NF-κB inhibitor suppresses experimental autoimmune neuritis in mice via declining macrophages polarization to M1 type

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Abstract

Guillain–Barre' syndrome (GBS) is an acute inflammatory and immune-mediated demyelinating disease of peripheral nervous system (PNS). Macrophages playing a central role in its animal model, experimental autoimmune neuritis (EAN) has been well-accepted. Additionally, NF-κB inhibitors has been used to treat cancers and showed beneficial effects. Here we investigated the therapeutic effect of M2 macrophage and NF-κB pathway is correlated with macrophages activation in experimental autoimmune neuritis (EAN) in C57BL/6 mice. We demonstrated that M2 macrophage transfusion can alleviate the clinical symptoms of EAN by reducing the proportion of M1 macrophage in the peak period, inhibiting the phosphorylation of NF-κB p65. The NF-κB inhibitor (BAY-11-7082) could alleviate the clinical symptoms of EAN and shorten the duration of symptoms by reducing the proportion of M1 macrophages and the expression of pro-inflammatory cytokines. Consequently, BAY-11-7082 exhibits strong potential as a therapeutic strategy for ameliorating EAN by influencing the balance of M1/M2 macrophages and inflammatory cytokines.

Introduction

Guillain–Barré syndrome (GBS) is a common autoimmune disease characterized pathologically by inflammation and demyelination in the peripheral nervous system (PNS) [1, 2]. Experimental autoimmune neuritis (EAN) is a classic animal model of GBS, which has been widely used in basic research of GBS [3, 4]. After activation, macrophages can be divided into pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages, and recently, new research found that M3 switch phenotype also existed [5, 6]. In GBS and EAN, M1 macrophages are involved in the inflammatory impairment of the myelin sheath through promoting cellular cytotoxicity and producing Th1 cytokines. M2 macrophages contribute to repairing myelin and axon by promoting Th2 immune response and the secretion of anti-inflammatory cytokines [7-9]. Several studies have demonstrated that the switch from M1 to M2 could effectively ameliorate the severity of EAN [7, 10, 11].

Nuclear factor-κB (NF-κB) is a inducible transcription factor expressed in a large number of cells and involved in immune and inflammatory responses [12]. Moreover, the activated form p65 of NF-κB has been observed in the sural nerve macrophages of acute and chronic inflammatory demyelinating polyneuropathy (AIDP, CIDP), and the sciatic nerves of rats with EAN, suggesting NF-κB attribute to the inflammatory demyelination. So far there are very few studies demonstrating the role of NF-κB in the course of macrophage polarized [13, 14]. However, the research results on the relationship between macrophages and NF-κB in this process have been controversial.

The objective of this study was to assess the effect of M2 macrophages in treating EAN and to explore the role of NF-κB regulating macrophage subtype. Our results showed that M2 macrophages is effective in

treating EAN by inhibiting the activation of NF-ϰB p65 and the production of pro-inflammatory cytokines. The inhibitor of NF-ϰB, BAY11-7082 attenuates the severity of EAN by inhibiting NF-ϰB pathway and the polarization of M1 macrophages.

Material and methods

Animals

Male C57BL/6J mice, 5-6 weeks old, were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were housed in pathogen-free conditions and fed with food and water ad libitum. The study was approved by the local ethics committee of The First Hospital of Jilin University, Changchun of China and all experimental procedures complied with the regulations for the management of laboratory animals in the Jilin Province (Ethical approval number: 2017-216).

Reagents

The neuritogenic P0 peptide 180-199 was synthesized by GenScript (USA). BAY11-7082 was purchased from Beyotime (China). Antibodies against NF-ϰB p65 and NF-ϰB phosphorylated p65 were purchased from Cell Signaling Technology (USA).

