BMSCs transplantation downregulated NF-κB signaling pathways by being induced to differentiate into neurons in Experimental autoimmune encephalomyelitis (EAE) animal model

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Research

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Abstract

Background: Multiple sclerosis (MS) is a complex, progressive neuroinflammatory disease associated with autoimmunity and poorly find an effective therapeutic strategy. Currently, experimental autoimmune encephalomyelitis (EAE) is widely used to study the pathogenesis of MS.

Methods: In our study, we performed flow cytometry to identify BMSCs. To systematically evaluate whether BMSCs can be differentiate into neuron cells, astrocytes and oligodendrocyte, we analyzed the biomarkers by immunofluorescence labeling. We demonstrated the effect of bone marrow mesenchymal stem Cells (BMSCs) transplant on the EAE animal model, and determined the expression of MAP-2, GFAP, and MBP in the cortex and hippocampus of mice.

Results: Our results showed that BMSCs could be induced to differentiate into neuron cells astrocytes and oligodendrocyte. BMSCs transplant improved the survival rate, neurological function scores, and obvious remyelination of mice in BMSCs transplantation group was significantly higher than EAE group ($P<0.01$). Morever, BMSCs transplant decreased the levels of IL-2, IL-10, IL-17 and IL-22 in the serum of EAE mice. Western blotting showed that the expression of NF-κB, IκB-α and IL-17 was decreased in BMSCs transplant group.

Conclusions: Herein, our results revealed that BMSCs were transplanted into the brain of EAE mice, differentiated into neurons, improved the survival rate, neurological function recovery and the extent of demyelination in EAE mice by downregulation of NF-κB signaling pathway.

Background

Multiple sclerosis (MS) is a chronic neuroinflammatory disease that is associated with autoimmunity in central nervous system (CNS)[1, 2]. Generally speaking, it is characterized by axon damage, demyelination, inflammatory infiltration and progressive neurological damage, leading to disability[3]. Morever, it is thought to be a multifocal demyelination disease in CNS[4]. Currently, although MS mainly depends on three types of drug treatment, there is less evidence that drugs are effective in the progression of MS[5]. Thus, finding an effective therapeutic strategy is very important for MS in clinical. Experimental autoimmune encephalomyelitis (EAE) is widely used to study the pathogenesis of MS, which represents both pathological and features of MS[6]. To some extent, EAE could effectively elucidate various pathological processes in MS, such as inflammation demyelination, axonal lesions[7].

Bone marrow mesenchymal stem Cells (BMSCs) are non-hematopoietic stromal cells that derived from bone marrow[8]. BMSCs differentiate into various cell types, which contribute to regeneration of tissues[9–11]. BMSCs also play immunomodulatory role through inhibiting T-cell activities[12]. In addition, BMSCs secrete growth factors to regulate hematopoietic stem/progenitor cell proliferation and differentiation[13]. Recently, some researchers have shifted the focus to stem cell-based therapy in the treatment of many diseases, including nervous system disease, indicating that stem cell may be associated with MS[14, 15]. In the acute and subacute phases, the tissues were selectively target

damaged by intravenous injection of BMSCs, which improved the recovery of neurological function, decreased inflammatory response and demyelination after EAE[16].

In our study, we found that BMSCs could differentiate into neurons, astrocytes and oligodendrocytes. At the same time, we established the EAE animal model by injected subcutaneously with MOG35-55 peptide. In addition, we performed BMSCs transplant and determined the effects of BMSCs on the progression of EAE in mice.

Materials And Methods

Cell culture

BMSCs purchased from Shanghai Baili Biotechnology Co., Ltd. were cultured at 37 °C and 5% CO_2 with DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA),100 U/mL penicillin and 100 U/mL streptomycin. Our previous study, we have evaluated phenotype of BMSCs by flow cytometric analysis.

Induced BMSCs to differentiate into neurons

BMSCs from the fifth generation were seeded in 6-well plates coated with poly ornithine and laminin at a density of 2×10^5 cells/ml. After 24 h, the DMEM/F12 medium were replaced with complete culture medium (Gibco, USA) containing 10 ng/ml bFGF. The complete culture medium was changed every 2 to 3 days. BMSCs in control group were not induced.

Induced BMSCs to differentiate into astrocytes

BMSCs from the fifth generation were seeded in 6-well plates coated with poly ornithine and laminin at a density of 2×10^5 cells/ml. After 24 h, the DMEM/F12 medium were replaced with DMEM/F12 medium (Gibco, USA) supplemented with 1% N_2 and 2% B27. The induced medium was changed every 2 to 3 days. BMSCs in control group were not induced.

Induced BMSCs to differentiate into oligodendrocyte

BMSCs from the fifth generation were seeded in 6-well plates coated with poly ornithine and laminin at a density of 2×10^5 cells/ml. After 24 h, the DMEM/F12 medium were replaced with NS medium (Gibco, USA) supplemented with 2% B27, 2 mM glutamine, 50U/mL penicillin, 50U/mL streptomycin, and 60 μg/mL T3. The NS medium was changed every 2 to 3 days. BMSCs in control group were not induced.

Animal and EAE model

C57BL/6 mice were purchased from Kunming Medical University. The mice were housed in sterile, constant temperature rooms at Kunming Medical University with a 12 h/12 h light/dark cycle, with free access to food and water. All experiments were performed in accordance with Ethics Committee of Kunming Medical University and approved by Ethics Committee of Kunming Medical University.

