

Promotion of microglial phagocytosis by tuftsin stimulates remyelination in experimental autoimmune encephalomyelitis

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Abstract. Microglia were once thought to serve a pathogenic role in demyelinating diseases, particularly in multiple sclerosis (MS). However, it has recently been shown that in the experimental autoimmune encephalomyelitis (EAE) model of MS, microglia could serve a protective role by promoting remyelination via the efficient removal of apoptotic cells, the phagocytosis of debris and the support of myelinating oligodendrocytes. The aim of the present study was to determine if the effect of microglia could promote the recovery of EAE and attenuate symptoms in EAE. The severity of EAE was assessed by clinical scores, pathologic changes revealed by luxol fast blue staining and immunohistochemical techniques. The results suggested that microglia reduced clinical scores in mice, suppressed ongoing severe EAE and promoted remyelination and recovery in EAE mice. In addition, following induction with tuftsin, the M1/M2 cytokine balance was shifted, downregulating the proinflammatory M1 response and upregulating the anti-inflammatory M2 response. Generally, microglia can stimulate remyelination, which serves a protective role in different phases of EAE and may represent a potential therapeutic strategy for the treatment of MS.

Introduction

Microglial cells are the resident immune cells of the central nervous system (CNS) and can quickly respond to CNS injury; activation occurs rapidly within hours (1). Once the microglia are activated, they can secrete and release cytokines and chemokines, such as interleukin (IL)-1, IL-2, IL-6, transforming growth factor (TGF) 131, and matrix metalloproteinase (MMP)9, and express

complement components and their receptors (2). Traditionally, microglial cell activity in the CNS has been considered detrimental, as they recruit and reactivate T cells in the CNS by secreting MMPs, disrupting the blood-brain barrier, upregulate major histocompatibility complex II molecules and exert OPC toxicity by producing inflammatory cytokines and reactive oxygen species (3). Microglia have been reported to participate in the pathogenesis of many human diseases, such as Alzheimer's disease, multiple sclerosis (MS), autoimmune diseases and other disorders (4). Particularly in MS, microglia have been reported to serve dual roles in demyelination in the CNS, including protective and pathogenic roles (5). However, previous studies have demonstrated that microglia may serve an important role in the remyelination process, through phagocytosing debris from cells and destroyed myelin, supporting myelinating oligodendrocytes and promoting axonal regeneration (6,7).

Tuftsin (Thr-Lys-Pro-Arg) was first described by Fridkin *et al* (8) and was thought to be a phagocytosis-stimulating factor for cells of monocytic origin. Tuftsin activates macrophages through binding to receptors that are expressed by cells of monocytic origin, including microglia (9).

The present study investigated the role of microglia in EAE progression. Tuftsin was used to alter the activation of microglia. Early administration of tuftsin altered the activation of microglia and attenuated the humoral immune responses associated with EAE progression to different degrees.

Materials and methods

Experimental animals. Adult (8-9 weeks old and 18-20 g) female C57BL/6 mice were purchased from North China University of Science and Technology. All mice were housed in a temperature-controlled room under a 12-h light/dark cycle for 4 weeks with food and water *ad libitum*. The mice were randomly divided into three groups; control group (n=12), experimental autoimmune encephalomyelitis group (EAE group, n=12) and Tuftsin group (n=12). All protocols were approved by the Animal Ethics Committee of the North China University of Science and Technology.

Induction of EAE in mice. EAE mice were induced with MOG35-55 (200 µg), and the mice were intraperitoneally

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injected with pertussis toxin (500 ng, List Biological Laboratories, Inc.) at 0 and 48 h following immunization. At least two investigators weighed and evaluated the animals for clinical scores in a blinded manner. There were two animals which appeared to be in intolerable distress and self-mutilated limbs; these were sacrificed using pentobarbital sodium (150 mg/kg).

Clinical assessment. Clinical scores (10) were determined in accordance with the following criteria: 0, healthy; 1, limp tail; 2, ataxia and/or paresis of the hind limbs; 3, paralysis of the hind limbs; 4, paresis and/or paralysis of the forelimbs; 5, moribund or dead.

Time-controlled drug delivery. The present study used ALZET mini-osmotic pumps to control drug delivery over time. The mice were injected with either PBS or 500 mM tuftsin [Gen Script (Nanjing) Co., Ltd.] at a rate of 0.25 ml/h (total volume was 100 ml). Pumps were implanted subcutaneously in the backs of anaesthetized mice on day 1 following immunization. On day 15, the pumps were replaced with fresh pumps and maintained thus until day 28.

