phalloidin and DAPI, according to the manufacturer's instructions. Tissue sections were observed using the Olympus microscope (Olympus BX51, Tokyo, Japan). TUNEL-positive nuclei were calculated in 15 randomly selected fields, averaged and expressed as a percentage of labeled nuclei within the fields.

Western blot analysis

The total protein content of spinal cords was extracted and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 5% (v/v) skim milk for 2 hours at room temperature and then incubated at 4°C overnight in primary antibody solution. Following a wash with 0.1% Tween in PBS for 4 times, the membranes were

incubated with the horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. The membranes were washed again to remove the unbound antibodies and detected by western blot detection system. The following antibodies were used: Anti-Beclin1 (1:1000, Proteintech), anti-LC3-II (1:1000, Sigma), anti-p62 (1:500, MBL), anti-cleaved caspase 3 (1:1000, CST), anti-Bcl-2 (1:500, Bioworld), anti-Bax (1:200, Bioworld), glyceraldehyde 3-phosphate dehydrogenase (1:500, Santa Cruz Biotechnology), and secondary antibody (1:1000, Santa Cruz Biotechnology). Positive signals were developed by EasyBlot ECL Kit (Sangon Biotech, Shanghai, China) and analyzed with ImageJ 1.49 (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

The spinal cords were fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) for 30 minutes on ice and were saturated in 30% (w/v) sucrose in PBS. After washing in PBS for 3 times, the tissues were then permeabilized with 0.3% (v/v) Triton X-100 in PBS for about 30 minutes at room temperature and blocked in 10% (v/v) equine serum (Beyotime, Haimen, China) for 30 minutes at 37°C. The tissues were double-stained with NeuN (Millipore, 1:200) and Bcl-2 (Bioworld, 1:150), left overnight at 4°C with a gentle shake, and then followed by incubation with fluorescent secondary antibody (Alexa Flour 488 or 594, Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 hour in the dark at room temperature. Cell nuclei were stained for 5 minutes using Hoechst 33258 (Beyotime). The fluorescent images were obtained using a confocal laser scanning biological microscope (FV500, Olympus, Japan).

Statistical analysis

Data are presented as mean ± standard deviation. Statistical analyses between two groups were analyzed by the Student's *t*-test, while differences between three or more groups were analyzed by one-way analysis of variance followed by the Newman-Keuls multiple comparison test. Data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The value of $p < 0.05$ was considered statistically significant.

RESULTS

Autophagic flux is impaired in the spinal cords of EAE mice

LC3-II is closely associated with autophagosome number, and Beclin1 is well known as autophagy-related gene. Suppression of LC3-II and Beclin1 expression reflects the impaired autophagy [12,13]. Western blotting was performed to monitor the levels of LC3-II and Beclin1 in the spinal cord of mice at the 13th, 20th, and 30th day following induction

of EAE. Downregulation of LC3-II was observed in EAE mice compared with the control group at each time point (Figure 1A and B). The level of Beclin1 was also downregulated in EAE mice but it was reversed to control level at the 30th day, as compared with control group (Figure 1C and D).

We further characterized total autophagic flux by comparing the difference in LC3-II level between control and EAE groups treated with or without CQ. CQ is a lysosomal protease inhibitor blocks the fusion of phagosomes and lysosomes at the terminal stage of the autophagy pathway [14,15]. We found that LC3-II level in control-CQ group was reduced compared with control-vehicle group. No remarkable change in LC3-II level between EAE-vehicle and EAE-CQ groups was observed (Figure 1E and F).

Influence of rapamycin and 3-MA on autophagic flux in EAE mice

To explore the effects of autophagy on EAE mice, rapamycin and 3-MA were used to induce and inhibit autophagy, respectively, following the EAE induction (Figure 2A). Afterward, autophagic flux was tested by detecting LC3-II, Beclin1, and p62 expressions using western blotting. p62 (SQSTM1/sequestosome 1) is conjugated to LC3, thus serving as an alternative indicator of autophagic flux [16]. As compared with EAE-vehicle mice, rapamycin treatment resulted in notable increases in LC3-II, Beclin1, and p62 protein levels in EAE-Rapa group (Figure 2B-E). On contrary, 3-MA significantly downregulated the expression of the three proteins in EAE-3-MA group, when compared with EAE-vehicle group.

