Adoptive Transfer of Immunomodulatory M2 Macrophages Suppresses Experimental Autoimmune Encephalomyelitis in C57BL/6 Mice via Blockading NF-ϰb Pathway

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Abstract

Objectives: The aims of the study were to further elucidate the roles of M1 and M2 macrophages in the pathogenesis of EAE and the effects of treatment with M2 macrophages that target certain pro-inflammatory cytokines and with immunomodulatory preparations that beneficially influence the disease course should be in focus of future therapeutic trials. Results: Enhanced the total number of macrophages at the onset of clinical signs in the EAE group, consistent with an increased proportion of M1 cells and low numbers of M2 cells. As the disease progressed and the symptoms worsened, M1 cells were decreased and M2 cells were gradually increased till the peak. In the recovery stage, M2 cell numbers were gradually decreased. Treatment with M2 macrophages inhibited the NF-ϰb pathway, alleviated the symptoms of EAE, reduced inflammatory cell infiltration and demyelination in the central nervous system, and decreased the numbers of macrophages in the spleens. BAY-11-7082, an NF-ϰb blocking agent, could reduce the total number of macrophages both in vivo and in vitro, effectively prevented the EAE development, and significantly inhibited the symptoms EAE in mice. Conclusions: Macrophages may play a crucial role in the pathogenesis of EAE, while M2 macrophages have anti-inflammatory effects. Transfer of M2 macrophages to EAE mice can block the NF-ϰb pathway successfully and relieve the EAE symptoms. Application of NF-ϰb blockers is useful in the prevention and treatment of EAE.

Introduction

Multiple sclerosis(MS) is an inflammatory demyelinating autoimmune disease in the central nervous system(CNS) and it affects approximately 2.5 million people worldwide and poses a major burden to the healthcare system[1,2]. Current therapeutic options for MS include corticosteroids, beta interferon, immunosuppressive agents and monoclonal antibodies like anti-CD20[3], however, these treatments are effective only in some patients, and have significant adverse effects.

The pathogenesis of MS is unclear. Many studies have shown that both patient characteristics[4], and environmental factors such as intestinal flora imbalance and also gene–environment(GxE) interactions can affect the development of MS[5,6].

Previous studies on model of MS, experimental autoimmune encephalomyelitis(EAE), have shown that macrophages may play important roles in the pathophysiology of MS[7]. At the onset of EAE and MS, macrophages from peripheral tissues enter into the CNS, resulting in the pathological changes and M1 macrophage increased[8,9]. M2 macrophages can antagonize and inhibit the inflammation caused by inflammatory molecules related to M1 macrophages[10-12]. Previous studies have reported that M2 microglia can promote recovery of motor function after spinal cord injury in mice and delay the progression of EAE[13,14]. Macrophages are homologous to microglia and are easier to obtain and culture. Therefore, promotion of

macrophage differentiation into M2 or enhancement of M2 macrophage are expected to be the novel strategies for the treatment of neuroinflammatory and immunological diseases in the future, which has attracted attention as a new method worldwide.

Nuclear factor kappa-light-chain-enhancer of activated B cells(NF-ϰb), a protein complex mainly in the form of p50/p65 isodimers, is a key intracellular signal transduction molecule that can regulate the expression of many molecules involved in the early stage of immune and inflammatory responses[15,16]. NF-ϰb played an important role in the immune response of the nervous system, which began to increase on the 9th day after EAE induction in Lewis rats, peaked at the 11th and 12th days, and gradually decreased after the 14th day, and the dynamic changes of NF-ϰb activity were consistent with the EAE symptoms[17,18]. M2 macrophages can down-regulate the signal transduction of NF-ϰb, which may have therapeutic effects on EAE via inhibiting NF-ϰb activity[10].

BAY-11-7082(Beyotime) is a kind of NF-ϰb pathway blocker that can completely and specifically abolish NF-ϰb DNA binding, which also an irreversible inhibitor of TNF-α-induced inhibitor of NF-ϰb(IϰB)-α phosphorylation that leading to NF-ϰb inactivation[19]. In the present study, we applied BAY-11-7082 in EAE mice to observe whether blocking NF-ϰb pathway can effectively alleviate the symptoms of EAE, and to explore the possible mechanisms behind the therapeutic role of BAY-11-7082 in EAE, in which investigation of its effect on macrophages is focused on.

Material and methods

Animals

Female C57BL/6 mice aged 6-8 weeks and weighing 18-20g were purchased from Vital River Laboratory Animal Technology Co., Ltd.(Beijing, China) and bred in our animal facility at The First Hospital of Jilin University. The mice were housed in pathogen-free conditions and had free access to food and water. All experiments were performed in accordance with the local ethics committee of The First Hospital of Jilin University, Changchun of China(Ethical approval number: 2017-216).

Induction of EAE and assessment of the clinical signs

The EAE and control induction and assessment of the clinical signs was the same with Xie's description[20].

