Journal of Neuroimmunology 333 (2019) 576967

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

# CCL26 and CCR3 are associated with the acute inflammatory response in the CNS in experimental autoimmune encephalomyelitis

Jifei Shou[a,1], Jing Peng[b,1], Zhikang Zhao[a], Xiaoxi Huang[a], Hui Li[a], Liheng Li[a], Xinxin Gao[a], Yanmeng Xing[a], Hongbo Liu[a,*]

[a] Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, PR China
[b] Department of Neurology, Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital, Shanghai, PR China

ABSTRACT

Chemokine ligand 26 (CCL26) is a member of the eotaxin family. It works by interacting exclusively with chemokine receptor 3 (CCR3) and acts as an eosinophil-selective chemoattractant. There is an emerging role for eotaxins in autoimmune diseases. Studies have reported that chemokine ligand 11 (CCL11) and CCL26 are upregulated in patients with neuromyelitis optica spectrum disorder (NMOSD) during remission, CCL26 levels appear to be decreased in relapsing-remitting multiple sclerosis (RRMS), whereas CCL26 levels are significantly increased in secondary progressive multiple sclerosis (SPMS), indicating that CCL26 participates in the pathogenesis of multiple sclerosis (MS). We investigated the levels of CCL26, CCR3 and claudin-5 (a marker of changes in BBB (blood-brain barrier) permeability) at different stages of experimental autoimmune encephalomyelitis (EAE) to explore the underlying immune mechanisms of EAE. Our results showed that the levels of CCL26 and CCR3 in EAE rats were significantly increased compared with those in the control group. The levels of CCL26 in the serum and in brain tissues as well as the protein expression of CCR3 in brain tissues were positively correlated with the inflammatory scores of brain tissues from EAE rats and were negatively correlated with the protein expression of claudin-5. We concluded that CCL26, which in turn binds to the receptor CCR3, showed pro-inflammatory effects and aggravated tissue damage involving BBB impairment, especially in the acute stage of EAE. Our study uncovers another possible immunopathological mechanism of MS and provides a possible target for immune therapy.

## 1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating autoimmune disease of the central nervous system (CNS). It occurs in young people, especially (Kan et al., 2014), and is characterized by a wide range of perivascular inflammatory cell infiltration, blood-brain barrier (BBB) damage and the demyelination of white matter within the CNS. A relapse-mitigation process occurs in the lesions (Marcus and Waubant, 2013). The infiltration of inflammatory cells into the CNS which triggers a cascade of inflammatory responses that leads to demyelination and neuroaxonal injury, is an essential step in the pathogenesis of MS and related animal models of experimental autoimmune encephalomyelitis (EAE) (Abramowski et al., 2014; Lassmann, 2010; Mecha et al., 2013).

Chemokines are small chemoattractant cytokines that can induce immune cell infiltration and accumulation in inflammatory tissue, and they have been suggested to contribute to neuroaxonal injury during EAE/MS. Among these cytokines, eotaxins and their emerging role have drawn much attention(D'Ambrosio et al., 2003; El Behi et al., 2005; Herrero-Herranz et al., 2008). The eotaxin gene family includes three members, eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26), that encode CC chemokines that stimulate the migration of eosinophils from vessels to target tissues by acting on C—C motif chemokine receptor 3 (CCR3).

Previous studies on eotaxins have mainly concentrated on the cells of the lung, gut and skin in asthma, inflammatory bowel disease and atopic dermatitis, respectively(Fujimoto et al., 2016; Takahashi et al., 2013). Recently, an important study showed that plasma CCL11 and CCL26 levels are upregulated during the remission stage in patients with neuromyelitis optica spectrum disorder (NMOSD). Furthermore, there is no correlation between CCL11 or CCL26 levels and clinical characteristics in patients with NMOSD, indicating that the two cytokines may be involved in the pathogenesis of NMOSD by aggravating inflammation and leading to relapse during the remission stage in patients with NMOSD(Tong et al., 2018). Several studies have indicated that the secretion of IL-4 and IL-13 stimulates CCL26, while IFN-γ can inhibit it. In addition, studies have observed that increased CSF levels of CCL26 are associated with the progression of mild cognitive impairment (MCI) to Alzheimer's disease (AD) (Hu et al., 2010; Westin et al., 2012) and are correlated with both age and CSF levels of tau proteins

* Corresponding author at: Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Jianshe East Road 1, Erqi District, Zhengzhou 450052, Henan, PR China.
*E-mail address:* liuhongbo6279@126.com (H. Liu).
[1] Contributed equally.

https://doi.org/10.1016/j.jneuroim.2019.576967
Received 10 March 2019; Received in revised form 13 May 2019; Accepted 15 May 2019

but do not correlate with the rate of cognitive decline, which indicates that CCL26 plays a role in neurodegenerative disorders.

A recent study demonstrated that plasma levels of CCL11 and CCL26 are increased in SPMS patients compared to RRMS patients. Interestingly, however, CCL11 and CCL26 actually appear to be decreased in RRMS patients when compared to healthy controls (HC), and CCL26 levels are significantly increased in SPMS patients, whereas CCL11 levels in SPMS patients appear to return to the levels in HC. These data indicate that CCL11 and CCL26 may participate in the pathogenesis of multiple sclerosis (MS). Whether CCL11 and CCL26 in the plasma and/or CSF, alone or in combination with other mediators, participate in the progression of MS is still unknown (Huber et al., 2014; Michael et al., 2013; Tejera-Alhambra et al., 2015).

Chemokine receptor expression generally correlates with ligand expression. In vitro studies in cells isolated from the CNS have demonstrated that CCR3, the receptor for eotaxins, is expressed on their membrane surfaces (van der Meer et al., 2000). These studies strongly support the view that the overproduction of eotaxins contributes to the abnormal accumulation of inflammatory cells and cytokines, which may participate in the destruction of the BBB and are linked to the pathogenesis of the central nervous system in autoimmune diseases. Brain microvascular endothelial cells and their tight junctions are the most important for the morphological basis of the BBB. Claudin-5 is a marker of changes in BBB permeability and participates in the inflammatory response in the CNS. However, the role of eotaxins in different neurological diseases and even in distinct stages of the same disease may differ (Huber et al., 2014; Michael et al., 2013; Tong et al., 2018; Westin et al., 2012). Hence, we investigated the levels of CCL26 and CCR3 in different stages of EAE and analysed their correlations with claudin-5 and inflammatory scores to explore the underlying immune mechanisms of EAE and search to new treatment targets for MS.

