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Metformin attenuates autoimmune disease of the neuromotor system in animal models of myasthenia gravis

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ABSTRACT

Metformin, the most widely used medicine for type 2 diabetes, displays anti-inflammatory functions *via* activating AMP-activated protein kinase (AMPK). Circulating autoantibodies and disequilibrium of helper T cells and regulatory T cells are pathological hallmarks of myasthenia gravis (MG). Rectify the imbalance of different T cell populations has become an important therapeutic strategy to treat MG. In this study, we assessed the effect of metformin on the development of autoimmunity using an experimental autoimmune myasthenia gravis (EAMG) rat model. We first provided evidence that oral administration of metformin attenuated the onset of EAMG. This effect was accompanied by a substantial decrease of circulating auto-antibody levels with no effect on blood glucose level. While metformin treatment *in vitro* showed little effect on inducible Treg, metformin strongly inhibited Th17 cell differentiation through the increase of reactive oxygen species and AMPK. Furthermore, an attenuation of antigen-induced IgG2b antibody production by two different doses of metformin was also observed in the AChR-specific recall response. In conclusion, the above results indicate that metformin may have therapeutic value for the clinical treatment of MG.

1. Introduction

Myasthenia gravis (MG) is a chronic autoimmune disorder of neuromuscular transmission [1]. The immunopathologic mechanisms that perpetuate the disease are directly related to the presence of specific autoantibodies produced by B cells [2]. The antibodies bind to the postsynaptic muscle end-plate and destroy postsynaptic molecules, including acetylcholine receptor (AChR), muscle specific kinase (MuSK) and other functionally related molecules. This process leads to impaired signal transduction and, consequently, incremental weakness and fatigability, particularly of the skeletal muscles – the hallmark symptoms of MG [2]. Several studies in experimental models have indicated that the Th17 subset, which is characterized based on the secretion of the proinflammatory cytokine IL-17, is involved in the process of producing antigen-specific antibodies by B cells [3–5]. In contrast, regulatory T cells (Treg), which are essential for the maintenance of immunologic self-tolerance, are defective in MG patients at regulating immune responses compared to those in healthy subjects [6,7]. Hence, disequilibrium of Th17 cells and regulatory T cells is considered to be the main driver in the immunopathogenesis of MG disease [8]. Together with autoreactive pathologic autoantibodies, autoantibody-producing B cells and their helper cells should be major treatment targets for MG.

Traditionally, metabolism is considered to be a mostly static process that is involved in maintaining cellular homeostasis. However, it is generally accepted that metabolic pathways are dynamically regulated in cells during developmental transitions [9–11]. Upon stimulation of antigen receptors, naïve $CD4^+$ T lymphocytes can proliferate and differentiate into distinct subsets of effector T cells, including Th1, Th2, Th17 or inducible Treg subsets [12]. Emerging evidence has demonstrated that coinciding with proliferation and differentiation, T cells

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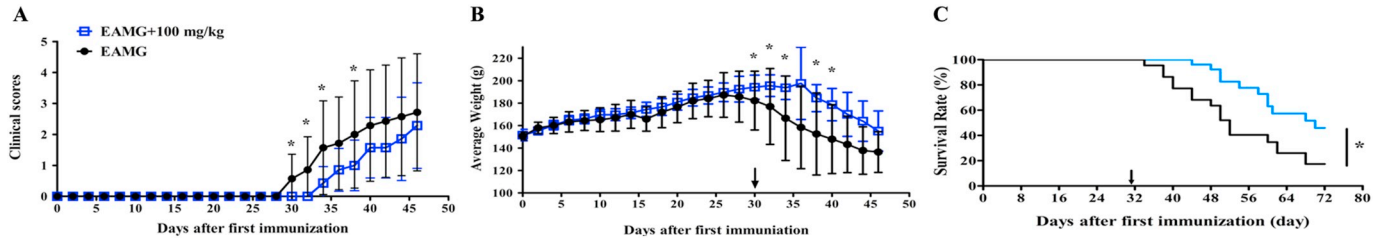

Fig. 1. Metformin ameliorates experimental autoimmune myasthenia gravis (EAMG) symptoms. Clinical manifestations (A), body weight (B) and survival rate (C) were measured in rats from the EAMG group (black) and the metformin (100 mg/kg)-administered group (blue). Arrows indicate the day that R-AChR$_{97–116}$ boosters were injected. Data are presented as the mean ± SD from 3 independent experiments with eight rats per condition per experiment, *, $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

undergo metabolic reprogramming to fulfill the bioenergetic and biosynthetic demand [13,14]. Pro-inflammatory CD4$^+$ T helper (Th) cells (Th1, Th2, and Th17) display a higher rate of glycolysis over mitochondrial metabolism, whereas Tregs prefer a mixed metabolism involving glycolysis, lipid oxidation, and oxidative phosphorylation (OXPHOS) [15,16]. Interrupting glycolysis during Th17 cell differentiation even inhibits the development of Th17 cells and favors the formation of Tregs [17].

Adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) serve as evolutionarily conserved energy sensors. They can connect cellular metabolic changes with signaling pathways that have the capacity to affect activation and differentiation processes within T cells [18,19]. The process of aerobic glycolysis is directly regulated by mTOR complex 1 (mTORC1) signaling through the transcriptional regulation of many key glycolytic enzymes [20,21]. mTORC1-deficient T cells fail to differentiate into Th1 and Th17 effector cells, which is associated with failure to upregulate lineage-specific transcription factors [22,23]. On the other hand, AMPK activation not only promotes glutamine-dependent OXPHOS for ATP production but also dampens mTORC1 activation and further suppresses IFN-γ cytokine mRNA translation [24,25]. AMPK also restrains the formation of Th17 cells by inhibiting acetyl-CoA carboxylase 1 (ACC1) [26,27]. In the context of development, helper T cells and Tregs are tightly linked to their metabolic state, and modulating AMPK activity can be suggested to regulate T-cell-driven inflammation in MG.