EAE model was induced by 125 μg of MOG35-55 peptide diluted with PBS(PH = 7.2) to 0.01 mol/mL, and mixed with complete Freund's adjuvant (CFA; Shanghai, China) including Mycobacterium tuberculosis. Each mouse was injected subcutaneously at one point in the groin and three points in the back. Subsequently, the mice were injected with 200 ng pertussis toxin (Thermo Fisher Scientific;Beijing, China). After 48 h, the mice were injected with 200 ng pertussis toxin again. On the 7th day, we also injected with 200 ng pertussis toxin into the mice. 30 mice were randomly divided into two groups. BMSCs cells were injected into the lateral ventricle by brain stereotactic technology.

Lifetime Analysis and Neurological Functional Measurement

Behavioral scores of EAE group and EAE + BMSCs transplantation group were graded daily according to the EAE model scoring standards. EAE model scoring standards as follows: 0, normal; 1, weakness of tail; 2.5: mild weakness of unilateral hind limbs; 3: significant weakness of unilateral hind limbs; 4: paralysis of unilateral hind limbs; 4.5: paralysis of unilateral hind limbs accompanied by weakness of opposite hind limbs or mild weakness of forelegs on the same side; 5: paralysis of bilateral hind limbs; 6: paralysis of bilateral hind limbs accompanied by paralysis of unilateral forelegs[17]. After 32days, the mice were anesthetized with 10% chloral hydrate. We collected serum from the mice and took cortex and hippocampus of brain tissues for the following experiments.

Immunofluorescence staining

After fixed with 4% paraformaldehyde, the cells/brain sections were penetrated with PBS containing 0.4% Triton X-100. Then, the cells/brain sections were blocked with 5% Bovine Serum Albumin (BSA; Beijing; China) at room temperature for 1 h. The primary antibodies were incubated overnight at 4℃. The secondary antibodies with fluorophores (1:1000, KPL) were incubated at 4℃ for 1 h. Then, nuclei staining with DAPI followed by capturing using a microscope (Olympus, Japan). We calculated the ratio of BMSCs to differentiate into neurons, astrocytes and oligodendrocytes, respectively. The primary antibodies were shown in Table.1.

RNA extraction and quantitative PCR

According to the instructions, total RNA of cells and brain tissues were extracted using Trizol Reagent (Lifetech, USA). We followed the instructions of the FastKing RT Kit (Fermentas; Shanghai, China) to synthesize the first strand of cDNA. Then, we performed quantitative PCR by SYBR Green master mix (KAPA; Shanghai, China). The primers were designed using beacon designer 7.90. The primer sequences were listed in Table.2. All experimental results were analyzed by 2-△△Ct method.

Luxol Fast Blue (LFB) staining

Paraffin sections of brain tissue were placed in a xylene solution for gradient hydration. Then, the sections were put into Luxol fast blue dye solution (Sigma, USA) in a 50–65℃ incubator overnight. Stained sections were taken out and passed through alcohol, water, and then added the color separation

solution. Sections were washed 3 times with water after differentiation, and then counterstained or gradient dehydrated.

ELISA Assay

The levels of IL-2, IL-10, IL-17 and IL-22 were detected by ELISA. According to the instructions of ELISA Kit, the supernatant was collected by centrifugation homogenate (5000 rpm for 15 minutes at 4 ℃). The levels of IL-2, IL-10, IL-17 and IL-22 were determined by ELISA Kit. We constructed standard curves by standard samples. Quantification of ELISA results were performed at 450 nm using an ELISA plate reader (Spectra Max 190, Molecular Devices, USA).

Western blotting

After the BMSCs transplantation were performed with for 32 days, protein was extracted from cortex and hippocampus tissues in lysis buffer. The protein concentration was determined by BCA protein kit. The protein samples were isolated from 10% twelve alkyl sulphate polyacrylamide gel and transferred to PVDF membrane. The primary antibodies of NF-κB (Proteintech; Beijing, China), IκBα (Proteintech; Beijing, China) and IL-17 (Abcam; Shanghai; China) were incubated overnight at 4℃, respectively. The secondary antibodies (Cell Signaling Technology; Beijing, China) were incubated at room temperature for 1 h. We used chemiluminescence detection reagent to detect immune-reactive bands. The optical density was detected by Image J. β-actin was used as a control to normalize the data.

Statistical analysis

GraphPad Prism5 software (GraphPad, USA) was used to conduct statistical analysis. One-way ANOVA and t test were used to analyze data. Data were presented as mean ± standard deviation (SD) and P value < 0.05 were considered as significant results.

Results

Characteristics of cultured BMSCs

Cultured BMSCs were observed by microscopy and flow cytometry analyses. Cultured BMSCs were adherent and showed with polygonal morphology. The morphology of BMSCs was likely fibroblasts after growing to the third generation (Fig. 1A). The surface marker expression of BMSCs were determined by flow cytometry (Fig. 1B-E). The result showed that cultured BMSCs expressed CD29 and CD90, while there was no expression of CD34 and CD45 on the surface of BMSCs. All the results were consistent with the previous studies[18, 19].

BMSCs were induced to differentiate into neuron cells

After the induced cells were fixed with 4% paraformaldehyde, the expression of MAP-2 and βIII-tubulin was detected by immunofluorescence staining (Fig. 2A). The result showed that MAP-2 and βIII-tubulin was expressed in the cytoplasm of induced BMSCs. Morever, yellow spots were also found in the

cytoplasm. We detected the expression of MAP-2 and βIII-tubulin mRNA by Q-PCR (Fig. 2B and C) ($P < 0.001$). The expression of MAP-2 and βIII-tubulin mRNA showed a significant increase after neuron induction, indicating that BMSCs were successfully induced into neuronal cells.

