ORIGINAL ARTICLES

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Therapeutic potential of artesunate in experimental autoimmune myasthenia gravis by upregulated T regulatory cells and regulation of Th1/Th2 cytokines

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Artesunate is a semi-synthetic derivative of a Chinese herb named *Artemisia annua* L. that is commonly used as an antimalarial agent in the history of traditional Chinese medicine. Many studies have reported artesunate possesses anti-inflammatory and immunoregulation properties. The present study was conducted to explore whether artesunate was effective in experimental autoimmune myasthenia gravis (EAMG) in Lewis rats. Our data showed that artesunate could improve the clinical symptoms and suppress the development of EAMG. Artesunate exerted its immunomodulatory effects by inhibiting lymphocyte proliferation and the expression of costimulatory molecules CD86, modulating Th1/Th2 cytokine expression levels, and enhancing the level of Treg cells. The final result of administration of artesunate was the decreased synthesis of anti-R97-116 IgG, IgG2a, and IgG2b antibodies. The treatment effect of artesunate was more obvious at dose of 10 mg/kg. These date suggest that artesunate might be a potential drug for the treatment of human myasthenia gravis (MG).

1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder characterized by dysfunction of neuromuscular junction transmission. The antibodies binding to acetylcholine receptor (AChR) cause complement cascade activation and postsynaptic membrane destruction, resulting in loss of AChR in the neuromuscular junction. The development of antibodies whose generation is T cell dependent results in a breakdown of the mechanism of tolerance and the clinical manifestation of weakness and fatigue of skeletal muscle (Phillips and Vincent 2016; Elson and Barker 2000). Commonly used therapies for MG include acetylcholinesterase inhibitors for symptomatic treatment, immunosuppressive medication (corticosteroids and immunosuppressants) for the treatment goals of full or nearly full physical function and high quality of life, intravenous immunoglobulin (IVIG) or plasmapheresis for short-term benefit, thymectomy for MG patients with thymoma or thymic hyperplasia, and monoclonal antibodies and so on (Gilhus 2016; Dalakas 2013; Diaz-Manera et al. 2012). But all those treatments have unavoidable severe side effects that impels us to study a more safe and effective therapeutic method. According to the nature of the currently observed antigenic targets, MG patients can be classified as different subgroups that present with anti-AChR antibodies, anti-muscle specific tyrosine kinase (MuSK) antibodies, anti-lipoprotein-related protein 4 (LRP4) antibodies, anti-titin and ryanodine receptor (RyR) antibodies and seronegative MG and so on. Among of them, around 85 to 90 % of MG patients have autoantibodies against AChR (Berrih-Aknin and Le Panse 2014; Hong et al. 2016). Because anti-AChR antibodies comprise the majority, so the EAMG model induced in rats by the synthetic peptide corresponding to region 97-116 of the rat acetylcholine receptor α subunit (R97-116) that shows clinical symptoms mimicking the human disease is used to evaluate new treatment perspectives in our study (Christadoss et al. 2000; Link and Xiao 2001; Fuchs et al. 2014;).

The use of herbals is on the rise in patients with autoimmune diseases, mainly because they are effective, inexpensive, and relatively safe. Artemisinin is isolated from *Artemisia annua* L., a herb with a long history as a remedy for fever, chills and malaria in the Chinese traditional medicinal system (Verma and Kumar 2016). Artemisinin and its derivatives including dihydroartemisinin, artemether, artesunate, arteether, sM735, SM905 and SM933 are collectively called artemisinin family drugs having shown different pharmacological properties (Hou and Huang 2016; Ansari et al. 2013). Among them, artesunate has received increasing attention. Besides antimalarial effects, other pharmacological actions of artesunate like antioxidant, anti-inflammatory, anti-allergic, antiangiogenic, anti-viral, anti-cancer and immunoregulation have been reported (Verma and Kumar 2016; Cheng et al. 2013; Wartenberg et al. 2003; Drouot et al. 2016; Sharma et al. 2014; Lai et al. 2013; Hou et al. 2014; Li et al. 2013). The anti-inflammatory and immunosuppressive activities make artesunate attractive as a potential novel therapeutic strategy for autoimmune diseases. So it was demonstrated that artesunate has a good therapeutic effect on some immunological diseases, such as systemic lupus erythematosus (Jin et al. 2009), collagen-induced arthritis (Zhu et al. 2016; Liu et al. 2017), experimental colitis (Yang et al. 2012) and delayed type hypersensitivity reaction (DTH) (Li et al. 2013) with little toxicity. In view of the current findings on artesunate in autoimmune diseases, we designed this study to evaluate whether artesunate would reduce the clinical symptoms of experimental autoimmune myasthenia gravis (EAMG). The satisfying results obtained finally suggest artesunate as a new therapeutic approach for MG in human.