Histological staining and immunohistochemistry. Spinal cords were obtained from anaesthetized mice, which were perfused intracardially with 4% paraformaldehyde. The samples underwent a dehydration in graded ethanol (70% ethanol 3-5 min; 80% ethanol 3-5 min; 90% ethanol 3-5 min; 95% ethanol 3-5 min). Paraffin-embedded tissue sections were cut in the coronal plane at a thickness of 5 μ m. Histological staining, including LFB staining, was performed to identify demyelination. The sections were left in LFB solution (Beijing Solarbio Science & Technology Co., Ltd.) at 56°C overnight, excess stain rinsed off with 95% ethyl alcohol and distilled water, differentiated in lithium carbonate solution for 30 sec and 70% ethyl alcohol for 30 sec, counterstained in cresyl violet solution (Guidechem) for 30-40 sec, rinsed in distilled water, differentiated in 95% ethyl alcohol for 5 min then placed in 100% alcohol for 5 min (twice) and finally two baths in xylene for 5 min each. Immunohistochemistry was performed with anti-myelin basic protein (MBP) antibodies to identify MBP (1:100; sc-271524, Santa Cruz Biotechnology, Inc.). Hematoxylin was used to stain cell morphology. The sections were observed under light microscope (magnification, x40) (11) and analyzed by Image 2 Pro plus 5.0 (Media Cybernetics, Inc.).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from the brain and spinal cord in all groups using the RNAeasy Micro kit purchased from OMEGA Company following the manufacturer's instructions. Reverse transcription was performed on 1 μ g of total RNA with an RT-PCR kit (Life Technologies; Thermo Fisher Scientific, Inc.), Purity quantification, cDNA synthesis and qPCR were performed according to the manufacturer's protocols. Reaction procedures were as follows: An initial step at 95°C for 5 min, 40 cycles of 94°C for 15 sec, and 60°C for 34 sec; reaction volume 20 μ l. The primer sequences were: GAPDH: Forward: 5'-TTCACCAACCATGGAGAAGGC-3', Reverse: 5'-GGCATG GACTGTGGTCATGA-3'; tumor necrosis factor (TNF)- α :

Forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3', Reverse: 5'-TGGG-AGTAGACAAGGTACAACCC-3'; IL-10: Forward: 5'-TGGCCACACTTGA-GAGCTGC-3', Reverse: 5'-TTCAGGGATGAAGCGGCTGG-3'; TGF- β : Forward: 5'-CCGCAACAACGCAATCTATG-3', Reverse: 5'-AGCCCT GTATTCCGTCTCCTT-3'.

qPCR was carried out using SYBR green mix (Roche, USA) and analyzed using the $2^{-\Delta\Delta C_q}$ method (11). The relative expression levels of the mRNAs were reported as fold changes vs. control.

Western blot analysis. Protein was extracted from the brain and spinal cord. The samples were lysed in Tissue Protein Lysis Solution (Thermo Fisher Scientific, Inc.). The protein concentration was determined using a BCA protein assay kit (OriGene Technologies, Inc.) and 25 μ g of protein was loaded per lane. The primary antibodies used were specific for β -actin (1:1,000; cat. no. AB8227, Abcam), for the detection of ionized calcium binding adaptor molecule 1 (iba-1; 1:1,000; no. AB5076, Abcam), and for the detection of MBP (1:500, no. sc-271524, Santa Cruz Biotechnology, Inc.). Protein extracts were separated by electrophoresis on 12% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes. The membranes were blocked in 5% non-fat milk for 2 h at room temperature and washed three times in PBS with Tween-20 (0.05%). The membranes were sequentially incubated at 4°C with primary antibodies (2 h) and secondary antibodies (1 h; 1:1,000; no. AB205718, Abcam) and enhanced chemiluminescence (ECL) solution. Finally, the images were captured and analyzed by using ImageJ v1.41 software (National Institutes of Health).

Statistical analysis. Data from experiments performed in triplicate were analyzed by One-way ANOVA, and multiple comparisons between groups were performed using the Student-Newman-Keuls method. The results are shown as the mean \pm standard error of mean. $P < 0.05$, $**P < 0.01$ or $***P < 0.001$ was considered to indicate a statistically significant difference.