Induction and clinical evaluation of EAN

EAN was induced by immunizing mice twice (days 0 and 8) by subcutaneous injection inoculum containing 150 ug P0 peptide 180-199 (GenScript, USA)) and 0.5 mg Mycobacterium tuberculosis (strain H37 RA; Difco, Franklin Lakes, NJ, USA) in 25 ml saline, and 25 ml Freund's incomplete adjuvant (FIA, ICN Biomedicals, Costa Mesa, CA, USA) into the back of mice. All mice were injected 400, 300, and 300 ng pertussis toxin (PTX, Merck, Whitehouse Station, NJ, USA) via tail veins on days -1, +1 and +3, respectively. Using a blinded protocol, the clinical signs of EAN were scored immediately before immunization (day 0) by two different examiners as follows until day 30: 0 = normal; 1 = less lively, reduced tonus of the tail; 2 = flaccid tail; 3 = abnormal gait; 4 = gait ataxia; 5 = mild paraparesis; 6 = moderate paraparesis; 7 = severe paraparesis; 0.5= intermediate clinical signs. The control group is treated in the same way as mentioned above, but without adding P0 peptide 180-199.

BAY11-7082 treats EAN

BAY11-7082 (Beyotime, China) was dissolved in 1% dimethyl sulfoxide (DMSO, USA) in PBS. EAN mice were divided into three groups. For preventative treatment, BAY11-7082 solution was administered by intraperitoneal injection (20 mg/kg) from day 1 to days 16 post-immunization (p.i.) by every two days. For therapeutic treatment, BAY11-7082 solution was administered by the same way as the preventative treatment at the same dose from the day on which the onset of symptoms was observed to day 30 by every two days. Control animals with EAN received the same volume of the vehicle solution (1% DMSO in PBS). Using the same protocol descripted as the above assessed the clinical signs of mice.

Flow cytometry

The spleens were removed under aseptic conditions at the different phase of disease (onset, peak and recovery), and splenocytes were harvested after lysing red blood cells. The spleen mononuclear cells (MNCs) with 1×10^6 resuspended in 100 ul PBS 1% bovine serum albumin (BSA) were first stained with F4/80, CD11b, CD206, Arg-1, and CD40, then fixed and permeabilized with the Fixation/Permeabilization Solution Kit for 20 minutes (min) (BD Pharmingen^{TM}), and then the stained with antibody for intracellular inductible nitric oxide synthase (iNOS).

The cultured macrophages were harvested and resuspended at 1×10^6 cells in PBS, then stained by F4/80-PE-CY7 (Biolegend), CD11b-FITC (BD PharmingenTM), CD206-BV650 (Biolegend), Arg-1-APC (R&D) for 45 min at room temperature (RT). Flow cytometric data were acquired using a FACS Aria flowcytometry (BD Biosciences) and analyzed with FlowJo software version 7.6.1 (flowjo.com).

Macrophage cultures in vitro and transfer to EAN mice

After lysis of the red blood cells, the collected bone marrow cells from femur and tibia were grown in complete RPMI-1640 medium (Gibco, Waltham, MA) containing 10% Fetal bovine serum (FBS, Sigma Aldrich, St Louis, MO), penicillin (100U/ml) and streptomycin (100U/ml). 1x10^6 cells was seeded into 96-well plates, then stimulated with macrophage colony-stimulating factor (M-CSF) (10 ng/ml; Pepro-Tech, Rocky Hill, NJ) for 48 hours (h), and afterwards LPS (for M1 macrophage) or IL-4 (for M2 macrophage) was added and incubated for another 48h. Thereafter, cells were washed for three times by phosphate buffer saline (PBS) to prepare for flow cytometry. The staining procedure was similar to that of the spleen cells as mentioned above. A part of cultured cells (M2 macrophage) stimulated with M-CSF and IL-4, respectively were harvested and incubated with P0 (20 μg/ml) peptide for 4 h at 37°C. After washed with PBS twice, the cells were transferred into EAN mice (0.1 ml, 1 × 10^6 cells for each mouse) via the caudal vein.

The other cultured cells (M1 and M2 macrophage) were added by Bay11-7082 (1 μM) and incubated for 48h, then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and Dimethylsulfoxide (150 μl) was added to each hole.