2. Investigations and results

2.1. Artesunate ameliorates the clinical symptoms of EAMG

To explore whether artesunate could ameliorate the severity of EAMG, rats in treatment groups were given artesunate by gavage at doses of 10 mg/kg and 100 mg/kg every day respectively from day 10 p.i., and the same volume of normal saline was given to EAMG rats. We assessed the clinical scores as the parameter of the severity of disease. Statistical analysis suggested that the rats in the artesunate treatment groups exhibited lower clinical scores when compared with rats in EAMG group from day 25 to day 43 p.i. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). The clinical severity of rats

in the high-dose group and the-low dose group differ significantly on day 25, 27 and 31 p.i.($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$) (Fig. 1).

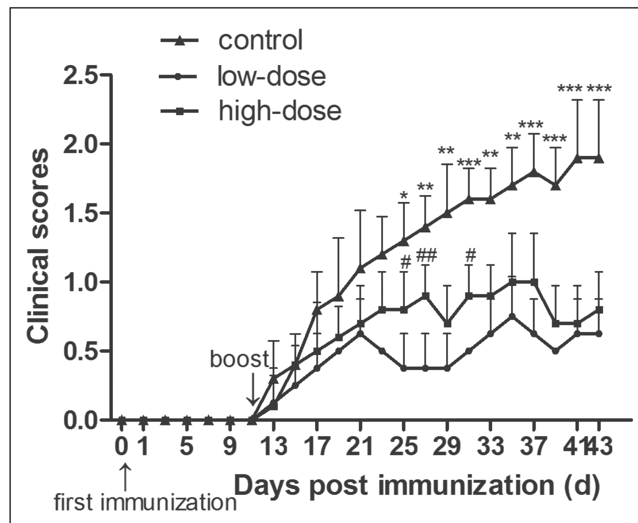

Fig. 1: Artesunate ameliorates the clinical symptom of EAMG. EAMG was induced by R97-116 peptide. From day 10 p.i., EAMG rats in three groups were administered intragastrically with normal saline, 10 mg/kg and 100 mg/kg artesunate respectively every day. The clinical scores of rats in control and artesunate-treated groups were assessed every day. The significant lower clinical scores of treatment groups than control group demonstrated artesunate suppressed the development of ongoing EAMG. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, the treatment groups versus control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, low-dose group versus high-dose group). The results are expressed as mean ± SD (n=5 rats/group).

2.2. Artesunate inhibits the proliferation of lymphocytes

In our study, lymphocyte proliferation was measured after 72 h of culture in the absence or presence of R97-116 antigen with CCK-8 assay to investigate the antigen specific lymphocyte responses among three groups. We observed that in the presence of R97-116 antigen, the differences of lymphocytes proliferation between artesunate-treated groups and control group were statistically significant ($p < 0.05$). For Con A stimulation, the proliferation of lymphocytes in two groups with artesunate intervention was also significantly inhibited ($p < 0.05$). There was no difference among the three groups when MNC cultured with RPMI 1640. The results showed that artesunate inhibited the proliferation of lymphocytes during EAMG (Fig. 2).

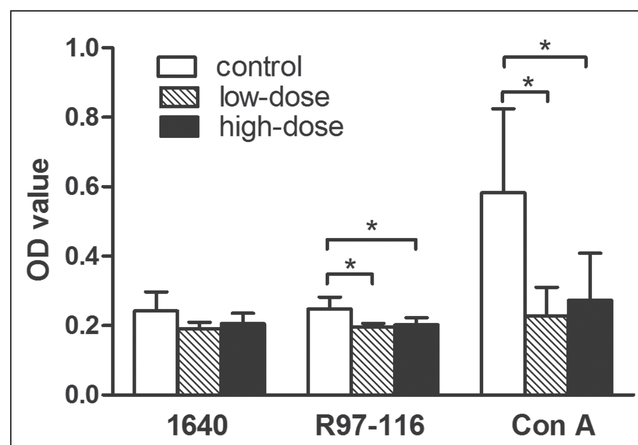

Fig. 2: Effect of artesunate treatment on lymphocyte proliferation in EAMG. The OD value was measured after culture of MNC with R97-116 antigen, Con A and RPMI 1640 for 72 h. Under the induction of R97-116 antigen and Con A, there were significant differences of OD value between artesunate-treated groups and control group. The differences of OD value among three groups weren't significant in the presence of RPMI 1640. The results are expressed as mean±SD (n=5 rats/group). Each experiment was repeated thrice ($^{*}p < 0.05$).

2.3. Artesunate inhibits the expression of CD86 on lymphocytes

CD80 and CD86 are molecules expressed on antigen presenting cells (APCs) that provide costimulatory signals necessary for T cell activation and survival. We detected the expression level of CD80 and CD86 by flow cytometry. Compared to the control group, the expression of CD86 of rats in artesunate-treated groups were obviously inhibited ($p < 0.01$ for both comparisons), but the difference between the low-dose and high-dose group had no statistical significance. The expression levels of CD80 among three groups were similar (Fig. 3).