Results

Effect of tuftsin on the activation of microglia in EAE. To explore the function of microglia in EAE, the expression of microglia in mice induced with tuftsin or PBS was first examined. EAE mice were induced with the MOG35-55 peptide. The expression levels of all groups were compared using western blotting at different time points of EAE. As shown in Fig. 1, western blots indicated increases in iba-1 and MBP expression in the spinal cords of tuftsin-treated mice compared with those of the EAE mice on day 7 ($P > 0.05$), day 14 ($P < 0.01$), day 21 ($P < 0.01$), and day 28 ($P < 0.01$). In a comparison of the EAE group and the control group, expression of iba-1 in EAE group demonstrated notably higher expression than the control group on day 14 ($P < 0.01$), day 21 ($P < 0.001$), and day 28 ($P < 0.001$); the EAE group demonstrated significantly higher expression of MBP compared with the control group on day 14 ($P < 0.001$), day 21 ($P < 0.001$) and day 28 ($P < 0.001$).

Effect of tuftsin on EAE clinical scores. Clinical scores are one of the validated behavioral methods used to evaluate

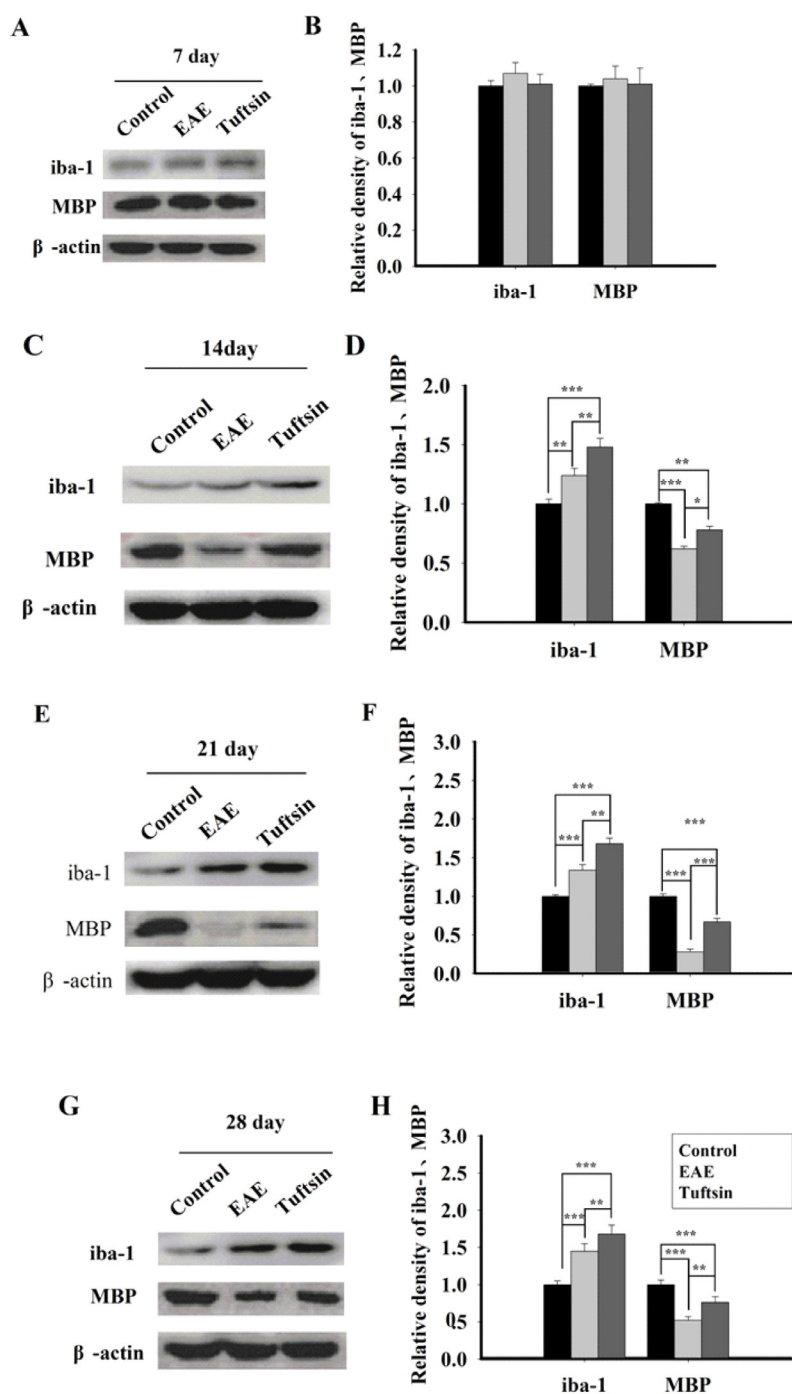


Figure 1. Expression of iba-1 was observed in all three groups. iba-1 and MBP expression of all groups at (A and B) 7 days, (C and D) 14 days, (E and F) 21 days and (G and H) 28 days. The results are shown as the mean \pm standard error of mean. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. iba-1, ionized calcium binding adaptor molecule 1; MBP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis.