BMSCs were induced to differentiate into astrocytes

We also detected the expression of GFAP by immunofluorescence staining after fixed with 4% paraformaldehyde (Fig. 3A). The result showed that GFPA were positive in the induced cells. Q-PCR showed that the levels of GFAP mRNA was significantly increased after BMSCs were induced (Fig. 3B) ($P < 0.001$). Taken together, BMSCs were induced to differentiate into astrocytes.

BMSCs were induced to differentiate into oligodendrocyte

Morever, we also induced to BMSCs differentiate into oligodendrocyte. Myelin Basic Protein (MPB) and Oligodendrocyte Marker (O4) were detected by immunofluorescence staining. As presented in Fig. 4A, MPB was observed in the nucleus of induced cells, while O4 was observed in the cytoplasm. We also detected the levels of MBP and O4 by Q-PCR(Fig. 4B-C)($P < 0.001$). The result showed that the levels of MBP and O4 was significantly increased after BMSCs were induced, which indicated that BMSCs were induced to differentiate into oligodendrocyte.

BMSCs transplant improved the survival rate, neurological function recovery and the extent of demyelination in EAE mice

In the study, 30 EAE mice were evaluated for survival analysis every week. Survival analysis showed that the survival rate of mice in BMSCs transplantation group was significantly higher than EAE group (Fig. 5A) ($P < 0.01$). To evaluate effect of the BMSCs transplantation, we also evaluated neurological function scores in EAE mice (Fig. 5B) ($P < 0.01$, $P < 0.001$). The result showed that neurological function scores of mice in BMSCs transplantation group was significantly decreased compared with EAE group. LFB staining of brain sections was performed, and the result showed that obvious remyelination was observed after BMSCs transplantation in the cortex and hippocampus of rats (Fig. 5C). In briefly, the survival rate, neurological function recovery and the extent of demyelination of EAE mice was improved by BMSCs transplant.

BMSCs transplant downregulated the expression of NF-κB signaling pathway in EAE mice

BMSCs transplant treatment reduced axonal loss and recovered neurological function by downregulation of NF-κB signaling pathway. Thus, we also determined the expression of MAP-2, GFAP, and MBP in the cortex and hippocampus of mice (Fig. 6A-C). As observed, the expression of MAP-2 was the most obvious in BMSCs transplant group, while there was no significant increase in the expression of MBP and GFAP. Furthermore, we measured the levels of IL-2, IL-10, IL-17 and IL-22 in the serum of EAE mice (Fig. 6D) ($P <$

0.01, P < 0.001). The levels of IL-2, IL-10, IL-17 and IL-22 in the serum of EAE mice was decreased significantly after BMSCs transplant. Western blotting showed that the expression of NF-κB, IκB-α and IL-17 was decreased in BMSCs transplant group compared with EAE group, while the expression of IκB-α was increased in BMSCs transplant group (Fig. 6E) (P < 0.01), indicating that BMSCs transplant improved the survival rate, neurological function recovery and the extent of demyelination in EAE mice through downregulation of NF-κB signaling pathway.

Discussion

MS is a complex neuroinflammatory disease caused by local inflammation and immune dysfunction, leading to demyelination and extensive mononuclear cell infiltration[20–22]. Generally speaking, MS is considered as a disease of central nervous system involved in $CD4^+$ T lymphocytes, including Th1 and Th17[23, 24]. Although the etiology still unclear, increasing evidence showed that genetic and environmental factors were associated with MS[25, 26]. It is well known that the complex pathogenesis of MS mainly includes demyelination and inflammation[27]. Transplantation or remyelination could promote the recovery of neurological function and demyelination of EAE[28, 29]. However, the transplantation of stem cells is the focus on the clinical treatment of MS[30, 31].

Increasing evidences have demonstrated that BMSCs promoted remyelinate axons and neurological function recovery in EAE animal model[32]. In this study, we have found that BMSCs could be induced to differentiate into neurons, astrocytes and oligodendrocytes. Our result demonstrated that BMSCs transplantation could improve the survival rate, neurological function recovery and demyelination in EAE mice by successfully inducing BMSCs to differentiate into neurons. Thus, improving the success rate of stem cell transplantation could be used as a means of clinical treatment of MS. Although stem cell transplantation is advanced, the success rate of stem cell transplantation is relatively low[33]. Some studies have evaluated the effect of stem cell transplantation on the clinical treatment of MS[34–36].

However, intravenous injection of BMSCs could not across the across the blood-brain barrier (BBB), which is a key problem in the future. In the present study, BMSCs were injected into the lateral ventricle to detect the markers of oligodendrocytes, neurons and astrocytes, indicating that BMSCs were induced to differentiate into neurons in EAE mice. In addition, there may be potential molecular mechanism in the pathogenesis of MS. In our study, we also found that BMSCs transplant downregulated the expression of NF-κB signaling pathway in EAE mice.

Our data showed that BMSCs transplant was benefit to the mice of EAE model, which was consistent with previous studies[37, 38]. However, the potential mechanisms for the effect of BMSCs transplantation on remyelination still unclear. BMSCs were induced to differentiate into neurons to improve the survival rate, neurological function recovery and the extent of demyelination in EAE mice. This may be caused by the activation of endogenous factors in the brain.

Morever, our results have demonstrated that the expression of NF-κB, IκB-α and IL-17 was decreased by BMSCs transplant, indicating that BMSCs transplant improved the survival rate, neurological function recovery and demyelination through downregulation of NF-κB signaling pathway. BMSCs transplantation could decrease neuroinflammatory response to promote axon regeneration, providing a therapeutic strategy for later clinical treatment. However, the way of BMSCs transplantation into human body needs to be further explored.