Macrophage cultures and transfer to EAE mice

Immunized C57BL/6 mice were sacrificed and bone marrow cells from femoral and tibial bones were collected in RPMI-1640 complete medium(Gibco, Waltham, MA) on days 2, 5, and 8 p.i. After red blood cell lysis, cells from the bone marrow were cultured in RPMI-1640 complete medium supplemented with penicillin and streptomycin(1%; Hyclone, Logan, UT), fetal bovine serum(10%; Sigma Aldrich, St Louis,MO), IL-4(10ng/ml; Pepro-Tech, Rocky Hill, NJ), and macrophage colony-stimulating factor(M-CSF) (10ng/ml; Pepro-Tech, Rocky Hill, NJ). On days 3, 5, and 7 post-culture, half of the previous medium was replaced with fresh medium and the concentrations of IL-4 and M-CSF were increased to 20ng/ml. M2 macrophages were harvested on day 8 post-culture using ethylene diamine tetra acetic acid(EDTA; Sigma) and incubated with MOG35–55(20μg/ml) peptide for 4h at 37°C. Then the cell concentration was set to 10×10^6/ml, and the cells were transferred to EAE mice(0.1ml, 1×10^6 cells/mouse) on days 10, 13 and 16 p.i. via the caudal vein. And the control group were treated with PBS. Experiments were replicated three times(n = 10).

Histological analysis

The spinal cord was removed from the spinal canal and embedded in paraffin. Multiple horizontal sections(5-6 μm thick) of the spinal cord were stained with hematoxylin-eosin(HE) and replicate sections were stained with luxol fast blue(LFB) violet for evaluation of the extent of inflammatory cell infiltration and demyelination. The number of infiltrated inflammatory cells and the degree of demyelination in each mouse were averaged from 10 complete cross-sections of the spinal cord.

Flow cytometric analysis

After the spleen was taken out, rinsed in RPMI-1640 complete medium,then lysed the red blood cell and adjusted the cell concentration to 1×10^6/ml with PBS and stained with the following antibodies: F4/80-PE-CY7(Biolegend), CD11b-FITC(BD Pharmingen^{TM}), CD206-BV650(Biolegend), Arg-1-APC(R&D), iNOS-PE(eBiosience), and CD40-BV421(BD Pharmingen TM). For detection of extracellular molecules, the cells were incubated for 30 min at 4°C with antibodies(F4/80, CD11b, CD206, Arg-1, CD40). For detection of intracellular molecules(iNOS), cells were permeabilized with the Fixation/Permeabilization Solution Kit(BD Pharmingen^{TM}) for 20 min, then incubated with the antibodies for 30 min at 4°C. In FACS analysis, both F4/80 and CD11b were positive to calculate the proportion of macrophages(Q2). In macrophages, iNOS or CD40 positive was identified as M1 macrophages(Q1+Q2+Q3), and CD206 or Arg-1 positive was identified as M2 macrophages(Q5+Q6+Q7)[7].

Cytokine cytometric bead array assay

Serum was obtained after centrifugation at $2092g$ for 10 min, and serum levels of IL-1β, IL-4, IL-6, IL-10, IL-12p70, IL-17A, and tumor necrosis factor(TNF) were measured using cytometric bead array(CBA) kits(BD Bioscience) according to the manufacturer's instructions. The CBA test was repeated twice(n=6) and results were expressed in pg/ml.

Western blotting

The spinal cords were cut into small fragments and added into the lysates, and a mixture of protein phosphatase inhibitors was added into the tissue cell lysates at a proportion of 1:100. The cracked samples were centrifuged for 3-5 minutes at 10,000-14,000$\times g$. The BCA Protein Quantitation Kit(Bioteke Corporation) was used to draw the standard curve and calculate the protein concentrations. For each 30μl protein sample, 10 μl of buffer was added and blended, and proteins were denatured by heating in a 100°C water bath for 3-5 minutes. After cooling to 25°C, the samples were centrifuged at 10,000-14,000rpm for 2-5 minutes. The supernatants were collected and directly used for electrophoresis.

Βλοςκαδε οφ τηε ΝΦ-κβ πατηωαψ το τρεατ ανδ πρεεντ EAE

The EAE mice were divided into three groups: 1) EAE control group(treated with PBS), 2) prevention group(treated with BAY-11-7082 on day 2, 5, 8, 11, 14, and 17p.i., 20mg/kg/i.p), and 3) treatment group(treated with BAY-11-7082 on days 10, 13, and 16 p.i, 20mg/kg/i.p). The weight and clinical score were recorded every day to observe the changes in the EAE symptoms. Experiments were replicated three times(n=10).

Culture of M1 and M2 macrophages

The detail methods were as same as the process of cultured M2 macrophage but M1 macrophage were added with LPS(10ng/ml, Solarbio) instead of IL-4. BAY-11-7082(1 μM) were added on the day 8 of cultures and control group were added with PBS, the wells were incubated for 48h, 20μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) solution was added to each well and incubated for 4h. Then, 150μl dimethylsulfoxide was added to each well and low-speed oscillation was performed on the shaker for 10min to fully dissolve the crystals. The absorbance value of each well was measured at 570nm by an enzyme-linked immunodetection.

Statistical analysis

All experiments were repeated three times with each group had 10 mice and data were expressed as mean ± standard error of mean(SEM). FLOWJO 7.6.1(Treestar) was used to analyze the flow cytometry data and GRAPHPAD PRISM 5.0(GraphPad Software Inc., San Diego, CA) was used to draw the graphs. Other statistical analyses were performed using SPSS 21.0(IBM Deutschland, Ehningen, Germany). The student's t-test was used for comparisons between two groups. All tests were two-tailed. $P<0.05$ was considered statistically significant, represented by *($P<0.05$), **($P<0.01$), ***($P<0.001$), and ****($P<0.0001$).

Results

Clinical course of EAE

The mice developed clinical signs of EAE on day 10(onset) p.i. and on day 22 p.i.(peak), the symptoms of EAE peaked, with an average score of 3.00±0.37. The signs began to ease gradually on day 25(recovery). The weight of EAE group gradually decreased until the lowest point on day 22 p.i.(peak), and then increased gradually, which was consistent with the changes of symptoms. Mice in the control group showed no clinical sign($P<0.0001$). (Figure 1a).