the symptoms of EAE in mice. Following induction of EAE with MOG35-55 or PBS, the symptoms of EAE mice with and without tuftsin treatment were compared by using clinical score testing. In Fig. 2A, we observed that in the acute phase of EAE, all groups had similar clinical scores. The clinical scores of the EAE and tuftsin groups changed from day 11; however, the EAE and tuftsin groups achieved a higher clinical score than the control group on day 14, day 21 and day 28 ($P < 0.05$). However, during the progression of EAE, the control group had no mice without the disease. Notably, EAE mice treated with tuftsin had

a significantly lower clinical score than did mice treated with PBS on day 14 ($P < 0.05$), day 21 ($P < 0.05$) and day 28 ($P < 0.05$). These results demonstrated that tuftsin treatment can ameliorate the behavior impairment induced by MOG35-55.

Remyelination was increased when the activation of microglia was stimulated A typical pathological change during EAE development is demyelination, which can be evaluated utilizing LFB staining. To investigate this aspect comprehensively, a histological examination of spinal cord tissue

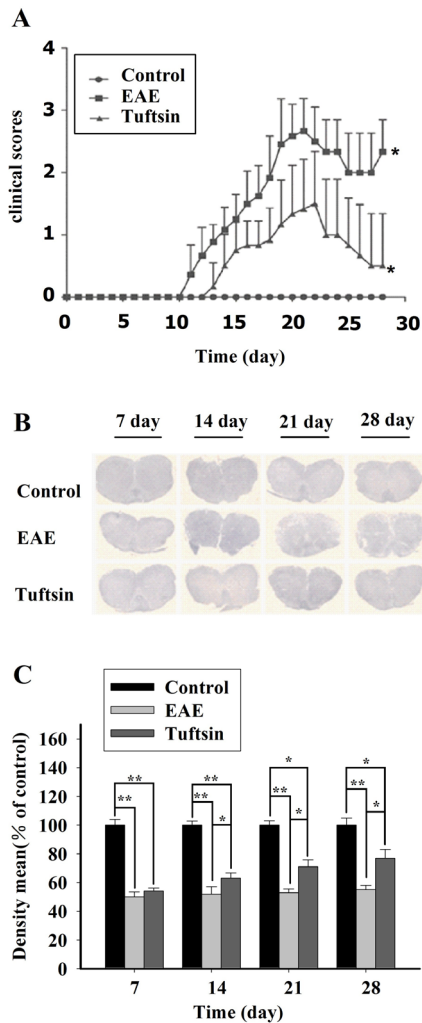


Figure 2. Effects of microglial cells exposed to tuftsin on EAE severity and demyelination. (A) Daily scores were averaged. Control (n=6); EAE (n=6); tuftsin (n=6) at day 7, day 14 ($P<0.05$), day 21 ($P<0.05$) and day 28 ($P<0.05$). (B) Demyelination lesions of the spinal cord in the three groups (magnification $\times 40$). Luxol fast blue staining at days 7, 14, 21 and 28. (C) Density mean (% of control). The results are shown as the mean \pm standard error of mean. * $P<0.05$, ** $P<0.01$. EAE, experimental autoimmune encephalomyelitis.

isolated from mice in the control, EAE and tuftsin groups was performed at different time points of the EAE process. The spinal cord tissue sections were subjected to LFB staining in which myelin is stained blue to green, immunohistochemical examination. As shown in Figs. 2B and C, and 3, significant demyelination was not observed in any group on day 7. At day 14, the clinical onset point, LFB staining of the EAE group demonstrated slight demyelination in the white matter. However, in the tuftsin group, the demyelination was less intense at the same time point. The structure of the myelin sheath in the control group, however, exhibited integrity. At the peak of the EAE process, day 21, a gradual but obvious change in the myelin sheath revealed severe deficiency in the EAE group. In contrast, demyelination was milder in the tuftsin group. On day 28, during the recovery stage of EAE, the loss of myelin did not continue but remyelination began, particularly in the tuftsin group, in which remyelination was more obvious than in the EAE group, showing that the structure of the myelin sheath was relatively intact. It should