These results suggest that autophagy was successfully induced by rapamycin, and was suppressed by 3-MA.

Influence of autophagy modulation on neuronal injury in EAE mice

To investigate the detailed functions of autophagy in EAE mice, histological evaluation was conducted in rapamycin and 3-MA treated EAE mice using H&E and LFB staining. We found that both, the population of infiltrated inflammatory cells and the area of the demyelinated lesion, were lower in EAE-rapamycin mice compared with EAE-vehicle group (Figure 3A-C). Nevertheless, significant increases in the number of infiltrated inflammatory cells and demyelinated lesion area were found in EAE-3-MA mice when compared with EAE-vehicle mice.

Nissl staining was performed to confirm whether induction or suppression of autophagy affected the neuronal injury in EAE mice. EAE-Rapa group exhibited an increase in the number of Nissl-stained cells compared with EAE-vehicle mice (Figure 3D and E). Unsurprisingly, the number of
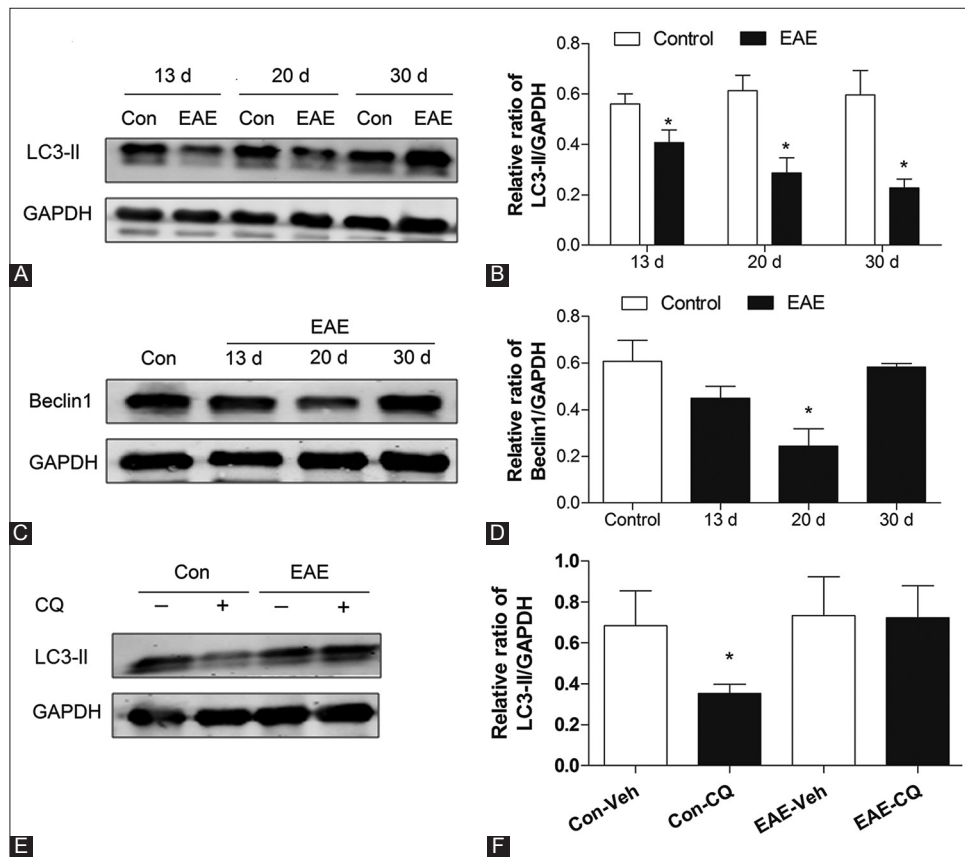
FIGURE 1. Autophagy flux is impaired in the spinal cords of experimental autoimmune encephalomyelitis (EAE) mice. (A and B) Western blot analysis of LC3-II protein levels in the spinal cords of EAE mice at day 13, 20 and 30 following the induction of EAE. (C and D) Western blot analysis of Beclin1 protein levels at 0, 13, 20 and 30 days post-immunization. (E and F) Western blot analysis of LC3-II protein levels in Control and EAE mice with or without chloroquine (CQ) treatment. n = 5 mice per group. *$p < 0.05$ compared with the control group.

Nissl-stained cells was remarkably decreased in EAE-3-MA group when compared with EAE-vehicle group.