Increased M1 macrophage at onset and M2 macrophage at peak of EAE

The percentage of macrophages in the spleen increased at the onset and decreased at the peak phase($P<0.0001$). At the recovery stage, the percentage of macrophages decreased continuously($P<0.001$). The percentage of M1 macrophages increased at the EAE onset($P<0.0001$), then decreased at the peak and recover($P<0.01$). Conversely, the percentage of M2 macrophages didn't increase at the onset($P>0.05$) and increased at the peak of EAE($P<0.0001$) and decreased a little at recover stage($P<0.05$), but still higher than normal(Figure 1b).

Pathological and inflammatory cytokine changes in EAE

The main pathological changes in MS are inflammation and demyelination in the white matter of the CNS, axonal injury and axonal loss, which can coexist and are often associated with diffuse lesions of the CNS[21]. As shown in Figure1c, a small number of monocyte and lymphocyte infiltrations could be seen in the spinal cord of EAE mice, with obvious perivascular inflammatory infiltrations by HE stainings. LFB stainings showed demyelination in the spinal cord.

The pro-inflammatory cytokines, IL-17A, IL-6, TNF-α, IL-12p70 and IL-1ß increased at the disease onset($P<0.0001$) and IL-17A, IL-6, TNF-α, IL-12p70 decrease at the peak hour($P<0.0001$). While anti-inflammatory cytokines, IL-4 and IL-10, significantly increased during the peak period($P<0.001$)(Figure1d).

Clinical course, pathological and inflammatory cytokine changes after M2 macrophage treatment

The proportion of M2 macrophages cultured *in vitro* was more than 97% determined by flow cytology(Figure 2a). Those were transfused into the mice when EAE mice showed clinical symptoms on day 10 p.i.(onset). EAE treated with M2 macrophage(M2-treated) and PBS developed the disease at the same time; however, the peak symptoms were lighter and recovered earlier in the M2-treated group than controls. Similarly, weight records of the two groups showed that the weight gradually decreased in the EAE control group until day 22p.i. and dropped to the lowest at the peak phase; while M2-treated group had lost weight until day 17p.i. and then their weight increased gradually. On day 22 p.i., EAE symptoms reached a peak with average scores of 3.08±0.20. The M2-treated group showed the highest score on day 17 p.i., with an average score of 2.36±0.26($P<0.0001$)(Figure 2b).

During the peak hour, the spinal cord was removed for pathological staining after perfusion. As shown in Figure 2c, the inflammatory cell infiltrations in spinal cords were less in the M2-treated group than in EAE control group measured by HE staining($P<0.0001$). LFB staining showed that spinal demyelination in the M2-treated group was significantly less severe than that in the EAE control group($P<0.0001$).

Additionally, pro-inflammatory cytokines, IL-6, IL-17A, TNF, IL-1ß and IL-12p70, are lower in the M2-treated group compared with the controls($P<0.01$). Anti-inflammatory cytokines, IL-4 and IL-10 are higher in M2 treated group($P<0.01$)(Figure 2d).

Decreased macrophages in spleens after M2 macrophage treatment

The macrophage percentages in the spleens were higher in EAE groups than that in M2-treated group at the peak of the EAE($P< 0.0001$). The percentage of M1 macrophages was lower in the M2-treated

group than in EAE group(P<0.05). The M2 macrophages were lower in the M2-treated group than in EAE group(P<0.0001).(Figure 3a)

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At the peak hour, phosphorylated and non-phosphorylated NF-κb p65 levels in the spinal cord were detected by western blotting. Phosphorylated p65 level was lower in the M2-treated group than in EAE group(Figure 3b).

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NF-κb level of spinal cord was detected in NF-κb blocker prevention and treatment group and EAE control group by western blot. As the Figure 4a shows, phosphorylated NF-κb level were lower in the EAE prevented and treated group than in the EAE control group(EAE vs. prevent: P<0.05).

We conducted experiments according to the grouping and administration methods introduced in the previous method. As shown in Figure 4b, the prevention group had a later disease onset than the EAE control group(P<0.001), with a lower symptom incidence(P<0.0001) and a lower score at the peak hour(P<0.0001). The treatment group had concurrent onset with EAE group; however, the score at peak hour was lower than EAE group(P<0.001).

After treated by the NF-κb blocker, the spinal inflammatory cell infiltrations were more severe in the EAE control groups than in the prevention and treatment groups(P<0.0001). LFB staining showed that spinal demyelination in the prevention and treatment groups was significantly less severe than that in the EAE control group(P<0.0001)(Figure 4c).

The pro-inflammatory cytokines, TNF-a, IL-6, IL-12p70 andIL-17A, were lower in the NF-κb blocker (prevent and treated) group than in the control group(P<0.05). Anti-inflammatory cytokines, IL-4 and IL-10 are higher in NF-κb prevent group(P<0.001), but no differences in treatment group(Figure 5a).

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After using the NF-κb blocker, the total macrophage percentages were lower in the NF-κb blocked (prevent and treated) groups than controls(P<0.01)(Figure 5b). Spleen M1 macrophages in the EAE control group were higher than that in the blocked group(P<0.01), and spleen M2 macrophages were higher in the EAE control group than in the blocked group(P<0.05)(Figure 5b).