## 2. Materials and methods

### 2.1. Animals and EAE introduction

Female Wistar rats (6–8 weeks old, weighing 180–200 g), were obtained from the Beijing Vital River Experimental Animal Company (China) and housed in the Henan Academy of Medical and Pharmaceutical Sciences in a pathogen-free environment. The process of EAE induction is briefly described below. Spinal cord homogenates from guinea pigs (Beijing Vital River Experimental Animal Company) weighing 300–350 g were emulsified with the same volume of complete Freund's adjuvant (CFA) (Sigma, St. Louis, MI, USA) containing 6 mg/ml bacillus Calmette-Guérin vaccine (Solarbio Bio-Technology Co., Shanghai, China). Thirty rats were subcutaneously injected into the foot at four separate sites with 0.5 ml of antigen emulsion. All experiments were approved by the Bioethics Committee of Zhengzhou University.

### 2.2. Clinical scoring and weight

Sixty female Wistar rats were randomly divided into two groups: the naïve control group and the EAE group. Immunized rats from the EAE group were randomly divided into three groups ($n$ = 10 in each group), according to the methods in study by Zhu L (Kan et al., 2017; Zhang et al., 2017): the early stage group (day 11 post immunization (p.i.)), the acute stage group (day 18 p.i.), and the remission stage group (day 24 p.i.).

The rats were weighed daily after the day of immunization, and the clinical signs of EAE were scored daily in a blinded fashion. The scoring criteria were as follows: 0 = no clinical score; 1 = loss of tail tone; 2 = hind limb weakness; 3 = hind limb paralysis; 4 = forelimb paralysis; and 5 = moribundity or death.

### 2.3. Histopathological evaluation

The EAE rats were anaesthetized with 10% chloral hydrate on day 11 p.i., on day 18 p.i. and on day 24 p.i. Then, the apical blood was collected, and the supernatant was collected after centrifugation. Precooled saline was used to rapidly perfuse the heart. Under sterile conditions, the brains were collected, embedded in paraffin, and processed for histological evaluation.

Inflammatory infiltration was determined by haematoxylin and eosin (H&E) staining. Histopathological examination was performed and scored in a blinded fashion as follows: 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, the organization of inflammatory infiltrates around blood vessels; and 3, extensive perivascular cuffing with extension into the parenchyma. Inflammation scores were calculated using Image-Pro Plus 5.0 (IPP5.0) software.

### 2.4. Immunohistochemistry

For immunohistochemistry, the brain tissues were dewaxed in xylol, rehydrated, and pretreated with 3% H2O2 to quench the endogenous peroxidase activity. After washing in PBS, nonspecific binding was blocked with bovine serum for 30 min at 37 °C, and the tissues were incubated with an anti-CCR3 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C overnight. Then, the sections were incubated with the corresponding secondary antibodies at 37 °C for 30 min. DAB (ZSGB-BIO Co., Ltd., Beijing, China) was employed to stain the sections after extensive washing. The integral optical density (IOD) of the positive cells, which was detected using a Biosens Digital Imaging System v1.6, represents CCR3 expression.

### 2.5. ELISA of serum CCL26 levels

Levels of CCL26 were detected by ELISA using a rat CCL26 ELISA Kit (Santa Cruz Biotechnology, Dallas, TX, USA). Experimental samples and standards were added to each standard well following the manufacturer's instructions. After incubation (30 min, 37 °C) and washing, chromogen solution A (50 μl) and chromogen solution B (50 μl) were added successively to each well hole. Afterwards, stop solution (50 μl) was added to end the reaction. The results were read within 15 min at 450 nm using a microtiter plate reader.

### 2.6. Immunofluorescence

Paraffin-embedded brain tissues from each group were cut into 5 μm-thick sections for immunofluorescence. Nonspecific binding was blocked with 3% bovine serum (Serotec, UK) and permeabilized with 0.3% Triton X-100 in 1% BSA in PBS for 30 min. The sections were incubated at 4 °C overnight with a claudin-5 antibody and then incubated with corresponding secondary antibodies at room temperature (RT) for 2 h. For each group, ten sections were examined in a blinded fashion. Image-Pro Plus 5.0 software was used to quantify the target protein expression.

### 2.7. Statistical analysis

All statistical analyses were performed with SPSS 17.0 (SPSS, IBM, USA) and GraphPad Prism 5.0 (La Jolla, CA, USA). All data are presented as the mean ± SD, and multiple comparisons were performed using the Kruskal-Wallis rank-sum test or ANOVA followed by the LSD-$t$-test. Correlation analysis was conducted using Pearson's correlation coefficient. For all values, $p < .05$ was considered to be significantly different.

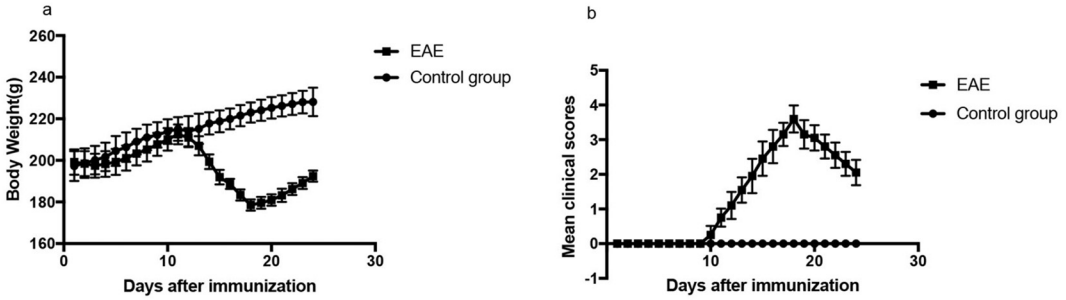



**Fig. 1.** Body weight and clinical scores of EAE rats were evaluated daily after immunization. a Body weight changes after the immunization of EAE rats ($n$ = 30) and naïve control rats (n = 30). b Clinical scores of EAE rats after the immunization of the two groups.

