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ORIGINAL ARTICLE

Immune characteristics study of AG490, a signal pathway inhibitor, in EAE model mice

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Abstract Multiple sclerosis (MS) is an autoimmune disease characterized by demyelination, axonal damage and progressive neurologic dysfunction in central nervous system (CNS). Many evidences show that B cells play an important role in the pathogenesis of MS. Follicular helper T cells (Tfh) secrete IL-21 to prompt the proliferation and differentiation of B cells in germinal center (GC) through clonal proliferation, somatic hypermutation, antibody class switching, antibody affinity maturation process. AG490 is a synthetic inhibitor to JAK-STAT signal pathway, which has been studied in inflammatory, tumor and autoimmune diseases. In the present study, the experimental mice were divided into 3 groups, vehicle group and AG490 group were given MOG35-55 to induce EAE model, from the third day after immunization, the mice were given vehicle or AG490 by intraperitoneal injection every other day. All mice were assessed clinical scores after immunization. On twentieth day, all mice were sacrificed, HE staining and solochrome cyanine staining were performed to evaluate inflammatory cells infiltration and demyelination, spleen sections were stained with PNA-FITC to analyze the difference in germinal center. Compared with vehicle group, the incidence of AG490 group was deceased, onset time was delayed, the severity was significantly reduced. The inflammatory cells and demyelination in AG490 group were lower than those in vehicle group. Immunofluorescence showed the fluorescence intensity of AG490 group was significantly lower than in the vehicle group, but higher than that of control group.

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1. Introduction

Multiple sclerosis (MS) is an autoimmune disease characterized by demyelination, axonal damage and progressive neurologic dysfunction in central nervous system (CNS) (Nylander and Hafler, 2012). Although there is no exact pathogenesis of MS, the abnormal CD4+ helper T cells (helper T lymphocytes, Th) is always considered as the main reason, meanwhile many evidences show that B cells play an important role in the pathogenesis of MS.

More than 95% of patients with MS have been found Oligoclonal bands (oligoclonal, bands, OCB) in their cerebrospinal fluid examination (Freedman et al., 2005). A large number of plasma cells have been found in acute and subacute plaque of MS around blood vessels. Demyelination is the main feature of brain lesions which has appeared as IgG deposition and complement regulatory in the diagnosis of patients (Breij et al., 2008). The studies also found that there are some relativities between antibody of virus antigen of serum and MS, such as the titers of 1-antibody of EB virus (Epstein-Barr virus, EBV) nuclear antigen were significantly higher than those in normal control group (Farrell et al., 2009). The study of pathology shows a substantial proportion of patients with secondary progressive multiple sclerosis (SPMS) in arachnoid membrane which has ectopic lymphoid follicles, it has the same structure as center germinal (GC) of B cell (Serafini et al., 2004; Magliozzi et al., 2007; Howell et al., 2011). Furthermore, the relapsing remitting multiple sclerosis (RRMS) patient's condition was significantly improved after B lymphocytes which can express the CD20 antigen was cleared selectively by Rituxan (Hauser et al., 2008). It has achieved similar affection in the experiment which used the second generation of Anti CD20 molecules (Barun and Bar-Or, 2012). In recent years, the researchers have found that except the function of humoral immunity and producing antibodies, the B cells also have negative immune regulation as the subgroup named regulatory B cell (Breg).

It has been found that Follicular helper T cells (Tfh) are the real T cells which participated in producing antibodies by B cells recently (King et al., 2008). IL-21 is the main effect factor of Tfh cells (Sarra et al., 2011). There are many cells that have IL-21R, for example, B cells, Tfh, Thl7 and NK cells, and the highest expression level are found in B cells (Spolski and Leonard, 2008). IL-21 is elevated in several autoimmune diseases, and the combination of IL-21 and IL-21R to activate JAK/STAT signaling pathway is considered to be the main cause of these disease. Tfh7 cells secrete IL-21 to prompt the proliferation and differentiation of B cells in germinal center (GC) through clonal proliferation, somatic hypermutation, antibody class switching, antibody affinity maturation process, and GC-B cells ultimately differentiate into long-lived memory B cells and plasma cells which play a very important role in humoral immune response (Schaerli et al., 2000).