Δεςρεασεδ μαςροπηαγε ια βλοςκινγ τηε ΝΦ-κβ πατηωαψ *ιν ιτρο*

In vitro proliferation and activity of M1(P<0.001) and M2 cells(P<0. 01) were higher in the control group than those in the NF-κb blocked group, which show that the blockade could block the production of M1 and M2 at the same time, and inhibition of M1 was more pronounced(Figure 5c).

Discussion

Macrophages are prominent innate immune cells and divided into M1 and M2 subtypes, and new research also found that M3 switch phenotype also exists[22,23]. Previous studies showed that increased macrophages were associated with neuronal damage in MS: In the acute phase, M1 macrophage were activated, along with the release of large amounts of pro-inflammatory cytokines, neuro-inflammation, CNS demyelination, and neuronal death[24-26]. However, as the disease progressed, M2 macrophages increased gradually, and the maximum levels of M2 and M1 macrophage reduced gradually from the peak of disease to the remission of EAE. In the late stages of the disease, M2 macrophages played a dominant role in the CNS and released a variety of anti-inflammatory cytokines, which reduced the inflammatory response and promoted tissue repair, while inhibiting the further progression of EAE[25]. Our results is consistent with the results of previous studies[27].

Recent studies have shown that some treatment for MS like glucocorticoids, fingolimod, glatirameracetate, teriflunomide and interferon-beta can downregulate expression M1 macrophage or activate M2 macrophage[28-32]. Previous studies have found that therapy with M2 macrophage or microglia can prevent the occurrence of type 1 diabetes and the motor neurons damage caused by spinal cord injured, and also alleviate EAE symptoms in rats[33,13,14]. However, there are the limited macrophages source and needed to expand more methods to obtain enough macrophages. In recent years, researchers have applied cytokines and compounds that can stimulate the differentiation of bone marrow stem cells into M2 macrophages, which have proven to be effective in transforming stem cells into M2 macrophage. In our study, IL-4 and M-CSF could successfully induce bone marrow stem cells to differentiate into M2 macrophages that have been transferred into EAE mice at the early stage of the disease. In comparison with the control group, the M2-treated group showed simultaneous development of symptoms, but their peak symptoms were alleviated, and symptom relief was faster. The degrees of spinal cord inflammation and the degrees of inflammation and demyelination in spinal codes and inflammatory factors secretion in blood were lower in M2-treated group than those in control group, which is similar to other results[34,12]. However, the difference from the previous studies is that our experiment is using the M2 macrophages induced from bone marrow stem cells and inject them by intravascular route, which will greatly facilitate future clinical operations and make cell therapy more likely. Our experiment also found that after treatment with M2 macrophage in EAE mice, the macrophages in the spleen were decreased, which included both M1 and M2 macrophages, however, the M2 macrophage decreased more significantly. Pro-inflammatory factors are decreased and anti-inflammatory factors are increased in peripheral blood.

It has been evidenced that once the inflammatory reaction leads to the immune system activation, and then the immune system tries to maintain the internal environment in balance by various ways. In our study, after a large number of M2 macrophages injected into EAE mice, the declined macrophage activation in the spleen of EAE mice through reducing M2 macrophages mainly was for restoring internal environment homeostasis. At the same time, due to the infusion of a large number of M2 macrophages, anti-inflammatory factors were significantly increased in the blood, promoting repairing the damaged tissue and alleviating the sings of EAE.

NF-ϰb is a key intracellular signal transduction molecule that can regulate the expression of many molecules involved in the early stage of immune and inflammatory responses. Induced NF-ϰb is mainly in the form of p50/p65 isodimers which p65 played a major role[15,16], and that is involved in the occurrence and development of MS. Previous research findings implied that changes in the activity or protein expression of NF-ϰb are closely related to the severity of EAE and the incidence of disease, clinical fraction, and inflammation of the CNS were all shown to decrease in mice with NF-ϰb defects[17,18]. Moreover, NF-ϰb also plays a central role in activating and differentiating T cells and blocking NF-ϰb function is an effective method to prevent autoimmune encephalitis[35].

After we applied M2 macrophages, the symptoms of the mice were reduced, and NF-kb expression in the spinal cord was also reduced too. Therefore, we wondered whether NF-kb was involved in the polarization of macrophages to M1 and M2 cells. Studies have shown that the NF-ϰB signal transduction pathway participates in the inflammatory immune response induced by macrophage[36]. NF-ϰB activity is closely related to the number, state, degree, and product of microglial activation, that is, the higher the NF-ϰB activity, the more activated microglial cells and the more inflammatory factors[37]. Therefore, we hypothesized that blocking NF-kb pathway can reduce the polarization of M1 macrophages, increase the number of M2 macrophages, and reduce the symptoms of EAE.

We applied a NF-ϰb blocker BAY-11-7082, which can block the phosphorylation of NF-ϰb65, and observed the effects on M1 and M2 macrophages *in vitro* and *in vivo* . The results showed that the severity of EAE was alleviated after the application of the blocker, and the therapeutic effect in the prevention group was better than that in the treatment group, indicating that the effect of early blockade was more obvious. The analysis of macrophages in the spleen of mice at the peak of EAE displayed that the proportion of total macrophages was lower in the NF-ϰb blockade group(prevented and treated group) than the control

group, in which the proportion of M1 macrophage declined significantly, however, the proportion of M2 decreased a bit. Meanwhile,*in vitro* experiments revealed that the blocker blocked the activity of both M1 and M2 macrophages simultaneously. However, the blockade of activity of M1 was more obvious than activity of M2, which is similar to *in vivo* study. Our results suggest that inhibition of NF-ϰb pathway can inhibit the generation of macrophages, especially M1 macrophage and pro-inflammatory factors. Therefore, early addition of this inhibitor can block the pro-inflammatory effect of M1 more effectively and reduce the inflammatory response in EAE mice.