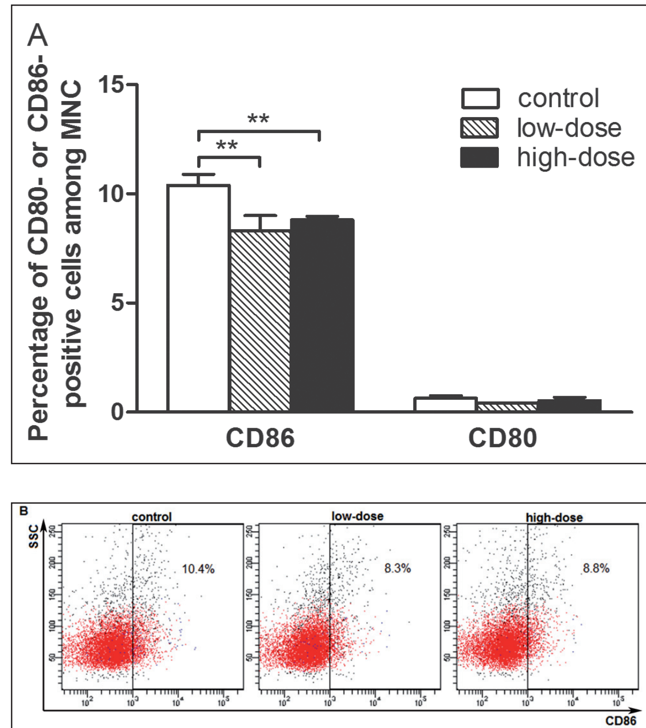

Fig. 3: The suppression of artesunate on costimulatory molecules on lymph node MNC. To investigate the effect of artesunate on the expression of costimulatory molecules CD80 and CD86, lymph MNC were analyzed by FACS after stained by PE-conjugated anti-CD80 and FITC-conjugated anti-CD86. Artesunate treatment at high dose and low dose both inhibited the expression of CD86 when compared with the control group. There were no significant differences of the expression of CD80 among three groups. The results are expressed as mean±SD (n=5 rats/group), and each experiment was repeated thrice ($^{**}p < 0.01$).

2.4. Artesunate treatment increases the number of Foxp3+ cells in spleen of EAMG rats

Treg cells are important for the maintenance of self-tolerance and play a negative regulatory role in autoimmune diseases. To know whether artesunate had a positive effect on Treg cells, we made the paraffin tissue sections of spleen to observe the number of $Foxp3^+$ cells. We found that artesunate treatment at high and low dose increased the number of $Foxp3^+$ cells when compared with normal saline treatment ($p < 0.01$ for both comparisons). There was no significant difference of $Foxp3^+$ cells numbers between the high dose group and the low dose group (Fig. 4).

2.5. Effect of artesunate treatment on the expression of TNF-α and IL-10

To further investigate the mechanism of artesunate on EAMG, flow cytometric analysis was applied to the detection of the production of TNF-α and IL-10 mainly secreted by T helper type 1 (Th1) and T helper type 2 (Th2) cells respectively. The results showed that artesunate in low dose could decrease the level of TNF-α^+ cells and enhance the expression of IL-10^+ cells when compared with normal saline treatment ($p < 0.001$ and $p < 0.01$ for the two

comparisons respectively), but high dose artesunate could not. It was shown that artesunate could regulate Th1 and Th2 responses synchronously in EAMG rats, and the effect was more significant in low dose (Fig. 5).