AG490 is a kind of artificial inhibitor of JAK/STAT signal pathway. It has the same structure as beta-parahydroxy-phenylpropionic acid, so it can combine with receptor tyrosine kinase competitively and then block tyrosine or serine phosphorylation in the special sites of JAK, finally blocking the activation of kinase and production of cytokine in the downstream. Previously, the study showed that AG490 can block the phosphorylation of JAK2 (Meydan et al., 1996), while the new research suggested it can also block JAK3 in T cells effectively (Cernkovich et al., 2008). At present, the research of AG490 is mainly focused on tumor and inflammation, meanwhile the results show that AG490 has good application prospects in some autoimmune diseases. In the present study, the immune characteristics of AG490 were evaluated in EAE model mice, the effect of AG490 to EAE was observed by clinical nerve function assessment and PNA-FITC immunofluorescent staining for spleen.

2. Materials and methods

2.1. Chemicals and reagents

MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK, with the purity >95%) was synthesized by Xi'an Lianmei (China). PTX was purchased by Alexis (Germany). Mycobacterium tuberculosis H37RA was provided by Difco (USA). CFA was purchased from SigmaAldrich (USA). Sterile PBS was purchased from Difco (USA). Fixing agent was purchased from LiankeBio (China). The 1640 complete medium and FBS were purchased from Gibco (USA). AG490 and DMSO were purchased from SigmaAldrich (USA).

2.2. Development of EAE animal model

2.2.1. Animal feeding and grouping

There are thirty-six unpregnancy female mice of clean grade C57BL/6 weighted 16–18 g from six to eight weeks old, provided by experimental animal center of Zhongshan University. Animals were kept under controlled conditions with the temperature maintained at 24 ± 2 °C. The mice were acclimatized to the housing environment for 1 week prior to the study. The rats were fasted but provided access to water overnight before testing began.

The number of 36 mice was adaptively fed for a week, divided with "g" as a unit of weight stratification then the experimental mice were divided into 3 groups (n = 12), normal control group (control group), vehicle control group (vehicle Group) and AG490 treatment group (AG490 group), vehicle group and AG490 group were given MOG 35–55 to induce EAE model, from the third day after immunization, the mice were given vehicle or AG490 by intraperitoneal injection every other day. The mice of control and vehicle groups were given intraperitoneal injection with an equal volume of 5% DMSO of sterile PBS.

2.2.2. Induction of EAE model

(1) Preparation of antigen adjuvant emulsion: MOG was diluted with aseptic PBS to 2 mg/ml, named A. The tuberculin H37RA was dissolved in Freund's adjuvant completely which named B with concentration of 10 mg/ml. The equal volumes of a liquid and the B solution were then emulsified by an electric emulsifying device for more than 30 min until the emulsion was formed, while the water can be kept in a small ball shape for a long time. Antigen adjuvant emulsion must be used within 12 h.

(2) The PTX (American Alexis) was diluted to 4 g/ml with a sterile endotoxin free PBS solution, kept at 4 °C.
(3) EAE induction: Animals were injected with 0.1 ml of antigen emulsion on both sides of the groin subcutaneous besides the abdomen. Then all the EAE model mice were intraperitoneally injected with PTX solution of 0.1 ml on the 0 day and at 48 h after processing, respectively. On the seven days, each mouse was injected with 0.1 ml antigen emulsion on both sides of the axillary subcutaneous to enhance the immune effect. Mice in control group were subcutaneously and intraperitoneally injected with the same dose of PBS.

2.2.3. Neurological function assessmenting

Blinding method was used to observe the animals after immunizing. Then the mice were weighed and got neurological function score with Knno methods (Kono et al., 1998).

Following the standard below:

Level 0: no disease; Level 1: mouse tail tension reduction or paralysis; Level 2: mouse tail paralysis, weakness of hind limbs; Level 3: paralysis of the hind limbs in mice; Level 4: mouse limb paralysis, man-made stand up cannot be reset; Level 5: mouse can be morbidity and then moribund.

2.3. Drawing materials

The materials also were taken at the peak period in each group. There are six mice that have been anesthetized by 0.1 ml of 10% chloral hydrate, then the heart was quickly exposed for cutting the right atrial appendage. 50 ml of precooling PBS was used to wash the whole body via the left ventricle till the liver and lungs becoming completely white. Another 100 ml of 4% poly formaldehyde was given for slow infusion, drawing was performed while the mouse to harden the bodyv. Spinal intact and the spleen are removed and placed in 4% poly formaldehyde at room temperature which are fixed 48 for hours, after 48 h, lumbar segments of the spinal cord and spleen were carefully isolated and then were taken to embed in paraffin.