The present study demonstrates that (a) macrophages are involved in the occurrence and development of EAE disease: M1 macrophages have pro-inflammatory effects, and M2 macrophages have anti-inflammatory effects; (b)bone marrow stem cells could be successfully induced to differentiate into M2 macrophages; (c) M2 macrophages blocked the NF-ϰb pathway and had a promising therapeutic effect on EAE, which enable rational therapeutic approaches for MS and other autoimmune diseases in the future; (d)NF-ϰb blockade effectively prevented EAE development and alleviated the severity of EAE via blockading NF-ϰb pathway. In the future, we will further study how the NF-ϰb pathway involving in the process of macrophage polarization.

Consent for Publication

All authors agree to the publication of this manuscript.

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Disclosure of conflict of interest

No competing interests to declare.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

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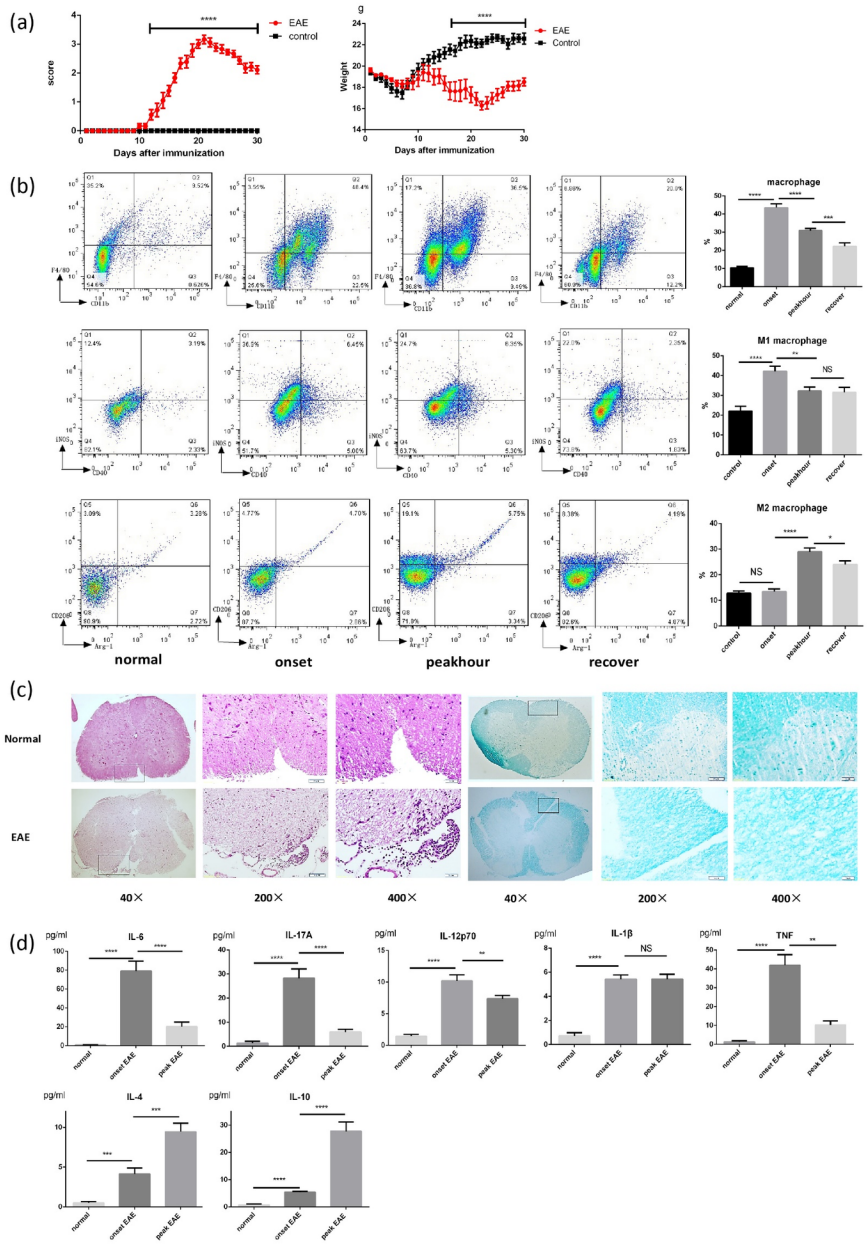


Figure 1. The clinical scores, macrophage percentages in spleen, pathology and cytokines changes in EAE. (a) EAE group developed clinical signs on day 10 (onset) p.i. On day 22 p.i., the symptoms of EAE peaked, then the signs began to ease gradually on day 25 (recovery). The control group had no symptoms. At the same time, the weight of EAE group gradually decreased until the lowest point on day 22 p.i., and then increased, which was consistent with the changes of symptoms. (b) Macrophage percentages in the spleens were measured at different stage of EAE: Macrophages in the spleen increased at the onset, then will gradually decreased at peakhour and recover stage; M1 macrophages increased at the onset, and decreased at the peak hour and recover; M2 macrophages increased at the peak hour, then decrease at recover. (c) Inflammatory cell infiltration around the blood vessels (HE stains) and demyelination in the spinal cord (LFB stains are more severe in the EAE mice than in the naïve mice (normal mice). (d) All the pro-inflammatory cytokines increased at the disease onset and IL-17A, IL-6, TNF-α, IL-12p70 decrease at the peak hour. While

anti-inflammatory cytokines, IL-4 and IL-10, significantly increased during the peak period.