Reactive oxygen species (ROS) generated in the mitochondria are the byproducts of aerobic metabolism. Disruption of electron transport in the electron transport chain (ETC), mainly NADH:ubiquinone oxidoreductase (complex I), results in a significantly increasing rate of mitochondrial ROS (mROS) production [28]. However, excessive mitochondrial ROS production triggers a feedback mechanism mediated by the activation of AMPK, causing an antioxidant response that dampens mitochondrial ROS production to regulate ROS levels [29]. A recent study showed that mitochondrial-derived ROS were essential for T cell activation by nuclear factor of activated T cells (NFAT) and promoted subsequent IL-2 production, whereas failure to attenuate accumulating ROS blocked T cell activation, which was partly mediated by decreasing mTOR that then allowed activated T cells to switch to glycolysis [30].

Metformin, the most commonly prescribed medicine for type 2 diabetes (T2D), has recently garnered more attention because of its anti-inflammatory effects *via* targeting NADH:ubiquinone oxidoreductase and subsequent activation of AMPK [31]. Although AMPK couples with mROS changes, whether metformin will act in this unconventional way is unclear. Studies have shown that metformin downregulates autoimmunity in an animal model of Roquin$^{san/san}$ mice, a murine model of systemic lupus erythematosus and multiple sclerosis [32,33].

Therefore, the objective of this study was to investigate the therapeutic potential and possible mechanisms of orally administered metformin on autoimmunity development using a classic model of human MG, the experimental autoimmune myasthenia gravis (EAMG) rats. Our findings verified that metformin attenuated the clinical manifestations of EAMG by impeding AChR antibody secretion in the serum of EAMG animals and Th17 differentiation without affecting Tregs by activating the AMPK pathway.

2. Materials and methods

2.1. Animals

Eight-week old 140–160 g female Lewis rats were purchased from Vital River (Beijing, China) and housed under specific pathogen-free conditions with a 12-h light-dark cycle. All experimental procedures were conducted in accordance with the principles outlined in the Harbin Medical University's Guide for the Care and Use of Laboratory Animals published by the China National Institute of Health. Studies were approved by the Institutional Animal Care and Use Committee of Harbin Medical University.

2.2. Induction, assessment, and treatment of EAMG rats

EAMG was induced by subcutaneous injection of 200 μL of an emulsion of 50 μg of R-AChR$_{97-116}$ (specific peptide corresponding to the rat AChRα subunit synthesized by Xi'an Lintai Bioscience & Technology Co., TD. Xi'an, China), *Mycobacterium tuberculosis* strain H37RA (Difco, Detroit, MI), 100 μL of incomplete Freund's adjuvant (IFA, Sigma-Aldrich, St. Louis, MO) and 100 μL of phosphate buffered saline (PBS) at the base of the tail on day 0. Then, on the 30th day, EAMG rats were boosted with the same emulsion without *Mycobacterium tuberculosis*. The healthy control group was immunized with the same dose of emulsion without R-AChR$_{97-116}$. To evaluate the effect of metformin on EAMG rats, metformin (metformin hydrochloride, TCI, America) (100 mg/kg body weight) or saline was administered orally daily from day 0 until the end of the study. Rats were weighed and scored every second day for clinical signs of EAMG until they were euthanized. Fatigability was evaluated after exercise (repetitive paw grips on the cage grid) for 30 s. Clinical signs were scored as follows [34]: 0, no disease; 1, mildly decreased activity and weak grip or cry, evident postexercise; 2, obvious clinical signs present before exercise (tremor, head down, hunched posture, weak grip); 3, severe clinical signs already present before exercise, no grip, moribund; and 4, dead. A score of 0.5, 1.5, 2.5, or 3.5 was assigned to rats with intermediate signs. The results are representative of the mean of the recording for each animal at each time point evaluation.

2.3. Measurement of AChR-specific autoantibody and glucose concentrations

To measure the AChRα subunit (R-AChR$_{97-116}$)-specific antibody in serum and cell culture supernatant, affinity-purified R-AChR$_{97-116}$ (2 μg/mL) in 100 μL of 0.1 M carbonate bicarbonate buffer (pH 9.6) was coated onto 96-well plates (Corning Costar 96-well plates, eBioscience, San Diego, CA) and incubated at 4 °C overnight, followed by a blocking

