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The adenosine A_{2A} receptor antagonist SCH58261 reduces macrophage/microglia activation and protects against experimental autoimmune encephalomyelitis in mice

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ABSTRACT

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system (CNS) affecting more than 2.5 million individuals worldwide. In the present study, myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) mice were treated with adenosine receptor A_{2A} antagonist SCH58261 at different periods of EAE development. The administration of SCH58261 at 11–28 days post-immunization (d.p.i.) with MOG improved the neurological deficits. This time window corresponds to the therapeutic time window for MS treatment. SCH58261 significantly reduced the CNS neuroinflammation including reduced local infiltration of inflammatory cells, demyelination, and the numbers of macrophage/microglia in the spinal cord. Importantly, SCH58261 ameliorated the EAE-induced neurobehavioral deficits. By contrast, the SCH58261 treatment was ineffective when administered at the beginning of the onset of EAE (i.e., 1–10 d.p.i). The identification of the effective therapeutic window of A_{2A} receptor antagonist provide insight into the role of A_{2A} receptor signaling in EAE, and support SCH58261 as a candidate for the treatment of MS in human.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system (CNS). It is characterized by paralysis, visual impairment, sensation problems, and lack of motor coordination (Ben-Zacharia, 2011; Steinman, 2001). Its pathological changes are considered as a two-stage process: The initial neuroinflammatory phase associated with blood-brain barrier damage, prominent infiltration of activated $CD4^+$ and $CD8^-$ T cells, and the presence of reactive astrocytes and proliferating oligodendrocytes. The degenerative phase is characterized by demyelination, axonal and neuronal loss, and accumulation of activated microglia and macrophages (Steinman, 2001; Neuhaus et al., 2003; Lopez-Diego and Weiner, 2008). Based on the multiple clinical presentations, MS is divided into 4 types of clinical courses: relapsing-remitting, secondary progressive, primary progressive, and progressive relapsing (Lublin et al., 2014). A classic animal model of MS—experimental autoimmune encephalomyelitis (EAE)—exhibits neuro-immune inflammatory responses in CNS associated with inflammatory demyelination pathological changes, and thus, is regarded as an animal model mimicking the primary progressing type of MS. It has been widely used as a research tool to search for new drugs to treat MS (Denic et al., 2011; Centonze et al., 2010; Li et al., 2012; Yao et al., 2012; Wang et al., 2014). However, the treatment for MS is largely limited to the management of symptoms (Thompson et al., 2010) and corticosteroid hormone treatment that are associated with many unwanted side-effects and limited efficacy. Thus, the development of effective potential disease-modifying pharmacological agents with preferred safety profile is vital.

Recently, animal studies suggested that adenosine A_{2A} receptors

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represent a novel therapeutic target for the treatment of MS (Yao et al., 2012; Liu et al., 2016, 2018; Ingwersen et al., 2016). Adenosine is an endogenous purine nucleoside, and the levels of extracellular adenosine increase rapidly and markedly during tissue inflammation. Increased extracellular adenosine as well as upregulated adenosine A_{2A} receptor ($A_{2A}R$) is proposed as a "STOP" signaling to effectively suppress the local inflammatory response to protect against excessive cellular damage to the surrounding tissues (Blackburn et al., 2009). For its ability to respond to locally increased extracellular adenosine and to target pathological inflammation, as well as the homeostasis modulation of immune functions with preferred safety profile, $A_{2A}R$ signaling has been considered and evaluated as a potential therapeutic target for inflammatory diseases such as MS (Yao et al., 2012; Wang et al., 2014; Mills et al., 2008, 2012).

The role of $A_{2A}R$ signaling in the development of EAE is complex and may exert multiple and sometimes opposite effects on immune and neuroinflammatory responses. We and others have recently demonstrated that the genetic inactivation of $A_{2A}Rs$ produced severer EAE pathology and neurological deficits with pronounced pro-inflammatory production and neurodegeneration with demyelination (Yao et al., 2012; Mills et al., 2012). Conversely, these findings also indicated that the treatment with SCH58261, an adenosine receptor antagonist specific for A_{2A} subtype, exerted a protective effect against EAE development (Mills et al., 2008, 2012). This apparent paradoxical effect reflects multiple and complex actions of A_{2A} receptor in regulating the EAE pathology. We hypothesized that the different effects of $A_{2A}R$ antagonists on the EAE pathology are dependent on the different periods of the EAE disease course. To this end, this study sought to determine the effect of $A_{2A}R$ antagonist SCH58261 on early immune induction period (i.e., 0–10 days post-immunization (d.p.i.)) or later neurobehavioral deficits period (i.e., 11–28 d.p.i.). We found that the effective therapeutic time window of $A_{2A}R$ antagonist SCH58261 was on the later neurobehavioral deficits period and was associated with the reduced activation of macrophage/microglia rather than on the early immune induction period.

2. Results

2.1. Administration of SCH58261 during 11–28 d.p.i. effectively improved the EAE-induced neurological deficits

To investigate the therapeutic time window of the selective $A_{2A}R$ antagonist SCH58261 on the development of EAE, we monitored and recorded the behavioral deficiency of EAE mice daily during the entire experimental period. At 13 d.p.i. after MOG_{35-55} treatment, mice began to display a progressive decrease in exploratory activity. On the 15 d.p.i., mice treated with MOG_{35-55} exhibited signs of muscle weakness, including flaccid tail and hind leg paralysis.

MOG_{35-55}-induced EAE in C57BL/6 mice is speculated to produce a single attack-disease course with no recurrence (Stark and Cross, 2006). Based on our previous studies (Li et al., 2012; Wang et al., 2014) and the pilot study showing the neurological deficits (e.g. ascending flaccid paralysis), we considered the 0–10 d.p.i. as the incubation period or immunoinduction period (corresponding to the initial non-symptom of EAE), and 11–28 d.p.i. as the neurobehavioral deficits period of EAE (Fig. 1A), which is slightly different from the EAE disease course 11–20 d.p.i. as we observed previously (Li et al., 2012; Wang et al., 2014).

Importantly, SCH58261 treatment during each period of EAE significantly improved the neurobehavioral deficits indicated by the reduced EAE scores (Fig. 1B) (two-way ANOVA analysis, $p = 0.001$). Compared to the EAE-DMSO group, the EAE-SCH 0–28d group (i.e., the SCH58261 treatment for the entire EAE disease course) markedly improved the behavioral deficit ($p = 0.001$), which was in agreement with the previous studies (Mills et al., 2008, 2012). Treatment with SCH58261 during the onset to peak stage of EAE alone (i.e., EAE-SCH 11–28d group) also showed the neuroprotective effect against EAE pathology ($p < 0.001$). On the other hand, mice administered SCH58261 during the early ten days (EAE-SCH 0–10d group) showed only a trend of alleviation without statistical significance ($p = 0.141$).

2.2. Administration of SCH58261 during the 11–28 d.p.i. reduced the infiltration of inflammatory cells, demyelination, and macrophage/microglia activation in the spinal cord

To further confirm the neuroprotection conferred by SCH58261 treatment, we examined the inflammatory cell infiltration, demyelination, and activation of glial cells in the spinal cord sections.

Consistent with the neurobehavioral deficits and EAE scores, the EAE-SCH 11–28d group showed a reduced inflammatory cell infiltration into the spinal cord as compared to the EAE-DMSO group ($p = 0.0072$) (Fig. 2A). Moreover, the EAE-SCH 0–28d group also displayed a reduction in inflammatory cell infiltration ($p = 0.0273$) as compared to the EAE-DMSO group. Consistent with the lack of behavioral effect, the EAE-SCH 0–10 d.p.i. group only showed a trend of reduction without statistical significance as compared to the EAE-DMSO group ($p = 0.9405$; Fig. 2A).

Similarly, LFB staining revealed a similar pattern of demyelination as that of inflammatory cells infiltration by showing that the treatment with SCH58261 attenuated demyelination in EAE. Specifically, the EAE-SCH 11–28d and the EAE-SCH 0–28 d.p.i. groups showed reduced demyelination; the EAE-SCH 11–28 d.p.i. showed a marked reduction than the EAE-DMSO group (Fig. 2B).

Furthermore, immunohistochemistry was used to determine the activation of astrocytes and macrophage/microglia involved in neuroprotection based on SCH58261 treatment. As shown in Fig. 3A, immunization with MOG_{35-55} increased the number of GFAP-positive astrocytes, which was not affected by the administration of SCH58261. Conversely, EAE pathology was associated with the markedly increased number of Iba-1-positive macrophage/microglia. SCH58261 treatment attenuated the macrophage/microglia activation in EAE mice (Fig. 3B); the EAE-SCH 11–28d and EAE-SCH 0–28d groups showed the decreased number of Iba-1-positive macrophage/microglia as compared to the EAE-DMSO group ($p = 0.0003$ and $p = 0.0008$, respectively).

2.3. Administration of SCH58261 during 11–28 d.p.i. suppressed the EAE-induced inflammation by reducing the pro-inflammatory cytokine IFN-γ

In order to elucidate the mechanism of SCH58261-mediated protection during EAE progression, we examined the expression of macrophage/microglia-related inflammatory cytokine IFN-γ in the cerebral cortexes. The expression level of IFN-γwas markedly increased in the EAE-DMSO group. The treatment with SCH58261 during the 11–28 d.p.i and 0–28 d.p.i. significantly reduced the increased level of IFN-γ in the cerebral cortex (Fig. 3C). The treatment with SCH58261 during the 0–10 d.p.i. did not exhibit any neuroprotective effect without the reduced level of IFN-γ in this group.