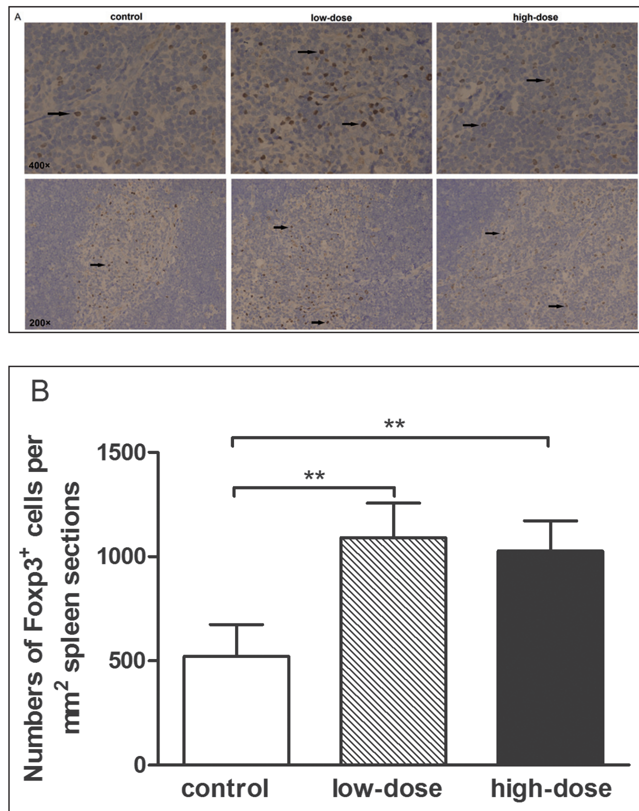

Fig. 4: Artesunate increases the number of Foxp3⁺ cells in spleen of EAMG. We assessed the number of Foxp3⁺ cells in spleen of EAMG rats by immunohistochemistry. A: Representative micrographs showed the Foxp3⁺ cells in spleen of rats in the three groups. The arrow referred to typical Foxp3⁺ cells and the nucleus was filled with brown. Original magnifications, 400× and 200×. B: The numbers of Foxp3⁺ cells per mm² spleen sections in the three groups. The results are expressed as mean±SD (n=5 rats/group) (**$p < 0.01$).

2.6. Artesunate reduces the level of serum anti-AChR antibodies

On day 43, we collected the blood sample from sacrificed rats to determine the level of serum anti-R97-116 IgG and its subclasses IgG1, IgG2a and IgG2b by ELISA. These data showed that treatment of artesunate resulted in a reduction of the level of serum anti-AChR IgG, IgG2a and IgG2b ($p < 0.05$, $p < 0.01$, and $p < 0.001$). Although the difference of serum anti-AChR IgG levels between the high dose and low dose group was small, but the two different doses of artesunate both had the inhibitory action on these antibodies. No significant differences in serum anti-R97-116 IgG1 antibodies was found among the three groups (Fig. 6).

3. Discussion

Artesunate is a semisynthetic derivative of artemisinin primarily used for the treatment of malaria. However, pharmacological research found some other potentially interesting properties. For autoimmune disorders, its anti-inflammatory and immunosuppressant activities are in the focus of research. Artesunate could directly regulate the activities of $CD4^+$ T cells and inhibit T lymphocyte proliferation (Li et al. 2013, Lee et al. 2015). Artesunate treatment resulted in decreased concentrations of proinflammatory cytokines IL-1β, IL-6, IL-8, TNF-α, IFN-γ and IL-17 in experimental colitis and human rheumatoid arthritis (Yang et al. 2012; Xu et al. 2007). In a mouse model of DTH, which was based on a T cell-mediated immune response, and in collagen-induced arthritis rats, intervention of artesunate increased the proportion of Tregs whose enhancement may regulate immune response negatively and protect individual from autoimmune (Li et al. 2013; Zhu et al. 2016; Liu et al. 2017; Shevach 2011). Jin et al. (2009) reported that the level of BAFF, the major B cell activation factor, was decreased in artesunate treated MRL/lpr murine model of systemic lupus erythematosus (Jin et al. 2009). Furthermore, we found that artesunate prevented the development of autoimmune arthritis in young K/BxN mice by inhibiting differentiation and proliferation of Germinal Center (GC) B cells, and suppressing the production of autoantibodies (Hou et al. 2014). In our rat models of EAMG, the immunoregulation function of artesunate was approved again as it inhibited the proliferation of lymphocytes and the production of the proinflammatory cytokine TNF-α, promoted IL-10 secretion and Foxp3⁺ cells production, and reduced the expression of costimulatory molecules CD86, accompanied by decrease of pathogenic anti-R97-116 IgG, IgG2a and IgG2b antibodies. In addition, we observed that these effects were not dose-dependent: artesunate in low dose (10 mg/kg) had a better therapeutic effectiveness than in the high dose (100 mg/kg).

MG is an antibody-mediated, T cell-dependent, complements involved autoimmune disease (De Baets et al. 2003; Soltys and Wu 2012) with the presence of signs such as diplopia, ptosis, without pupillary abnormalities, bulbar symptoms (dysphagia, dysphonia, dysarthria, chewing difficulty), weakness and fatigue of skeletal muscles that worsen with activity and as the day progresses and relieve after a rest or treatment of acetylcholinesterase inhibitors (Berrih-Aknin et al. 2014; Silvestri and Wolfe 2012). As with many autoimmune diseases, MG has a multifactorial etiology. Immune responses deregulated under the influence of the comprehensive effect of genetic, environmental, and behavioral factors (Cavalcante et al. 2013; Bright 2007). After ingestion and processing of antigens, APCs transmit signals by the first signal molecules MHC (especially MHC class II) and the second, costimulatory molecules (CD40, CD80 and CD86) to activate naive $CD4^+$ T cells, inducing the proliferation and differentiation of $CD4^+$ T cell into effector Th cells that can be divided to subpopulations according to cytokine profiles and functions (Lee et al. 2015). All cells, cytokines and chemokines participated in the inflammatory process can mediate tissue damage in autoimmune diseases. Any method that interferes with these networks may have therapeutic potential for the treatment of MG. Lack of surface molecule MHC class II and costimulatory molecules CD80 and CD86 that play a key role in the activation of $CD4^+$ T cells can result in T cell anergy or immune tolerance. We observed that the level of CD86 was decreased, the proliferation of lymphocytes was inhibited and the clinical scores were lowered after administration of artesunate in this study. This indicated that artesunate exerted its immunoregulation to improve clinical symptoms of EAMG rats by interposing in the process of antigen presentation and cell proliferation.