Cytokine cytometric bead array (CBA)

Serum was obtained by centrifugation of blood samples at 3000 rpm for 15 min at 4 °C. The levels of IL-1β, IL-4, IL-6, IL-10, IL-12p70, L-17A, and tumor necrosis factor (TNF)-a were measured using CBA kits (BD Bioscience, USA) as the manufacturer's instructions.

Western blot analysis

The samples of protein from sciatic nerves were loaded into 12% SDS-PAGE gels, then transferred to PVDF membrane (Millipore Billerica, MA, USA). The following antibodies were used: rabbit anti-NF-κB p65 and anti-NF-κB p-p65 monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA, 1:1000); β-actin (Bioss, China, 1:2000) and the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Bioss, China, 1:1000). The blots were developed with enhanced chemiluminescence kit (Amersham Imager 600, GE, USA). Densitometric analysis of western blots were done using ImageJ software.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) or Student's t-test was used to evaluate the differences between the two groups. For all statistical analyses, the level of significance was set at $p < 0.05$.

Results

Clinical course of EAN

All mice immunized with P0 peptide 180-199 in combination with FCA acquired EAN. In our experiment, the onset of clinical symptom of EAN in mice occurred on day 10 p.i. After the peak phase (at day 15 p.i.), the scores of severity of EAN decreased. On day 14 EAN mice started to recovery. The other mice immunized with PBS and complete Freund's adjuvant (FCA) as the controls did not observe the symptoms of EAN (**Figure 1**).

M2 macrophages treatment attenuates clinical severity in EAN

On the 8th day after cultures of M2 macrophages induced by M-CSF and IL-4 in vitro, we evaluated the proportions of macrophages and M2 macrophages by flow cytometric analysis. The proportions of macrophages and M2 macrophages were identified as 98.48% ± 0.189% and 94.60% ± 1.407%, respectively (Figure 2a).

M2 macrophages were administered by the caudal vein from days 8 to day 14 p.i. (0.1 ml, 1 × 10^6 cells, once two days) to treated EAN mice. Our results showed that that the clinical scores of EAN were significantly light in M2 macrophages-treated group from days 8 to day 20 p.i. compared to the control EAN group (p¡0.05 on each time point) (Figure 2b). Additionally, the duration of symptoms was markedly shorter in M2 macrophages treated group than the control group received PBS.

M2 μαςροπηαγες τρεατμεντ ρεδυςεδ τηε εξπρεσσιον οφ M1 μαςροπηαγες ανδ ρεδυςεδ τηε αςτιατιον οφ NΦ-ϰB ιν EAN

The data of flow cytometric displayed reduced expression of M1 macrophages (both CD40 and iNOS positive) compared to the control group (P¡0.05), and elevated expression of M2 macrophages (either CD206 or Arg-1 positive) from spleen MNCs (P¡0.05) (Figure 2c and d).

Western blotting results indicated that M2 macrophages treatment reduced the expression of NF-ϰB p-p65 in sciatic nerves of EAN mice on the peak phase compared to the control group (P¡0.05) (Figure 2e).

M2 macrophages treatment inhibits the levels of inflammatory cytokines in serum

When compared to the control group, a significant reduction in the expressions of inflammatory cytokines including IL-1β, IL-17A, TNF-α and IL-6 were observed in the M2 macrophages treatment group (p¡0.05). By contrast, the levels of the anti-inflammatory cytokines IL-4 and IL-10 in M2 macrophages treatment group tended to rise. However, there was no significant difference regarding the expression of IL-4, IL-10 and IL-12 between the control and M2 treatment groups (Figure 2f).

BAY11-7082 treatment suppresses the severity of EAN in mice

BAY11-7082 was applied by two different patterns to treat EAN mice (Fig. 3a). For the preventative group, BAY11-7082 was administered from immunized day to the onset of clinical signs of EAN (day 8 p.i). For the therapeutic group, the same dose of BAY11-7082 was administered from the onset of clinical signs to the peak phase. Our results showed that the scores of severity of EAN were decreased in both treated groups with BAY11-7082 compared to control EAN groups. The clinical scores of preventative group were more significant. However, the onset of EAN was delayed in the preventative group, and the duration of symptoms was shorter than both therapeutic and control groups. (Figure 3a).