There are also six mice anesthetized then put in 75% ethanol for 5 min, on the right side of the ice to take the spleen and half of the spleen was cut which is put in 10% FBS containing cold spleen at the 1640 culture medium to isolate spleen cells. The other half of the spleen was put in freezing tubes within liquid nitrogen and then transferred into refrigerator with −80 °C for storage.

2.4. General pathology

2.4.1. HE staining

The lumbar spinal cord was embedded in paraffin which was cut continuously by freezing microtome with the thickness was about 4–5 μm. Put the slide into xylene - xylene - 100% alcohol - ethanol (100%–95% alcohol - 90% alcohol - 80% alcohol - 70% alcohol in turn every process for 10 min. Then follow the steps which are manual dewaxing-distilled water cleaning-hematoxylin staining and where it should be continued for 10 min to stain the nuclear. Thirdly using water to clean and dividing in 0.5% hydrochloric acid alcohol to wash out the nonspecific blue. Meanwhile soaking for 30 min to blue again, staining cytoplasm by 0.5% eosin counterstain for 20–30 s, washing nonspecific red by 80% alcohol. Finally, sliding slide into 70% alcohol - 80% alcohol - 90% alcohol - 95% alcohol - ethanol (100%–100% alcohol-xylene– xylene in turn and every step last 5 min to dewater, observing after mounting neutral resin.

Following the standard to observe infiltration of inflammatory cell (O'Neill et al., 2006):

0 point: no cell infiltration; 1 point: the infiltration of inflammatory cells appeared in the spinal membrane; 2 points: inflammatory cell aggregation and infiltration in 1–2 blood vessels; 3 points: inflammatory cell aggregation in 3–4 blood vessels and/or a large range of 1 parenchymal involvement cell infiltration; 4 points: a large number of cell infiltration involving albar over 20% of the region.

2.4.2. Myelin staining

Lumbar spinal cord of mice in every group was embedded and made into series sections which were about 4–5 μm thick then dried at 37 °C deg and stained with solochrome cyanine so that the condition can be observed.

Following were the steps: soaking the paraffin in water; staining with solochrome cyanine for 20–30 min; watering till them be blue; put the slices into ferric alum for 5–10 min; soaking the slices in ferricyanic acid borax; dehydration and washing.

Following the standard of Kuerten to score myelinoclasis (Kuerten et al., 2007):

1 point: a little of subpial demyelination; 2 points: obvious demyelination around the subpial or vascular; 3 points: the integration of the myelin sheath around the vascular or subpial; 4 points: a large number of demyelination around perivascular or subpialcumulationa half of spinal cord, and with CNS of inflammatory cell infiltration; 5 points: extensive perivascular and subpial demyelination, the transverse section of the spinal cord involvement, and with CNS parenchymal infiltration of inflammatory cells.

2.5. Immunofluorescence

(1) Preparation of paraffin sections of mouse spleen tissue; (2) The paraffin section was put in the oven of 60 °C for 60 min, to conventionally dewax; (3) The paraffin sections was put in 0.1% Triton-100 solution (dissolved in PBS) in room temperature static for 5 min; (4) PNA-FITC (100 μg/ml) was dropped on the paraffin section to incubate for 30 min avoiding light 37 °C , washed with PBS for 3 min × 3; (5) Adding DAPI solution, light 37 °C and incubated for 10 min PBS wash 3 min × 3; (6) Glycerol under fluorescence microscope.

2.6. Statistical analysis

Statistical analysis was carried out by SPSS16.0, all data were expressed in this standard which is mean ± SD. A one-way ANOVA was used for pairwise comparisons while the results meeting normality and homoscedasticity, the least significant difference method (LSD) was also used and was considered statistically significant while $P < 0.05$. Kruskal-Wallis was used for nonparametric test while not meeting the normality or homoscedasticity, Mann-Whitney *U*-test was used for pairwise comparisons and was considered statistically significant while is $P < 0.05$.