In addition, compared with EAE-vehicle group, EAE-Rapa group showed obvious postponed onset of disease symptoms, a declined disease incidence, and an improved clinical score, which were in agreement with previous studies [17]. In contrast, compared with EAE-vehicle mice, the clinical score of EAE-3-MA mice showed almost no difference during the early stages but showed higher neurological scores during the acute and chronic stages (Figure 3F).

Overall, we inferred that autophagy restoration might ameliorate inflammation, demyelination, and neuronal injury, as well as improve clinical score in EAE mice.

Autophagy modulation may be associated with the apoptotic pathway in EAE mice

To see whether the alteration of autophagy affect EAE-induced apoptosis, we first conducted TUNEL staining to assess apoptotic activity in the spinal cords of EAE mice. A marked increase of apoptotic cell number was observed in EAE-vehicle group when compared with the control group. Rapamycin treatment decreased TUNEL-positive cells number in EAE-Rapa group, while 3-MA treatment increased

TUNEL-positive cells in EAE-3-MA group compared to the EAE-vehicle group (Figure 4A and B).

Next, we analyzed the expression of apoptosis-associated proteins to further clarify the role of autophagy in EAE-induced apoptosis. Western blotting showed that rapamycin treatment significantly upregulated Bcl-2, and downregulated Bax and cleaved caspase-3 to some extent in EAE-Rapa group (Figure 4C-E). In contrast, 3-MA exhibited a pro-apoptotic effect in EAE-3-MA group.

We conducted immunofluorescent colocalization of Bcl-2 and NeuN to further confirm the influence of autophagy on neuronal apoptosis in EAE mice. The results in Figure 4F show an increase in the number of Bcl-2-positive neurons in EAE-Rapa group compared with EAE-vehicle group, whereas 3-MA treatment decreased the number of Bcl-2-positive neurons in EAE-3-MA group.

To summarize, these data indicated that the effects of autophagy regulation on neurons are mediated through interaction between autophagy and pro-apoptotic signals in EAE mice.

DISCUSSION

Although axonal-neuronal degeneration was first associated with MS more than 100 years ago, researchers have been
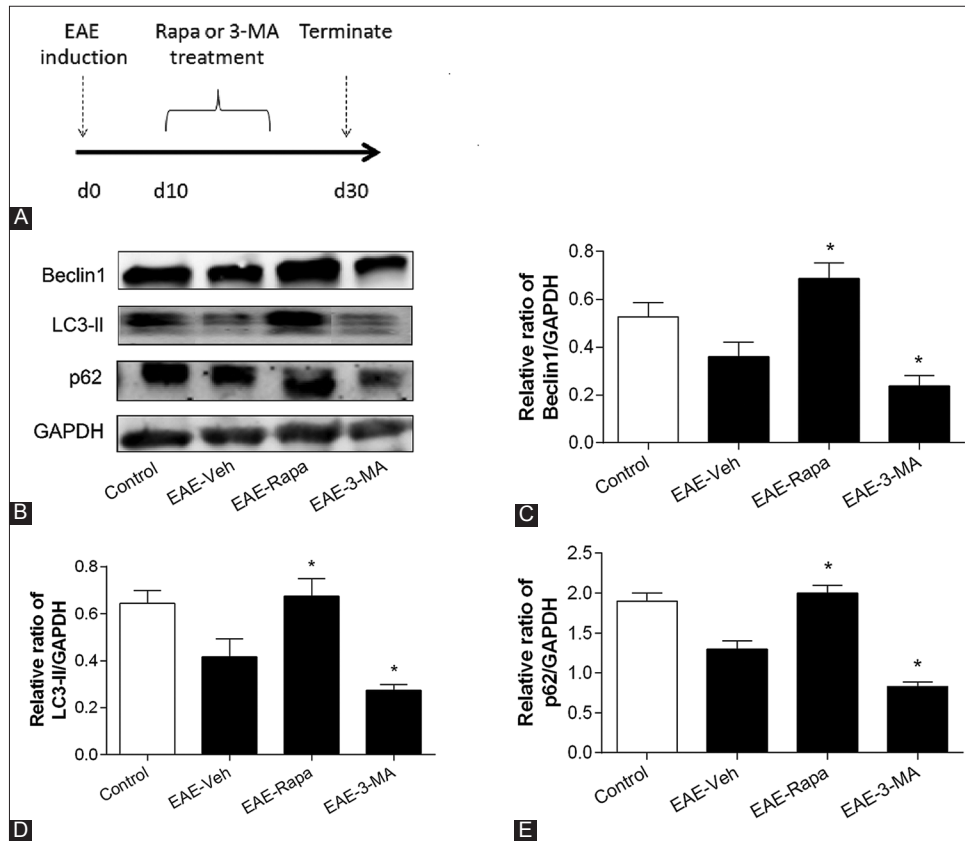
FIGURE 2. Induction and inhibition of autophagy in experimental autoimmune encephalomyelitis (EAE) mice. (A) EAE injury was induced in C57BL/6J mice by immunization with MOG35-55 emulsified in complete Freund's adjuvant, and administered with rapamy-cin (1 mg/kg) [EAE-Rapa group], 3-methyladenine (3-MA) (1.5 mg/kg) [EAE-3-MA group], or vehicle [EAE-Veh group] for 10 days from the 10th day after the EAE induction. (B) Western blot analysis of Beclin1, LC3-II, and p62 in the spinal cords of mice in control, EAE-vehicle, EAE-Rapa, and EAE-3-MA groups. Quantitation of (C) Beclin1, (D) LC3-II, and (E) p62 protein expressions. n = 5 mice per group. *$p < 0.05$ compared with EAE-vehicle group. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