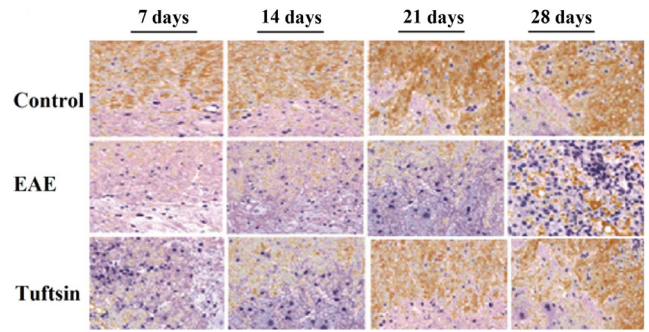


Figure 3. Immunohistochemistry and western blot analyses for the myelin sheath using anti-MBP. Immunohistochemistry for the myelin sheath using MBP in spinal cord sections (magnification, $\times 40$). The white matter indicated by MBP and the amount of MBP differed at various stages of EAE. MBP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis.

be noted that the structure of myelin in the control group remained unaltered.

Microglia subset alteration alleviates EAE symptoms. Microglia were demonstrated to have M1 and M2 subsets, which represent pro- and anti-inflammatory populations, respectively (12). $\text{TNF-}\alpha$ and nitric oxide were secreted by M1 microglia, while neuroprotective M2 microglia produced protective cytokines, including IL-10 and $\text{TGF-}\beta$. M2 microglia have been reported to serve a protective role in EAE by ameliorating experimental autoimmune encephalomyelitis (13). To test whether M1 or M2 microglia contributed to the severity of EAE during development, the concentrations of cytokines produced by M1 or M2 microglia were detected. Total RNA was extracted from the brains from each group and RT-qPCR performed to determine the microglial phenotype based on $\text{TNF-}\alpha$ levels for M1, and IL-10 and $\text{TGF-}\beta$ levels for M2. Fig. 4 shows that the amounts of $\text{TNF-}\alpha$ and $\text{TGF-}\beta$ were reduced in the tuftsin group and that the level of IL-10 was increased in the EAE group. The levels of $\text{TNF-}\alpha$, IL-10 and $\text{TGF-}\beta$ were increased at day 14 in the tuftsin group and EAE group, and the levels of IL-10 and $\text{TGF-}\beta$ in the tuftsin-treatment group exceeded those in the EAE group ($P<0.01$ and $P<0.05$, respectively), but the level of $\text{TNF-}\alpha$ was less than that in the EAE group ($P<0.01$). At days 21 and 28, a comparison of the levels of IL-10 and $\text{TGF-}\beta$ in the tuftsin treatment group with those in the EAE group revealed that the former increased more than the latter ($P<0.001$, $P<0.001$); however, the level of $\text{TNF-}\alpha$ demonstrated the opposite pattern. The control group generated little $\text{TNF-}\alpha$, IL-10 and $\text{TGF-}\beta$. Collectively, these data indicated that microglial activation did not contribute to the development of EAE but rather the secretion of protective cytokines, which ameliorated the progression of clinical EAE. Thus, these experiments indicated that microglia released anti-inflammatory cytokines, improving remyelination.

Discussion

The present study observed the role of microglia in EAE progression and the findings demonstrated that demyelination increased during EAE progression before peaking. By increasing microglial activation, the disease severity of EAE