Conclusion

In conclusion, our results revealed that BMSCs were transplanted into the brain of EAE mice, differentiated into neurons, improved the survival rate, neurological function recovery and the extent of demyelination in EAE mice by downregulation of NF-κB signaling pathway.

Abbreviations

MS: Multiple sclerosis; EAE: Experimental autoimmune encephalomyelitis; BMSCs: Bone marrow mesenchymal stem Cells; CNS: Central nervous system; MAP-2: Microtubule-associated protein 2;GFAP: Gial fibrillary acidic protein; MPB: Myelin Basic Protein; O4:Oligodendrocyte Marker ;LFB: Luxol Fast Blue

Declarations

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Authors' contributions

Jia Geng conceived and supervised the study. Jia Geng and Guo-yi Liu a designed the experiment. Guo-yi Liu, Fan-yi Kong, Shu Ma and Li-yan Fu performed the experiments and analyzed data. Jia Geng wrote the manuscript with support from Guo-yi Liu and Fan-yi Kong. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal experiments was approved by the Ethics Committee of Kunming Medical University and approved by Ethics Committee of Kunming Medical University

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table.1 Primary antibodies

Primary antibodies	Company	Dilution
MAP-2	Abcam	1:600
βIII-tubulin	Abcam	1:400
GFAP	Abcam	1:300
MBP (Myelin Basic Protein)	Abcam	1:400
O4(Oligodendrocyte Marker)	Sigma	1:500

Table.2 Primer sequences

Gene	Sequence (5′~3′)	Company
GFAP	AATCACAAGGTCACAAGA	Invitrogen
	GGCGTTCCATTTACAATC	
β tubulin	TCAAGATGTCCTCCACCTT	Invitrogen
	GTGAACTGCTCGGAGATG	
MAP-2	AAGGTGAACAAGAGAAAGA	Invitrogen
	GAGAAGGAGGCAGATTAG	
MBP	ACTATAAATCGGCTCACAAG	Invitrogen
	AGCGACTATCTCTTCCTC	
O4	CCTTGTTGCCACCATCTG	Invitrogen
	CATACAGGGAGTAGCCAAAG	
GAPDH	AAAGGGTCATCATCTCTG	Invitrogen
	GCTGTTGTCATACTTCTC	

Figures

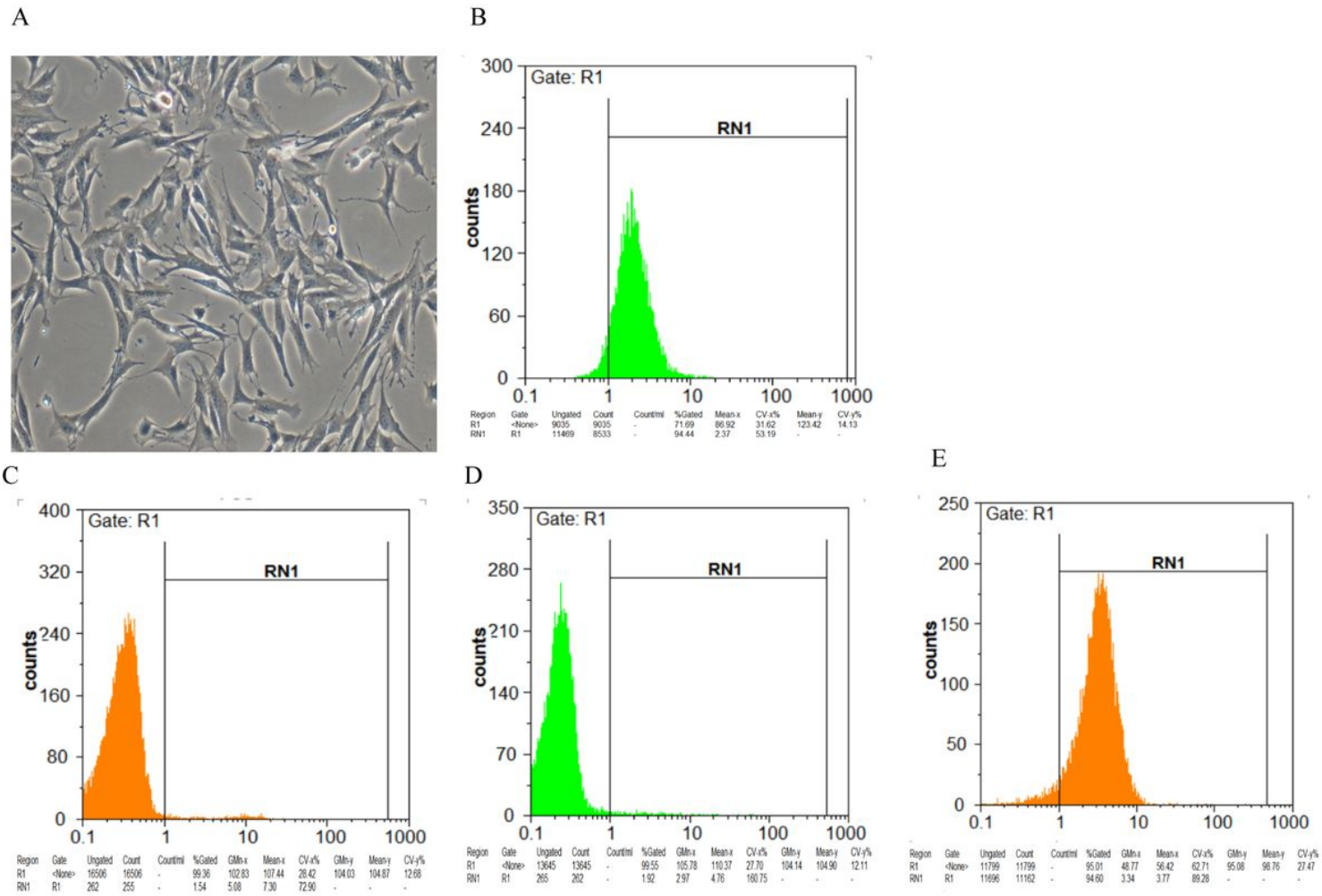

Figure 1

BMSCs morphology and surface maker expression. (A)BMSCs morphology was assessed by microscopy. (B-E) CD29, CD34, CD45 and CD90 surface marker expression was determined by flow cytometry.