3. Results

3.1. General situation, incidence and clinical score of mice

There was no abnormal nervous system performance in normal control group (control group) in the process of experiment. The general situation of nosopoietic murine appearance changed significantly, such as the appearance of hair was not smooth, eat less, weight loss, reaction to the outside stimulation unresponsive, showed the tail down to the floor, gait unsteady, limb weakness, then developed to limb paralysis, involving bilateral hindlimb, serious mice appear quadriplegia. Immunohistochemistry results showed, compared with vehicle group (22.58 ± 9.21 vs 7.5 ± 3.40), the expressions were decreased in AG490 group, have statistical significance ($P = 0.01$) (Table 1 and Fig. 1). Solvent control group (Vehicle Group) mice on the 12th day of onset, the median time to onset of 12.83 + 1.47 days. The mice in AG490 treatment group on the 14th day of onset, the median time to onset of 16.16 + 1.21 days, obviously express later than vehicle group which has statistical significance (Table 1 and Fig. 1). Thus, compared with the vehicle group, AG490 treatment group can effectively reduce the incidence of EAE mice, delay time of onset, and can significantly reduce the severity of the disease.

3.2. Pathological changes of myeloid tissue in mice

HE staining showed that the vehicle group in the myeloid tissue of mice have infiltration of a large number of inflammatory cell, inflammatory cells mainly gathered around the spinal pia mater and vascular, forming the structure like "cuff" (Fig. 2). Inflammatory cells were significantly reduced which was dealt with AG490 and was significantly lower compared with vehicle group (2.67 ± 0.47 vs 1.50 ± 0.50) with statistical significance ($P = 0.011$) (Table 1 and Fig 3). Solochrome cyanine staining showed that vehicle group mouse had myelinoclasis in substantia alba medullae spinalis and a large number of inflammatory

Table 1 The clinical features and pathological score of vehicle group and AG490 group.

Processing	Onset	Cumulative score	Inflammation	Demyelination	Incidence
Vehicle	12.83 ± 1.47	22.58 ± 9.21	2.67 ± 0.47	3.50 ± 0.5	91.70%
AG490	16.16 ± 1.21**	7.5 ± 3.40**	1.50 ± 0.50*	1.67 ± 0.75**	62.20%*

* Compared with the vehicle group, $P < 0.05$.
** Compared with the vehicle group, $P < 0.01$.

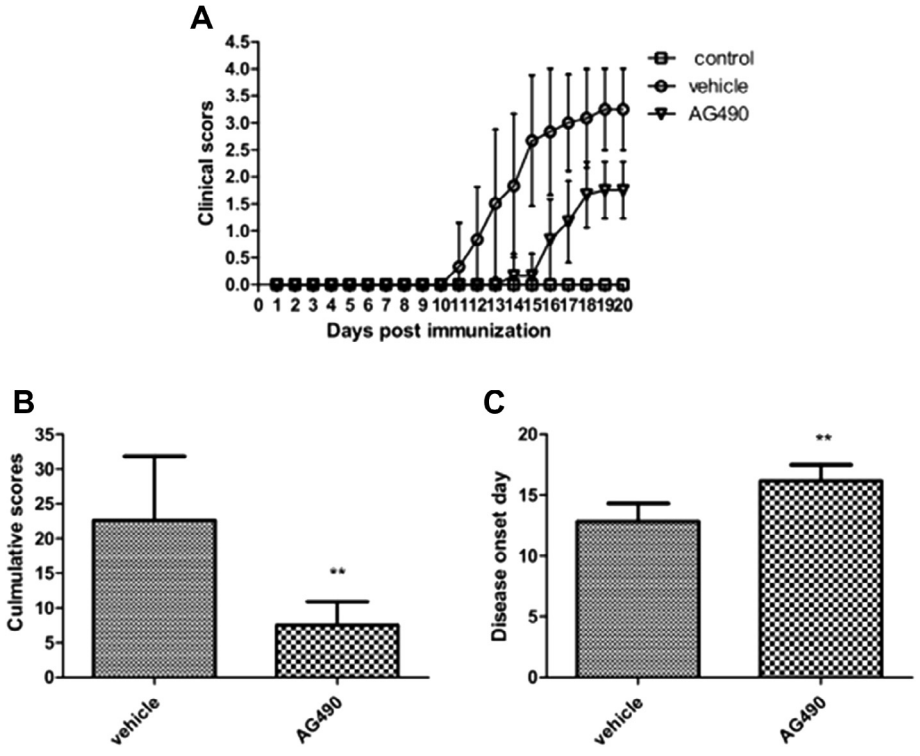

Figure 1 AG490 delayed the onset time of EAE mice and significantly improved its neurological function. Clinical score, B: vehicle group and AG490 treatment group, the cumulative clinical score, the average onset time of C: vehicle and AG490 treatment group were all the mice before and after A: immunization. *: compared with the vehicle group, $P < 0.05$, **: compared with the vehicle group, $P < 0.01$.