2.4. Administration of SCH58261 after the onset of EAE also improved the EAE-induced neurological deficits

Since the $A_{2A}R$ antagonist SCH58261 was effective at 11–28 d.p.i. in reducing the behavioral deficits, we further explored whether SCH58261 treatment after the onset of behavioral deficit could still confer protects against EAE pathology. As shown in Fig. 4B, SCH58261 treatment after the onset of behavioral deficit significantly reduced the neurological behavior scores when comparing the EAE-SCH group with the EAE-DMSO group (two-way ANOVA, followed by the LSD test, $F(1,22) = 4.785$, $p = 0.0396$); the improvement was observed on the 6th day after onset of EAE and SCH58261 treatment.

2.5. Administration of SCH58261 after the onset of EAE reduced the inflammatory cells infiltration and demyelination in the spinal cord

As shown in Fig. 4, a large number of inflammatory cells infiltration and severe demyelination was found in the EAE-DMSO mice, and the administration of SCH58261 after onset of EAE reduced the infiltration of inflammatory cells (Fig. 4C) and demyelination in the spinal cords (Fig. 4D), which was consistent with the behavioral improvement by

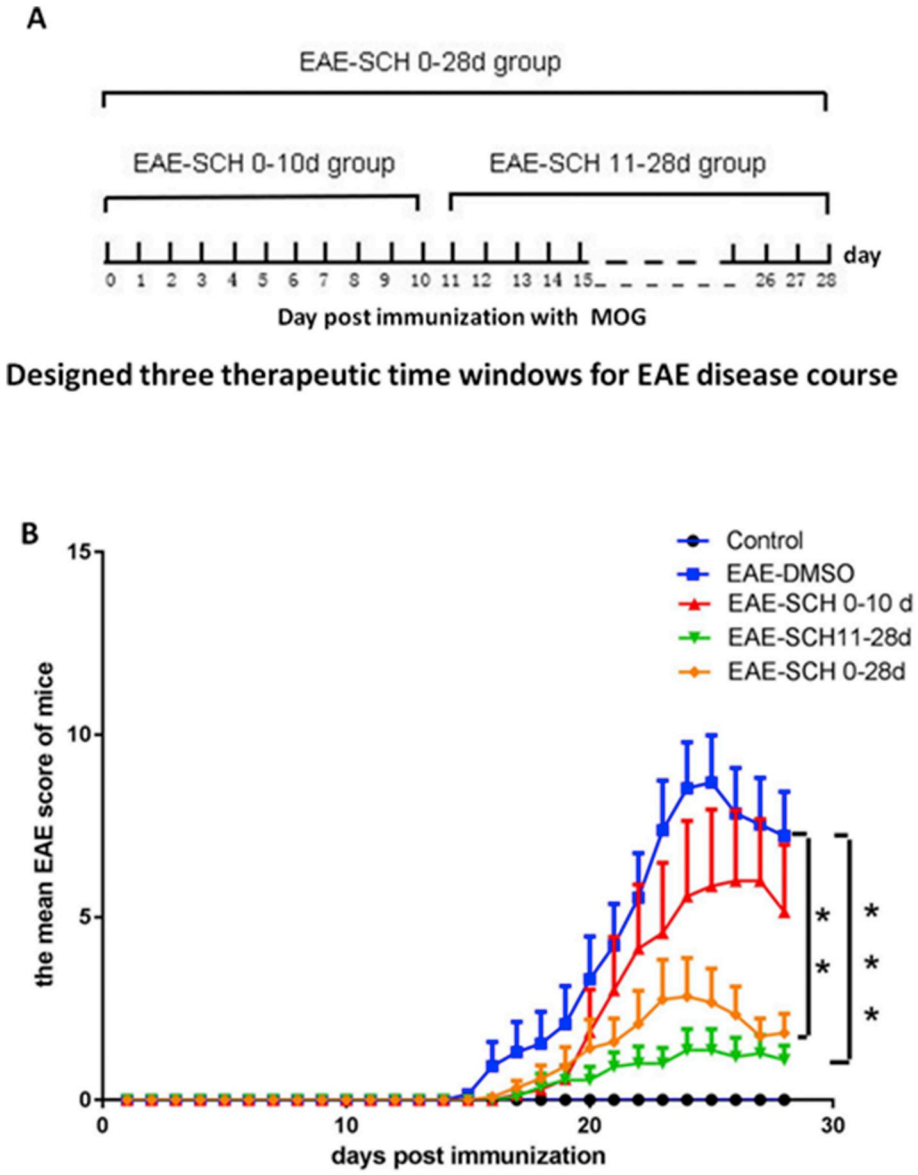

Fig. 1. The effect of $A_{2A}R$-antagonist SCH58261 treatment at different periods of EAE disease course. (A) Schematic showing three therapeutic time windows when $A_{2A}R$ antagonist SCH58261 was administered to mice at different periods. Therapeutic window 1: EAE-SCH 0–28 d group, total EAE course from the immune induction and the onset to development; therapeutic window 2: EAE-SCH 0–10 d group, at the early period of an immune inductive responses; therapeutic window 3: EAE-SCH 11–28 d group, at the later period of an onset-to-peak of neurobehavioral deficits. (B) EAE mice in different groups were treated with $A_{2A}R$ antagonist SCH58261 during 0–10 d.p.i., 0–28 d.p.i., and 11–28 d.p.i., or with DMSO and no treatment, respectively. The day when mice received MOG treatment was considered as day 0. The neurobehavioral deficit of EAE mice was monitored daily, and the mean EAE score was calculated. Error bars represent the SEM. Two-way ANOVA analysis revealed a significant effect of treatment ($F(3,27) = 6.766$, $p = 0.001$). ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

SCH58261 treatment after the EAE onset. Quantitative analysis showed that the number of infiltrating inflammatory cells in the EAE-SCH group declined as compared to the EAE-DMSO group ($t(19) = 2.517$, $p = 0.0210$). Meanwhile, the EAE-SCH group showed a significant reduction of demyelination ($t(19) = 3.296$, $p = 0.0038$). These results confirmed that SCH58261 exerted a neuroprotective effect after EAE onset by intervening the inflammatory and demyelination processes.

2.6. Administration of SCH58261 after the onset of EAE modulated Iba-1 positive macrophage/microglia activation

We also determined whether macrophage/microglia activation could be affected by SCH58261 treatment after the onset of the behavioral deficit by immunofluorescence double staining. We found that the treatment with SCH58261 for 10 days markedly decreased the number of Iba-1-positive cells as compared to the EAE-DMSO group (Fig. 5A and B). In addition, SCH58261 treatment for 10 days, after the onset of the behavioral deficit, decreased the expression of inducible nitric oxide synthase (iNOS) in macrophage/microglia ($t(12) = 2.682$, $p = 0.020$) as compared to the EAE-DMSO group.

2.7. Administration of SCH58261 after EAE mice showed that the neurobehavioral deficits suppressed the EAE-induced pro-inflammatory cytokine IFN-γ

Since the treatment with SCH58261 during the 11–28 d.p.i. of EAE reduced the level of IFN-γ in the cerebral cortex, we examined whether the same phenomenon occurred when the treatment with SCH58261 after the onset of EAE mice showed neurobehavioral deficits. As expected, SCH58261 treatment after EAE mice showed neurobehavioral deficits also remarkably downregulated the secretion of IFN-γ in the EAE mice cerebral cortexes (Fig. 5C).

3. Discussion

The most significant finding in the present study was the demonstration thatf the $A_{2A}R$ antagonist could protect against EAE pathology when administered at the neurobehavioral deficits period (i.e. 11–28 d.p.i., when mice developed distinct motor and other symptoms with pronounced neuroinflammatory demyelination pathology in CNS) but not at the initial incubation period of EAE with pronounced immune induction period or specific lymphocyte proliferation cellular pathology in bone marrow-derived cells (i.e. 0–10 d.p.i.). The study also defined the potential therapeutic effect and time window, whereby the $A_{2A}R$ antagonist SCH58261 exerted a protective effect against EAE pathology. While adenosine signaling is critical