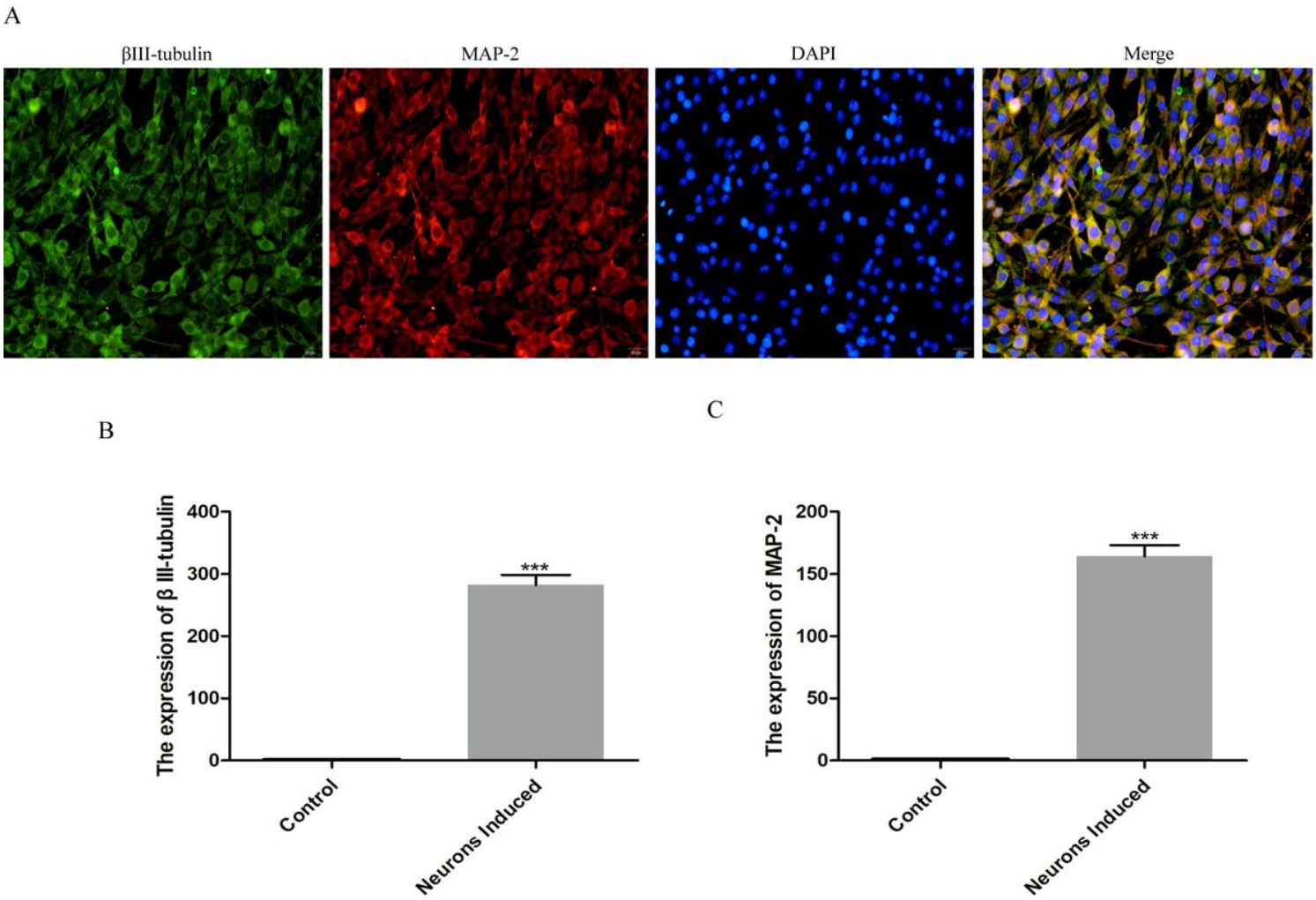

Figure 2

BMSCs were induced to differentiate into neuron cells. (A)The expression of MAP-2 and βIII-tubulin was detected by immunofluorescence staining. (B)We detected the expression of MAP-2 and βIII-tubulin mRNA by Q-PCR (P<0.001). The expression of MAP-2 and βIII-tubulin mRNA showed a significant increase after neuron induction, indicating that BMSCs were successfully induced into neuronal cells. Data were expressed as mean ± SD. **P < 0.01, ***P<0.001.