## 3. Results

### 3.1. Weight and clinical scoring

The body weights of the naïve control rats increased gradually, while that of the EAE rats was reduced on day 11 p.i., and a significant difference in body weight was observed between the two groups beginning on the 18th day ($p < .05$ , Fig. 1a). On day 10 p.i., we observed that the EAE rats developed a progressive disease course, while the clinical scores were significantly reduced. The naïve control group showed no neurological defects, and their clinical score was 0. There was a significant difference between the two groups on the 18th day ($p < 0.05$, Fig. 1b).

### 3.2. CNS histopathologic changes

To observe the relationship between disease progression and CNS inflammatory infiltration, the hippocampal fimbria from the brains of all rats were examined by H&E staining. Inflammatory cell infiltration and the demyelination of the CNS are markers of EAE and MS. In the immune system, a small number of autonomous cells can control a large number of nonspecific cells, triggering the immune cascade and causing myelin damage and demyelination. Eliminating these autoreactive cells may result in a rapid and significant regression of the whole inflammatory cascade. We observed that, in the hippocampal regions of the brain tissues of the naïve control group, there was no inflammatory cell infiltration, the nerve cells were fragmented and necrotic, glial cells were found in the white matter, and there was an inflammation score of 0, which was consistent with the lack of clinical symptoms in the group. In the EAE rats, there was a large number of infiltrating inflammatory cells surrounding small blood vessels with typical cuffing. The inflammatory scores of the hippocampal areas of the brain tissues of the EAE rats were significantly higher compared with those of the naïve control group, and the difference was statistically significant ($P < .01$, Fig. 2a, b, c, d).

As shown in Fig. 2c, on day 18 p.i. (the acute stage), the EAE rats reached a maximum inflammatory score of 2.47 ± 0.23. Compared with those of the early stage group (1.51 ± 0.47, Fig. 2b) and the remission stage group (1.91 ± 0.56, Fig. 2d), the inflammatory scores of rats in the acute phase group increased significantly, and the difference was statistically significant ($P < .05$, Fig. 2e).

### 3.3. Protein expression of claudin-5 in brain tissue

Brain microvascular endothelial cells and their tight junctions are the first barrier of the BBB. Changes in tight junctions and their associated proteins may lead to changes in BBB permeability. Claudin-5, which is a specific indicator of the integrity of the BBB, is an important component of the BBB. The results showed that, in the EAE group, claudin-5 protein expression in brain tissue was lower than that in the naïve control group ($P < 0.01$, Fig. 3a, b, c, d). Furthermore, we observed that, in the acute stage, claudin-5 protein expression decreased significantly (13.39 ± 1.28, Fig. 3c) compared with that in the early stage group (25.93 ± 2.46, Fig. 3b) and the remission stage group (21.19 ± 1.25, Fig. 3d), as shown in Fig. 3e, and the difference was statistically significant.

### 3.4. Levels of CCL26 in the serum and brain tissues and the protein expression of CCR3 in brain tissues

We detected the levels of CCL26 and the protein expression of CCR3 by ELISA and immunohistochemistry. As shown in Fig. 4a and b, the CCL26 levels in the serum and brain tissues were significantly higher in all EAE groups than in the naïve group (all $P < .01$). The levels of CCL26 and CCR3 in the acute stage group were higher than those in the early stage group and the remission stage group (both $P < .05$, Fig. 4a, b, Fig. 5e). The number of CCR3-positive cells in the EAE group showed a significant increase compared to that in the naïve group (all $P < .01$, Fig. 5e). Furthermore, significantly higher CCR3 expression was observed in the acute stage group ($P < .05$, Fig. 5a, b, c, d, e).

### 3.5. Correlation analysis

To analyse whether the increased expression of CCL26 and CCR3 in the CNS is related to enhanced EAE severity, correlation analysis was performed for all EAE groups (early stage, acute stage and remission stage). Our results showed that inflammation scores were positively correlated with CCL26 and CCR3 protein levels in all EAE groups ($r = 0.817$, $r = 0.735$, $r = 0.847$, all $P < .01$; Fig. 6a, b, c). In contrast, claudin-5 protein levels were negatively correlated with CCL26 and CCR3 protein levels and inflammation scores ($r = -0.881$, $r = -0.856$, $r = -0.900$, $r = -0.837$, $P < .01$–$0.05$; Fig. 6e, f, g, d). Furthermore, the levels of CCL26 in the serum were positively correlated with the levels of CCL26 in brain tissues ($r = 0.908$, $P < .01$; Fig. 6 h).

Together, these results show that there is a close correlation between disease severity in EAE rats and the levels of CCL26 and CCR3.

## 4. Discussion

Multiple sclerosis is an autoimmune inflammatory demyelinating disease characterized by wide perivascular inflammatory cell infiltration, BBB damage and white matter demyelination in the central nervous system (Bendfeldt et al., 2012). Experimental autoimmune encephalomyelitis (EAE) is an ideal animal model to study MS immune pathogenesis and to guide clinical treatments. Studies have shown that there is obvious damage to the blood-brain barrier during the course of EAE. The BBB is mainly composed of cerebral microvascular endothelial cells, a basement membrane, astrocytes, and other cells, among which brain microvascular endothelial cells and their tight junction are the most important for the morphological basis of the BBB. Additionally, damage to tight junctions and related proteins may result