According to the results of Western blotting, the expression of p-NF-ϰB p65 was inhabited in the preventative group treated by BAY11-7082 compared to the control group. (Figure 3b).

BAY11-7082 promotes M2 macrophagepolarization in EAN mice

The proportion of M1 and M2 cells in spleens of EAN mice received BAY11-7082 in treatment/preventative groups was investigated via flow cytometry analysis. The results indicated that the proportion of M2 was elevated significantly in the preventative group compared to the control group (P¡0.01). Interestingly, the proportion of M1 was reduced significantly in the preventative group compared to the control group (P¡0.05), suggesting that BAY11-7082 is able to promote macrophage polarization to M2 type (Fig. 3c).

To explore the mechanisms behind the effects of BAY11-7082 suppressing EAN, M1 and M2 macrophages were cultured in vitro and half of them were added by BAY11-7082 as experimental group. The activity and proliferation of M1 and M2 macrophages were evaluated by MTT method. The results showed that the BAY11-7082 inhibited the activity and proliferation of M1 macrophages significantly compared to the control cultures without BAY11-7082 ((P¡0.001), whereas, the BAY11-7082 upregulated the activity and proliferation of M2 in spite of the differences is not significant compared to control culture group.(Fig. 4d-e)

BAY11-7082 alters the expression of cytokines

The expression of inflammatory cytokines shapes the outcome of EAN [15]. We used the CBA to confirm the relevant changes in cytokines of EAN after treatments with BAY11-7082. That is, the expressions of TNF-α, IL-6 and IL-12 were greatly reduced in BAY11-7082 preventative group compared to control and therapeutic groups ($p<0.01$) (Fig. 3f). However, there were no marked differences for the expression of IL-4, IL-10, IL-1β and IL-17A among the three groups (Fig. 3f). Besides, the differences regarding the expression of cytokines that were mentioned on above in the control and therapeutic groups were meaningless.

Discussion

In this study, we used EAN mice to explore the effects of M2 macrophages and BAY11-7082 as an NF-κB inhibitor on this disease. Our results displayed that M2 macrophages ameliorated the clinical signs and reduced the duration of symptoms of EAN in mice by inhibiting pro-inflammatory M1 macrophages and cytokines accumulation. Additionally, the M2 macrophage polarization was increased in M2 treatment group, which was related to inhibition of NF-κB signaling pathway. BAY11-7082 could also diminish EAN symptoms by reducing the expression of TNF-α, IL-6 and IL-12, delaying the onset of EAN in preventive group. Further, BAY11-7082 greatly increased the percentage of M2 macrophage and reduced the percentage of M1 macrophage in preventive group. In vitro, BAY11-7082 inhibited the activity and proliferation of M1 macrophages. Overall, these results demonstrated the NF-κB signaling pathway may be involved in the pathogenesis of EAN by regulating the polarization of macrophages and inhibiting the expression of inflammatory cytokines. The results of adoptive transfer of M2 macrophages into EAN mice displayed the marked beneficial effects on EAN by inhibiting the activation of NF-κB.

Macrophages are broadly divided into two phenotypes: pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2) [14]. M1 macrophages secrete pro-inflammatory cytokines that cause tissue damage and disease development, whereas M2 macrophages express high levels of anti-inflammatory molecule to reduce inflammation and promote disease recovery [14, 16]. Macrophages play either a pro-inflammatory or anti-inflammatory role in the different stages of GBS [17]. M1 cells promote the expression of major histocompatibility complex-II (MHC-II), adhesion molecules, reactive oxygen intermediates (ROI), and inflammatory cytokines, resulting inflammation, broken brood–nerve barrier (BNB), and demyelination [3]. In contrast, M2 macrophages exert a neuroprotective role in the pathogenicity of EAN [18]. M2 macrophages may contribute to the spontaneous re-myelination and regeneration of the axon [19, 20] by promoting T cell apoptosis, suppressing inflammatory responses[9], clearing myelin and axonal debris [16], and inducing the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β[21].