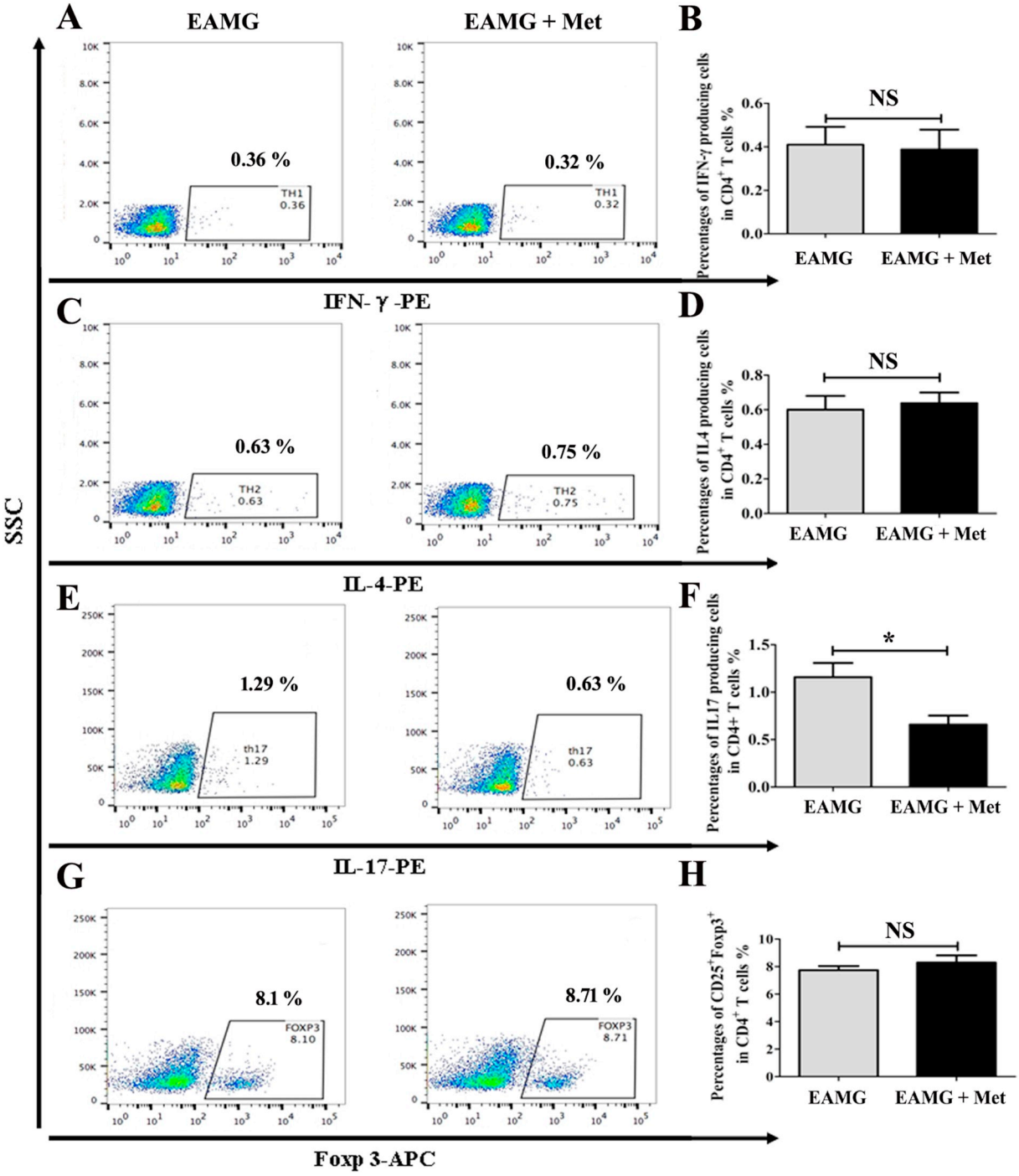

Fig. 2. Effects of metformin on Th subtypes *in vivo*. T lymphocytes from different groups of EAMG rats were collected for the detection of Th subtypes *in vivo*. Flow cytometry analysis illustrated the percentages of IFN-γ (A–B), IL-4 (C–D), IL-17A (E–F), CD25 and Foxp3 (G–H) in $CD4^+$ T cells with or without metformin administration. Graphs demonstrate the mean ± SD from two independent experiments with eight rats per condition per experiment, *, $P < 0.05$, NS, no significant difference.

step with 5% fetal bovine serum (FBS) at room temperature (RT) for 2 h. Serum samples derived from all animals at different time points were diluted 1:5000 in PBS (pH 7.4) containing 5% FBS. Diluted serum or cell culture supernatant was incubated without dilution at 37 °C for 2 h. The plates were washed with PBS-T (PBS 0.05% Tween 20) and incubated with rabbit anti-rat IgG antibody (rabbit anti-rat IgG, Immunoway) at RT for 120 min. Then, peroxidase-conjugated goat anti-rabbit IgG (ZSGB-BIO, China) was added and incubated for 30 min at 37 °C. Finally, TMB substrate solution (eBioscience) was added to colorize the plate at RT for 10 min, and the reaction was stopped by adding 2 M H_2SO_4. The absorbance values were measured at 450 nm on an ELISA plate reader (Bio-Rad Laboratories, Inc. Hercules, CA). Total serum glucose levels were measured using commercial kits from Asan Pharmaceutical (Hwaseong-si Gyeonggi-do, Korea).

2.4. $CD4^+$ T and $B220^+$ B cell isolation

Axillary, inguinal, popliteal, and paraaortic lymph nodes and splenic cells were dissected seven days after the first immunization. Mononuclear cells were harvested by passing cell suspensions through a