paying little attention to this aspect of the disease [18]. In recent years, pharmacological modulation of autophagy has been considered as a potential therapy for prevention or treatment of neurodegenerative conditions. Therefore, characterization of autophagic activity and elucidation of underlying mechanisms of autophagy in MS are necessary. In the current study, we found that the autophagy markers LC3-II and Beclin1 were decreased in EAE mice while the downregulation of LC3-II was recovered when CQ was present. These findings indicate that autophagy might be implicated in the pathogenesis of neurodegeneration in EAE mice. Furthermore, we found that autophagy induced by rapamycin could ameliorate inflammation, demyelination, and neuronal injury, as well as improve clinical score in EAE mice. In addition, rapamycin-induced autophagy suppressed cell apoptosis by decreasing Bax/Bcl-2 ratio and inactivating cleaved caspase-3. Conversely, the effect of 3-MA, the autophagy inhibitor, resulted in completely opposite results in EAE mice.

Beclin1 is a key component of theVps34/Class III PtdIns 3-kinase complex involved in the formation and maturation of autophagosome. We found that Beclin1 level was reduced in the spinal cords of EAE mice. We hypothesized that the reduced Beclin1 level was the result of a negative feedback

secondary to the blockage of autophagosomal degradation; this protected against apoptotic and autophagic cell death [19]. A recent study demonstrated that Beclin1-deficient mice were resistant to EAE [20], implicating that the suppression of Beclin1 is possibly an adaptive or self-protective response to EAE-induced injury. Additionally, defective LC3-II was found in EAE mice in the current study. The decrease of LC3-II might have resulted from decreased autophagosome formation and increased autophagosome degradation. Altogether, these findings were consistence with the previous study [10] where impaired autophagic flux was observed in EAE mice.