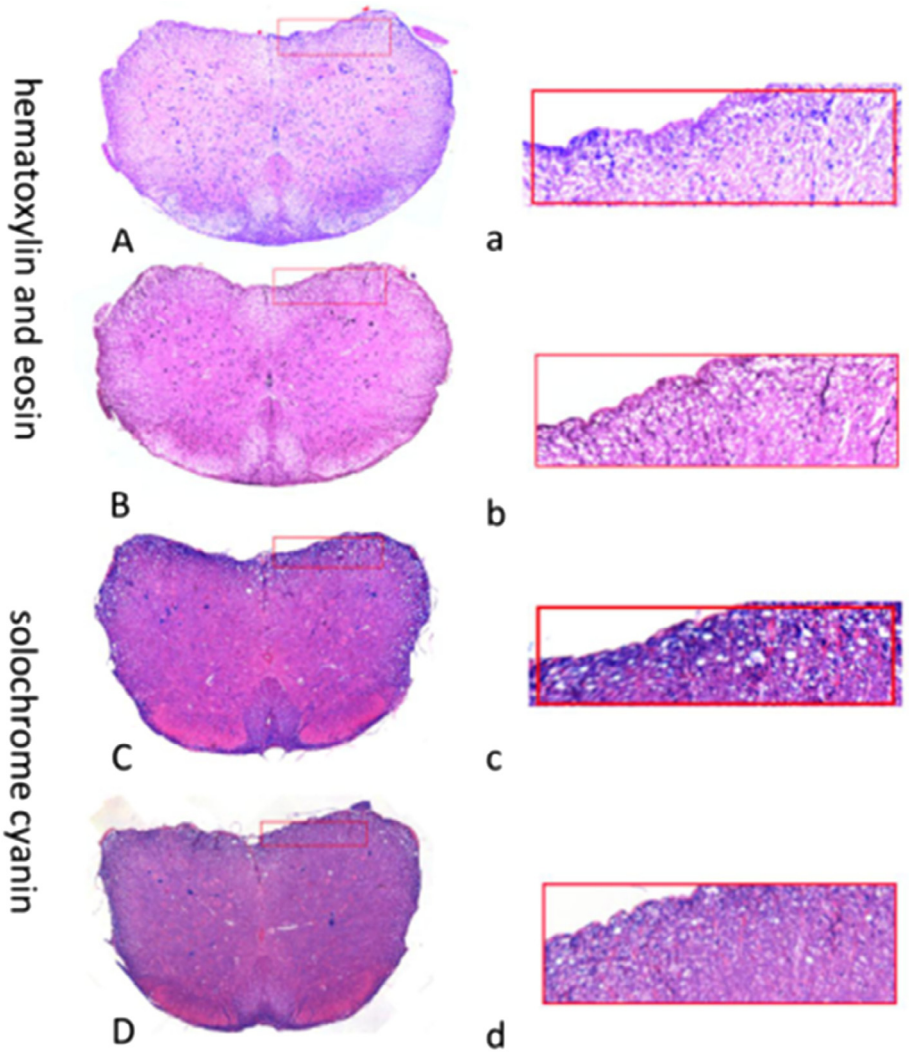

Figure 2 AG490 significantly reduced the pathological changes in EAE mice, inflammatory cell infiltration and changes in the lumbar spinal cord were significantly reduced. A, B, for HE staining, C, D for the sand LuoLuo cyanine dye; a, B and C AG490 group mice, B, D vehicle mice; a, B, C, D for corresponding local amplification. Six mice in each group.

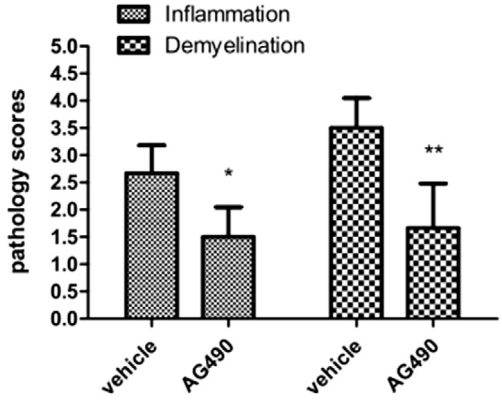

Figure 3 AG490 significantly reduced the inflammatory score and the score of the EAE mice, *: compared with the *P* group, vehicle <0.05, **: compared with the *P* group, vehicle <0.01. Six mice in each group.

cell infiltrating around it. Inflammatory cells were significantly reduced which was dealt with AG490 and was significantly lower compared with vehicle group (3.50 ± 0.50 vs 1.67 ± 0.75) also with statistical significance ($P = 0.006$) (Table 1 and Fig. 3).