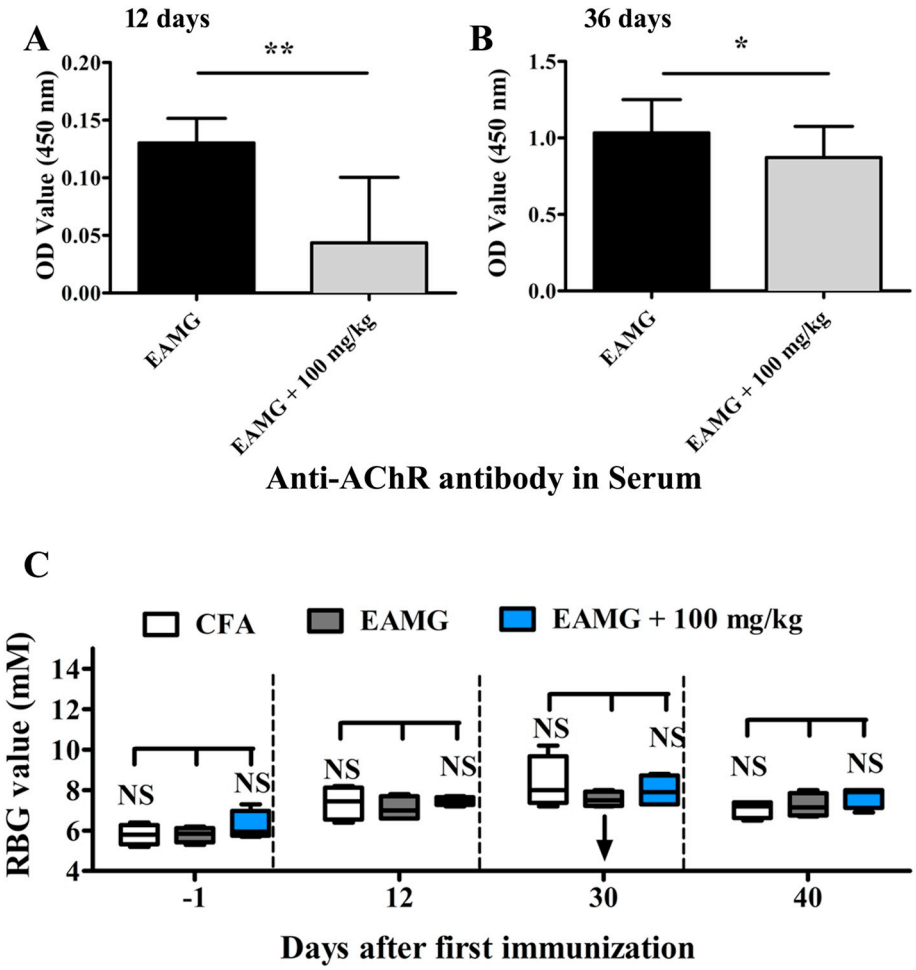

Fig. 3. Effects of metformin on anti-AChR antibody production and blood glucose levels. (A–B) Anti-AChR antibody titers in serum on both the 12th day and 36th day after the first immunization in the 100 mg/kg metformin-treated groups were decreased compared with those in the EAMG groups. (C) There was no significant difference in blood glucose levels among rats from the CFA, EAMG and metformin-treated groups at different time-points. Data are representative of three independent experiments and are expressed as the mean ± SD of six rats/group, *, $P < 0.05$, **, $P < 0.01$, NS, no significant difference.

40-μm nylon mesh. $CD4^+$ T cells and $B220^+$ B cells were subsequently isolated by performing a negative selection with a MagCellect Rat $CD4^+$ T Cell Isolation Kit or MagCellect Rat B cell Isolation Kit (R&D Systems, Inc., USA) according to the manufacturer's instructions. Cells were cultured in lymphocyte-culture medium consisting of RPMI 1640 (Sigma-Aldrich, St. Louis, MO) with 10% FBS (Gibco, Paisley, UK), 1% penicillin–streptomycin (Gibco), 1% L-glutamine (Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), 1% nonessential amino acids (Sigma-Aldrich), 2×10^{-5} M 2-mercaptoethanol (2-ME, Amresco, Solon, OH, USA) and R-$AChR_{97–116}$ at a final concentration of 10 μg/mL.

2.5. CCK-8 analysis

A total of 200 μL of 1 × 10 [6]/mL lymphocytes derived from EAMG rats was plated into 96-well plates with different concentrations of metformin and incubated for 48 h. At the end of incubation, 20 μL of Cell Counting Kit-8 (CCK-8, Dojindo, Japan) reagent was added to the medium and then incubated for an additional 90 min. The absorbance values were measured at 450 nm on an ELISA plate reader (Bio-Rad Laboratories, Inc. Hercules, CA).

2.6. Th17 and iTreg differentiation

For the differentiation assay, 1×10^6 purified $CD4^+$ T cells in 0.5 mL of culture medium were plated into 24-well plates coated with anti-rat CD3 (purified anti-rat CD3 antibody, Biolegend, San Diego, CA) (2 μg/mL), anti-rat CD28 (purified anti-rat CD28 antibody, Biolegend, San Diego, CA) (1 μg/mL) and 10 μg/mL R-$AChR_{97–116}$. For Th17 differentiation, purified $CD4^+$ T cells (2×10^6 cells/mL) were stimulated for 3 d in the presence of transforming growth factor-β1 (2 ng/mL, PeproTech, PeproTech Inc., USA) and IL-6 (50 ng/mL, PeproTech, PeproTech Inc., USA). For iTreg differentiation, purified $CD4^+$ T cells (2×10^6 cells/mL) were stimulated for 3 d in the presence of transforming growth factor-β1 (6 ng/mL, PeproTech, PeproTech Inc., USA).