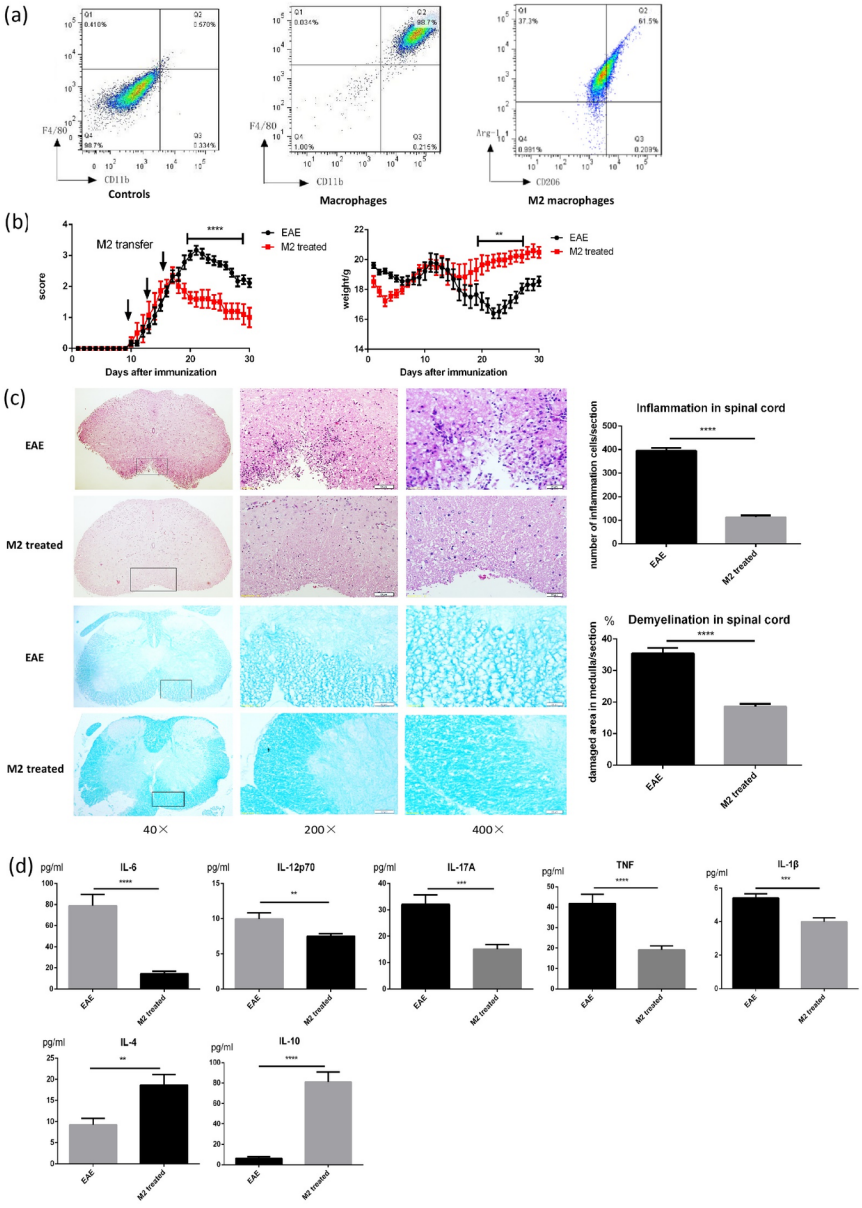


Figure 2. Treated EAE with M2 macrophage that inducing in vitro and observed pathological and cytokines changes in M2-treated and control groups. (a) The proportions of macrophages and M2 macrophages were 98.48% ± 0.189% and 97.60% ± 1.407%, respectively. IL-4 and M-CSF can induce the M2 macrophages efficiently. (b) M2 macrophages were harvested on day 8 post-culture and transferred to EAE mice on days 10, 13, and 16 p.i. via the caudal vein. After adoptive transfer of M2 macrophages, the weight of the animals in the M2-treated group gradually increased after day 17 with the score decreased, while, the weight began to increase from day 22 in the control group. (c) During the peak hour, inflammatory cell infiltration and demyelination in the spinal cord are more severe in the controls than in the M2-treated group. (d) Pro-inflammatory cytokines, IL-6, IL-17A, TNF, IL-1ß and IL-12p70, are lower in the M2-treated group compared with the controls. Anti-inflammatory cytokines, IL-4 and IL-10 are higher in M2 treated group.