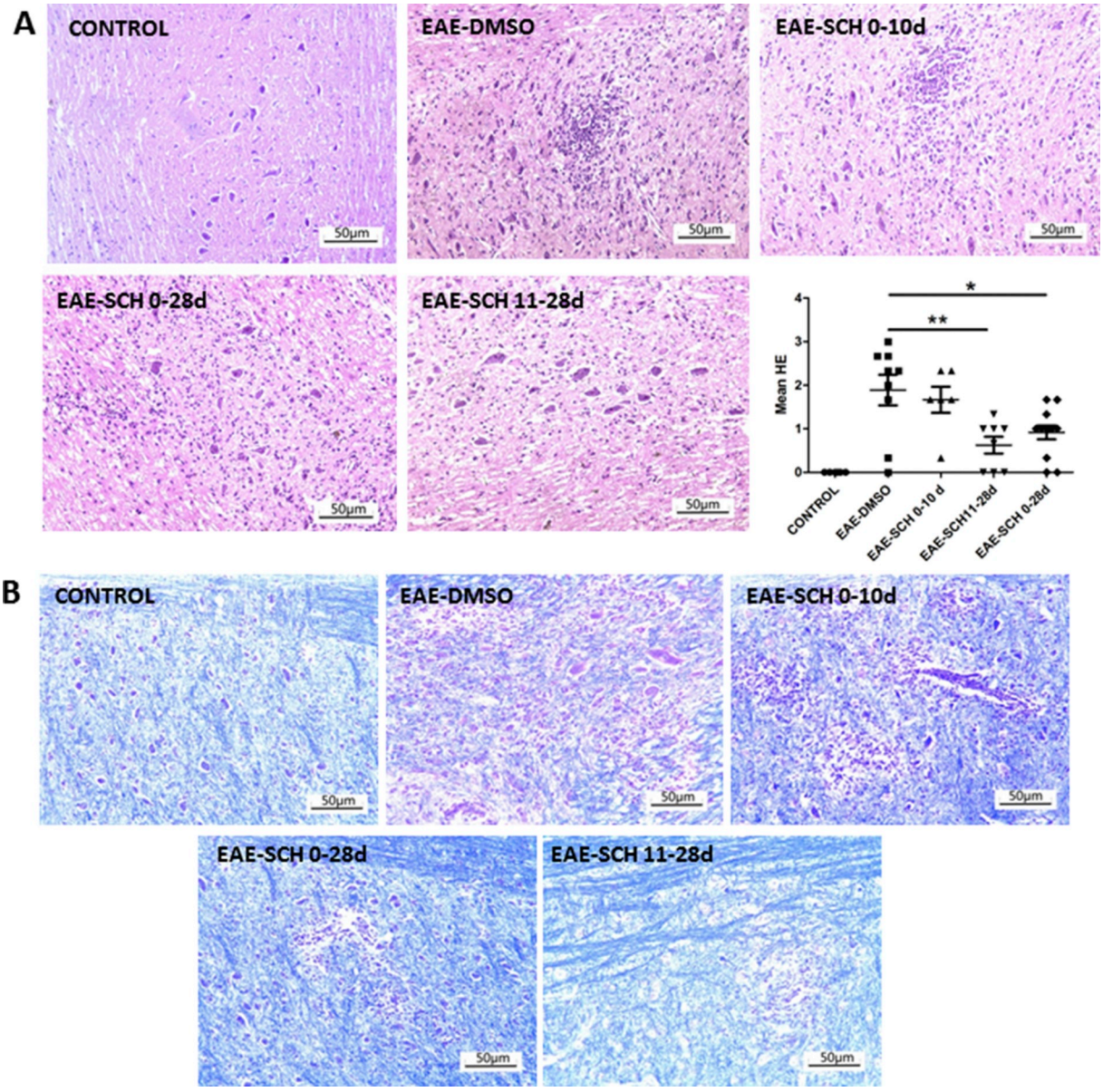

Fig. 2. The effect of treatment with $A_{2A}R$-antagonist SCH58261 at different periods of EAE on the histopathological changes in the spinal cord sections. (A) The effect of treatment with $A_{2A}R$-antagonist SCH58261 at different periods of EAE on the infiltration of inflammatory cells in the spinal cord. Representative images from paraffin-embedding tissue sections of the spinal cord (stained by HE 200 × , scale bar = 50 μm). (A′): Quantitative analysis of the pathological scores. One-way ANOVA analysis revealed a significant effect of treatment ($F(3,31) = 5.562$, $p = 0.0036$). Control group (n = 6); EAE-DMSO group (n = 9); EAE-SCH0-10d group (n = 6); EAE-SCH0-28d group (n = 12); EAE-SCH11-28d group (n = 8). Five fields per spinal cord in each group were analyzed at 400 × magnification for H&E staining. Error bars represent the SEM. * indicates $p < 0.05$, while ** indicates $p < 0.01$. (B) The effect of treatment with $A_{2A}R$-antagonist SCH58261 at different periods of EAE on demyelination changes in the spinal cord sections. Representative images obtained from paraffin-embedding in tissue sections of the spinal cord (stained by LFB 200 × , scale bar = 50 μm).

to the development of EAE pathology, the mechanism underlying $A_{2A}R$ signaling in the development of EAE is complex and is yet to be elucidated. Our recent study with $A_{2A}R$ KO revealed that the genetic inactivation of $A_{2A}R$ exacerbates EAE pathology with the increased behavioral deficit, increased inflammatory infiltration, overexpression of proinflammatory cytokines, and exacerbated demyelination in $A_{2A}R$ KO mice as compared to the WT mice (Yao et al., 2012). This phenomenon suggested that $A_{2A}R$ signaling is critical for the maintenance of neuronal integrity in response to EAE insults. Consistent with this, a recently study has shown that activation of A_{2A} signaling by selective $A_{2A}R$ agonist CGS21680 inhibits the EAE progression by suppressing the specific lymphocyte proliferation, reducing the infiltration of $CD4^+$ T lymphocytes, increasing intracellular calcium levels (Liu et al., 2016), and reducing the effects of Th1 stimulation on the blood-brain barrier permeability (Liu et al., 2018). These effects of the $A_{2A}R$ signaling are likely attributed to the $A_{2A}Rs$ in bone marrow-derived cells since $A_{2A}R^{-/-}$ mice (recipient) that receive wild-type (donor) bone marrow are protected from the EAE development (Mills et al., 2012). These findings are in agreement with the experimental findings from several peripheral tissues that $A_{2A}R$ signaling on lymphocytes exhibit anti-inflammatory effects (Blackburn et al., 2009). Furthermore, a previous study indicated that contrary to the $A_{2A}R$ KO, treatment with $A_{2A}R$ antagonist SCH58261 confers a protective effect against EAE pathology (Mills et al., 2008). Our results validated this finding and further demonstrated that $A_{2A}R$ antagonist confers the protective activity during the neurobehavioral deficit period of EAE. This phenomenon is partially explained by the opposite effect of $A_{2A}R$ signaling on non-immune cells within the CNS than that in the bone marrow-derived cells (Mills et al., 2012). Despite the opposite effects of $A_{2A}R$ signaling in the immune and non-immune cells and the exacerbated damage in the genetic $A_{2A}R$ KO, pharmacological blockade of the $A_{2A}R$ produces overall (predominantly) protective effects. Thus, the pharmacological