Ιν αγρεεμεντ ωιτη τηοσε φινδινγς, τηε πρεσεντ στυδψ σηοωεδ τηατ τηε ιμπροεδ ουτςομε οφ EAN ωας ασσοςιατεδ ωιτη τηε ηιγηερ προπορτιον οφ M2 μαςροπηαγες ανδ τηε M2 μαςροπηαγε πολαριζατιον ωας ινςρεασεδ ιν M2 τρεατμεντ γρουπ. Ιν αδδιτιον, τηε ρολε οφ ΒΑΨ11-7082 ινηιβιτινγ EAN ςλεαρλψ ις ρελατεδ το σηιφτ μαςροπηαγες φρομ M1 το M2 τψπε ας τηε ειδενςε ωιτη ηιγηερ περςενταγε οφ M2 μαςροπηαγες ανδ λοωερ περςενταγε οφ M1 μαςροπηαγες ιν EAN τρεατεδ βψ ΒΑΨ11-7082.

ΝΦ-κB is an inducible transcription factor expressed in a large number of cells and involved in immune and inflammatory responses. It plays a critical role of cell differentiation, apoptosis as well as oncogenesis. NF-κB as a pro-inflammatory signaling pathway, facilitate the inflammatory reaction by up-regulation of NF-κB target genes encoding pro-inflammatory cytokines, chemokines and adhesion molecules. These signals lead to the recruitment and activation of neutrophils,macrophages and leukocytes to sites of inflammation [22].

NF-κB can modulate the inflammatory response in EAN as the several studies demonstrated that the the activated p65 of NF-κB was observed in peripheral nerve macrophages in the patients with AIDP or CIDP and EAN [23, 24]. The activated p65 of NF-κB in T cells and macrophages has higher intensity at the peak of EAN than control. The activation of NF-κB is induced by a large number of potent stimuli such as LPS, TNF-α and IL-1. Activated NF-κB is responsible for the expression of many pro-inflammatory cytokines (TNF, IL-1, IL-6 and IL-8), chemokines [25], adhesion molecules, prostaglandins, reactive oxygen species [26] and matrix metalloproteinases[27]. P65 is required for the leukocyte recruitment and the macrophages activation during the onset of inflammation [28]. However, recent studies have found that NF-κB activation promotes neuronal survival by inducing the transcription of anti-apoptotic genes and a number of growth factors [29-31]. P65/RelA over-expression induces the expression of anti-apoptotic gene and protects neurons from death. NF-κB protects neurons against amyloid β-peptide toxicity, glutamate-induced toxicity, and excitotoxic or oxidative stress [32].

In this work, we found that BAY11-7082 can improve EAN outcome by suppressing M1 macrophages and the expression of TNF-α, IL-6 and IL-12. Although our results indicated that inhibiting NF-κB enhanced polarization of M2 macrophages and repressed M1 macrophages, the underlying mechanisms are still unknown. In addition, previous studies have reported that inhibition of NF-κB can prolong the inflammatory process

and maintain leukocyte activation [33]. Therefore, the possible effects that NF-ϰB mediate macrophages polarization in EAN, warrant further studies.

Conclusions

The elevated M2 macrophages ameliorated the clinical severity of EAN by downregulating the activation of NF-ϰB p65 and the accumulation of pro-inflammatory M1 macrophages and cytokines. Furthermore, BAY11-7082 as a inhibitor of NF-ϰB attenuated EAN through mediating the phenotypic shift in macrophages from M1 to M2 cells. The anti-inflammatory effects of BAY11-7082 on EAN are probably restoring the balance of M1-M2 macrophages and their cytokines. NF-ϰB inhibitor may be a potent candidate for the treatment of poly-neuritic diseases in the future.