3.3. The formation of GC in the spleen of each group was analyzed by immunofluorescence microscopy PNA-FITC staining

B cell can be shown by PNA (+) when it was in the early stages. The weakest PNA (+) green fluorescent in the control group suggesting the less non-immuned mice, meanwhile the strongest green fluorescent in the vehicle group suggesting the large number of GC cells in EAE model mice. In AG490 treatment group, the green fluorescence of PNA (+) compared with the vehicle group decreased significantly but still higher than the control group (Fig. 4).

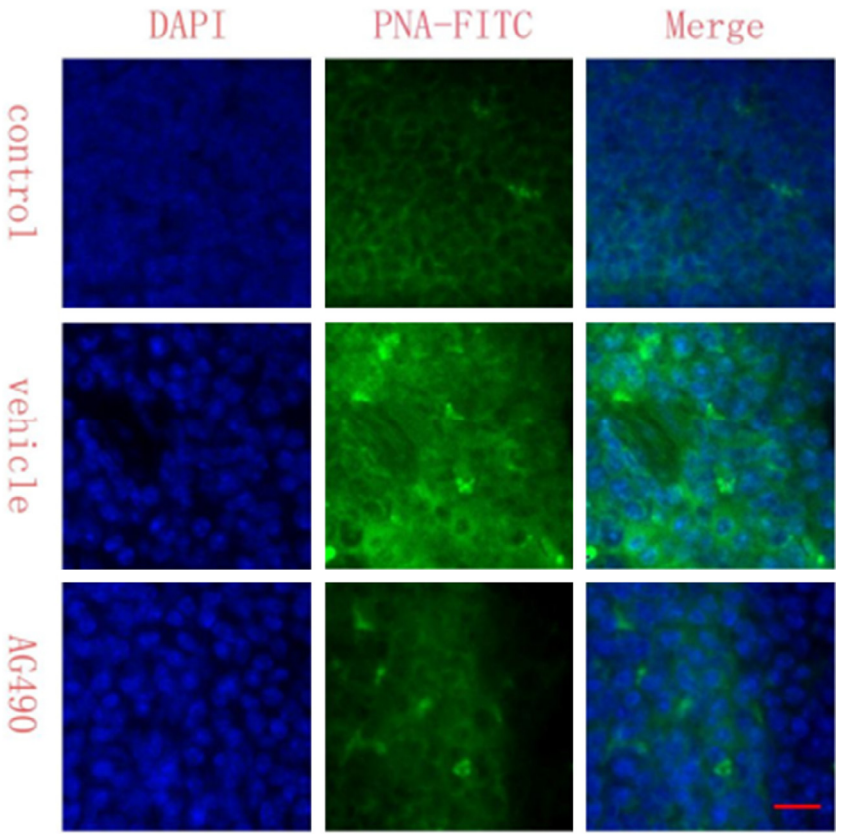

Figure 4 The spleen sections of each group PNA-FITC staining immunofluorescence microscopy results (400×, Bar = 100 m), AG490 significantly reduced the formation of GC in EAE mice. PNA and fluorescein FITC connection, PNA positive cells showed green fluorescence, DAPI staining showed blue. Six mice in each group.

4. Discussion

4.1. Development of EAE model

EAE are MS disease models that are induced by myelin antigen and peptide in experimental animals. It is considered as the ideal experimental tool of MS which has the similar aspects in clinical manifestations and pathology with MS. There are three myelin antigens which is consisted of myelin protein protein (PLP), myelin basic protein (MBP) and myelin glycoprotein (MOG) and induce EAE. MOG as transmembrane protein can be expressed out of myelin membrane and oligodendroglia which is only the total myelin protein 0.05–0.1% several times, but it has high immunogenicity and it as the key part can cause cerebritis (Mendel et al., 1995). The background of C57BL/6 mice has been clarified and incidence of a disease is really high which has a wide application in EAE. Meanwhile the mice have been immunized by MOG35-55 which have the similar nosogenesis with MS.