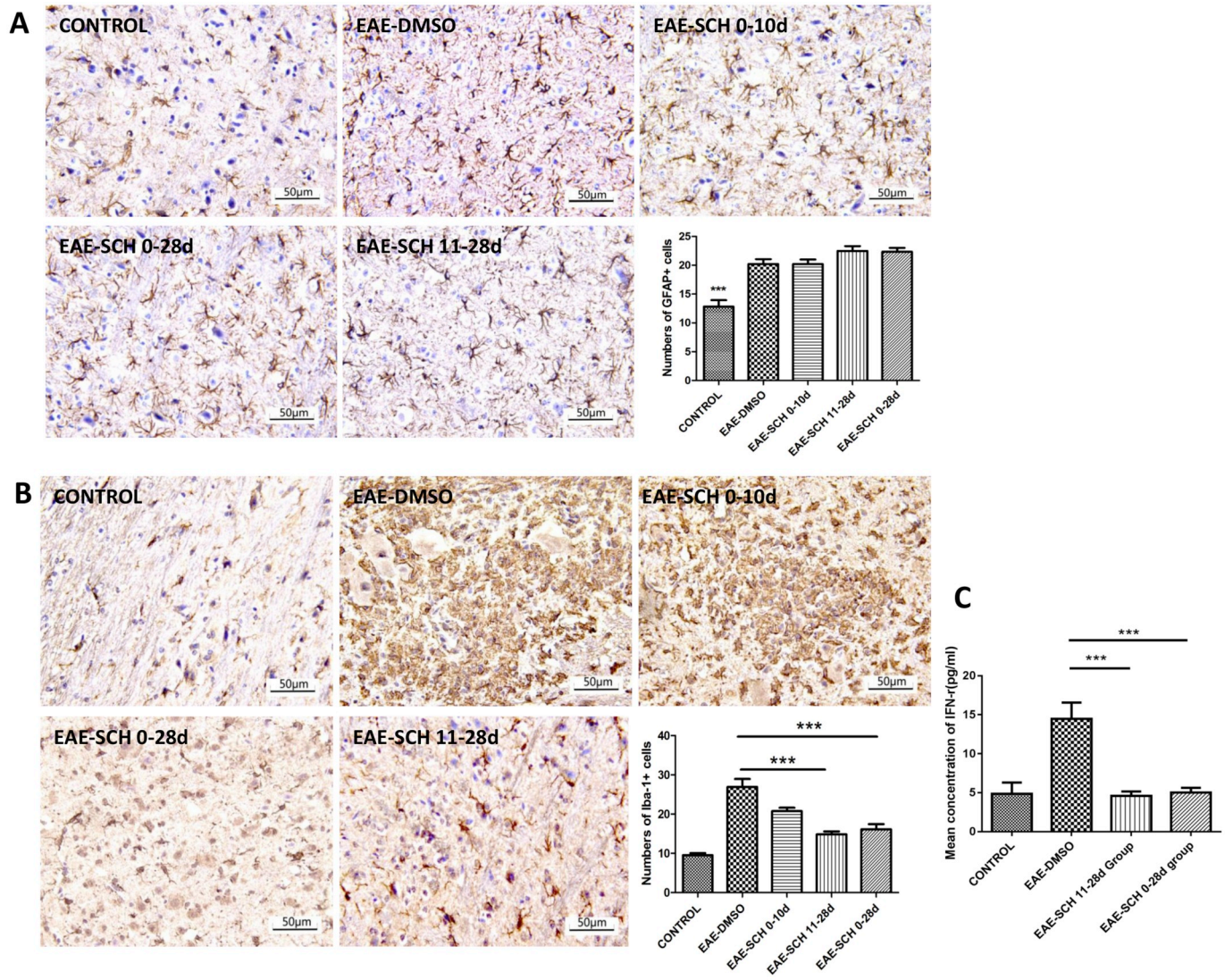

Fig. 3. (A) The effect of treatment with A_{2A}R-antagonist SCH58261 at different periods of EAE on the population of GFAP-positive astrocytes in the spinal cord sections. Representative images taken from paraffin-embedding tissue sections of the spinal cord (stained by immunohistochemical technique (GFAP-positive cells) 400 ×, scale bar = 50 μm). (a) Five fields per spinal cord in each group were analyzed at 400 × magnification for GFAP staining. Error bars represent the SEM. One-way ANOVA analysis did not reveal any significant effect of the treatment ($F(3,26) = 2.086$, $p = 0.1266$). Control group (n = 6); EAE-DMSO group (n = 6); EAE-SCH0-10d group (n = 7); EAE-SCH0-28d group (n = 9); EAE-SCH11-28d group (n = 8). **(B) The effect of treatment with A_{2A}R-antagonist SCH58261 at different periods of EAE on the population of Iba-1 positive macrophage/microglia in the spinal cord sections**. Representative images were obtained from paraffin-embedding tissue sections of the spinal cord (stained by immunohistochemical technique (Iba-1 positive cell) 400 ×, scale bar = 50 μm). (a) Five fields per spinal cord in each group were analyzed at 400 × magnification for Iba-1 staining. Error bars represent the SEM. *** indicates $p < 0.001$. One-way ANOVA analysis revealed the significant effect of treatment ($F(3,28) = 9.909$, $p = 0.0001$). Control group (n = 6); EAE-DMSO group (n = 9); EAE-SCH0-10d group (n = 6); EAE-SCH0-28d group (n = 9); EAE-SCH11-28d group (n = 8). **(C) The effect of administration of SCH58261 at different disease courses on the secretion of IFN-γ in the cerebral cortex of EAE mice.** Four groups of C57BL/6 female mice received different CFA/MOG and SCH58261/DMSO at different disease courses of the EAE model as described in the Methods. Mouse cerebral cortexes were dissected and secretion levels of IFN-γ were determined by ELISIA. *** indicates $p < 0.001$. One-way ANOVA followed by LSD post-hoc comparison. ($F(2,24) = 18.27$, $p < 0.0001$). Control group (n = 6); EAE-DMSO group (n = 9); EAE-SCH0-28d group (n = 9); EAE-SCH11-28d group (n = 9).

blockade of the A_{2A}R signaling represents a novel therapeutic strategy for the treatment of MS (Ingwersen et al., 2016; Mills et al., 2008, 2012).

Since our findings demonstrated that the SCH58261 was effective during the neurological deficit period of EAE, corresponding to the neuroinflammatory phase of MS, A_{2A}R antagonist should be administered at the neurodegenerative phase of MS pathology in future. Whether A_{2A}R antagonist treatment is effective on the degenerative phase of other types of EAE model (i.e., relapsing-remitting, secondary progressive, and progressive relapsing) needs to be investigated further. Conversely, SCH58261 treatment at the incubation period of EAE or the immune induction period was ineffective. The present study demonstrated that the administration of SCH58261 starting from the occurrence of EAE neurobehavioral deficits could protect against EAE. This phenomenon indicated that the effective therapeutic time window with A_{2A}R antagonist SCH58261 could confer protection against EAE pathology even at the relatively later period, i.e., after the onset of behavioral deficit in mice. Similarly, we found that caffeine treatment at the behavioral deficits period or during the entire course of EAE protected against EAE pathology (Wang et al., 2014). These distinct effects of the A_{2A}R signaling in EAE were consistent with the recent findings that preventive EAE treatment with A_{2A}R-specific agonist inhibits the myelin-specific T cell proliferation ex vivo and ameliorates the disease, while application of the same agonist after disease onset exacerbates the non-remitting EAE progression and results in severer tissue destruction (Ingwersen et al., 2016). Together, these findings establish that the A_{2A}R antagonists confer neuroprotection against EAE pathology by specifically acting during the neurobehavioral deficits period after the onset of EAE. This is critical for the drug development since the desirable treatment window is indicative of the potential therapeutic effect of A_{2A}R antagonist

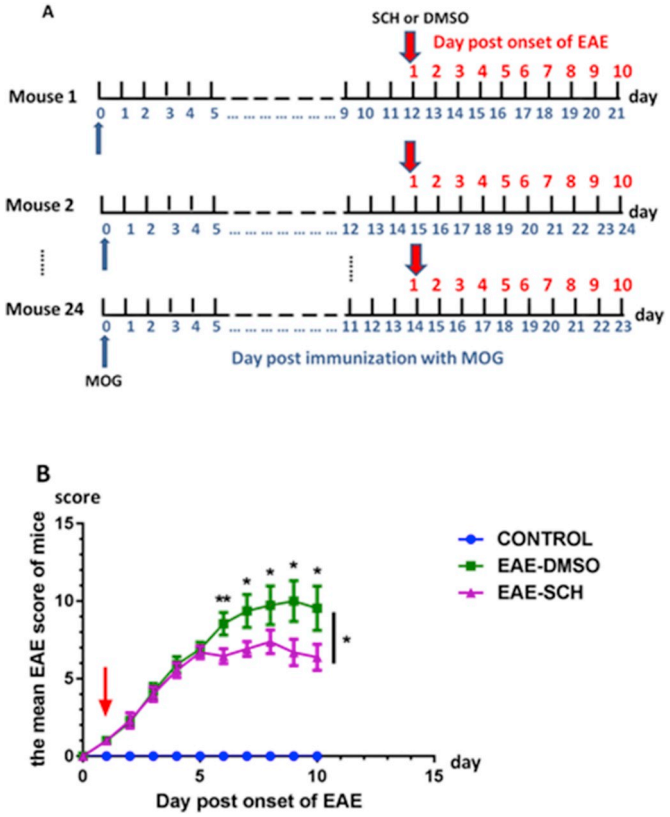

Fig. 4. (A) When EAE mice showed neurobehavioral deficits and were treated with A_{2A}R-antagonist SCH58261. EAE mice were treated with A_{2A}R-antagonist SCH58261 or DMSO for ten days after showing neurobehavioral deficits (i.e., half paralyzed tail). Schematic diagram showing d.p.i. by MOG injection, and showed the days after onset of EAE when mice showed neurobehavioral deficits and were administered SCH58261 or DMSO (red arrows indicated the first day when EAE mice showed neurobehavioral deficits and the beginning of treatment with SCH58261 or DMSO; blue arrows indicated the day when mice were treated with MOG). **(B) Effect of treatment with A_{2A}R-antagonist SCH58261 after EAE mice showed neurobehavioral deficits**. The day when EAE mice showed neurobehavioral deficits was defined as the first day after the onset of EAE. The behavioral score was monitored daily, and mean EAE score was calculated. Error bars represent the SEM. Arrow indicates the first day when EAE mice showed neurobehavioral deficits and the beginning of treatment with SCH58261 or DMSO. Two-way ANOVA analysis revealed a significant effect of treatment ($F(1,22) = 4.785$, $p = 0.0396$)). * indicates $p < 0.05$; ** indicates $p < 0.01$. **(C) The effect of treatment with A_{2A}R-antagonist SCH58261 after the onset of EAE on the infiltration of inflammatory cells in mice spinal cords**. Representative images from frozen tissue sections of the spinal cord. (A) H&E staining exhibited the infiltration of inflammatory cells into EAE mice lumbar spinal cord. (C′) Quantitative analysis of the pathological scores. Four fields per spinal cord in each group were analyzed at 400 × magnification for H&E staining. Error bars represent the SEM (Control group n = 11; EAE-DMSO group n = 10; EAE-SCH group n = 11). * indicates $p < 0.05$. T-test analysis revealed a significant effect of treatment ($t(19) = 2.517$, $p = 0.0210$). **(D) The effect of treatment with A_{2A}R-antagonist SCH58261 after the onset of EAE on demyelination in mice spinal cords**. LFB staining exhibited the level of demyelination in mice lumbar spinal cord (scale bars = 100 μm). (D′) Quantitative analysis of the pathological scores. Four fields per spinal cord in each group were analyzed at 400 × magnification for LFB staining. Error bars represent the SEM (Control group n = 11; EAE-DMSO group n = 10; EAE-SCH group n = 11). * indicates $p < 0.05$. T-test analysis revealed a significant effect of treatment ($t(19) = 3.296$, $p = 0.0038$).

has a potential therapeutic effect on the symptomatic active phase of MS, which can be used as a candidate for the development of new drug and the treatment strategies for MS.