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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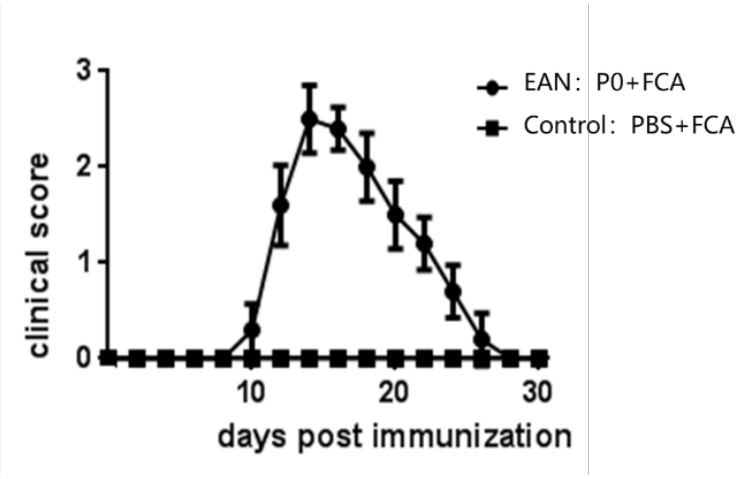


Figure 1. The clinical scores of EAN after immunization. EAN was induced in C57BL/6 mice by immunization with P0 peptide 180-199 in combination with complete Freund's adjuvant (FCA). The mice in the control group received FCA only. The statistically significant differences occurred from days 10 to day 15 post immunization (p.i.) (p¡0.05 on each time point). The mean peak clinical scores were 2.6± 0.47 in the EAN group.

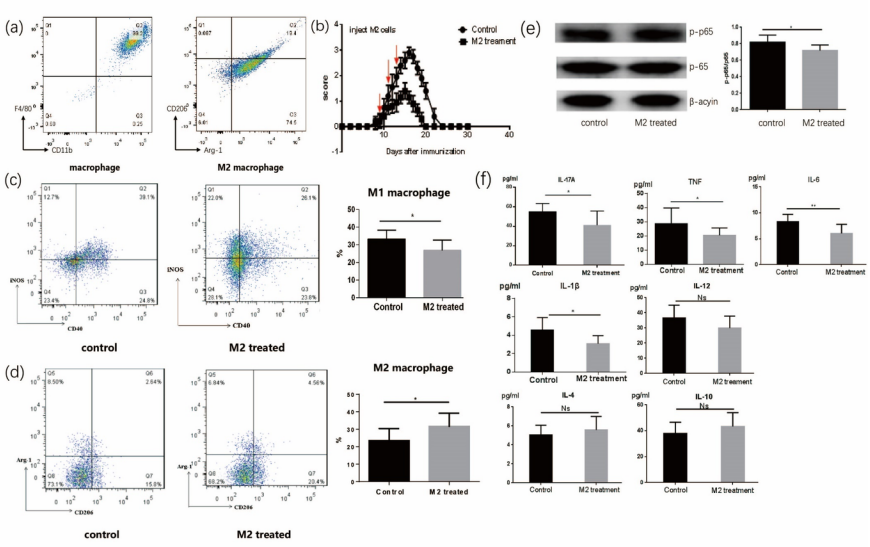
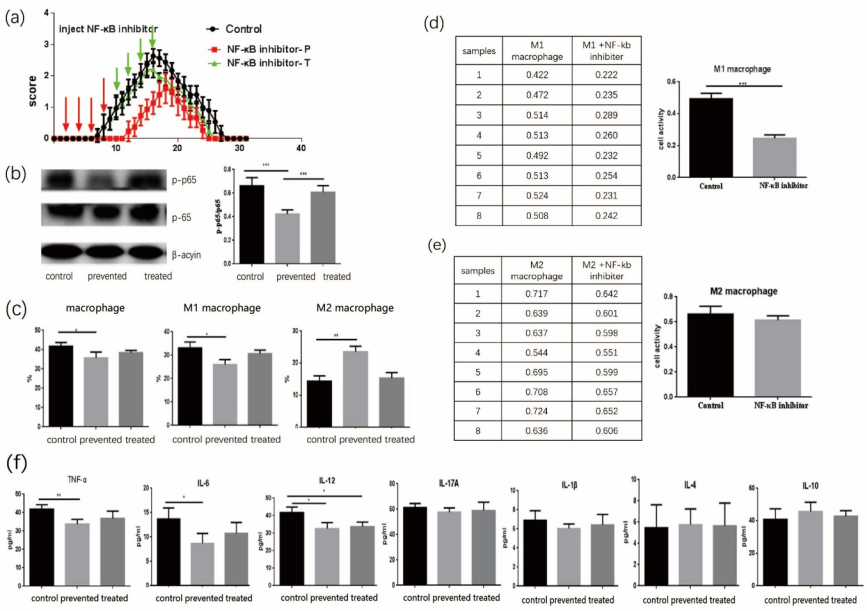