In this study, we analyzed whether autophagy is functionally relevant to the process of neurodegeneration in EAE mouse model, comparing the results between rapamycin- and 3-MA-treated EAE mice. Previous evidence suggested that when etiologic factors of the disease impair the efficacy of autophagy, neurodegeneration and neuronal death subsequently occur [21]. The present study demonstrated that the restoration of autophagy could ameliorate inflammation, demyelination, and neuronal injury, as well as the abnormal clinical score in EAE mice, which strongly links autophagy to neuroprotection. Given that pharmacological modulators have complex actions outside autophagy, further studies are
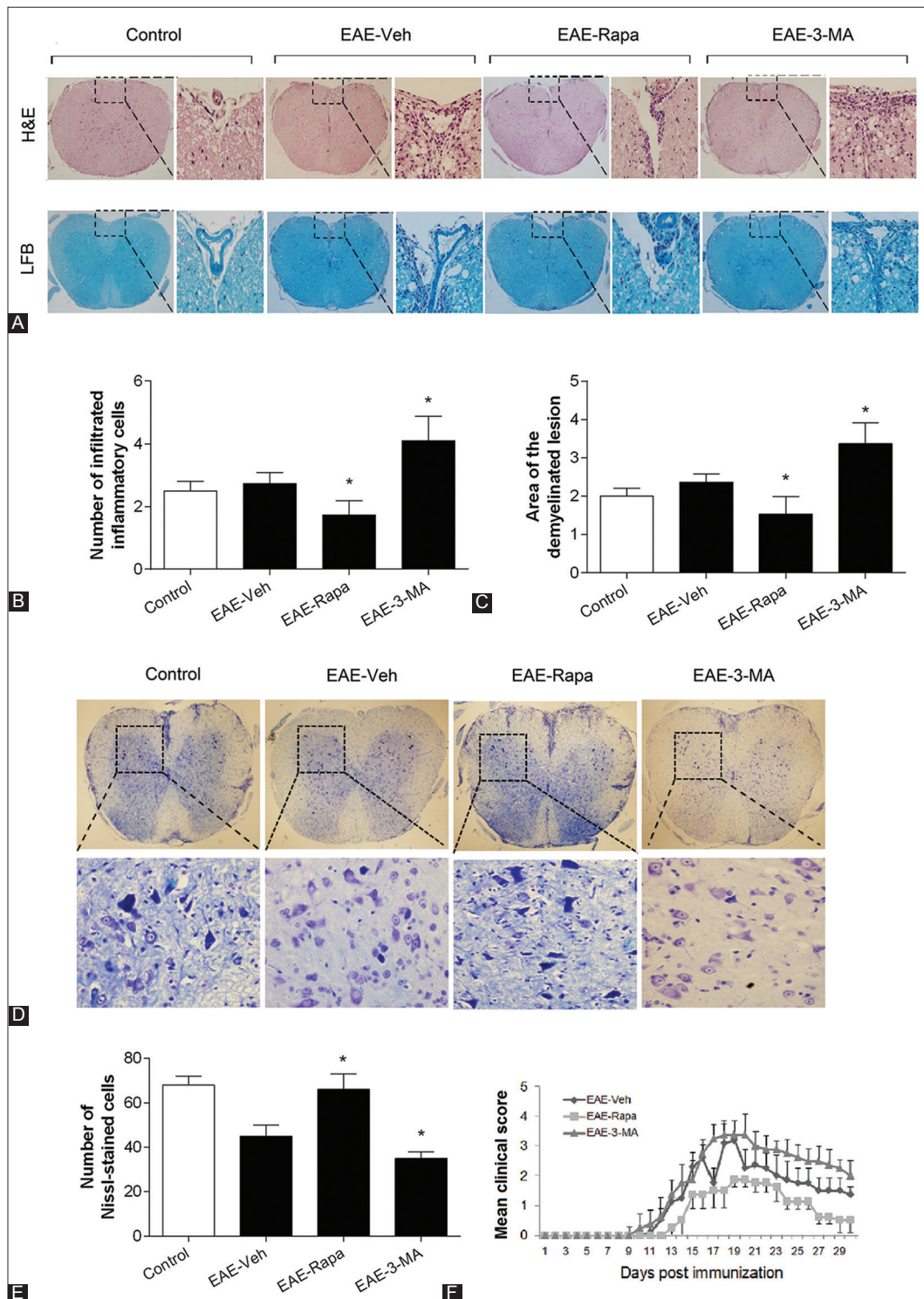
FIGURE 3. Effects of autophagy modulation on neuronal injury. (A) The lumbar spinal cords were sampled at the 20th day after experimental autoimmune encephalomyelitis (EAE) induction from control, EAE-vehicle, EAE-rapamycin (Rapa) and EAE-3-methyladenine (3-MA) groups. Tissues were made into paraffin sections and stained with H&E and Luxol fast blue. Quantitation of (B) infiltrated inflammatory cells, and (C) demyelinated lesion area. (D) Nissl staining was performed in the lumbar spinal cord from the four groups of mice. (E) Quantitation of Nissl-stained cells. (F) Comparison of the clinical scores of EAE mice treated with rapamycin or 3-MA. For clinical scores, n = 15 mice per group; while all others were n = 5 mice per group. *$p < 0.05$ compared with the EAE-vehicle group.

required to investigate the effects of genetic or RNAi-based approaches in MOG-induced EAE model.