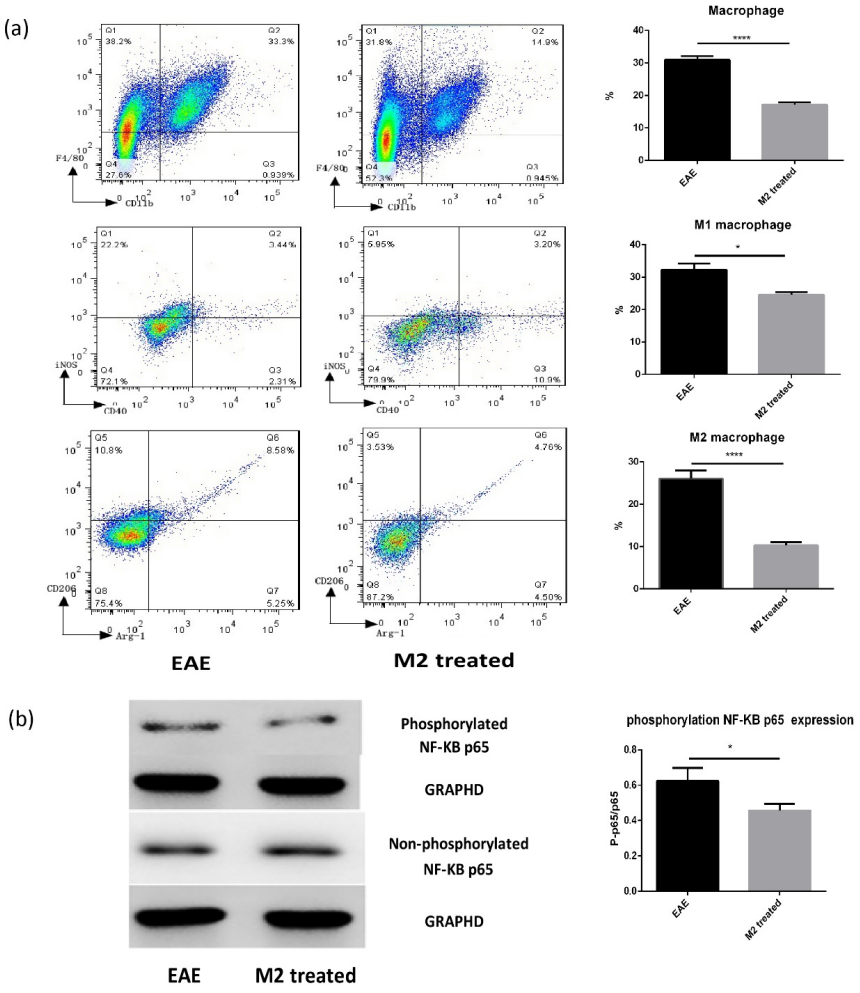


Figure 3. Macrophages and the activity of NF-ϰb changes in M2-treated and EAE control mice. (a) Macrophage percentage is lower in the M2-treated group than those in the EAE groups at peak hour; M1 macrophages are lower in the M2-treated group than in the control groups; M2 macrophages are lower in the M2-treated group than in the EAE groups. (b) Expression the ratio of phosphorylated in total NF-ϰb is lower in the M2-treated group than in the EAE group.