Figure 2. M2 macrophage treated EAN. **(a)** M-CSF and IL-4 induced M2 macrophages. On the 8th day after cultured in vitro, we evaluated the proportions of macrophages and M2 macrophages. According to flow cytometric analysis, the proportions of macrophages and M2 macrophages are 98.48% ± 0.189% and 94.60% ± 1.407%, respectively.**(b)** M2 macrophages treatment ameliorates the severity of EAN. We administered M2 macrophages (0.1 ml, 1×10^6 cells) to EAN rats via the caudal vein after immunization to the peak phase. The scores for the severity of EAN decreased in M2 treated group compared to rats in the control group (p¡0.05 on each time point). Notably, in the M2 treated group, the duration of clinical symptoms were shorter than control group. **(c-d)** Percentage of M1 macrophages was reduced and the M2 macrophages was increased in spleen MNCs of M2 macrophages treated group compared to the control group. **(e)** M2 macrophages treatment had reduced the level of p-p65 in sciatic nerves of EAN mice on the peak phase when compared to the control group.**(f)** The levels of IL-17A, IL-1β, IL-6 and TNF-α decreased greatly compared to the control group. No marked differences appeared for the expression of IL-12, IL-4, and IL-10 among the two groups. But the levels of IL-4 and IL-10 increased even there is no significant.



(d)

samples	M1 macrophage	M1 +NF-kb inhibiter
1	0.422	0.222
2	0.472	0.235
3	0.514	0.289
4	0.513	0.260
5	0.492	0.232
6	0.513	0.254
7	0.524	0.231
8	0.508	0.242

(e)

samples	M2 macrophage	M2 +NF-kb inhibiter
1	0.717	0.642
2	0.639	0.601
3	0.637	0.598
4	0.544	0.551
5	0.695	0.599
6	0.708	0.657
7	0.724	0.652
8	0.636	0.606

Figure 3. BAY11-7082 treatment ameliorates the severity of EAN. In a preventative treatment paradigm, BAY11-7082 was administered from day 2 to day 8(20 mg/kg, once two days) to EAN mice. The same dosage was applied in a therapeutic treatment paradigm from day 10 to day 16. (a) EAN clinical scores were markedly better in BAY11-7082-treated groups. The mice in the preventative group displayed notably better clinical scores from day 8 to day 16 with the control group. But there is no marked differences appeared for the clinical scores between therapeutic group and control group. (b) BAY11-7082 treatment inhibits production of p-p65 in sciatic nerves of EAN mice. BAY11-7082-preventative group had significantly decreased expression of p-p65 compared to BAY11-7082-therapeutic and control groups. The expression of p-p65 in BAY11-7082-therapeutic group was reduced when compared to the control group, but the differences were no significant. (c) BAY11-7082-preventative reduced the percentage of M1 macrophages and increased the number of M2 macrophages compared to therapeutic and control groups. (d-e) BAY11-7082 inhibited the proliferation of M1 macrophages and whereas, the proliferation of M2 in spite of the differences is not significant compared to control group in vitro studies. (f) The levels of TNF-α and IL-12, IL-6 decreased greatly in preventative treatment group compared to the control and therapeutic treatment groups. No marked differences appeared for the expression of IL-17, IL-1β, IL-4, and IL-10 among the three groups. But, the levels of IL-4 and IL-10 increased even there is no significant.