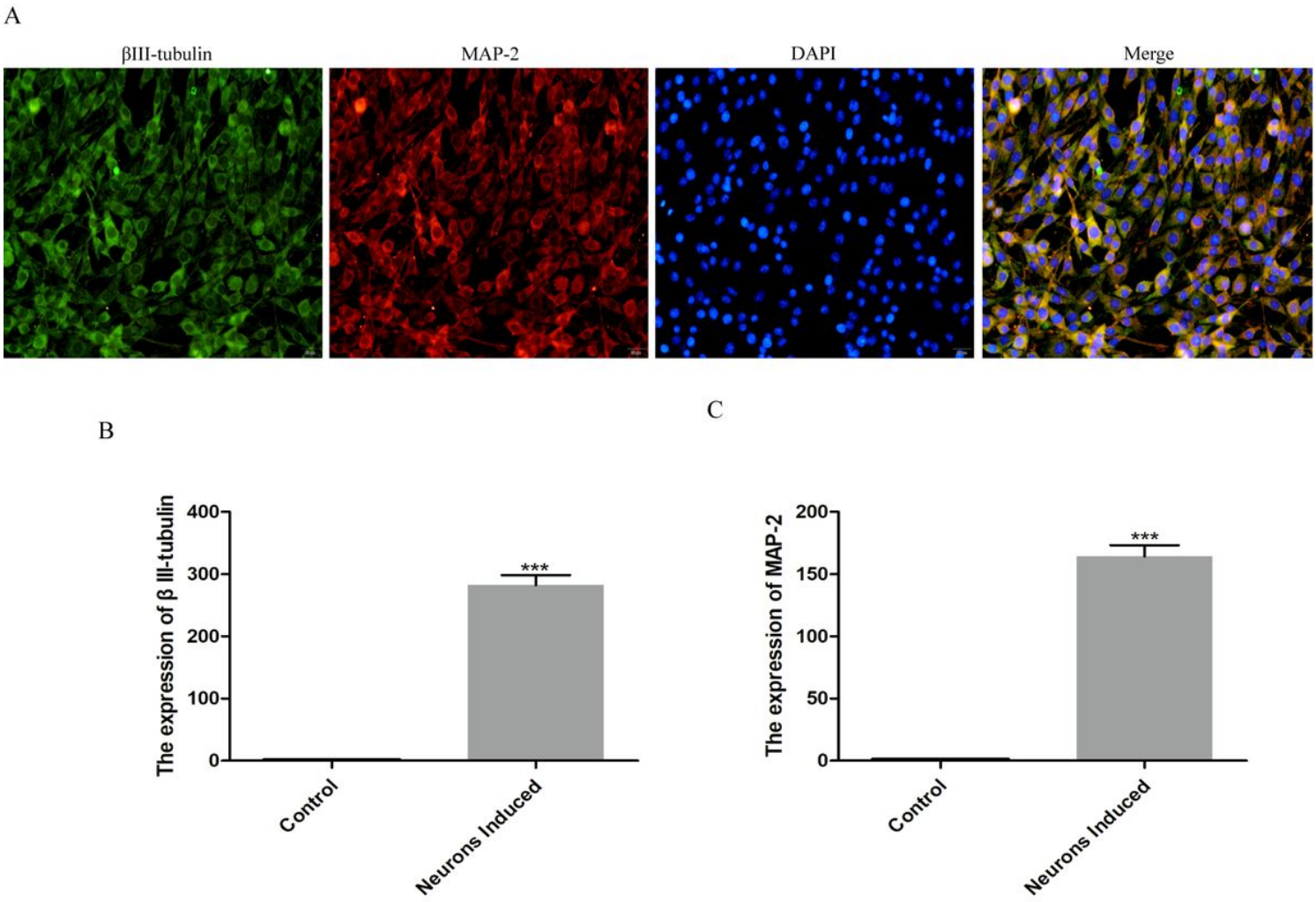

Figure 3

BMSCs were induced to differentiate into astrocytes. (A)The expression of GFAP was observed by immunofluorescence staining after fixed with 4% paraformaldehyde. (B)The levels of GFAP mRNA was significantly increased by Q-PCR after BMSCs were induced (P<0.001). Data were expressed as mean ± SD. ***P<0.001.