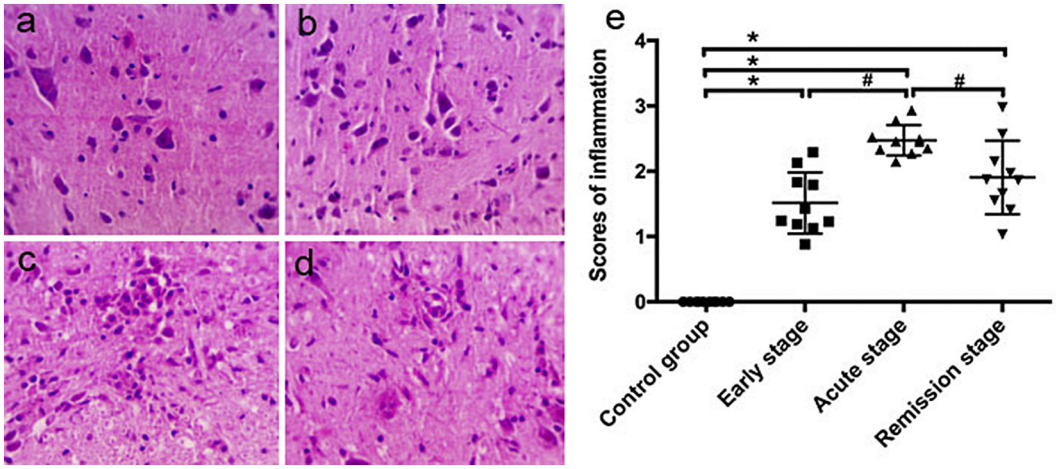



**Fig. 2.** H&E staining was performed to detect inflammation. Digital images were collected under bright-field settings using a 40× objective. a Image of H&E staining of the hippocampal fimbria of the brains of naïve control rats. b Image of H&E staining of the hippocampal fimbria of the brains of EAE rats in the early stage. c Image of H&E staining of the hippocampal fimbria of the brains of EAE rats in the acute stage. d Image of H&E staining of the hippocampal fimbria of the brains of EAE rats in the remission stage. e Comparison of the mean inflammation scores among the different groups ($n$ = 10 in each group). * $P < 0.01$, compared to the naïve group; # $P < 0.05$, compared to the early stage group and remission stage group.

in changes in BBB permeability (Errede et al., 2012).

Argaw et al. (Argaw et al., 2009) found that the protein expression of claudin-5 in the brain tissue of EAE rats is lower than that in naïve control rats, indicating that increased BBB permeability may be related to the decrease of claudin-5 expression. Liebner et al. (Liebner et al., 2000) knocked out the claudin-5 gene in mice and found that there are indeed varying degrees of BBB defects in the brain tissues of these mice.

Our study results showed that claudin-5 protein expression in the brain tissues of the EAE rats in different stages of the disease was lower than that in the naïve control group. Compared to that in the early stage and remission stage, claudin-5 protein expression was significantly reduced in the acute stage, confirming that there is damage to the continuity of the blood-brain barrier in the pathogenesis of EAE, consistent with previous research results. The mechanism of BBB dysfunction in different diseases is mainly related to decreases in tight junction (Chehade et al., 2002), the elevation of inflammatory response (Chen et al., 2018; Wang et al., 2012), oxidative stress (Giacco and Brownlee, 2010; Qian et al., 2017; Sharma et al., 2018), and an increase in matrix metalloproteinases (MMPs) in the blood plasma (Navaratna et al., 2013).

Several studies have demonstrated that multiple inflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), may participate in the destruction of the BBB and are linked to the pathogenesis of the central nervous system in autoimmune disease(Wang et al., 2012). For example, in C57BL6 EAE rats, the inflammatory response provokes alterations and decreases in tight junction proteins and triggers the visible leakage of IgG (Shrestha et al., 2014). The elevation of matrix metalloproteinase-2 (MMP-2) induced by IL-6 signalling is detected in NMO patients with BBB disruption (Uchida et al., 2017). A recent study found that the production of IL-6 induced by TNF-α elevates the permeability of brain endothelial cells in vitro (Rochfort et al., 2016). Increasing evidence has shown that inflammatory cytokines that result in BBB damage may participate in the pathogenic mechanism of many neurological diseases such as MS, NMO, epilepsy and dementia (Liu et al., 2016; Peng et al., 2018; Wang et al., 2016; Yang et al., 2017). However, the pathological mechanism of BBB leakage remains unclear.

In our study, the results show that the protein expression of claudin-5 in the EAE group was negatively correlated with the inflammation scores in the EAE group. Meanwhile, in the EAE group, we found that the levels of CCL26 in the serum and brain tissues and the number of CCR3-positive cells in the brain tissues were negatively correlated with

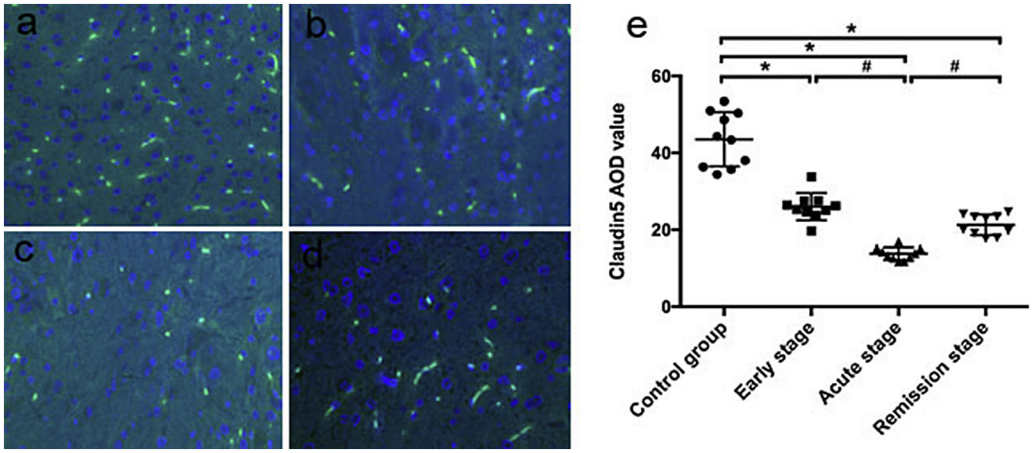



**Fig. 3.** The protein expression of claudin-5 was assessed by immunofluorescence analysis of rat brain tissues. a Immunofluorescence images of claudin-5 in naïve control rats. b Immunofluorescence images of claudin-5 in EAE rats in the early stage. c Immunofluorescence images of claudin-5 in EAE rats in the acute stage. d Immunofluorescence images of claudin-5 in EAE rats in the remission stage. e Comparison of the average optical density (AOD) values of claudin-5 expression among the different groups ($n$ = 10 in each group). * $P < 0.01$, compared to the naïve group; # $P < 0.05$, compared to the early stage group and remission stage group.