Bcl-2 family members play a key role in the control of neuronal cell death [22]. The Bcl-2 protein blocks apoptosis by interaction with Bax [23], and suppresses autophagic activity by binding to Beclin1 [24]. In the current study, rapamycin decreased the numbers of TUNEL-positive cells, as well as the Bax/Bcl-2 ratio following the EAE induction. The ratio of Bax to Bcl-2 decreased, indicating that the cells were less susceptible to apoptotic stimuli, as described in another study [25].
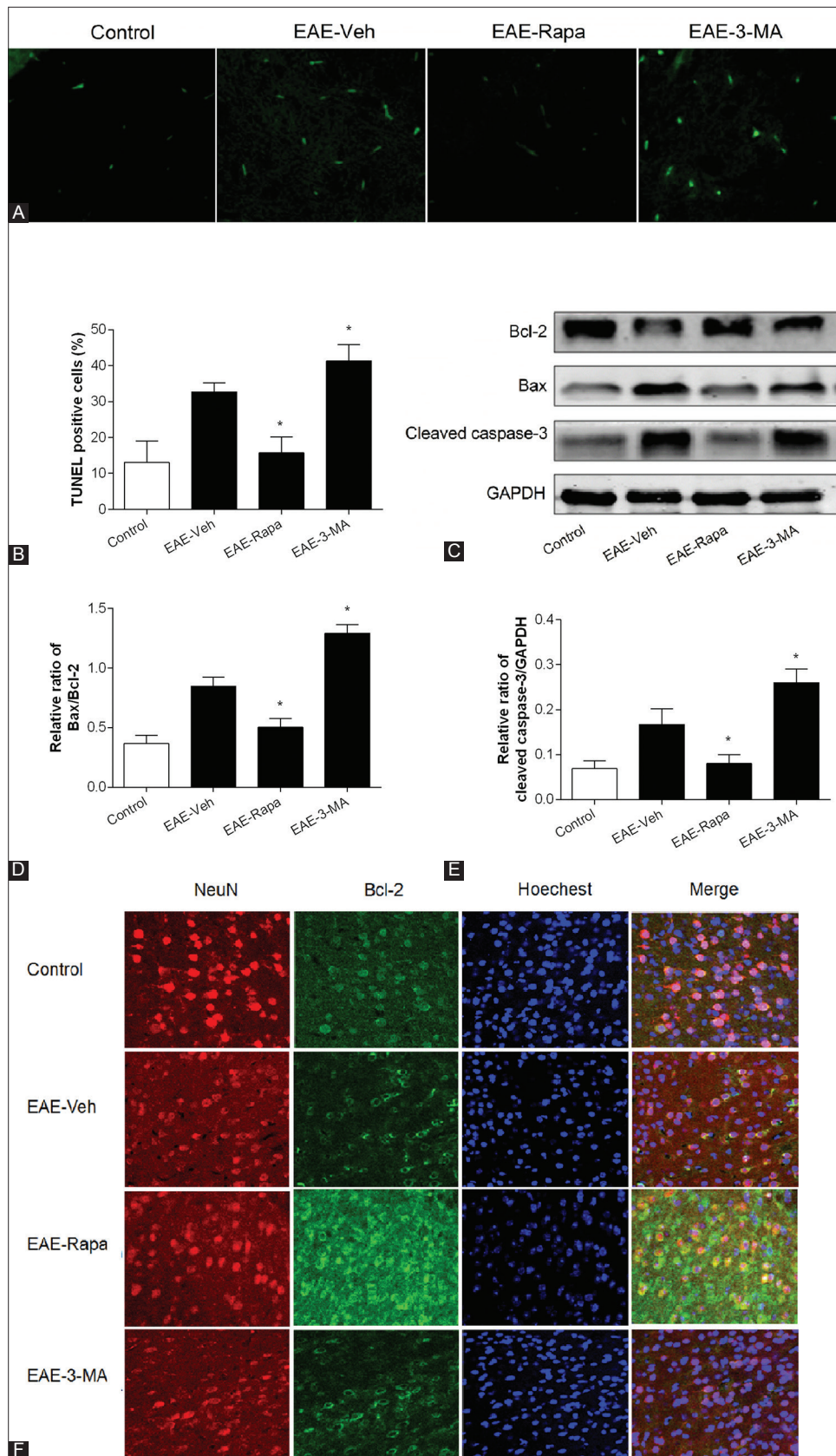
FIGURE 4. Induction and inhibition of autophagy affect apoptosis pathway in experimental autoimmune encephalomyelitis (EAE) mice. (A) Terminal-deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) staining identified apoptotic cell death at day 20 post-immunization in control, EAE-vehicle, EAE-rapamycin (Rapa) and EAE-3-methyladenine (3-MA) mice. (B) Quantitation of TUNEL positive cells. (C) Western blot analysis of protein levels of Bcl-2, Bax, and cleaved caspase-3 in the four groups of mice. Quantitation of (D) Bax/Bcl-2 ratio and (E) cleaved caspase-3. (F) Immunofluorescent colocalization of Bcl-2 and NeuN in the four groups. n = 5 mice per group. *p < 0.05 compared with EAE-vehicle group. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