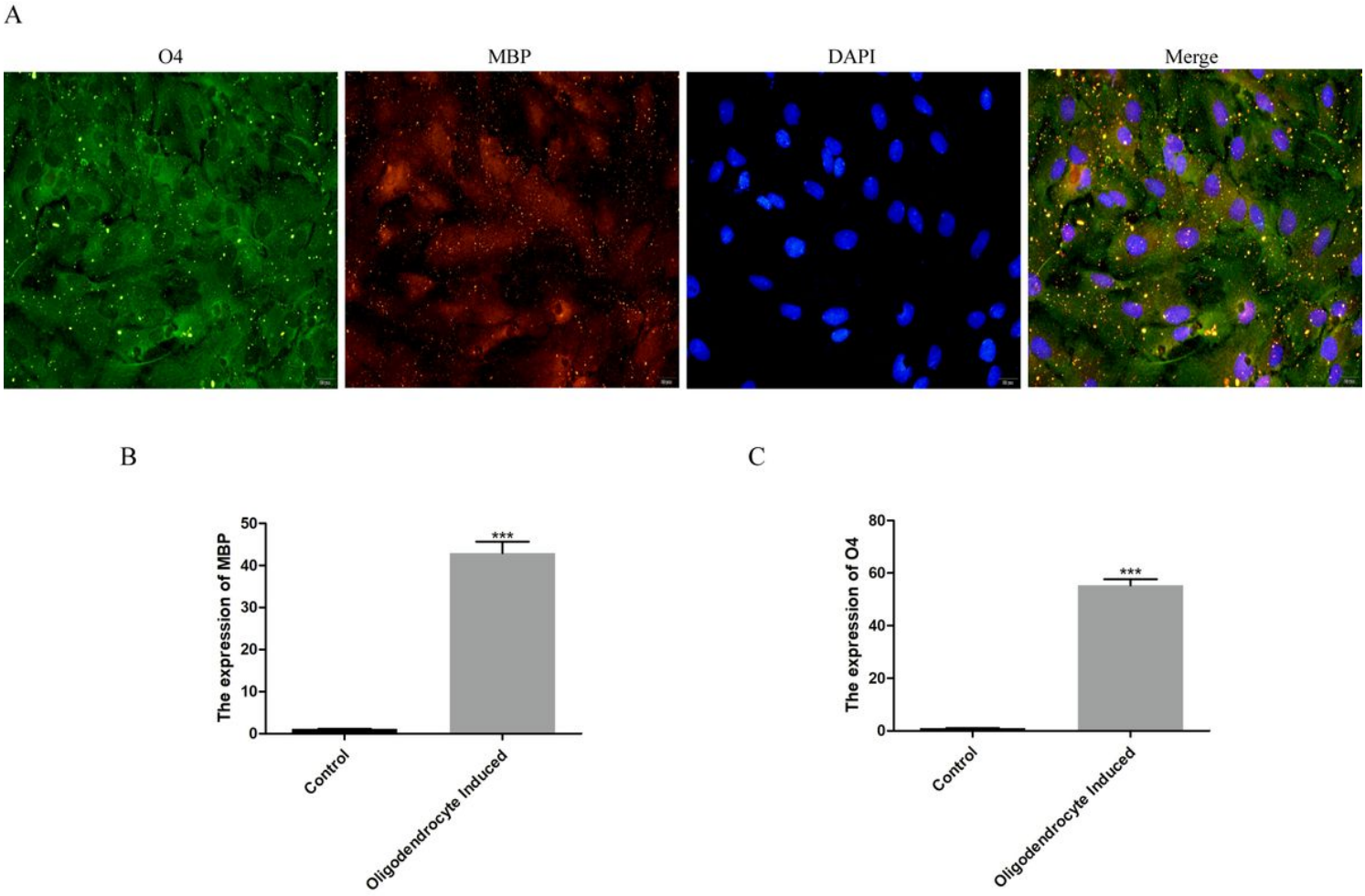

Figure 4

BMSCs were induced to differentiate into oligodendrocyte. (A)Myelin Basic Protein (MPB) and Oligodendrocyte Marker (O4) were detected by immunofluorescence staining. (B-C) We also detected the levels of MBP and O4 by Q-PCR. Data were expressed as mean ± SD. ***P<0.001.