4.2. AG490 in medical treatment

At present, AG490 has shown good application prospects in the study of tumor, inflammation and some autoimmune diseases. In vitro study showed that AG490 not only can inhibit the proliferation and induced apoptosis of tumor cells, but also can block the JAK2/STAT3 signal of colorectal cancer cells. In macrophages, AG490 inhibited the production of IFN-γ and nitric oxide by nitric oxide synthase, and reduced the release of TNF-α. In animal models, the number of CD4+CD25+Foxp3+ cells has been in type I diabetic mouse model by increasing the dependent dose model of AG490 which affects the development of the disease. Induced shock response by yeast polysaccharide, AG490 decreased the severity of the inflammatory response and prevented severe renal dysfunction. In model that collagen induces arthritis, AG490 induced the formation of Treg and inhibited the differentiation of Th17 by inhibiting the JAK2/STAT3 signaling pathway, which significantly reduced the severity of the disease. AG490 is still rarely seen in the study of EAE. The present study shows that AG490 can inhibit the invasion of T cells and delay or inhibit the occurrence of antigen specificity (Constantin et al., 1998).

With the IC_{50} of 10 M, the range of maximum effective concentration is 50–100 M equaling to 20–40 mg/kg of AG490 (Seeger et al., 2007). In this study, each mouse was given 500 g, which is equal to 25 mg/kg. Our results showed that the experimental mice had the good tolerance dose. AG490 had significant neuroprotection which significantly delayed the onset time, significantly reduced the disease and treated the pathogenesis of EAE mice. Lumbar segments of the spinal cord were stained by HE and sand LuoLuo cyanine also confirmed that infiltration and demyelination degree of AG490 group mice inflammatory cells decreased significantly. The phosphorylation of STAT3 was obviously inhibited, and the

number of B, Tfh cells, plasma cells and so on were decreased significantly.

4.3. Effect of AG490 on Tfh

Chemokine receptor CXCR5 sustained high expression trend by Tfh, which are migrated and located in folliculi lymphaticus by chemokine CXCL13. These cells express ICOS, PD1, CD40L and other molecules on the surface and secret IL-21 cytokines and interact with B cells, B cells of auxiliary GC are proliferation and differentiation, experiencing clonal proliferation and somatic high mutation, antibody class switching, antibody affinity maturation and process, then ultimately differentiate into long-lived memory B cells and plasma cells, in the humoral immune response plays an important role (Ge et al., 2015). TFH becoming a bridge between humoral immunity and cellular immunity that is produced by auxiliary B cells in real meaning. Th1, Th2 and Th17 cells in GC formation and provide assistance to B cells are not necessarily required. The differentiation of Th2, Th17 and Th1 were dependent on T-bet, GATA-3, ROR, Foxp3, Treg and T, respectively, and the transcription factors were not highly expressed in Tfh cells. Research shows B cell lymphoma factor 6 (B cell lymphoma 6 bcl-6) is TFH transcription factor, bcl-6 defect of T cells could not differentiate into TFH cells, almost cannot produce the GC reaction, confirmed that bcl-6 exist (Peng et al., 2014).

Although there are other CD4+ T cell subsets that may also turn into TFH cells in some special or high GC reaction environment, as isolated CD4+ T cell subsets that have been recognized gradually (Xiao et al., 2013). Recently, studies have shown that transcription factor B lymphocytes induce mature protein (lymphocyte-induced maturation protein-1 B, Blimp-1) to inhibit the role of Bcl-6, thus inhibiting the differentiation of Tfh.

In MS patients, several studies have reported that the ratio of Tfh in peripheral blood is higher than that in healthy controls. Our study showed that the ratio of Tfh was significantly higher in the vehicle group than in the control group, whereas the Tfh ratio was significantly decreased in the AG490 treated mice compared with the vehicle group. Results of western blot also showed that the number of different groups of TFH transcription factor bcl-6 expression trend and its corresponding TFH ratio trend is similar to that of AG490 inhibits TFH differentiation was further confirmed, reducing the TFH, which can achieve the purpose of restraining the inflammation and improving the symptoms.

References

Barun, B., Bar-Or, A., 2012. Treatment of multiple sclerosis with anti-CD20 antibodies. Clin. Immunol. (Orlando, Fla.) 142, 31–37.