Most autoimmune diseases are thought to be a result of a loss of self-tolerance, which allows for the development and function of autoreactive lymphocytes. Tregs are critical for the maintenance of immune tolerance and prevention excessive immune reaction by suppressing the activation, proliferation, and cytokine production of autoreactive T cells positively (Huang et al. 2015; Thiruppathi et al. 2012 b). Another possible reason for which Tregs protect the body from the autoimmune diseases is the suppression and destruction of autoreactive B cells, which result in the reduction of autoantibody production and subsequent complement fixation (Zhao et al. 2006; Iikuni et al. 2009). The forkhead/winged helix transcription factor Foxp3 expressed specifically in Tregs can convert naive T cells to a regulatory T phenotype functionally similar to naturally occurring $CD4^+CD25^+$ regulatory T cells. Thus Foxp3 plays an important role in the development and function of Tregs cells (Hori et al. 2003; Fontenot and Rudensky 2005; Hori and Sakaguchi 2004). The number of $CD4^+CD25^+$ Tregs in MG patients and EAMG rats was lower than in healthy controls. After immune suppression therapy or thymus resection, the number of $CD4^+CD25^+$ Treg rose (Balandina et al. 2005; Battaglia et al. 2005; Fattorossi et al. 2005; Sun et al. 2004; Masuda et al. 2010;

Fig. 5: Effect of artesunate treatment on the levels of TNF-α and IL-10. To explore the effect of artesunate on the responses of Th1 and Th2 in EAMG rats, we examined the number of TNF-α⁺ and IL-10⁺ cells by flow cytometry. The results showed that artesunate treatment could inhibit the expression of TNF-α⁺ cells and enhance the number of IL-10⁺ cells. While rats in the high-dose group had lower numbers of TNF-α⁺ and higher numbers of IL-10⁺ cells than control group without significant differences. The results are expressed as mean ± SD (n=5 rats/group), and each experiment was repeated thrice (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Aricha et al. 2008). Current studies also supported the point that the function of Treg cells from EAMG was deficient, which could result in a failure to suppress autoreactive cells. For instance, Treg cells from EAMG rats had poorer ability than Treg cells from controls in suppressing the proliferation of $CD4^+$ T effector and B cells. Moreover, $CD4^+CD25^+$ cells from EAMG rats exhibited an elevated extent of apoptosis and expressed upregulated levels of Fas and Th17-associated cytokines (Gertel-Lapter et al. 2013). *In vitro* proliferation assays, Treg-mediated suppression of responder T (Tresp) cells was impaired in MG patients. It was associated with a reduced expression of Foxp3 in isolated Tregs (Thiruppathi et al. 2012 a). In addition, the increase of Treg migration to the

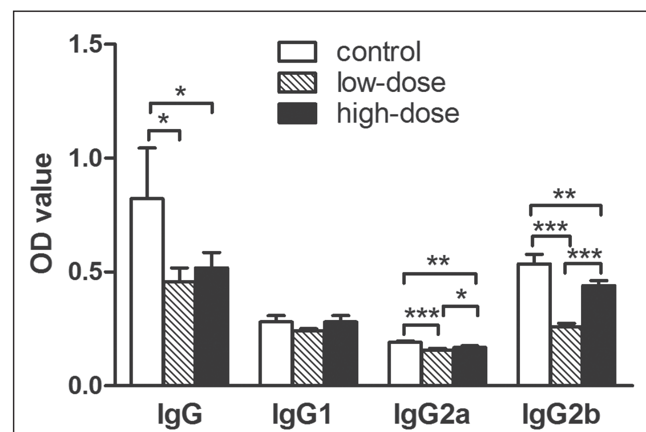

Fig. 6: Artesunate decreases the level of serum anti-AChR antibodies. On day 43 p.i., we collected blood samples to detect the production of anti-R97-116 peptide IgG antibody and its subtypes IgG1, IgG2a, and IgG2b by ELISA. The production of anti-R97-116 IgG, IgG2a and IgG2b in EAMG rats was significantly inhibited by artesunate at dose of 10 mg/kg and 100 mg/kg, and the differences between rats in the low-dose group and the high-dose group were significant. But the level of IgG1 among three groups didn't differ significantly. The results are expressed as mean ± SD (n=5 rats/group), and each experiment was performed four times (* $p < 0.05$, ** $p < 0.01$, ** $p < 0.001$)

GC could suppress B-cell responses to acetylcholine receptor and attenuate the muscular weakness of MG (Liu et al. 2010). To sum up, numerical, functional, and/or migratory deficits in $CD4^+CD25^+$ Tregs are all linked to the pathogenesis of MG (Danikowski et al. 2017; Alahgholi-Hajibehzad et al. 2015). We detected directly the number of $Foxp3^+$ cells in groups treated with or without artesunate by immunohistochemistry analysis. The data showed that rats with intervention of artesunate had a higher number of $Foxp3^+$ cells than the control group. This indicates that artesunate could rectify Treg abnormalities by enhancing the number of Treg and the expression of Foxp3 to regulate the immune responses.