In addition, the expression of cleaved caspase-3 proteins was downregulated in EAE-Rapa mice. Caspases (cysteine-aspartic proteases) are essential enzymes in apoptosis. As a key executioner caspase, caspase-3 was shown to be significantly increased in EAE mice [26]. Caspase-3 is also involved in neuronal apoptotic death [27], and suppression of caspase-3 activation plays a neuroprotective role in various animal models of MS [28,29].

Taken together, in this report, we have provided evidence about defective autophagy pathways responsible for neuronal damage in EAE mice. The study also indicated that rapamycin alleviated neurotoxicity, at least partly, through the enhancement of autophagic activity in EAE mice and that pharmacological activation of autophagy may protect against neuronal injury. Current insights suggest that drugs with neuroprotective properties should be used in combination with basic treatments to prevent chronic neurological disability in MS patients [30]. Our results imply that autophagy modulation could be an optimal therapeutic strategy.

ACKNOWLEDGMENTS

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

REFERENCES

[1]   Obert D, Helms G, Sättler MB, Jung K, Kretzschmar B, Bähr M, et al. Brain metabolite changes in patients with relapsing-remitting and secondary progressive multiple sclerosis: A two-year follow-up study. PLoS One 2016;11(9):e0162583.
https://doi.org/10.1371/journal.pone.0162583.

[2]   Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: Results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. Neurology 1996;46(4):907-11.
https://doi.org/10.1212/WNL.46.4.907.

[3]   Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature 2011;469(7330):323-35.
https://doi.org/10.1038/nature09782.

[4]   Bhattacharya A, Eissa NT. Autophagy and autoimmunity crosstalks. Front Immunol 2013;4:88.
https://doi.org/10.3389/fimmu.2013.00088.

[5]   Wong E, Cuervo AM. Autophagy gone awry in neurodegenerative diseases. Nat Neurosci 2010;13(7):805-11.
https://doi.org/10.1038/nn.2575.

[6]   Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 2006;441(7095):880-4.
https://doi.org/10.1038/nature04723.

[7]   Martinez-Vicente M, Cuervo AM. Autophagy and neurodegeneration: When the cleaning crew goes on strike. Lancet Neurol 2007;6(4):352-61.
https://doi.org/10.1016/S1474-4422(07)70076-5.

[8]   Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 2006;441(7095):885-9.
https://doi.org/10.1038/nature04724.

[9]   Li Z, Chen L, Niu X, Liu J, Ping M, Li R, et al. Immunomodulatory synergy by combining atorvastatin and rapamycin in the treatment of experimental autoimmune encephalomyelitis (EAE). J Neuroimmunol 2012;250(1-2):9-17.
https://doi.org/10.1016/j.jneuroim.2012.05.008.

[10] Zhen C, Feng X, Li Z, Wang Y, Li B, Li L, et al. Suppression of murine experimental autoimmune encephalomyelitis development by 1,25-dihydroxyvitamin D3 with autophagy modulation. J Neuroimmunol 2015;280:1-7.
https://doi.org/10.1016/j.jneuroim.2015.01.012.

[11] Zhang X, Chen S, Song L, Tang Y, Shen Y, Jia L, et al. MTOR-independent, autophagic enhancer trehalose prolongs motor neuron survival and ameliorates the autophagic flux defect in a mouse model of amyotrophic lateral sclerosis. Autophagy 2014;10(4):588-602.
https://doi.org/10.4161/auto.27710.

[12] Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000;19(21):5720-8.
https://doi.org/10.1093/emboj/19.21.5720.