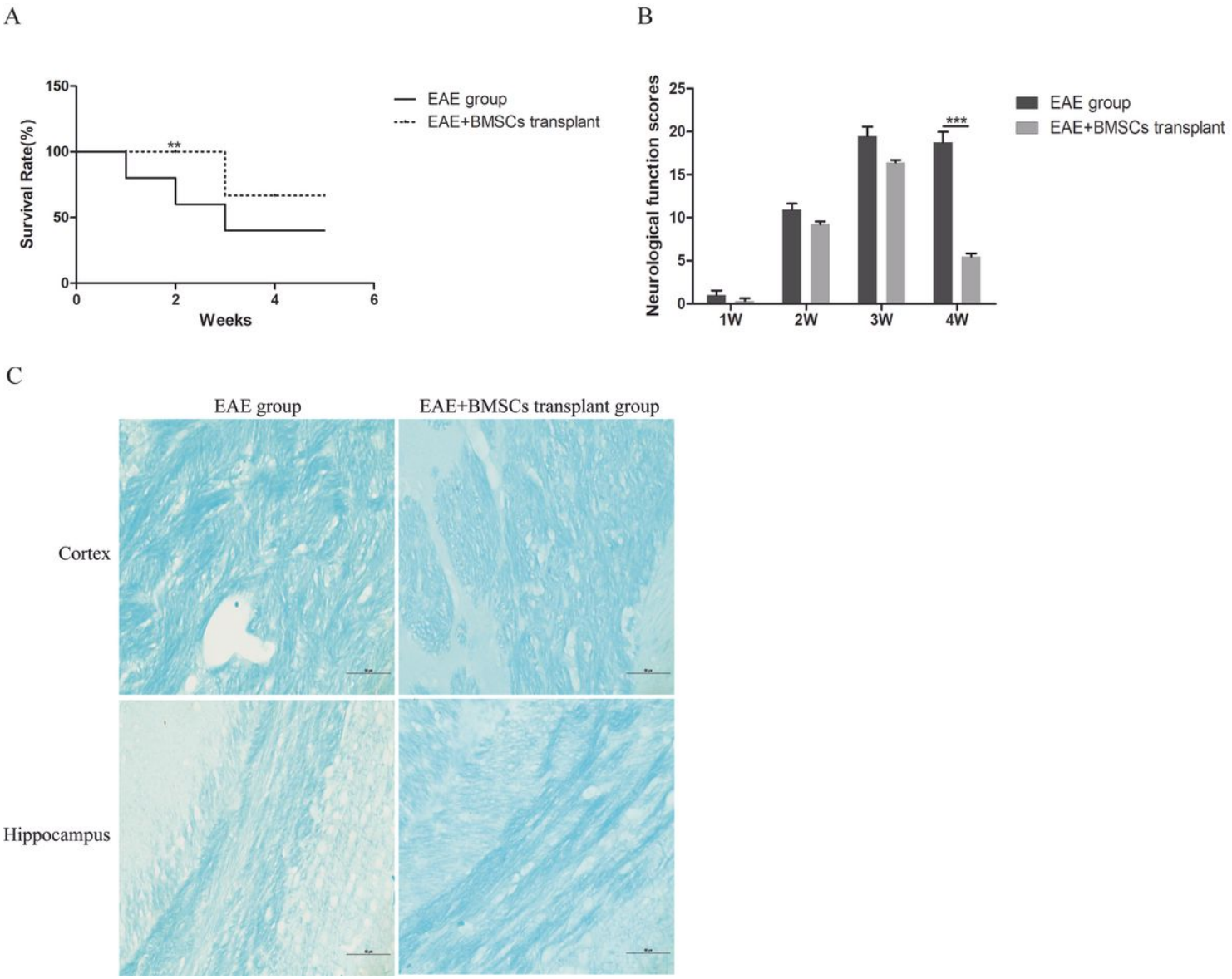

Figure 5

BMSCs transplant improved the survival rate, neurological function recovery and the extent of demyelination in EAE mice. (A)The survival rate of mice was analyzed by the survival curve. (B) The neurological function scores were evaluated in EAE mice. (C)LFB staining of brain sections was performed after BMSCs transplantation in the cortex and hippocampus of rats. Data were expressed as mean ± SD. **P<0.01, ***P<0.001.

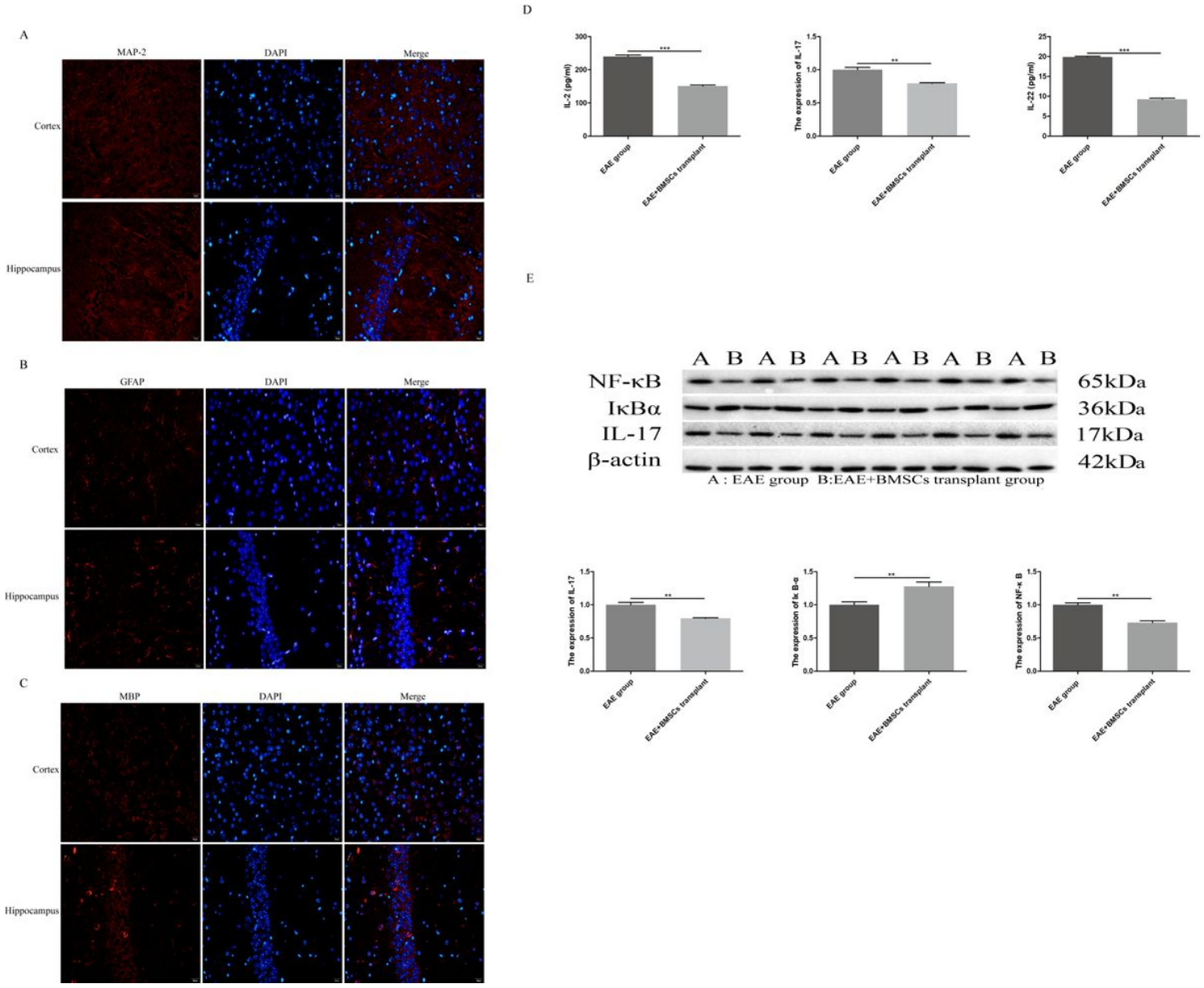

Figure 6

BMSCs transplant downregulated the expression of NF-κB signaling pathway in EAE mice. (A-C) The expression of MAP-2, GFAP, and MBP were determined in the cortex and hippocampus of mice. (D) The levels of IL-2, IL-10, IL-17 and IL-22 were measured in the serum of EAE mice. (E) The expression of NF-κB, IκB-α and IL-17 was detected in BMSCs transplant group by western blotting. Data were expressed as mean ± SD. **P<0.01, ***P<0.001.