As a potent pro-inflammatory cytokine and a key immune signaling molecule, TNF-α mainly produced by Th1 cells is strongly induced after infection or injury. It is vital to regulate both pro- and anti- inflammatory mediators (Feldmann and Maini 2003). TNF-α plays an important role in the induction and development of MG. It was reported that TNF-α significantly increased in the whole thymus and the sera of MG patients, thus causing the impairment of Treg cells and immunoregulatory defect (Gradolatto et al. 2014). There was a direct correlation between the level of TNF-α and the clinical severity of MG patients (Tüzün et al. 2005). In addition, TNF-α could direct B cell maturation, and the low levels of TNF-α contributed to the production of low affinity antibodies (Duan et al. 2002). In view of the pathogenicity of TNF-α, TNF-α-blocking agents were tried to be applied to clinical therapy of MG patients. Results showed TNF-α-blocking agents had significant clinical efficacy and were proposed for MG treatment (Lee et al. 2009). Intraperitoneal injection of anti-TNF-α antibodies on EAMG rats effectively decreased mortality and severity of clinical muscle weakness (Duan et al. 2002). Etanercept, a TNF-α receptor blocker, is currently used in the treatment of MG (Rowin 2008). All this highlights the importance of TNF-α inhibition in MG treatment. In our study, artesunate exhibited its anti-inflammatory and immunomodulatory activities by decreasing the level of TNF-α, which was in accordance with results showing artesunate's effect on experimental models of Crohn's disease and inflammation (such as gastric mucosal damage, acute lung injury and atherosclerosis lesion formation) (Verma and Kumar 2016; Yang et al. 2012; Zhao et al. 2017; Jiang et al. 2016).

IL-10 is an important pleiotropic cytokine secreted by Th2 cells, B cells, and macrophages, with a broad spectrum of biological activities including inhibition of cytokine production of Th1 cells, down-regulatory effects on APC function with decreased expression of MHC class II and costimulatory molecules, suppression of antigen-specific T-cell proliferation, as well as stimulation of B cell differentiation and the humoral immune response at the same time (Duan et al. 2002; Sabat et al. 2010; Moore et al. 2001). The roles of IL-10 on experimental autoimmune responses are complex and conflicting. Zhang et al. found that IL-10 aggravated EAMG and the level of IL-10 mRNA in EAMG rats treated with immunomodulatory drugs was lower than in the control group (Zhang et al. 2001, 1997). The lower morbidity and severity of EAMG symptoms in $IL\text{-}10^{-/-}$ mice was attributed to reduced numbers of B cells and decreased autoantibody production (Poussin et al. 2000). These data indicate that IL-10 plays a promoting role in EAMG and MG. But on the other hand, IL-10 deficiency in mice led to increased susceptibility to autoimmune disorders (Guo 2016). Huang et al. (2000) found that mRNA expression of IL-10 was lower in MG patients, especially in non-thymectomized patients compared with healthy controls. With administration of dual altered peptide, the clinical symptoms of EAMG were improved, accompanied by elevated secretion of IL-10 (Paas-Rozner et al. 2001). TNF $receptor\text{-}1^{-/-}$ mice that were resistant to EAMG showed increased IL-10 expression (Wang et al. 2000). Dendritic cells (DCs) treated with IL-10 (IL-10-DCs) effectively suppressed the development of EAMG by conversion of immature DCs into tolerogenic APCs on the effect of IL-10 (Duan et al. 2004). The above results and the higher expression of IL-10 in artesunate-treated rats compared to EAMG rats in our experiments support the protective effect and negative regulatory function of IL-10 in the development of MG and EAMG. In addition, recent studies suggested that IL-10 was important for potentiating the induction and maintaining the suppressive function of Tregs (Hsu et al. 2015; Chaudhry et al. 2011; Murai et al. 2009). Maybe this can provide another explanation for the positive fluctuation relationship between IL-10 and Foxp3 expression in this study.