2.7. Intracellular staining

The following antibodies were used: anti-rat-CD4-FITC, CD4-PERCP, B220-PE, CD25-PERCP, MitoSOX-PE, IFN-γ-PE, IL-4-PE, IL-17-APC, FoxP3-APC, and CFSE-FITC (all from eBioscience, San Diego, CA). To measure intracellular cytokines, differentiating Th17 cells (day 3) and iTreg cells (day 3) were collected and stimulated for 4 to 6 h with PMA (50 ng/mL, Sigma-Aldrich) and ionomycin (1 μg/mL, Enzo) in the presence of Brefeldin A (BFA, eBioscience, San Diego, CA), permeabilized by applying Cytofix/Cytoperm (BD Biosciences, San Jose, CA), and then stained with the appropriate antibodies. To identify committed cells, transcription factor staining was conducted using the Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA) and staining for Foxp3. Data were acquired on a BD FACSVerse flow cytometer and analyzed using FlowJo X (TreeStar software). To detect

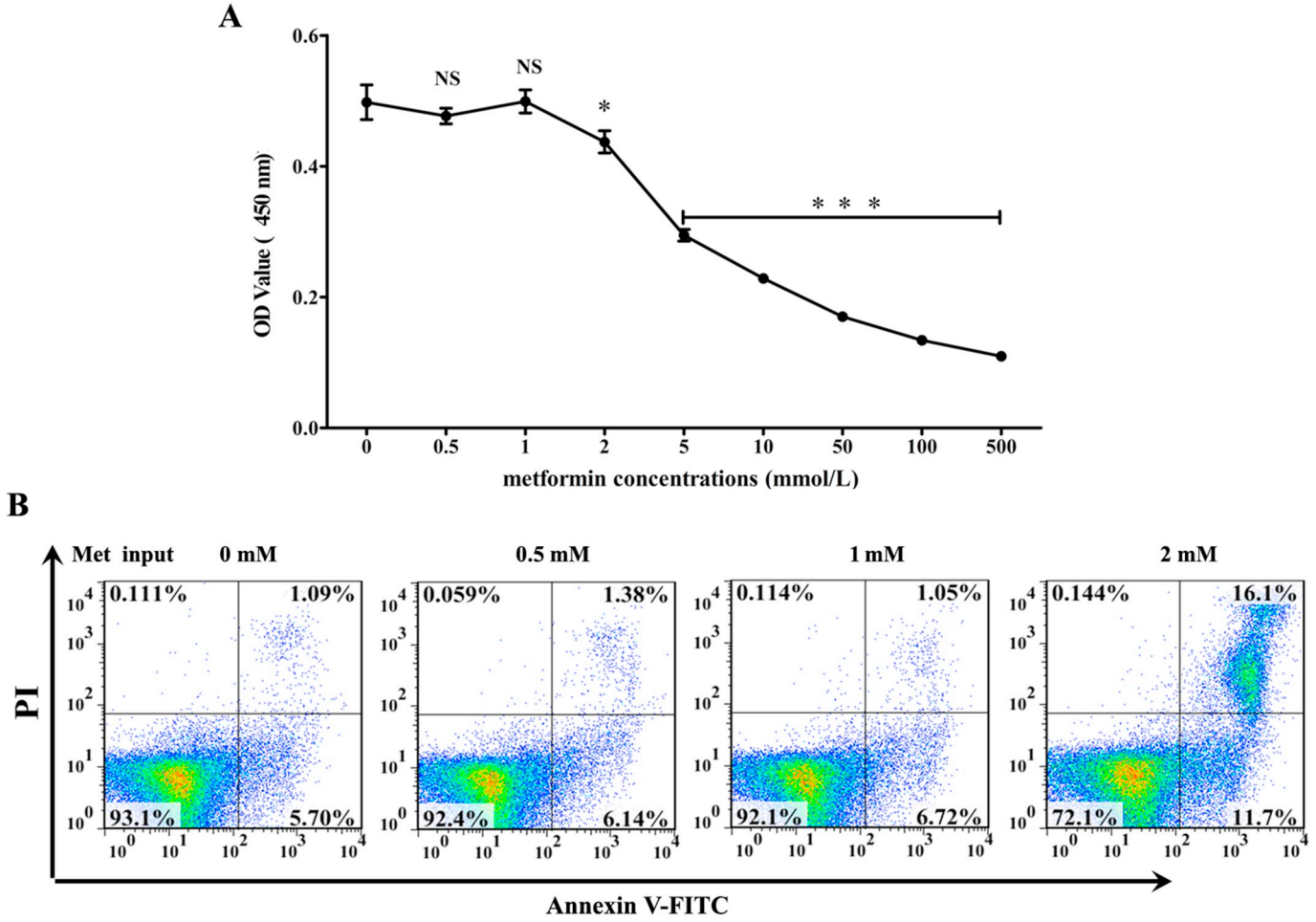

Fig. 4. High concentrations of metformin inhibit lymphocyte proliferation. (A) Cell proliferation of AChR-specific lymphocytes with different concentrations of metformin was assayed with a CCK-8 kit. (B) Lymphocyte apoptosis and necrosis induced by various concentrations of metformin (0.5 mM to 2 mM) were assessed *via* Annexin V-FITC/PI staining detection. Data are shown as the mean ± SD from three independent experiments, *, $P < 0.05$, *** P, < 0.001, NS, no significant difference.

ROS in mitochondria, MitoSOX staining (Invitrogen, CA) was performed as described without the stimulation of PMA, ionomycin and BFA [35]. To detect cell proliferation, CFSE staining was conducted as described prior to incubation [36].