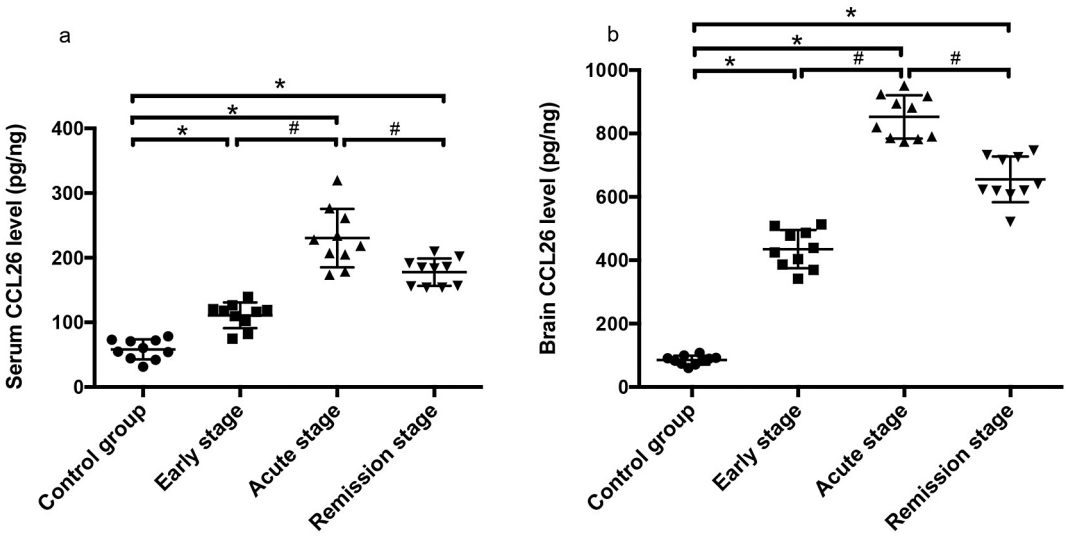



**Fig. 4.** Levels of CCL26 in the rat serum and brain tissues were detected by ELISA. Sera and brain tissues were harvested from naïve rats and EAE rats. a Serum CCL26 levels in the different groups. b Brain CCL26 levels in the different groups ($n$ = 10 each group). * P < 0.01, compared to the naïve group; # P < 0.05, compared to the early stage group and remission group.

the protein expression of claudin-5, indicating that the inflammatory response caused by CCL26, which in turn binds to its receptor (CCR3), may also mediate the mechanism of BBB damage and that the increase in BBB permeability and the increase in the inflammation score of EAE rats is consistent with the progression of EAE.

Chae (Chae et al., 2005) found that a single-nucleotide polymorphism (SNP) of the CCL26 gene is closely related to susceptibility for diseases, such as rheumatoid arthritis (RA). Ogilvie et al. (Ogilvie et al., 2001) reported that CCL26 is a natural antagonist of CCR2 and that CCL2 synergistically inhibits the migration of monocytes to inflammatory tissues. In addition, CCL26 is another agonist of CX3CR1 that not only gathers CCR3-expressing cells, such as eosinophils, mast cells and Th2 cells but also amasses CX3CR1-expressing cells, such as terminally differentiated CD8 + T cells and CD16 + NK cells (Nakayama et al., 2010). It appears that CCL26 may play a dual role in the pathogenesis of allergic and other diseases.

In previous research, MS was recognized as a Th1 disease. Chemokines play an important role in immunoregulatory activities, such as cytokine production and Th1/Th2 cell induction. For example, CXCR3 and CCR5 are expressed on Th1 cells, while CCR3 and CCR4 are expressed on Th2 cells. CCR3 and its major ligands, CCL13 and eotaxins (CCL11, CCL24, and CCL26), are members of the seven transmembrane domain G protein-coupled receptor family. Whether CCL26, which in turn binds to its receptor (CCR3), can induce immune cell infiltration and accumulation in inflammatory tissue, activate suppressor cells and cause changes in the Th1/Th2 balance is an urgent question. Thus, whether Th2 cells are equally well recruited to the CNS by selective chemokines provides important information about the immunopathology of this Th1 disease.

Research has revealed that the plasma levels of CCL11 and CCL26 are upregulated in patients during the remission stage of NMOSD, and there is no correlation between CCL11 levels, CCL26 levels, and clinical characteristics in patients with NMOSD, indicating that the two cytokines may be involved in the pathogenesis of NMOSD. In addition, a recent study demonstrated that CCL11 levels are decreased during the relapse stage of MS, perhaps in an attempt to limit progressive damage.