[13] Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, Juin P, et al. Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. EMBO J 2007;26(10):2527-39.
https://doi.org/10.1038/sj.emboj.7601689.

[14] Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 2005;5(9):726-34.
https://doi.org/10.1038/nrc1692.

[15] Solomon VR, Lee H. Chloroquine and its analogs: A new promise of an old drug for effective and safe cancer therapies. Eur J Pharmacol 2009;625(1-3):220-33.
https://doi.org/10.1016/j.ejphar.2009.06.063.

[16] Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 2005;171(4):603-14.
https://doi.org/10.1083/jcb.200507002.

[17] Donia M, Mangano K, Amoroso A, Mazzarino MC, Imbesi R, Castrogiovanni P, et al. Treatment with rapamycin ameliorates clinical and histological signs of protracted relapsing experimental allergic encephalomyelitis in Dark Agouti rats and induces expansion of peripheral CD4 CD25 Foxp3 regulatory T cells. J Autoimmun 2009;33(2):135-40.
https://doi.org/10.1016/j.jaut.2009.06.003.

[18] Bjartmar C, Trapp BD. Axonal and neuronal degeneration in multiple sclerosis: Mechanisms and functional consequences. Curr Opin Neurol 2001;14(3):271-8.
https://doi.org/10.1097/00019052-200106000-00003.

[19] Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: Crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 2007;8(9):741-52.
https://doi.org/10.1038/nrm2239.

[20] Kovacs JR, Li C, Yang Q, Li G, Garcia IG, Ju S, et al. Autophagy promotes T-cell survival through degradation of proteins of the cell death machinery. Cell Death Differ 2012;19(1):144-52.
https://doi.org/10.1038/cdd.2011.78.

[21] Boland B, Nixon RA. Neuronal macroautophagy: From development to degeneration. Mol Aspects Med 2006;27(5-6):503-19.
https://doi.org/10.1016/j.mam.2006.08.009.

[22] Akhtar RS, Ness JM, Roth KA. Bcl-2 family regulation of neuronal development and neurodegeneration. Biochim Biophys Acta

2004;1644(2-3):189-203.
https://doi.org/10.1016/j.bbamcr.2003.10.013.

[23] Walensky LD. BCL-2 in the crosshairs: Tipping the balance of life and death. Cell Death Differ 2006;13(8):1339-50.
https://doi.org/10.1038/sj.cdd.4401992.

[24] Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 2005;122(6):927-39.
https://doi.org/10.1016/j.cell.2005.07.002.

[25] Raisova M, Hossini AM, Eberle J, Riebeling C, Wieder T, Sturm I, et al. The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. J Invest Dermatol 2001;117(2):333-40.
https://doi.org/10.1046/j.0022-202x.2001.01409.x.

[26] Das A, Guyton MK, Matzelle DD, Ray SK, Banik NL. Time-dependent increases in protease activities for neuronal apoptosis in spinal cords of Lewis rats during development of acute experimental autoimmune encephalomyelitis. J Neurosci Res 2008;86(13):2992-3001.

https://doi.org/10.1002/jnr.21737.

[27] Kingham PJ, Cuzner ML, Pocock JM. Apoptotic pathways mobilized in microglia and neurones as a consequence of chromogranin A-induced microglial activation. J Neurochem 1999;73(2):538-47.
https://doi.org/10.1046/j.1471-4159.1999.0730538.x.

[28] Guyton MK, Das A, Samantaray S, Wallace GC 4th, Butler JT, Ray SK, et al. Calpeptin attenuated inflammation, cell death, and axonal damage in animal model of multiple sclerosis. J Neurosci Res 2010;88(11):2398-408.
https://doi.org/10.1002/jnr.22408.

[29] Ahmed Z, Doward AI, Pryce G, Taylor DL, Pocock JM, Leonard JP, et al. A role for caspase-1 and -3 in the pathology of experimental allergic encephalomyelitis: Inflammation versus degeneration. Am J Pathol 2002;161(5):1577-86.
https://doi.org/10.1016/S0002-9440(10)64436-7.

[30] Luessi F, Siffrin V, Zipp F. Neurodegeneration in multiple sclerosis: Novel treatment strategies. Expert Rev Neurother 2012;12(9):1061-76.
https://doi.org/10.1586/ern.12.59.