Furthermore, these complex and opposite effects of the A_{2A}R signaling at the different periods of the disease likely reflect distinct cellular elements involved in these activities, depending on the cellular basis and disease course. In the current study, SCH58261 was found to be effective when the drug was administered during the neurobehavioral deficits period or after the onset of EAE. One of the underlying mechanisms may be associated with the A_{2A}R antagonist-induced reduction in the population and activity of Iba-1 positive macrophage/microglia. Macrophage/microglia specialized immune functions in the CNS, thereby playing a crucial role in neuroinflammation (Tansey and Goldberg, 2010; Reitz et al., 2011). A previous study showed that A_{2A} receptor antagonist (ZM-241385) could prevent A_{2A} receptor agonist (CGS21680) potentiation lipopolysaccharide-induced NO release in the in vitro mixed glial (microglia and astrocyte) cultures. Double immunofluorescence demonstrated that NOS-II immuno-staining co-localized with the microglial marker (Saura et al., 2005). In addition, removal of the endogenous extracellular adenosine or blocking A_{2A}R with SCH58261 prevented the secretion of LPS-induced increase of both brain-derived neurotrophic factor (BDNF) and proliferation of microglia (Gomes et al., 2013). Thus, we speculated that one of the underlying mechanism of A_{2A}R antagonist exerting a neuroprotection effect during EAE progress is related to the reduction in the population and suppression of the activity of Iba-1-positive macrophage/microglia. Whether SCH58261 provides a neuroprotective effect by acting on oligodendrocytes and astrocytes needs further study. The neurobehavioral deficits period of EAE corresponds to the appearance of neurological deficiency, resulting from immune neuro-inflammation, demyelination, substantial axonal damage, and oligodendrocyte apoptosis in the EAE model. One of the key pathogenesis mechanisms in the neurobehavioral deficits period is the accumulation of activated microglia and macrophages in the development of autoimmune demyelination (Lopez-Diego and Weiner, 2008). The activation of macrophage/microglia is closely associated with the development of histopathological lesions and progression of EAE; for instance, macrophage/microglia that can release several cytotoxic molecules, such as IFN-γ. The release of IFN-γ can activate microglial cells, leading to feed-forward regulation of inflammation. The current study showed that SCH58261 attenuated the activation of Iba-1 positive macrophage/microglia accompanied by the significantly suppressed level of IFN-γ. In addition, the activation of microglia and macrophages contributes to inflammatory demyelination during MS. Recent evidence has shown that macrophages are involved in demyelination while microglia are involved in myelin debris clearance (Ingwersen et al., 2016; Yamasaki et al., 2014). The activation of A_{2A}R inhibits the myelin uptake by both microglia and macrophages (Ingwersen et al., 2016). Nitric oxide synthase (iNOS) catalyzes the NO synthesis, a marker of pro-inflammatory macrophages/microglia, which might participate in the neurodegenerative process of MS when activity increases (Boven et al., 2006; Mantovani et al., 2004; Mosser and Edwards, 2008). Evidence has shown that a strong immunoreactivity for iNOS is diffusely distributed in the center and at the outer edge of the active demyelinating lesions (Mikita et al., 2011). The elevated levels of *iNOS* mRNA are correlated with the severity of the clinical signs during the development of EAE (Gordon, 2003; Oleszak et al., 1998; Raes et al., 2007). The current result showed that the administration of A_{2A}R antagonist SCH58261 was predisposed to a decline in the expression of iNOS in macrophage/microglia.

4. Materials and methods

4.1. Animals

C57BL/6J female mice (aged 8–10 weeks) were obtained from the Laboratory Animal Center of Wenzhou Medical University, China. All animal procedures in the present study were conducted according to the protocol approved by the Institutional Animals Care and Use Committee at Wenzhou Medical University, China, which adhered to the NIH Guide for the Care and Use of Laboratory Animals.

4.2. Induction of EAE

EAE was induced as described previously (Li et al., 2012; Yao et al., 2012; Wang et al., 2014). Briefly, the mice were immunized with $MOG_{35–55}$ (AC Scientific, China) oligodendrocyte glycoprotein, emulsified in incomplete Freund's adjuvant (Sigma, USA) ($MOG_{35–55}$-CFA) supplemented

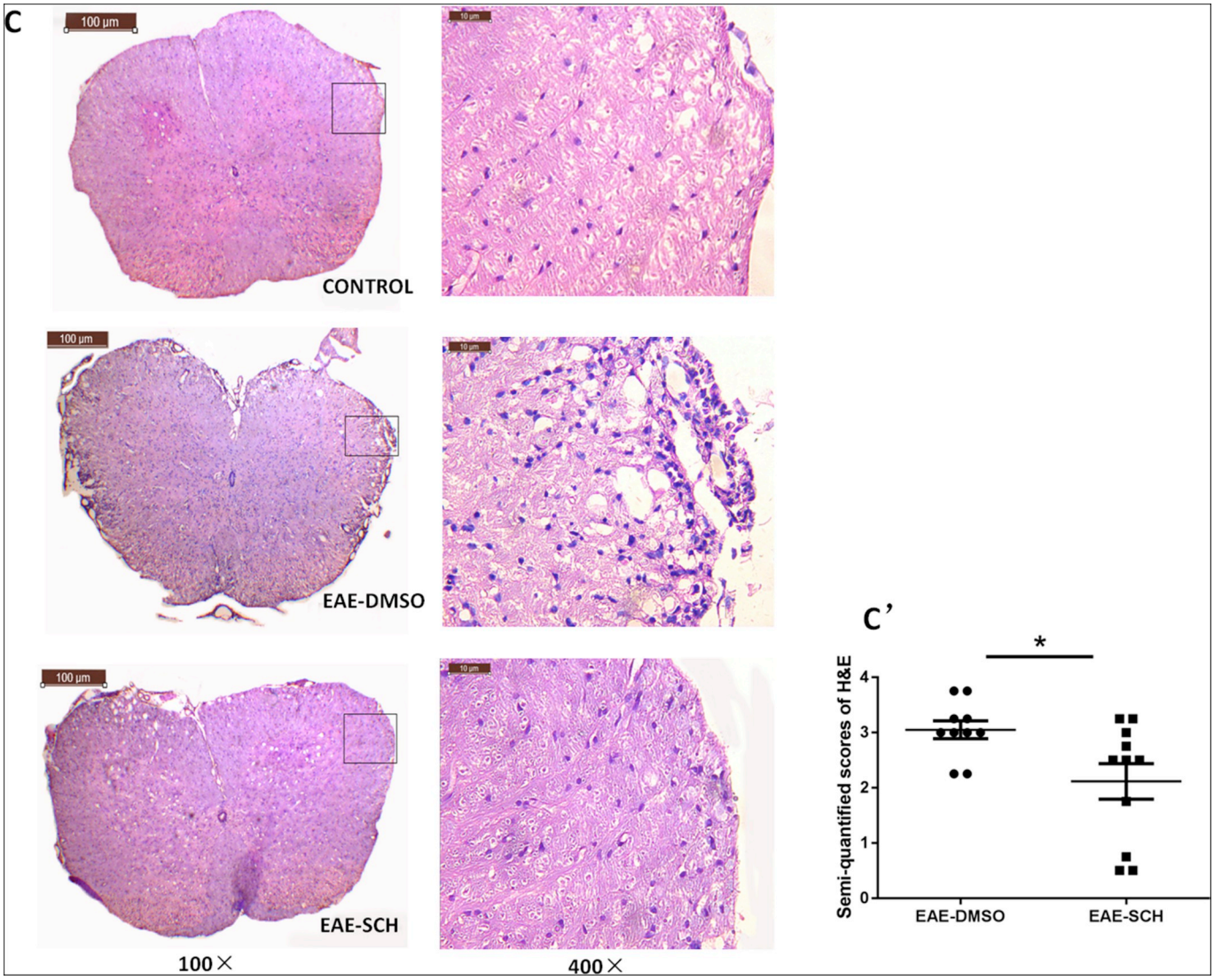

Fig. 4. (*continued*)

with 8 mg/ml *mycobacterium tuberculosis* H37Ra. Each mouse was subcutaneously injected with 200 μg $MOG_{35–55}$-CFA in the flanks. In addition, pertussis toxin (200 ng, Sigma) was injected intraperitoneally immediately and 48 h after immunization. The neurobehavioral deficits of EAE score and body weight were examined daily by an investigator who was blind to the treatment until the mice were killed.

4.3. SCH58261 treatment and group

Mice were treated with SCH58261 (2 mg/kg/d, Sigma, USA, i.p.) in DMSO or DMSO alone from the initial day of EAE induction and continued throughout the experiment. The doses of SCH58261 were selected based on the previous study, which showed protective effects of SCH58261 during EAE progression (Mills et al., 2008). In the first protocol, we determined the effective therapeutic time-window for SCH58261-mediated protection against EAE, we randomly divided the mice into five groups according to SCH58261 treatment schedule: (1) Control group (n = 6): Control mice received no treatment but were housed in the same condition as the experimental group; (2) EAE-DMSO group (n = 13): EAE mice received DMSO (rather than SCH58261) treatment; (3) EAE-SCH 0–10d group (n = 7): EAE mice received SCH58261 from the 1st to 10th d.p.i; (4) EAE-SCH11–28d group (n = 11): EAE mice received SCH58261 from the 11th–28th day of immunization; (5) EAE-SCH 0–28d group (n = 12): EAE mice received SCH58261 from the 1st to 28th day of immunization.