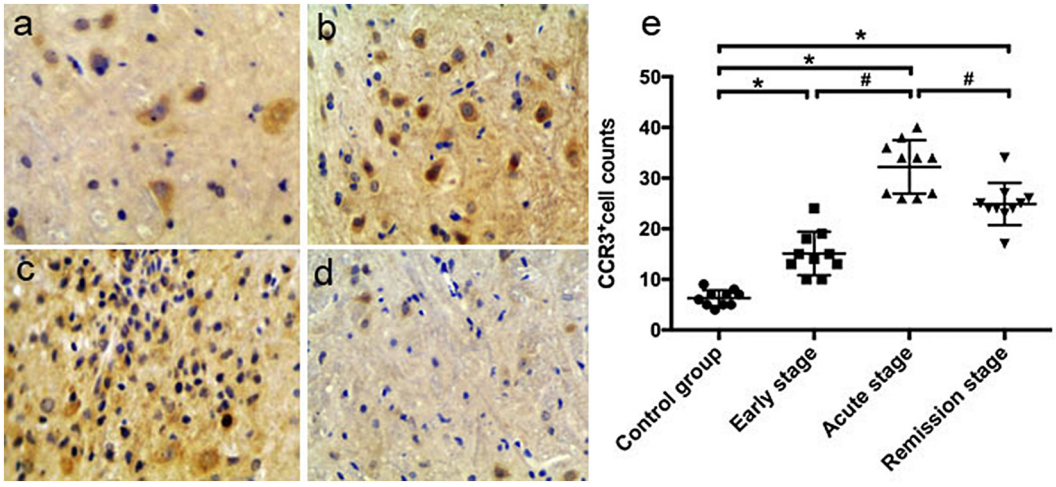
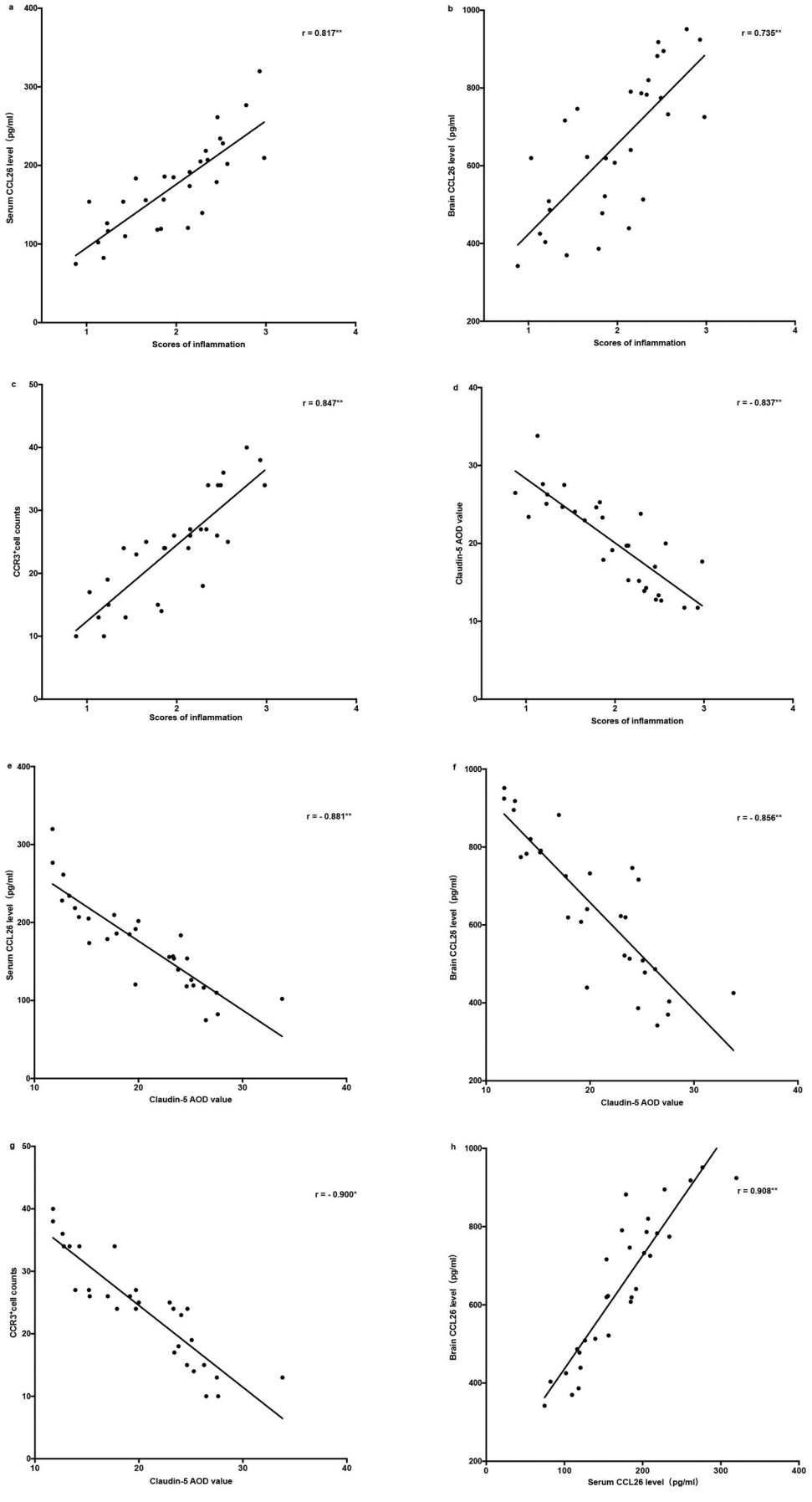



**Fig. 5.** CCR3-positive cells were detected by immunohistochemistry. Brain tissues were harvested, and transverse sections were stained with anti-CCR3 antibodies. Digital images were collected under bright-field settings using a 40× objective. a Immunohistochemistry images of CCR3-positive cells in naïve control rats. b Immunohistochemistry images of CCR3 -positive cells in EAE rats in the early stage. c Immunohistochemistry images of CCR3 -positive cells in EAE rats in the acute stage. d Immunohistochemistry images of CCR3-positive cells in EAE rats in the remission stage e Comparison of CCR3-positive cell counts among the different groups (n = 10 each group). * P < 0.01, compared to the naïve group; # P < 0.05, compared to the early stage group and remission stage group.




(caption on next page)

**Fig. 6.** Scatter plots displaying the CCL26, CCR3 and claudin-5 protein levels and inflammation scores in all EAE groups ($n = 30$). a, b, c, d The correlation between CCL26, CCR3 and claudin-5 protein levels and inflammation scores. e, f, g The correlation between CCL26, CCR3 and claudin-5 protein levels. h The correlation between the levels of CCL26 and CCR3. r correlation coefficient, *P < 0.05 , **P < 0.01.

A study also showed that plasma levels of CCL11 and CCL26 are increased in SPMS patients compared to RRMS patients. Interestingly, however, when compared to those in healthy controls (HC), CCL11 and CCL26 levels actually appear to be decreased in RRMS patients. CCL26 levels are significantly increased in SPMS patients, whereas CCL11 levels in SPMS patients appear to return to the levels in HC. These data indicate that CCL11 and CCL26 participate in the pathogenesis of MS and play complex and multiple roles in immune responses. Similarly, our results found that the levels of CCL26 in the serum and in brain tissues and the number of CCR3-positive cells in brain tissues were elevated in the EAE groups compared with the naïve control group. Meanwhile, we also found that, when the serum levels of CCL26 rose, the levels of CCL26 in the brain were also elevated, and the brain CCL26 levels were positively correlated with the serum levels. We hypothesize that serum CCL26 can enter the brain through the impaired BBB and bind to CCR3 to induce inflammation responses in lesions. Therefore, the serum CCL26 level may be an indicator of the brain CCL26 level and a biomarker of inflammation in the CNS.