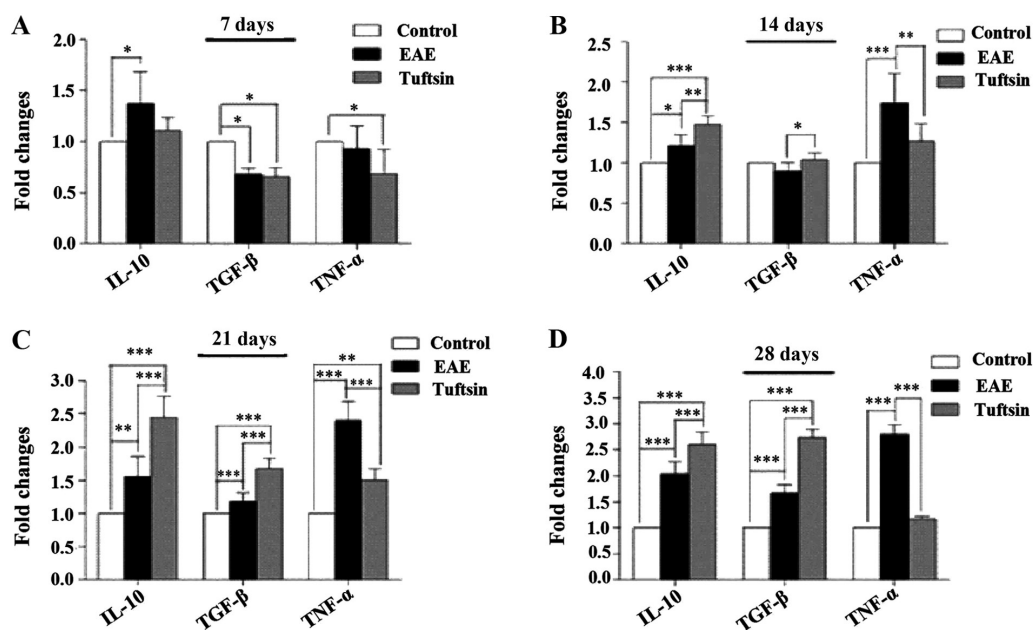


Figure 4. RT-qPCR was performed on RNA from spinal cord homogenates extracted from all three groups at various time points. The proinflammatory cytokine TNF- α and the anti-inflammatory cytokines IL-10 and TGF- β produced by microglial cells were quantified at (A) 7, (B) 14, (C) 21 and (D) 28 days. n=6. The results are shown as the mean \pm standard error of mean. *P<0.05, **P<0.01 and ***P<0.001. RT-qPCR, reverse transcription-quantitative PCR; TNF, tumor necrosis factor; IL, interleukin; TGF, transforming growth factor.

could be suppressed in the chronic phase. However, the recovery of EAE mice occurred earlier in the tuftsin-treated group than in the EAE group, correlating with microglial activation. Generally, activation of microglia reduced EAE severity and advanced the recovery of EAE in C57BL/6 mice.

To investigate the implications of microglia for MS, EAE was induced with MOG35-55 in C57B6/L mice, which is the commonest animal model used to study MS (14). Then, clinical score assessment, a widely accepted behavioral method for evaluating EAE symptoms, was used to observe the variation among the mice. In the present study, mice subjected to MOG35-55 exhibited weakness in the tail and hind limbs. The results of subsequent clinical score testing demonstrated that the mice treated with tuftsin achieved markedly better scores relating to limb weakness and performance compared with their counterparts which did not receive tuftsin, especially during the recovery phase.

Subsequently, the variation of pathology, including LFB staining, was assessed in the spinal cord because LFB is considered an index of demyelination, which is a widely accepted model for assessing the degree of demyelination at the pathology level (15). LFB staining revealed not only intense mitochondrial staining but also staining for demyelinating fibers both peripherally and in the CNS (16), which was widespread in myelin staining. An increase in myelination in the EAE group was detected compared with the control group and the tuftsin group, which could promote microglia; this effect decreased in the spinal cord compared with the EAE group, especially during the recovery phase. These data indicated that in the recovery phase, microglia could improve remyelination and attenuate the severity of EAE symptoms.

The role of microglia in demyelinating neurodegenerative diseases, MS and its animal model EAE is still controversial (6). Previous data have shown that microglia

serve a detrimental role and encompass a wide range of harmful factors, such as serving a toxic role in neurons and oligodendrocyte precursor cells, and releasing proteases, inflammatory cytokines and free radicals (17), as well as recruiting and promoting T lymphocyte reactivity in the CNS (18,19). Emerging evidence, however, has demonstrated that microglia serve beneficial roles in the brain during neurodegenerative disease (5), particularly in the recovery of EAE progression. The precise mechanisms involved in the protective role of microglia remain unclear, but evidence indicates that microglia serve a vital role, including axonal regeneration, assistance with remyelination, efficient removal of injured myelin debris and release of trophic support factors (6). By phagocytosing cellular debris (20) and releasing trophic support factors and anti-inflammatory cytokines, microglia can protect neurons from injury and repair the injured brain and spinal cord (21). Furthermore, all of these effects contribute to myelin regeneration in MS and EAE, in which remyelination occurs spontaneously (22).