MG is an autoantibody-mediated disease. The main symptoms of MG are caused by the effect of autoantibodies to AChR on the muscle end plates. Destruction of the postsynaptic membrane, antigenic modulation, competition at ligand-binding sites and steric hindrance (Huijbers et al. 2014) are essential effector mechanisms of autoantibodies that downregulate the AChR and lead to the dysfunction of neuromuscular junction transmission. IgG antibodies have many isotypes. Among of them, the IgG1 subclass is beneficial for preventing autoimmune diseases, but the observation that rat IgG1 also activates complement causes a controversy about the effect of IgG1 on MG or EAMG, while IgG2 (including IgG2a and IgG2b) is associated with the pathogenesis of MG. The concentrations of IgG1 and IgG2 are, respectively, related to Th2-type and Th1-type inflammatory immune responses (Chae et al. 2012). Research had suggested that Th1 cells secrete TNF-α to promote the production of IgG2 antibodies (Conti-Fine et al. 2006). In our study, we noticed a statistically significant reduction of anti R97-116 IgG, IgG2a and IgG2b antibody levels in treatment groups with decreased expression of TNF-α. Thus it is stated that artesunate could inhibit the production of IgG2a and IgG2b antibodies by reducing the secretion of TNF-α.

Our results suggest that artesunate, especially in low doses, could protect against the development of EAMG by its immunoregulatory effect. The specific mechanism includes inhibiting lymphocyte proliferation, reducing the expression of costimulatory molecules CD86, regulating Th1/Th2 cytokine expression levels and enhancing the level of Treg cells, which finally resulted in a decreased production of anti-R97-116 IgG, IgG2a, and IgG2b antibodies. In conclusion, these data demonstrated that artesunate suppressed the development of EAMG. The immunosuppressive biological activity of artesunate might provide an effective treatment for human MG.

4. Experimental

4.1. Animals and main reagents

Female Lewis rats, 6- to 8-weeks-old, weighing 140-160 g, were acquired from Beijing Vital River Laboratory Animal Technology Co. Ltd., and allocated in cages of the local animal house under specific pathogen-free conditions. Rats took food and water freely in the experimental period with alternate 12 h light and dark. All experiments in this study were approved by the institutional animal ethics committee. R97-116 peptide (DGDFAIVKFTKVLLDYTGHI) purified from rats was synthesized by CL. (Xian, China) Bio-Scientific Co. Ltd. Artesunate (98% purity) was provided by Guangxi Guilin Pharmaceutical Co., Ltd. Incomplete Freund's adjuvant (IFA) and *Mycobacterium tuberculosis* (MTB) H37Ra were purchased from Sigma-Aldrich (St. Louis, MO, USA) and BD Pharmingen (BD Biosciences, San Jose, CA, USA) respectively.

4.2. Induction of EAMG

Female Lewis rats were subcutaneously injected with 50 µg R97-116 synthetic peptide emulsified in 1 mg MTB H37Ra and IFA in the total volume of 200 µl into both hind footpads on the day that was recorded as the day 0 after initial immunization. Then the same dosage of hybrid emulsion was injected along the back on day 11 post immunization (p.i.) as the second immunization.

All rats were randomly divided into three groups with the minimum number to obtain a meaningful interpretation of data (5 rats/group). From day 10 p.i., rats in treatment groups were intragastrically treated with artesunate at doses of 10 mg/kg and 100 mg/kg every day until day 43 p.i. The same volume of normal saline was given to EAMG rats.

4.3. Clinical evaluation

From the initial immunization, evaluation of clinical manifestations was performed in a double-blind fashion every other day until sacrifice of rats. The clinical scores of rats were evaluated according to the signs of tremor, hunched posture, muscle strength, and fatigability. Fatigability was assessed after exercise (repetitive paw grips on the cage grid) for 30 s. The severity of clinical symptoms was mainly graded as follows (Baggi et al. 2004): grade 0: normal strength and no abnormalities; grade 1: mildly decreased activity and weak grip or cry, more evident at the end of testing; grade 2: clinical signs present before exercise (tremor, head down, hunched posture, weak grip); grade 3: severe clinical signs present before exercise, no grip, moribund; grade

4: dead. Intermediate scores of 0.5 increment were given to rats with intermediate signs. Results are expressed as the mean of the evaluations recorded for each animal at each time point.

4.4. Preparation of mononuclear cells

On day 43 p.i., inguinal lymph nodes were removed from sacrificed rats under aseptic conditions, cut into small particles with scissors and ground adequately to obtain the mononuclear cells (MNC). Cells were washed three times and resuspended to the concentration of 2 x 10^6/ml in complete medium containing 1% (v/v) penicillin/streptomycin (Millipore, Phillipsburg, NJ, USA), 10% (v/v) fetal bovine serum (FBS; Gibco, Grand Land, NY, USA) and 89% RPMI 1640 (HyClone, Beijing, China).