We also designed the second protocol to determine the effect of SCH58261 treatment. Firstly, the mice were injected with MOG_{35-55} to induce the EAE model; the neurobehavioral deficits were observed from the 0 to 28th d.p.i. When the mice showed neurobehavioral deficits (flaccid tail), they were randomly divided into two groups: (1) EAE-SCH group (n = 13): When mice showed behavioral deficits in several days post immunization(half paralyzed tail) indicated the EAE onset, and the mice were treated with SCH58261 by intraperitoneal injection for 10 days consecutively (most EAE mice reached the peak period); (2) EAE-DMSO group (n = 11): EAE mice were treated with DMSO after the EAE onset for the next 10 days as a positive control group; (3) Control group (n = 12): Mice were injected with saline (instead of MOG_{35-55}) and raised in the same condition as the other groups. All mice were sacrificed on the 10th day post onset of EAE, respectively.

4.4. Behavioral evaluation of EAE

The day of $MOG_{35–55}$-CFA immunization was regarded as day 0 d.p.i. The neurobehavioral deficits were scored twice a day for each mouse according to the previously established criteria (Yao et al., 2012; Wang et al., 2014; Weaver et al., 2005) as following: tail: 0, no signs; 1, half paralyzed tail; 2, fully paralyzed tail; Limbs: 0, no signs; 1, weak or altered gait; 2, paresis; 3, fully paralyzed limb. Each of the hind- and forelimbs were assessed separately. Thus, a fully paralyzed quadriplegic animal would attain a score of 14, and mortality equates to a score of 15. The mice were

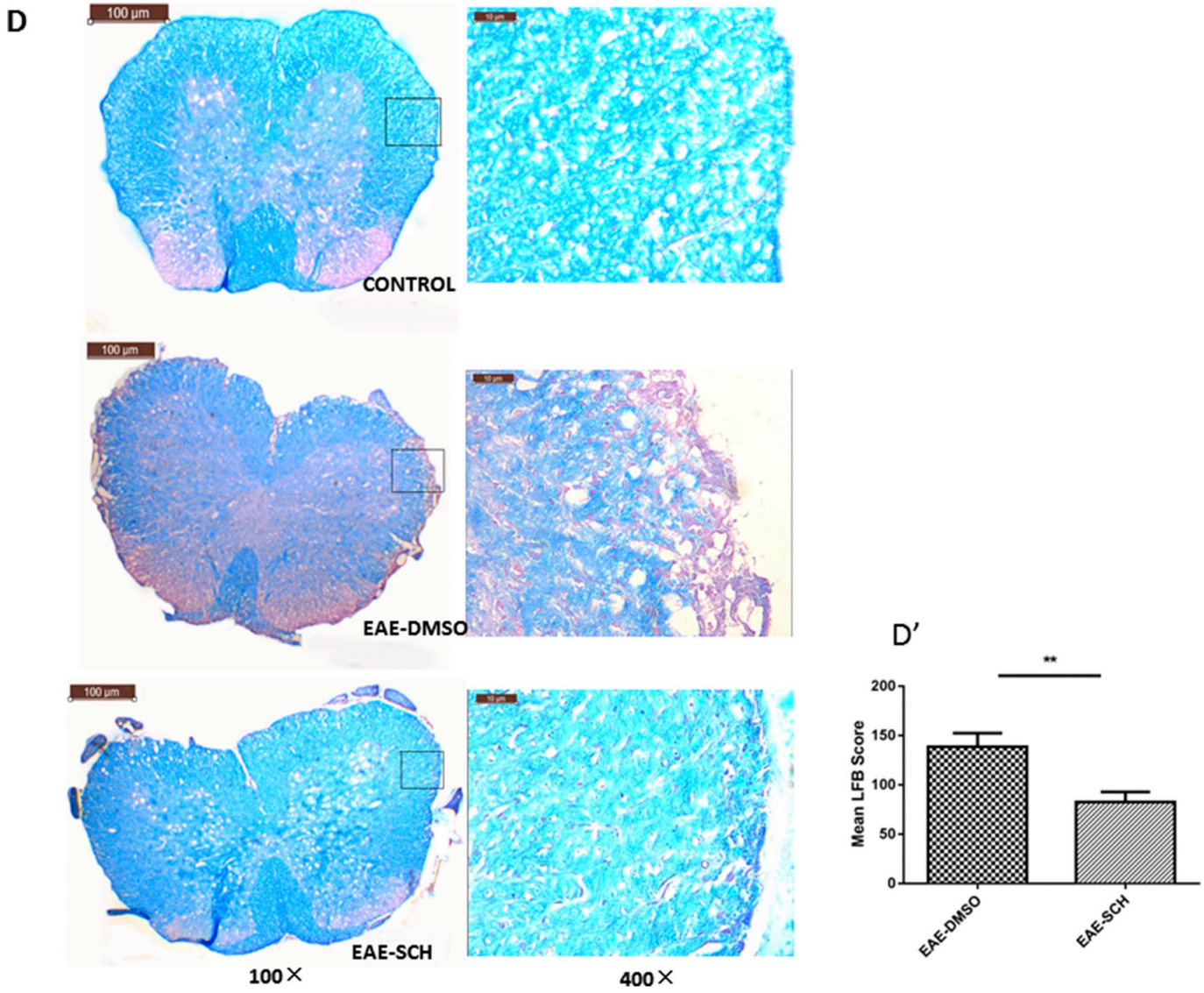

Fig. 4. (*continued*)

sacrificed after the appearance of a peak (At 28th d.p.i, when the EAE mice showed the highest EAE scores based on the pilot study) in neurobehavioral deficits for histological and biochemical analysis.

4.5. Histological analyses

In the first protocol at the 28th d.p.i or in the second protocol at the 10th day post onset of EAE, all the experimental mice were sacrificed, and the cerebral cortex and the spinal cords were dissected out. Subsequently, the brain tissues were frozen in liquid nitrogen and stored at −80 °C for further analysis, while the lumbar and thoracic spinal cords were dissected out for processing by routine paraffin-embedding for hematoxylin-eosin staining and Luxol Fast Blue (LFB) staining.

4.5.1. H&E staining for inflammatory cells infiltration

The sections were stained with hematoxylin-eosin, as described previously (Yao et al., 2012; Wang et al., 2014; Okuda et al., 1999). We first calculated the number of nuclei in each microscopic view at 400 × high magnification in each group and then computed their mean values. The mean values of the nuclei of the experimental groups at high magnification were subtracted from the mean values of the control groups, and the mean numbers of infiltrating immune cells were presented. The sections were semi-quantified as follows: 0 = no inflammation; 1, cellular infiltrates only in the perivascular areas and meninges; 2, mild cellular infiltrates in the parenchyma (1–10/section); 3, moderate cellular infiltrates in the parenchyma (11–100/section); 4, marked cellular infiltrates in the parenchyma (> 100/section) (Okuda et al., 1999).

4.5.2. LFB staining for myelin

Spinal cord sections were stained with LFB to detect myelin damage using a previously published protocol (Li et al., 2012; Wang et al., 2011a, 2011b). Briefly, after a step of removing the lipids, sections were immersed in LFB solution at 56 °C overnight (14 h) and rinsed with 95% ethanol and distilled water to remove excess staining. Then, the slides were incubated in lithium carbonate solution for 30 s and then in 70% ethyl alcohol for 30 s. Next, the slides were rinsed in distilled water. The differentiation was verified under a microscope to ensure that myelin was sharply stained. Subsequently, the sections were then mounted for examined by light microscopy. Image Pro Plus 6.0 was used for quantitative analysis.

4.5.3. Immunohistochemical analysis of glial activation

Sections of lumbar spinal cords were dehydrated through a graded series of ethanol, followed by treated with 0.3% H_2O_2 to inactivate the endogenous peroxidase with high pressure to retrieve antigen. Then, the sections were blocked with 5% BSA and incubated with goat anti-Iba-1 polyclonal antibody (1:100, Abcam, USA) and rabbit anti-GFAP monoclonal antibody (1:100, Zhongshan Gold Bridge Biotechnology Co. Ltd, China) overnight at 4 °C. After washing with PBS, sections were