One mechanism of remyelination is the efficient clearance of myelin debris that contains inhibitory factors for axonal growth, which is a critical step during the remyelination process (23). Microglia, the resident immune cells in the CNS, could serve a phagocytic role similar to that of other cells of monocytic origin. Microglial phagocytosis has been suggested to remove myelin debris and apoptotic cell debris during demyelination and is beneficial to promoting regeneration (21). Independent studies have shown that microglia function through lectin-, integrin-, and phosphatidylserine-mediated recognition of apoptotic neurons, followed by phagocytosis and clearance of apoptotic neurons (24). In the present study, administering tuftsin promoted phagocytosis for microglia expressing tuftsin receptors and enhanced the phagocytosis of microglia. Therefore, tuftsin treatment may upregulate the role

of phagocytosis by combining with its receptor on microglia, which could efficiently clear the debris from disrupted myelin sheaths and injured neurons and may maintain an environment favorable for remyelination.

One important factor in remyelination is oligodendrocyte precursor cells (25), which migrate to the demyelination lesion, differentiate into myelinating oligodendrocytes and synthesize myelin (26). A previous study demonstrated that remyelination occurs during demyelination progression, in which endogenous oligodendrocyte precursor cells (OPCs) intend to remyelinate, but this process fails (25). In addition, blocking the differentiation of oligodendroglial progenitor cells causes remyelination failure during MS progression (27). A study indicated that different states of microglia were related to oligodendrogenesis by producing IGF-1 and downregulating TNF- α (28). Microglia have been demonstrated to present a cytokine and chemokine repertoire, which enables them to activate and recruit endogenous OPCs to the MS lesion and transmit trophic support during remyelination.

Inflammatory autoimmune attack against myelin in the CNS leads to myelin destruction and demyelinated lesion formation (plaques) in MS (29). Thus, inhibition of the inflammatory response, instead of improving anti-inflammation, contributes to remyelination. Microglia serve an important role in the pathology and pathogenesis of MS. A previous study suggested that microglia might express immune receptors, nod-like receptors (NLRs), which are intracellular receptors capable of suppressing inflammation and ameliorating EAE symptoms (30). In addition, deregulation of cytokines (M1 vs. M2) has been reported to be involved in the pathogenesis of remyelination (31). A previous study demonstrated that microglia/macrophages contribute to driving oligodendrocyte differentiation during CNS remyelination by shifting the phenotype from pro-inflammatory M1 to anti-inflammatory M2, which triggers remyelination (32). The M2 phenotype of microglia secrete IL-10 and TGF- β , providing evidence for the induction of an anti-inflammatory M2 phenotype to suppress innate immunity in affected brain tissue (31). TGF- β is a signature of microglia and exerts a protective effect against various neuronal insults (33). Therefore, the M1/M2 balance in EAE was determined by measuring TNF- α as an M1 cytokine and IL-10 and TGF- β as M2 cytokines. In the present study, EAE induced a decrease in IL-10 and TGF- β but an increase in TNF- α in the spinal cord; however, tuftsin treatment normalized these cytokine levels. On the other hand, a previous study indicated that inflammation can convert a non-remyelinating context to a remyelinating context (34).

Together, the results the present study illustrated the possibility that microglia may be activated towards an anti-inflammatory state by shifting to the M2 mode, generating a properly regulated immunosuppressive response. The findings indicated that microglial activation could significantly enhance remyelination induced by anti-immunization in mice with EAE. Diminishing the response to inflammation and enhancing white matter lesions may result from the neuroprotective effect of microglia. More studies are required to detail the mechanisms of microglial activation at the molecular level

by ameliorating neurologic deficits. The results of the present study provide useful information for considering the intervention of microglial activation as an alternative treatment choice for multiple sclerosis.

As for the concentration of the tuftsin, it was thought to be the optimal concentration. At this concentration, there were very few adverse reactions and no mortality in the animals, and this concentration has a good therapeutic effect. However, there were two animals which appeared to be in intolerable distress and self-mutilated limbs and culled for this reason. Of course, in future experiments, we will continue to explore the concentration of the tuftsin to find the more appropriate concentration.

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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.

Authors' contributions

ZB and FF designed the research study. YL performed the experiments and research. JH analyzed the data and wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All protocols were approved by the Animal Ethics Committee of the North China University of Science and Technology, and animal handling was carried out according to the guidelines from the National Institutes of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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