Breij, E.C., Brink, B.P., Veerhuis, R., Van den Berg, C., Vloet, R., Yan, R., Dijkstra, C.D., Van der Valk, P., Bo, L., 2008. Homogeneity of active demyelinating lesions in established multiple sclerosis. Ann. Neurol. 63, 16–25.

Cernkovich, E.R., Deng, J., Bond, M.C., 2008. Adipose-specific disruption of signal transducer and activator of transcription 3 increases body weight and adiposity. Endocrinology 149, 1581–1590.

Constantin, G., Brocke, S., Izikson, A., et al, 1998. Tyrphostin AG490, a tyrosine kinase inhibitor, blocks actively induced experimental autoimmune encephalomyelitis. Eur. J. Immunol. 28, 3523–3529.

Farrell, R.A., Antony, D., Wall, G.R., Clark, D.A., Fisniku, L., Swanton, J., Khaleeli, Z., Schmierer, K., Miller, D.H., Giovannoni, G., 2009. Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI. Neurology 73, 32–38.

Freedman, M.S., Thompson, E.J., Deisenhammer, F., Giovannoni, G., Grimsley, G., Keir, G., Ohman, S., Racke, M.K., Sharief, M., Sindic, C.J., Sellebjerg, F., Tourtellotte, W.W., 2005. Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. Arch. Neurol. 62, 865–870.

Ge, S., Peng, W., Li, D., et al, 2015. Study on antibacterial molecular drugs in Eucalyptus granlla wood extractives by GC-MS. Pak. J. Pharm. Sci. 28, 1445–1448.

Hauser, S.L., Waubant, E., Arnold, D.L., 2008. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. New Engl. J. Med. 358, 676–688.

Howell, O.W., Reeves, C.A., Nicholas, R., et al, 2011. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. Brain: J. Neurol. 134, 2755–2771.

King, C., Tangye, S.G., Mackay, C.R., 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu. Rev. Immunol. 26, 741–766.

Kono, D.H., Urban, J.L., Horvath, S.J., et al, 1998. Two minor determinants of myelin basic protein induce experimental allergic encephalomyelitis in SJL/J mice. J. Exp. Med. 168, 213–227.

Kuerten, S., Kostova-Bales, D.A., Frenzel, L.P., et al, 2007. MP4- and MOG:35–55-induced EAE in C57BL/6 mice differentially targets brain, spinal cord and cerebellum. J. Neuroimmunol. 189, 31–40.

Magliozzi, R., Howell, O., Vora, A., et al, 2007. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain: J. Neurol. 130, 1089–1104.

Mendel, I., Kerlero de Rosbo, N., Ben-Nun, A., 1995. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. Eur. J. Immunol. 25, 1951–1959.

Meydan, N., Grunberger, T., Dadi, H., 1996. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. Nature 379, 645–648.

Nylander, A., Hafler, D.A., 2012. Multiple sclerosis. J. Clin. Investig. 122, 1180–1188.

O'Neill, E.J., Day, M.J., Wraith, D.C., 2006. IL-10 is essential for disease protection following intranasal peptide administration in the C57BL/6 model of EAE. J. Neuroimmunol. 178, 1–8.

Peng, W., Wang, L., Zhang, M., 2014. Separation characteristics of lignin from Eucalyptus camaldulensis lignin celluloses for biomedical cellulose. Pak. J. Pharm. Sci. 27, 723–728.

Sarra, M., Franze, E., Pallone, F., Monteleone, G., 2011. Targeting interleukin-21 in inflammatory diseases. Expert Opin. Therap. Targets 15, 695–702.

Schaerli, P., Willimann, K., Lang, A.B., et al, 2000. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J. Exp. Med. 192, 1553–1562.

Seeger, F.H., Zeiher, A.M., Dimmeler, S., 2007. Cell-enhancement strategies for the treatment of ischemic heart disease. Nat. Clin. Pract. Cardiovasc. Med. 4 (Suppl. 1), S110–113.

Serafini, B., Rosicarelli, B., Magliozzi, R., Stigliano, E., Aloisi, F., 2004. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. Brain Pathol. (Zurich, Switzerland) 14, 164–174.

Spolski, R., Leonard, W.J., 2008. Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu. Rev. Immunol. 26, 57–79.

Xiao, Z.P., Peng, Z.Y., Dong, J.J., et al, 2013. Synthesis, molecular docking and kinetic properties of beta-hydroxy-beta-phenylpropionyl-hydroxamic acids as Helicobacter pylori urease inhibitors. Eur. J. Med. Chem. 68, 212–221.