2.8. Western blot assays

For the detection of AMPK and ROS protein alterations after metformin addition, a total of 5×10^7 AChR-specific $CD4^+$ T cells at a concentration of 5×10 [6]/mL were incubated with or without the addition of 0.5 mM or 1 mM metformin for 48 h. RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the collection of cell proteins. For the western blot assay, rabbit-anti-rat-AMPK and -mTOR antibodies were purchased from Beyotime Biotechnology (1:2000, Shanghai, China), anti-GAPDH and anti-β-actin antibodies (1:2000) were purchased from Santa Cruz Biotechnology (China), and horseradish peroxidase-conjugated goat-anti-rabbit IgG (1:1000, Beyotime Biotechnology, Shanghai, China) was selected as the secondary antibody. The data were normalized to the levels of β-actin or GAPDH, and the degree of immunoreactivity was expressed relative to the corresponding control.

2.9. Statistical analysis

GraphPad Prism 5 software (GraphPad, San Diego, CA) was used for statistical analyses. Data are expressed as the mean ± SD. An unpaired Student's *t*-test was used to compare numerical data between two groups, while one-way analysis of variance (ANOVA) was used to compare data from multiple groups. The significant differences between groups in the clinical score, body weight, and anti-AChR antibody level were analyzed with two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test. A two-tailed P value of <0.05 was considered statistically significant.

3. Results

3.1. Metformin attenuates EAMG disease progression

The EAMG rat model was induced by the R-$AChR_{97\text{-}116}$ peptide as described [34]. To evaluate the therapeutic potential of metformin on EAMG disease, metformin dissolved in saline or saline only was orally administered daily until the end of this study (gavage, 100 mg/kg) starting from the first immunization. Clinical scores and body weight were monitored every other day, and the survival rate of all rats was also recorded. Compared with the EAMG group, an obvious alleviation of clinical disease scores and a delay in the onset of the disease (Fig. 1A) were observed in the 100 mg/kg metformin administration group (*, $P < 0.05$). Body weight loss, which is accompanied by clinical symptoms, was also reversed by 100 mg/kg metformin treatment (Fig. 1B, *, $P < 0.05$). Meanwhile, the survival rate was also significantly elevated after 100 mg/kg metformin administration (Fig. 1C, *, $P < 0.05$). These data implied that metformin had good therapeutic potential in