Furthermore, the levels of CCL26 and CCR3 were significantly elevated in the brain tissues of EAE rats in the acute stage, when the EAE rats had severe clinical symptoms. Notably, the levels of CCL26 in the serum and brain tissues and the number of CCR3-positive cells in brain tissues were positively correlated with inflammatory scores. This finding may imply that CCL26, which in turn binds to its receptor (CCR3), may play a pro-inflammatory role in EAE, which is consistent with previous studies in different autoimmune diseases, and may play a more important role during the acute stage. On the other hand, the CCL26-CCR3 reaction in EAE brains induces immune cell infiltration, promotes inflammatory responses and aggravates clinical disability. Furthermore, CCR3, as a Th2-associated chemokine receptor, may participate in the immunopathology of EAE, thus representing another possible immune characteristic of MS and reminding us that the Th2-associated immune response plays a key role in the process of MS.

These results combined with the study on claudin-5 led us to speculate that the inflammatory effects of CCL26 and CCR3 induce inflammatory cells and cytokines in the CNS and exacerbate local immune effects and tissue damage, further aggravating BBB damage and exacerbating the inflammatory response in the CNS.

## 5. Conclusions

In summary, our study demonstrates that CCL26 and CCR3 show pro-inflammatory effects in the acute stage of EAE and promote the progression of the disease and tissue damage, which are involved in changes to the BBB. Further studies should be performed to clarify the pro-inflammatory effects and immunopathological mechanisms in MS and its distinct subtypes.

## Acknowledgements

This work was supported by Nature Science Foundation of Henan Province (No.162300410308).

## References

Abramowski, P., Steinbach, K., Zander, A.R., Martin, R., 2014. Immunomodulatory effects of the ether phospholipid edelfosine in experimental autoimmune encephalomyelitis. J. Neuroimmunol. 274, 111–124.
Argaw, A.T., Gurfein, B.T., Zhang, Y., Zameer, A., John, G.R., 2009. VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proc. Natl. Acad. Sci. U. S. A. 106, 1977–1982.
Bendfeldt, K., Kloppel, S., Nichols, T.E., Smieskova, R., Kuster, P., Traud, S., et al., 2012. Multivariate pattern classification of gray matter pathology in multiple sclerosis. Neuroimage. 60, 400–408.
Chae, S.C., Park, Y.R., Shim, S.C., Lee, I.K., Chung, H.T., 2005. Eotaxin-3 gene polymorphisms are associated with rheumatoid arthritis in a Korean population. Hum. Immunol. 66, 314–320.
Chehade, J.M., Haas, M.J., Mooradian, A.D., 2002. Diabetes-related changes in rat cerebral occludin and zonula occludens-1 (ZO-1) expression. Neurochem. Res. 27, 249–252.
Chen, M., Peng, J., Wei, W., Wang, R., Xu, H., Liu, H., 2018. A novel ETFDH mutation in an adult patient with late-onset riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency. Int. J. Neurosci. 128, 291–294.
D'Ambrosio, D., Panina-Bordignon, P., Sinigaglia, F., 2003. Chemokine receptors in inflammation: an overview. J. Immunol. Methods 273, 3–13.
El Behi, M., Dubucquoi, S., Lefranc, D., Zephir, H., De Seze, J., Vermersch, P., et al., 2005. New insights into cell responses involved in experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol. Lett. 96, 11–26.
Errede, M., Girolamo, F., Ferrara, G., Strippoli, M., Morando, S., Boldrin, V., et al., 2012. Blood-brain barrier alterations in the cerebral cortex in experimental autoimmune encephalomyelitis. J. Neuropathol. Exp. Neurol. 71, 840–854.
Fujimoto, T., Imaeda, H., Takahashi, K., Nishida, A., Shioya, M., Inatomi, O., et al., 2016. Eotaxin-3 (CCL26) expression in human pancreatic myofibroblasts. Pancreas 45, 420–424.
Giacco, F., Brownlee, M., 2010. Oxidative stress and diabetic complications. Circ. Res. 107, 1058–1070.
Herrero-Herranz, E., Pardo, L.A., Gold, R., Linker, R.A., 2008. Pattern of axonal injury in murine myelin oligodendrocyte glycoprotein induced experimental autoimmune encephalomyelitis: implications for multiple sclerosis. Neurobiol. Dis. 30, 162–173.
Hu, W.T., Chen-Plotkin, A., Arnold, S.E., Grossman, M., Clark, C.M., Shaw, L.M., et al., 2010. Novel CSF biomarkers for Alzheimer's disease and mild cognitive impairment. Acta Neuropathol. 119, 669–678.
Huber, A.K., Wang, L., Han, P., Zhang, X., Ekholm, S., Srinivasan, A., et al., 2014. Dysregulation of the IL-23/IL-17 axis and myeloid factors in secondary progressive MS. Neurology. 83, 1500–1507.
Kan, Q.C., Zhang, S., Xu, Y.M., Zhang, G.X., Zhu, L., 2014. Matrine regulates glutamate-related excitotoxic factors in experimental autoimmune encephalomyelitis. Neurosci. Lett. 560, 92–97.
Kan, Q.C., Zhang, H.J., Zhang, Y., Li, X., Xu, Y.M., Thome, R., et al., 2017. Matrine treatment blocks NogoA-induced neural inhibitory signaling pathway in ongoing experimental autoimmune encephalomyelitis. Mol. Neurobiol. 54, 8404–8418.
Lassmann, H., 2010. Axonal and neuronal pathology in multiple sclerosis: what have we learnt from animal models. Exp. Neurol. 225, 2–8.
Liebner, S., Fischmann, A., Rascher, G., Duffner, F., Grote, E.H., Kalbacher, H., et al., 2000. Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. Acta Neuropathol. 100, 323–331.
Liu, H., Xing, Y., Guo, Y., Liu, P., Zhang, H., Xue, B., et al., 2016. Polymorphisms in exon 2 of CD1 genes are associated with susceptibility to Guillain-Barre syndrome. J. Neurol. Sci. 369, 39–42.
Marcus, J.F., Waubant, E.L., 2013. Updates on clinically isolated syndrome and diagnostic criteria for multiple sclerosis. Neurohospitalist. 3, 65–80.
Mecha, M., Carrillo-Salinas, F.J., Mestre, L., Feliu, A., Guaza, C., 2013. Viral models of multiple sclerosis: neurodegeneration and demyelination in mice infected with Theiler's virus. Prog. Neurobiol. 101-102, 46–64.
Michael, B.D., Elsone, L., Griffiths, M.J., Faragher, B., Borrow, R., Solomon, T., et al., 2013. Post-acute serum eosinophil and neutrophil-associated cytokine/chemokine profile can distinguish between patients with neuromyelitis optica and multiple sclerosis; and identifies potential pathophysiological mechanisms - a pilot study. Cytokine. 64, 90–96.
Nakayama, T., Watanabe, Y., Oiso, N., Higuchi, T., Shigeta, A., Mizuguchi, N., et al., 2010. Eotaxin-3/CC chemokine ligand 26 is a functional ligand for CX3CR1. J. Immunol. (Baltimore, Md : 1950) 185, 6472–6479.
Navaratna, D., Fan, X., Leung, W., Lok, J., Guo, S., Xing, C., et al., 2013. Cerebrovascular degradation of TRKB by MMP9 in the diabetic brain. J. Clin. Invest. 123, 3373–3377.
Ogilvie, P., Bardi, G., Clark-Lewis, I., Baggiolini, M., Uguccioni, M., 2001. Eotaxin is a natural antagonist for CCR2 and an agonist for CCR5. Blood. 97, 1920–1924.
Peng, J., Zhang, H., Liu, P., Chen, M., Xue, B., Wang, R., et al., 2018. IL-23 and IL-27 levels in serum are associated with the process and the recovery of Guillain-Barre syndrome. Sci. Rep. 8, 2824.
Qian, J., Wu, C., Peng, J., Liu, H., 2017. Bilateral paramedian thalamic and midbrain infarction due to occlusion of the artery of percheron in an elderly male: a case report. Neurol. Sci. 38, 1123–1126.
Rochfort, K.D., Collins, L.E., McLoughlin, A., Cummins, P.M., 2016. Tumour necrosis factor-alpha-mediated disruption of cerebrovascular endothelial barrier integrity in vitro involves the production of proinflammatory interleukin-6. J. Neurochem. 136, 564–572.
Sharma, A., Tate, M., Mathew, G., Vince, J.E., Ritchie, R.H., de Haan, J.B., 2018. Oxidative stress and NLRP3-inflammasome activity as significant drivers of diabetic cardiovascular complications: therapeutic implications. Front. Physiol. 9, 114.
Shrestha, B., Paul, D., Pachter, J.S., 2014. Alterations in tight junction protein and IgG permeability accompany leukocyte extravasation across the choroid plexus during neuroinflammation. J. Neuropathol. Exp. Neurol. 73, 1047–1061.
Takahashi, K., Imaeda, H., Fujimoto, T., Ban, H., Bamba, S., Tsujikawa, T., et al., 2013.