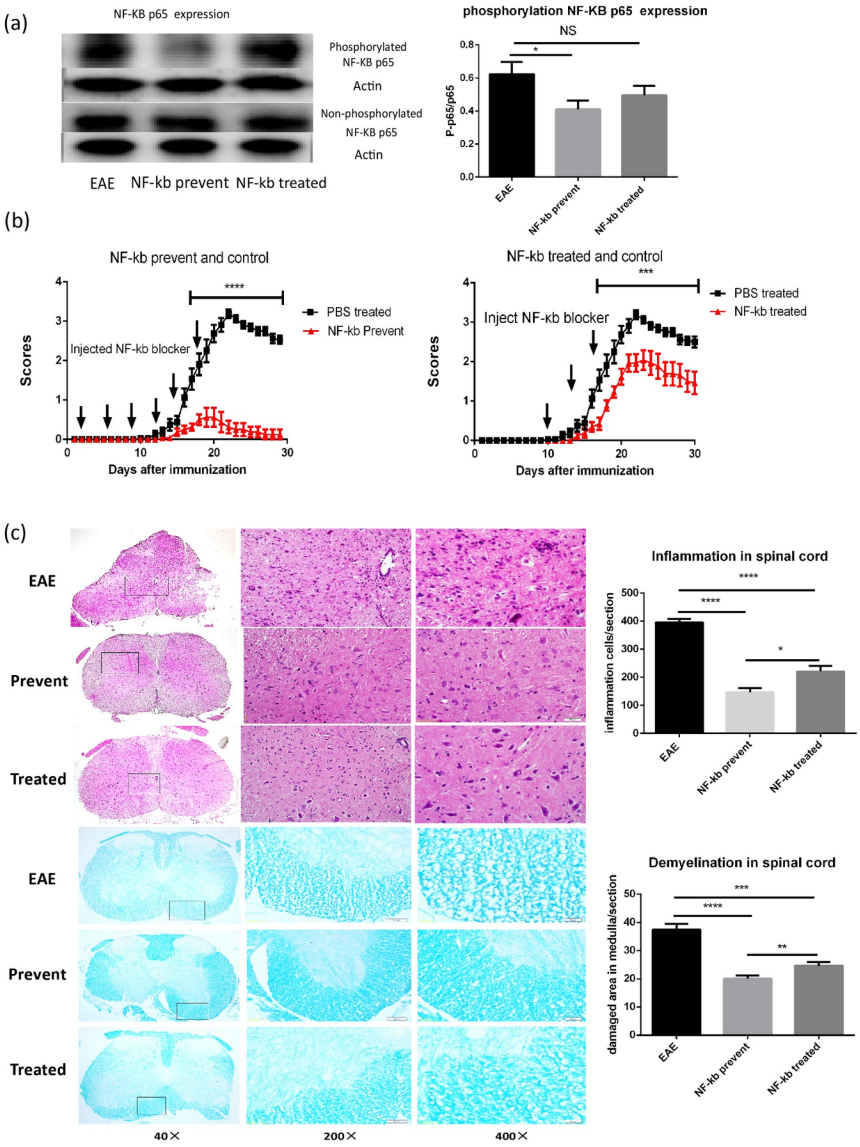
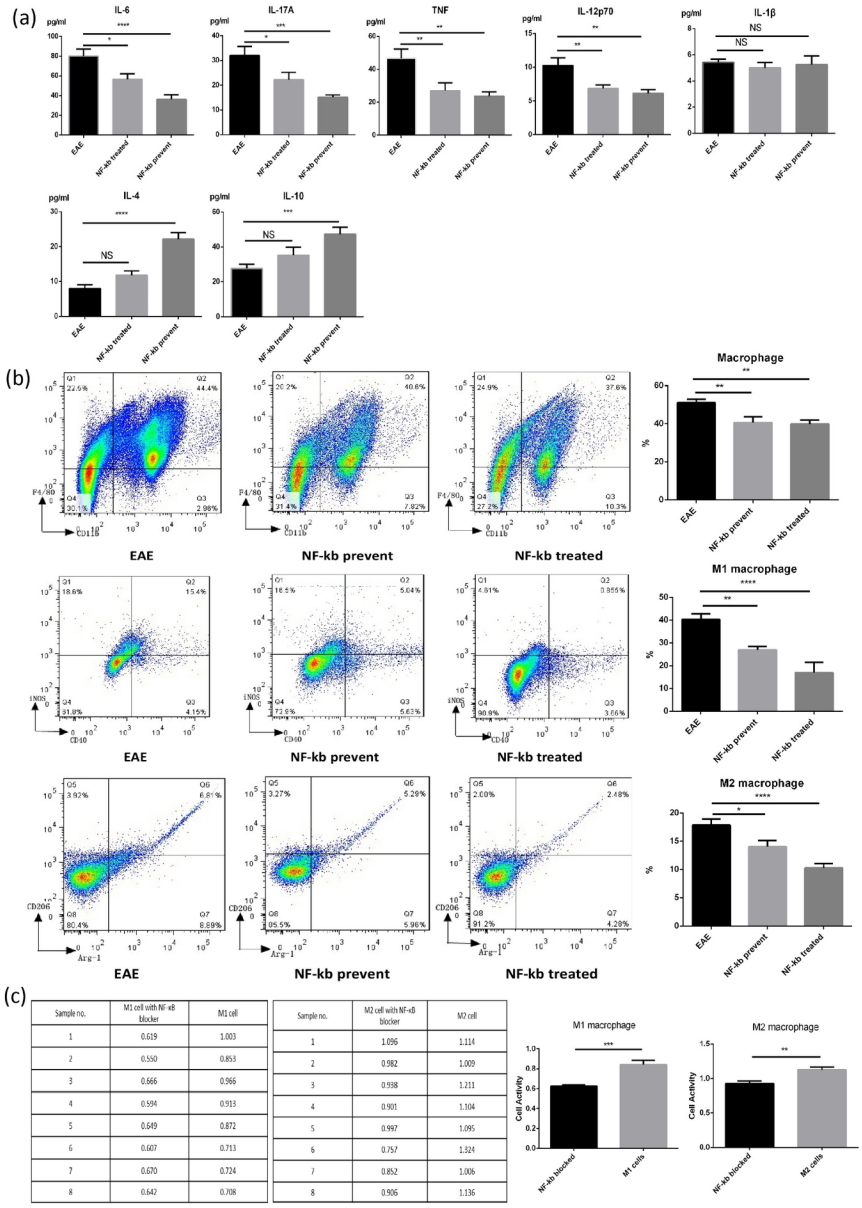


Figure 4. NF-ϰb activity in the spinal cord, EAE score and pathological differences between the EAE and NF-ϰb blocked groups. (a) Phosphorylated NF-ϰb level is lower in the blocked group than in the EAE group and the prevention group is statistically different from the EAE group. (b) Intravenous BAY-11-7082 injection every 3 days from day 2 after the immunization until the peak hour showed that the prevention group had a lower score at the peak phase; Intravenous BAY-11-7082 injection every 3 days from day 10 after the immunization until the peak hour showed that the score was lower in the BAY-11-7082-injected treatment group compared with the EAE group. (c) Inflammatory cell infiltration and structural damage in the spinal cord are more severe in controls than in the prevention and treatment groups.



Sample no.	M1 cell with NF-κB blocker	M1 cell
1	0.619	1.003
2	0.550	0.853
3	0.666	0.966
4	0.594	0.913
5	0.649	0.872
6	0.607	0.713
7	0.670	0.724
8	0.642	0.708

Sample no.	M2 cell with NF-κB blocker	M2 cell
1	1.096	1.114
2	0.982	1.009
3	0.938	1.211
4	0.901	1.104
5	0.997	1.095
6	0.757	1.324
7	0.852	1.006
8	0.906	1.136

Figure 5. Cytokines and macrophage changes *in vitro* and*in vivo* after NF-ϰb pathway blockade. (a)The cytokine levels (IL-6, IL-17A, TNF and IL-12p70) are lower in the NF-ϰb blockade group than in the EAE group. Anti-inflammatory cytokines, IL-4 and IL-10 are higher in NF-ϰb blockade group. The results in the prevention group are more significantly different. (b) Macrophages decreased in the spleen after the NF-ϰb blockade; Spleen M1 macrophages were higher in the EAE group than in the blockade group; Spleen M2 macrophages were higher in the EAE group than in the NF-ϰb blockade group. (c) *In vitro*proliferation and activity of M1 and M2 cells are higher in the control group than those in the NF-ϰb blockade group.