4.5. Cell proliferation

MNC suspension in 200-μl aliquots with cell concentration of 2 x 10^6/ml from the three groups were cultured in 96-well plates in triplicates, followed by the addition of 10 μl R97-116 peptide (10 μg/ml) as the irritant. Positive controls and negative controls were incubated with equal amounts of concanavalin A (Con A) and RPMI 1640 respectively. After 72 h of incubation at 37 °C, cells were incubated with 10 μl Cell Counting Kit-8 (CCK-8) solution for further 4 h. The optical density (OD) was measured at the wavelength of 450 nm on a microplate reader and results were expressed as OD values±standard deviation (SD).

4.6. Flow cytometric analysis

For cell surface staining, after washing with 0.5% bovine serum albumin (BSA, Sigma-Aldrich) and centrifugation, lymph node MNC were marked with PE-labeled anti-rat CD80 (BioLegend, San Diego, CA, USA) and FITC-labeled anti-rat CD86 (BioLegend). Before analysis by FACSAria ™ II flow cytometer, cells were resuspended by phosphate buffered saline (PBS) for 30 min at 4 °C. For intracellular cytokine analysis, MNC were cultured with leukocyte activation cocktail (BD Golgiplug) in 24-well plates in an incubator with 5 % CO_2 at 37 °C for 4-6 h to stimulate the secretion of intracellular cytokines. Then cells were fixed with 2% paraformaldehyde at 4 °C and permeabilized with 0.5% saponin at room temperature for 20 min. Then anti-IL-10-PE (BD Pharmingen) and anti-TNF-α-FITC (BioLegend) were used for intracellular staining at 4 °C in dark for 30 min. After resuspention, the cells were analyzed by flow cytometry. For assessment of regulatory T cell (Treg) expression, MNC were stained extracellularly by PE-conjugated anti-rat CD25 (eBioscience, San Diego, CA, USA) and FITC-conjugated anti-rat CD4 (eBioscience). Fixation and permeabilization were performed with specialized Fixation/Permeabilization fluid (eBioscience) at 4 °C in dark overnight subsequently. After washing and centrifugation, cells were stained with PE-Cy5-conjugated anti-rat Foxp3 (eBioscience) at 4 °C in dark for 30 min. Flow cytometric analysis of samples was conducted later.

4.7. Immunohistochemistry

Referring to the operation process reported by Xu et al. (2014), segments of spleen from sacrificed rats under aseptic conditions were dissected and made to paraffin tissue sections (5 μm) that were deparaffinized and hydrated subsequently. The sections were treated with 0.3 % hydrogen peroxide for 20 min to block endogenous peroxidase activity and washed three times in PBS. Then sections were boiled in citrate buffer for antigen retrieval and incubated overnight at 4 °C with mouse anti-rat Foxp3 antibody (1:100; eBioscience). After staining with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Zhongshan Goldenbridge Biotechnology, Beijing, China), development with diaminobenzidine (DAB) substrate (Zhongshan Goldenbridge Biotechnology) was behind to detect the number of $Foxp3^+$ cells. As negative controls for immunostaining, the primary antibody was omitted. The tissue areas were measured by image analysis in five sections per spleen, and the results were expressed as the number of positive cells per square millimeter tissue section.

4.8. ELISA for serum anti-R97-116 antibodies

Heart blood was collected rapidly from sacrificed rats on day 43 p.i. to detect levels of anti-R97-116 antibodies by enzyme linked immunosorbent assay (ELISA) with serum samples acquired from blood after high speed centrifugation. The 96-well plates were coated with triplicate aliquots of R97-116 peptide solution (final concentration: 5 g/ml) overnight at 4 °C. Washing with PBST (PBS containing 0.05 % Tween20) three times later, 200 μl PBS containing 10% FBS and 0.05% Tween20 was added for block at 37 °C for 1.5 h. Incubation of serum samples of each rat at a dilution of 1:100 (in PBST) for 2 h at 37 °C was followed. After washing three times, the plates were incubated with diluted biotin labeled Rabbit anti rat IgG (1:3000 in PBST; Biosynthesis Biotechnology, Beijing, China) at 37 °C for 1 h, and the same steps were operated with dilution of 1:1000, 1:500 and 1:500 for IgG1, IgG2a and IgG2b (all from BioLegend) respectively. HRP labeled streptavidin (1:1000 in PBST; Biosynthesis Biotechnology) was used then at 37 °C for 30 min, followed by the color development with tetramethylbenzidine (TMB) substrate (Tiangen Biotechnology, Beijing, China) in dark. Finally, the OD value was measured at 450 nm by a ELISA microplate reader after adjunction of the stop buffer (1M H2SO4).

4.9. Statistical analysis

Data analysis was performed with SPSS version 17.0 software. Results were expressed as mean±SD. Comparisons among three groups were analyzed by one-factor analysis of variance (ANOVA), and Least Significant Difference (LSD) test was used for pairwise comparisons among groups. Data conversion was necessary for variance heterogeneity. If the variance homogeneity was still not met, non-parametric rank sum test was employed for multiple comparisons. A value of p less than 0.05 was considered to have statistical significance.

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