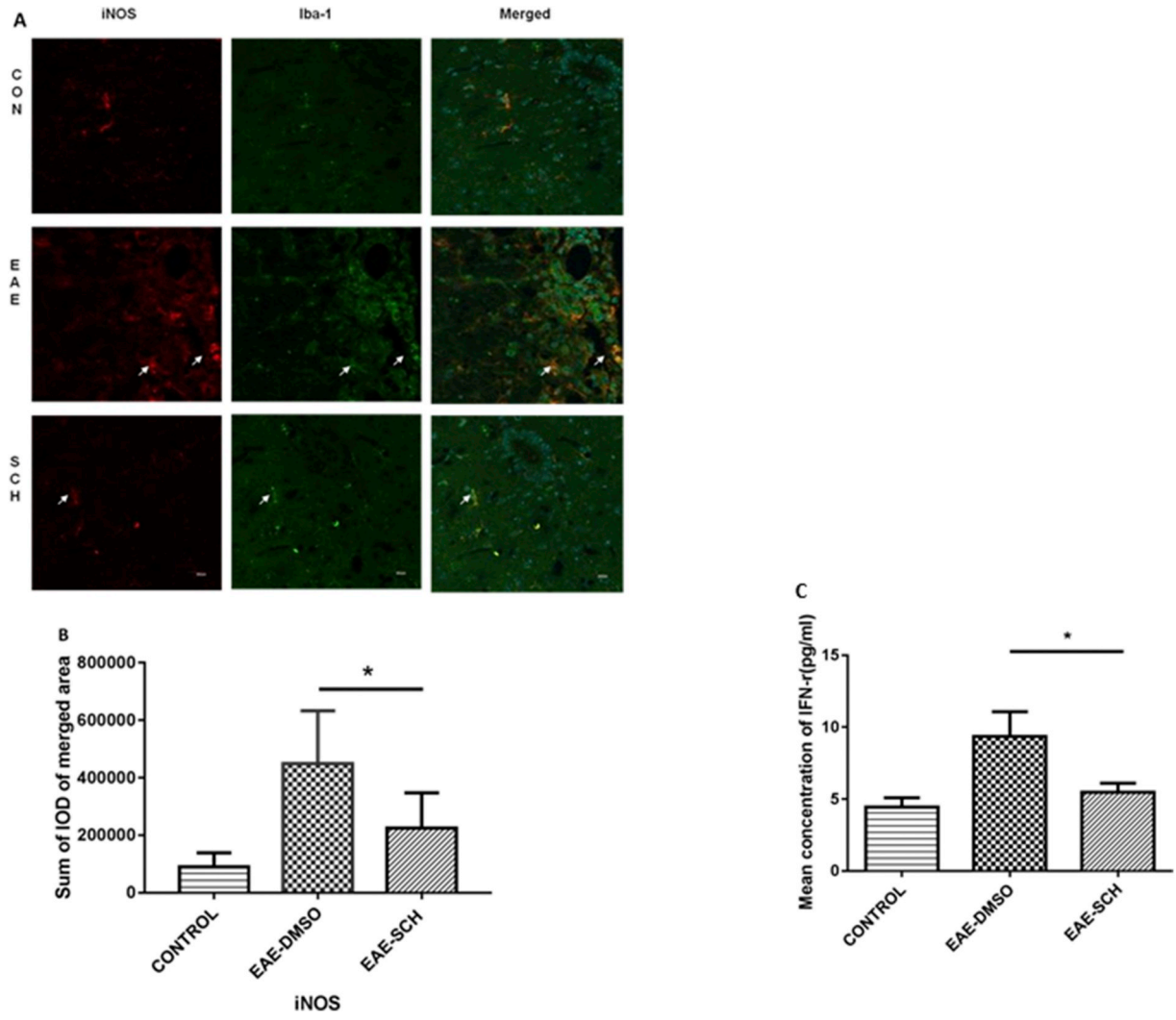

Fig. 5. (A) and (B) The effect of treatment with A_{2A}R-antagonist SCH58261 after the onset of EAE on the activation of macrophage/microglia in the spinal cords. (A) Immunofluorescence double staining showed the expression of iNOS in Iba-1-positive microglia/macrophages (600 ×). Arrows in panels indicate positive cells. Treatment with SCH58261 for ten days markedly decreased the expression of iNOS. (B) Four fields per spinal cord per mouse were analyzed at 600 × magnification for both IBA-1- and iNOS-positive staining (Control group n = 8; EAE-DMSO group n = 7; EAE-SCH group n = 7) ($t(12) = 2.682$, $p = 0.020$). * indicates $p < 0.05$. **(C) The effect of administration of SCH58261 after EAE mice showed neurobehavioral deficits on the secretion of IFN-γ in the EAE mice cerebral cortexes**. Three groups of C57BL/6 female mice received different CFA/MOG and SCH58261/DMSO as described in the Methods. Mouse cerebral cortexes were dissected and secretions of IFN-γ were determined by ELISIA (Control group n = 6; EAE-DMSO group n = 5; EAE-SCH group n = 7) ($t(10) = 2.404$, $p = 0.037$). * indicates $p < 0.05$.

incubated with biotinylated anti-goat or anti-rabbit IgG, followed by SABC reagent and diaminobenzidine for visualization. The number of positive cells in the white matter of every section in five random high power fields (400 ×) was counted by two observers, who were blinded to the treatment group, and averaged.

4.6. ELISA analyses of IFN-γ levels

To detect the secretion levels of IFN-γ in mice cerebral cortexes, EAE mice cerebral cortexes were dissected out and homogenized in cell-lysis buffer supplemented with phenyl-methyl-sulphonyl fluoride. After centrifugation, the supernatants were used for the determination of IFN-γ levels by ELISA according to the manufacturer's protocol (Westang Biotech, Shanghai, China).

4.7. Immunofluorescence double-staining

On the 10th day post-onset of EAE, the mice were perfused with ice-cold 4% paraformaldehyde solution. Spinal cords were post-fixed overnight and cryoprotected in 18% sucrose solution for a minimum of 48 h. Spinal cords were cut with a cryostat and 30-μm-thick coronal sections were collected. Then, the sections were placed in the anti-freeze solution and stored at −20 °C until assayed.

Subsequently, the coronal sections were placed for 30 min at room temperature and fixed with 4% acetone for 10 min. After washing in 0.1 M PBS, the slices were incubated in 3% H_2O_2 for 10 min to inactivate the endogenous peroxidase. After rinsing in PBS, the sections were incubated with 10% FBS for 10 min at room temperature, followed by overnight incubation at 4 °C with rabbit anti-iNOS polyclonal antibody (1:100, Abcam) and goat anti-Iba-1 polyclonal antibody (1:100, Abcam). After rinsing in PBS, the sections were co-incubated for 30 min at room temperature in the dark with fluorescent secondary antibodies: FITC-Affini Pure Donkey Anti-Goat IgG (1:100, Jackson, USA) and Cy3-AffiniPure Donkey Anti-Rabbit IgG (1:100, Jackson). After extensive washings, the slices were incubated with DAPI (1:500) for 1 min at 37 °C in the dark. After final washing, the slices were mounted with glycerinum and visualized under confocal laser scanning microscope FV 10i (Olympus, Japan). The expression of proteins in each section was analyzed by software Image-Pro Plus 6.0. Finally, we used confocal laser microscopy scanning to determine whether or not iNOS was expressed in the macrophage/microglia after treatment with SCH58261.

4.8. Statistical analyses

The SPSS16.0 statistical program and GraphPad Prism 7 were used for statistical analysis. All data are presented as mean ± SEM unless otherwise stated. Mouse EAE neurological behavioral deficit scores and histopathological scores were analyzed by two-way ANOVA (with two factors, i.e. treatment and disease course), followed by LSD *post-hoc* comparisons and Mann-Whitney U-tests. The comparisons among multiple disease courses with single treatment factor were analyzed by one-way ANOVA, while those between two groups with single treatment factor were analyzed by *t*-test; $p < 0.05$ was considered as statistically significant.

5. Conclusions

In the present study, we demonstrated that administration of A_{2A}-specific receptor antagonist SCH58261 during the neurobehavioral deficits period of EAE or after the onset of EAE provides neuroprotection and attenuates the neurological deficits symptoms. The protective effect of SCH58261 was associated with the inactivation of macrophage/microglia and the reduction of iNOS expression. This mechanistic understanding of A_{2A}-specific receptor antagonist SCH58261 against EAE pathology might advance the prospective treatment strategies for MS.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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References

Ben-Zacharia, A.B., 2011. Therapeutics for multiple sclerosis symptoms. Mt. Sinai J. Med. J. Transl. Personal. Med. 78 (2), 176–191.

Blackburn, M.R., Vance, C.O., Morschl, E., Wilson, C.N., 2009. Adenosine receptors and inflammation. Handb. Exp. Pharmacol. 193, 215–269. https://doi.org/10.1007/978-3-540-89615-9_8.

Boven, L.A., Van Meurs, M., Van Zwam, M., Wierenga-Wolf, A., Hintzen, R.Q., Boot, R.G., Aerts, J.M., Amor, S., Nieuwenhuis, E.E., Laman, J.D., 2006. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain : J. Neurol. 129 (Pt 2), 517–526. https://doi.org/10.1093/brain/awh707.

Centonze, D., Muzio, L., Rossi, S., Furlan, R., Bernardi, G., Martino, G., 2010. The link between inflammation, synaptic transmission and neurodegeneration in multiple sclerosis. Cell Death Differ. 17 (7), 1083–1091. https://doi.org/10.1038/cdd.2009.179.

Denic, A., Johnson, A.J., Bieber, A.J., Warrington, A.E., Rodriguez, M., Pirko, I., 2011. The relevance of animal models in multiple sclerosis research. Pathophysiology 18 (1), 21–29. https://doi.org/10.1016/j.pathophys.2010.04.004.

Gomes, C., Ferreira, R., George, J., Sanches, R., Rodrigues, D.I., Goncalves, N., Cunha, R.A., 2013. Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: a2A receptor blockade prevents BDNF release and proliferation of microglia. J. Neuroinflammation 10, 16. https://doi.org/10.1186/1742-2094-10-16.

Gordon, S., 2003. Alternative activation of macrophages. Nat. Rev. Immunol. 3 (1), 23–35. https://doi.org/10.1038/nri978.

Ingwersen, J., Wingerath, B., Graf, J., Lepka, K., Hofrichter, M., Schroter, F., Wedekind, F., Bauer, A., Schrader, J., Hartung, H.P., Prozorovski, T., Aktas, O., 2016. Dual roles of the adenosine A2a receptor in autoimmune neuroinflammation. J. Neuroinflammation 13, 48. https://doi.org/10.1186/s12974-016-0512-z.