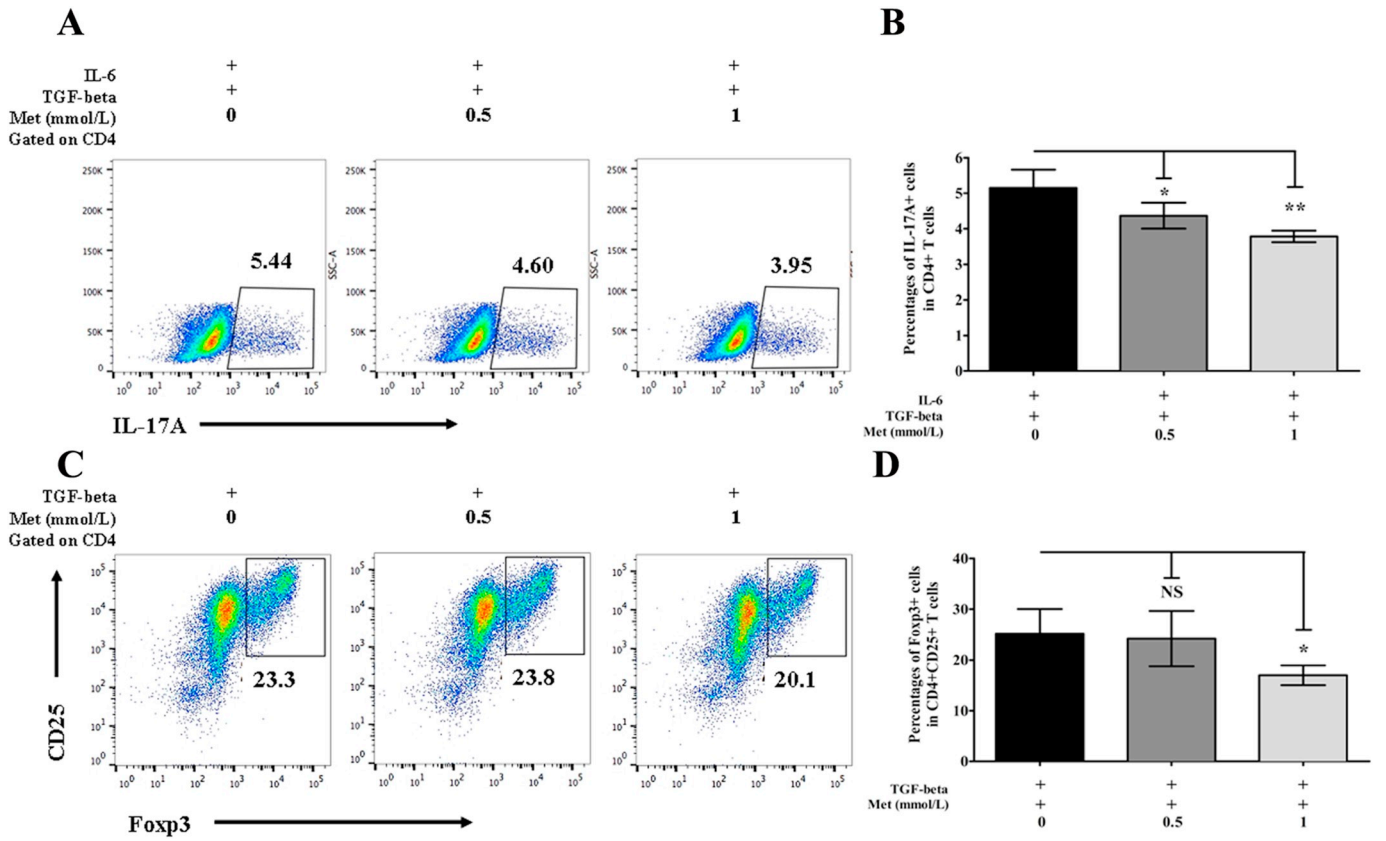

Fig. 5. Metformin could modulate the differentiation of AChR-specific-Th17 and Treg subsets *in vitro*. Flow cytometry analysis illustrated the percentages of interleukin-17A (IL-17) expression (A), CD25 and forkhead box P3 (Foxp3) expression (C) in $CD4^+$ T cells in the presence of various concentrations of metformin. Graphs depict the mean ± SD of the percentages of Th17 cells (B) and Treg cells (D) in the lymph nodes obtained from four symptomatic EAMG rats, *, $P < 0.05$, ** P, < 0.01, NS, no significant difference.

attenuating EAMG disease progression.

3.2. Effects of metformin on Th subtypes in vivo

T lymphocytes of spleens and lymph nodes from different groups of EAMG rats were collected for the detection of the effects of metformin on Th subtypes *in vivo*. The data we obtained are described as follows: both IFN-γ- and IL-4-secreting $CD4^+$ T cells, known as Th1 and Th2, were hardly changed after metformin treatment (Fig. 2A–D, NS, no significant difference), while Th17 cells, which are classified by the specific production of IL-17, were significantly downregulated in metformin-treated rats (Fig. 2E–F, *, $P < 0.05$). Although we did not observe any obvious changes in the proportion of regulatory T cells ($CD4^+CD25^+Foxp3^+$Treg) (Fig. 2G–H, NS, no significant difference), the decrease in Th17 cells overall, resulted in an increase in the Treg/Th17 ratio, which may also contribute to the alleviation of this disease, since the shift in the balance between Th17 cells and Treg cells is an important feature of autoimmune diseases; hence, the derailed Treg/Th17 balance may contribute to disease progression and therefore could function as a prognostic marker.

3.3. Metformin inhibits autoantibody production with no effect on blood glucose levels

The hallmark of EAMG disease is the presence of autoimmune antibodies in the serum, leading to tissue damage [2]. Therefore, we tested the effect of metformin on the levels of AChR-specific autoimmune antibody in the serum. As shown in Fig. 3A–B, metformin significantly decreased the serum level of anti-AChR antibody on both the 12th day and 36th day after the initial immunization (*, $P < 0.05$, **, $P < 0.01$). Although metformin has been widely used to control blood glucose in type 2 diabetes, it has been reported that it has no effect on blood glucose levels in nondiabetic human subjects. In our test, the irrelevance of the blood glucose level and EAMG disease was concluded because no difference was observed in blood glucose levels between CFA and EAMG rats. Additionally, metformin treatment attenuated EAMG disease progression with no effect on blood glucose levels of EAMG rats (Fig. 3C, NS, no significant difference).

3.4. Metformin inhibits lymphocyte proliferation by inducing cell apoptosis and cell death

To evaluate the effects of metformin on AChR-specific lymphocytes, an *in vitro* assay was first conducted to clarify the dosage of metformin. Total splenic cells were collected and stimulated by AChR peptide with different concentrations of metformin. After 72 h of incubation, 10 μL of CCK-8 was added to each well to detect the effect on proliferative capacity. The data in Fig. 4A show that 0.5 mM and 1 mM had no effect on cell proliferation, while dosages of 2 mM and higher could inhibit cell proliferation (*, $P < 0.05$, ***, $P < 0.001$) by inducing cell apoptosis and cell death (Fig. 4B).

3.5. Metformin modulates AChR-specific-Th17 and Treg differentiation by promoting the formation of ROS in mitochondria

As we know, a deficiency of helper T cells results in a dysfunction of antibody secretion in MG, implying an indispensable role of helper T cells in assisting antibody secretion of B cells. To explore whether metformin could modulate T cell differentiation *in vitro*, lymph node MNCs were obtained from symptomatic EAMG rats and cultured under