Regulation of eotaxin-3/CC chemokine ligand 26 expression by T helper type 2 cytokines in human colonic myofibroblasts. Clin. Exp. Immunol. 173, 323–331.
Tejera-Alhambra, M., Casrouge, A., de Andres, C., Seyfferth, A., Ramos-Medina, R., Alonso, B., et al., 2015. Plasma biomarkers discriminate clinical forms of multiple sclerosis. PLoS One 10, e0128952.
Tong, Y., Yang, T., Wang, J., Zhao, T., Wang, L., Kang, Y., et al., 2018. Elevated plasma chemokines for eosinophils in neuromyelitis optica spectrum disorders during remission. Front. Neurol. 9, 44.
Uchida, T., Mori, M., Uzawa, A., Masuda, H., Muto, M., Ohtani, R., et al., 2017. Increased cerebrospinal fluid metalloproteinase-2 and interleukin-6 are associated with albumin quotient in neuromyelitis optica: their possible role on blood-brain barrier disruption. Multiple Scler. (Houndmills, Basingstoke, England) 23, 1072–1084.
van der Meer, P., Ulrich, A.M., Gonzalez-Scarano, F., Lavi, E., 2000. Immunohistochemical analysis of CCR2, CCR3, CCR5, and CXCR4 in the human brain: potential mechanisms for HIV dementia. Exp. Mol. Pathol. 69, 192–201.
Wang, J., Li, G., Wang, Z., Zhang, X., Yao, L., Wang, F., et al., 2012. High glucose-induced expression of inflammatory cytokines and reactive oxygen species in cultured astrocytes. Neuroscience 202, 58–68.
Wang, J., Fu, X., Yu, L., Li, N., Wang, M., Liu, X., et al., 2016. Preconditioning with VEGF enhances angiogenic and neuroprotective effects of bone marrow mononuclear cell transplantation in a rat model of chronic cerebral hypoperfusion. Mol. Neurobiol. 53, 6057–6068.
Westin, K., Buchhave, P., Nielsen, H., Minthon, L., Janciauskiene, S., Hansson, O., 2012. CCL2 is associated with a faster rate of cognitive decline during early stages of Alzheimer's disease. PLoS One 7, e30525.
Yang, J., Li, Q., Wang, Z., Qi, C., Han, X., Lan, X., et al., 2017. Multimodality MRI assessment of grey and white matter injury and blood-brain barrier disruption after intracerebral haemorrhage in mice. Sci. Rep. 7, 40358.
Zhang, M.L., Zhang, X.J., Kang, J., Zhang, H.J., Chen, X.L., Liu, N., et al., 2017. Matrine promotes NT3 expression in CNS cells in experimental autoimmune encephalomyelitis. Neurosci. Lett. 649, 100–106.