Li, F., Zhang, Z.X., Liu, Y.F., Xu, H.Q., Hou, S.T., Zheng, R.Y., 2012. 2-BFI ameliorates EAE-induced mouse spinal cord damage: effective therapeutic time window and possible mechanisms. Brain Res. 1483, 13–19. https://doi.org/10.1016/j.brainres.2012.09.016.

Liu, Y., Zou, H., Zhao, P., Sun, B., Wang, J., Kong, Q., Mu, L., Zhao, S., Wang, G., Wang, D., Zhang, Y., Zhao, J., Yin, P., Liu, L., Zhao, X., Li, H., 2016. Activation of the adenosine A2A receptor attenuates experimental autoimmune encephalomyelitis and is associated with increased intracellular calcium levels. Neuroscience 330, 150–161. https://doi.org/10.1016/j.neuroscience.2016.05.028.

Liu, Y., Alahiri, M., Ulloa, B., Xie, B., Sadiq, S.A., 2018. Adenosine A2A receptor agonist ameliorates EAE and correlates with Th1 cytokine-induced blood brain barrier dysfunction via suppression of MLCK signaling pathway. Immun. Inflamm. Disord. 6 (1), 72–80. https://doi.org/10.1002/iid3.187.

Lopez-Diego, R.S., Weiner, H.L., 2008. Novel therapeutic strategies for multiple sclerosis–a multifaceted adversary. Nat. Rev. Drug Discov. 7 (11), 909–925. https://doi.org/10.1038/nrd2358.

Lublin, F.D., Reingold, S.C., Cohen, J.A., Cutter, G.R., Sorensen, P.S., Thompson, A.J., Wolinsky, J.S., Balcer, L.J., Banwell, B., Barkhof, F., Bebo Jr., B., Calabresi, P.A., Clanet, M., Comi, G., Fox, R.J., Freedman, M.S., Goodman, A.D., Inglese, M., Kappos, L., Kieseier, B.C., Lincoln, J.A., Lubetzki, C., Miller, A.E., Montalban, X., O'Connor, P.W., Petkau, J., Pozzilli, C., Rudick, R.A., Sormani, M.P., Stuve, O., Waubant, E., Polman, C.H., 2014. Defining the clinical course of multiple sclerosis: the 2013 revisions. Neurology 83 (3), 278–286. https://doi.org/10.1212/WNL.0000000000000560.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M., 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25 (12), 677–686. https://doi.org/10.1016/j.it.2004.09.015.

Mikita, J., Dubourdieu-Cassagno, N., Deloire, M.S., Vekris, A., Biran, M., Raffard, G., Brochet, B., Canron, M.H., Franconi, J.M., Boiziau, C., Petry, K.G., 2011. Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. Mult. Scler. 17 (1), 2–15. https://doi.org/10.1177/1352458510379243.

Mills, J.H., Thompson, L.F., Mueller, C., Waickman, A.T., Jalkanen, S., Niemela, J., Airas, L., Bynoe, M.S., 2008. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. Proc. Natl. Acad. Sci. Unit. States Am. 105 (27), 9325–9330.

Mills, J.H., Kim, D.G., Krenz, A., Chen, J.F., Bynoe, M.S., 2012. A2A adenosine receptor signaling in lymphocytes and the central nervous system regulates inflammation during experimental autoimmune encephalomyelitis. J. Immunol. 188 (11), 5713–5722. https://doi.org/10.4049/jimmunol.1200545.

Mosser, D.M., Edwards, J.P., 2008. Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. 8 (12), 958–969. https://doi.org/10.1038/nri2448.

Neuhaus, O., Archelos, J.J., Hartung, H.P., 2003. Immunomodulation in multiple sclerosis: from immunosuppression to neuroprotection. Trends Pharmacol. Sci. 24 (3), 131–138. https://doi.org/10.1016/S0165-6147(03)00028-2.

Okuda, Y., Sakoda, S., Fujimura, H., Saeki, Y., Kishimoto, T., Yanagihara, T., 1999. IL-6 plays a crucial role in the induction phase of myelin oligodendrocyte glycoprotein 35–55 induced experimental autoimmune encephalomyelitis. J. Neuroimmunol. 101 (2), 188–196.

Oleszak, E.L., Zaczynska, E., Bhattacharjee, M., Butunoi, C., Legido, A., Katsetos, C.D., 1998. Inducible nitric oxide synthase and nitrotyrosine are found in monocytes/macrophages and/or astrocytes in acute, but not in chronic, multiple sclerosis. Clin. Diagn. Lab. Immunol. 5 (4), 438–445.

Raes, G., Beschin, A., Ghassabeh, G.H., De Baetselier, P., 2007. Alternatively activated macrophages in protozoan infections. Curr. Opin. Immunol. 19 (4), 454–459. https://doi.org/10.1016/j.coi.2007.05.007.

Reitz, C., Brayne, C., Mayeux, R., 2011. Epidemiology of alzheimer disease. Nat. Rev. Neurol. 7 (3), 137–152. https://doi.org/10.1038/nrneurol.2011.2.

Saura, J., Angulo, E., Ejarque, A., Casado, V., Tusell, J.M., Moratalla, R., Chen, J.F., Schwarzschild, M.A., Lluis, C., Franco, R., Serratosa, J., 2005. Adenosine A2A receptor stimulation potentiates nitric oxide release by activated microglia. J. Neurochem. 95 (4), 919–929. https://doi.org/10.1111/j.1471-4159.2005.03395.x.

Stark, J.L., Cross, A.H., 2006. Differential expression of suppressors of cytokine signaling-1 and -3 and related cytokines in central nervous system during remitting versus non-remitting forms of experimental autoimmune encephalomyelitis. Int. Immunol. 18 (2), 347–353. https://doi.org/10.1093/intimm/dxh373.

Steinman, L., 2001. Multiple sclerosis: a two-stage disease. Nat. Immunol. 2 (9), 762–764. https://doi.org/10.1038/ni0901-762.

Tansey, M.G., Goldberg, M.S., 2010. Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. Neurobiol. Dis. 37 (3), 510–518.

Thompson, A.J., Toosy, A.T., Ciccarelli, O., 2010. Pharmacological management of symptoms in multiple sclerosis: current approaches and future directions. Lancet Neurol. 9 (12), 1182–1199. https://doi.org/10.1016/S1474-4422(10)70249-0.

Wang, Z.W., Wang, P., Lin, F.H., Li, X.L., Li, X.F., O'Byrne, K.T., Hou, S.T., Zheng, R.Y., 2011a. Early-life exposure to lipopolysaccharide reduces the severity of experimental autoimmune encephalomyelitis in adulthood and correlated with increased urine corticosterone and apoptotic CD4+ T cells. Neuroscience 193, 283–290. https://doi.org/10.1016/j.neuroscience.2011.07.047.

Wang, P., Wang, Z.W., Lin, F.H., Han, Z., Hou, S.T., Zheng, R.Y., 2011b. 2-BFI attenuates experimental autoimmune encephalomyelitis-induced spinal cord injury with enhanced B-CK, CaATPase, but reduced calpain activity. Biochem. Biophys. Res. Commun. 406 (1), 152–157. https://doi.org/10.1016/j.bbrc.2011.02.018.

Wang, T., Xi, N.N., Chen, Y., Shang, X.F., Hu, Q., Chen, J.F., Zheng, R.Y., 2014. Chronic caffeine treatment protects against experimental autoimmune encephalomyelitis in mice: therapeutic window and receptor subtype mechanism. Neuropharmacology 86, 203–211. https://doi.org/10.1016/j.neuropharm.2014.06.029.

Weaver, J.G., Tarze, A., Moffat, T.C., Lebras, M., Deniaud, A., Brenner, C., Bren, G.D., Morin, M.Y., Phenix, B.N., Dong, L., Jiang, S.X., Sim, V.L., Zurakowski, B., Lallier, J., Hardin, H., Wettstein, P., van Heeswijk, R.P., Douen, A., Kroemer, R.T., Hou, S.T., Bennett, S.A., Lynch, D.H., Kroemer, G., Badley, A.D., 2005. Inhibition of adenine nucleotide translocator pore function and protection against apoptosis in vivo by an HIV protease inhibitor. J. Clin. Investig. 115 (7), 1828–1838. https://doi.org/10.1172/JCI22954.

Yamasaki, R., Lu, H., Butovsky, O., Ohno, N., Rietsch, A.M., Cialic, R., Wu, P.M., Doykan, C.E., Lin, J., Cotleur, A.C., Kidd, G., Zorlu, M.M., Sun, N., Hu, W., Liu, L., Lee, J.C., Taylor, S.E., Uehlein, L., Dixon, D., Gu, J., Floruta, C.M., Zhu, M., Charo, I.F., Weiner, H.L., Ransohoff, R.M., 2014. Differential roles of microglia and monocytes in the inflamed central nervous system. J. Exp. Med. 211 (8), 1533–1549. https://doi.org/10.1084/jem.20132477.

Yao, S.Q., Li, Z.Z., Huang, Q.Y., Li, F., Wang, Z.W., Augusto, E., He, J.C., Wang, X.T., Chen, J.F., Zheng, R.Y., 2012. Genetic inactivation of the adenosine A2A receptor exacerbates brain damage in mice with experimental autoimmune encephalomyelitis. J. Neurochem. 123 (1), 100–112.