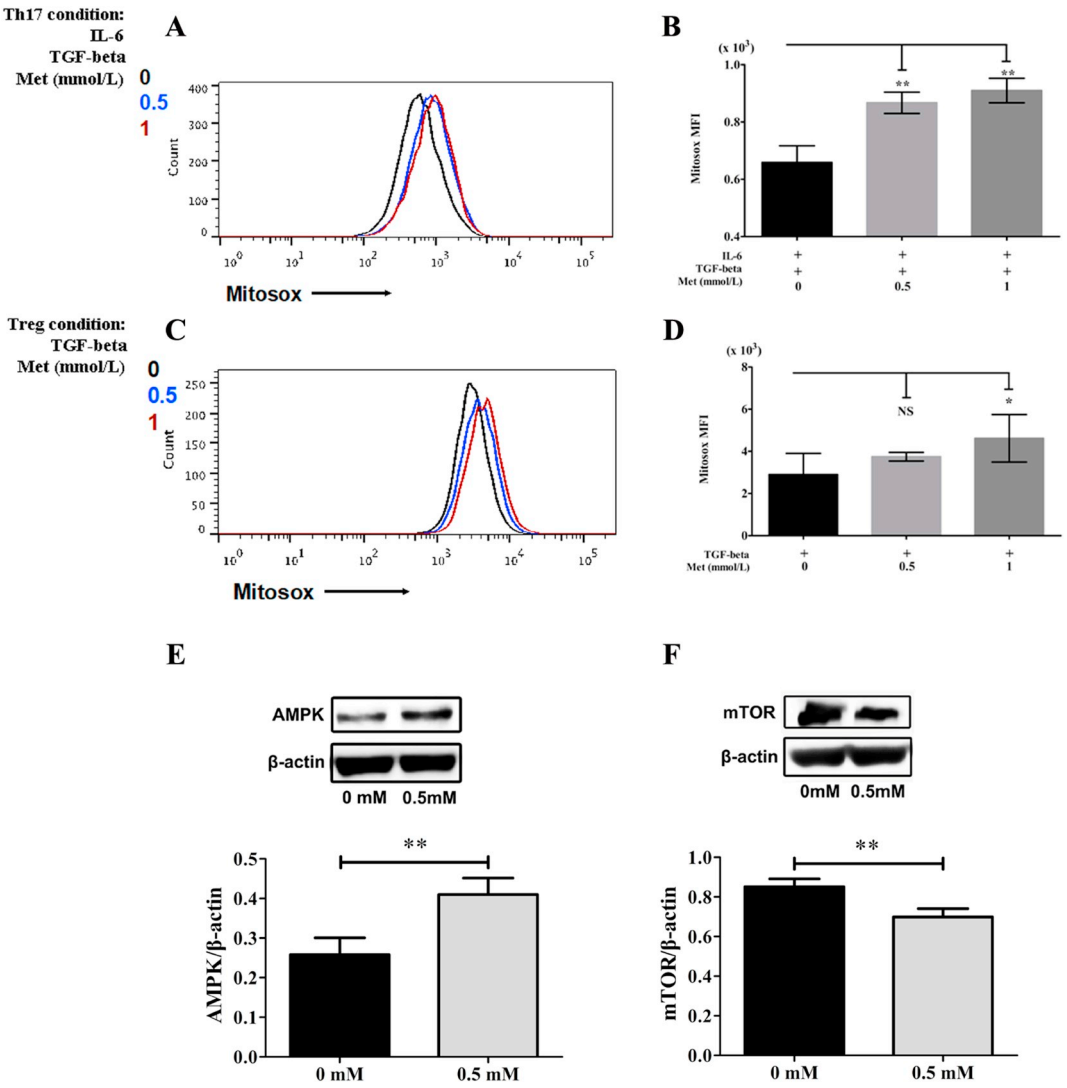

Fig. 6. Mitochondrial ROS accumulation in lymphocytes is affected by metformin. ROS levels were detected by flow cytometry after 3 days of polarization under Th17 (A) and Treg (C) polarized conditions in the presence of metformin. Histograms represent the mean ± SD of the mean fluorescence intensity (MFI) for MitoSOX in Th17 (B) and Treg (D) polarized conditions from three independent experiments. AMPK (E) and mTOR (F) protein levels were detected by western blot assay from two independent experiments, *, $P < 0.05$, **, $P < 0.01$, NS, no significant difference.

Th17-inducing conditions or Treg-inducing conditions in the presence of metformin (0.5–1 mM) or vehicle (dimethyl sulfoxide). Differentiation of IL-17-expressing $CD4^+$ T cells (Th17 cells) was significantly suppressed by metformin regardless of dose (Fig. 5A–B, *, $P < 0.05$, **, $P < 0.01$), whereas $CD4^+CD25^+Foxp3^+$ Treg cells were unaffected in the presence of 0.5 mM metformin and decreased in the presence of 1 mM metformin (Fig. 5C–D, *, $P < 0.05$). These results indicated that metformin could regulate Th17 and Treg balance in a dose-dependent manner.

AMPK activation, which leads to increased mitochondrial production of ROS, is the primary pathway that is activated by metformin [37,38]. To determine whether metformin acted in part through ROS generation, ROS levels in mitochondria were examined in T cells that experienced Th17 or Treg skewing conditions. The results indicated that under Th17 polarized conditions, both 0.5 mM and 1 mM metformin increased MitoSOX staining, indicating a generation of ROS in mitochondria (Fig. 6A–B, **, $P < 0.01$), whereas only 1 mM metformin upregulated MitoSOX staining under Treg skewing conditions. The MitoSOX value under Treg skewing conditions showed little change with 0.5 mM metformin incubation (Fig. 6C–D, *, $P < 0.05$, NS, no significant difference). In addition, 0.5 mM metformin significantly activated the AMPK pathway (Fig. 6E, **, $P < 0.01$), resulting in a reduction of mTOR expression (Fig. 6F, **, $P < 0.01$). These data suggested that metformin induced ROS production that Tregs were programmed to manage (MET 0.5 mM), whereas Th17 cells were less able to handle ROS stress caused by activation of AMPK by metformin. These results are consistent with previous studies showing that Teffs may be more sensitive to ROS stress than Tregs [39,40].

3.6. Metformin directly inhibits the antibody production by B lymphocytes

Antigen-specific antibodies produced by B cells are mainly dependent on T cell help [3–5]. Although direct effects of metformin on T cells were observed in the above tests, we wanted to know if AChR-specific B cells could also be directly affected by metformin. As shown in Fig. 7A, B cell proliferation was significantly inhibited by 0.5 mM and above dosages of metformin in the CFSE assay (**, $P < 0.01$, ***, $P < 0.001$). The percentages of IgG2b-secreting B cells ($CD45R^+IgG2b^+$) were not altered by lower dosages (0.5 to 2 mM) of metformin addition, but elevated by 5 mM metformin due to the cytotoxicity of higher dosages of metformin on cell proliferation in Fig. 4A (Fig. 7B, NS, no significant difference, **, $P < 0.01$). Besides, the ratios

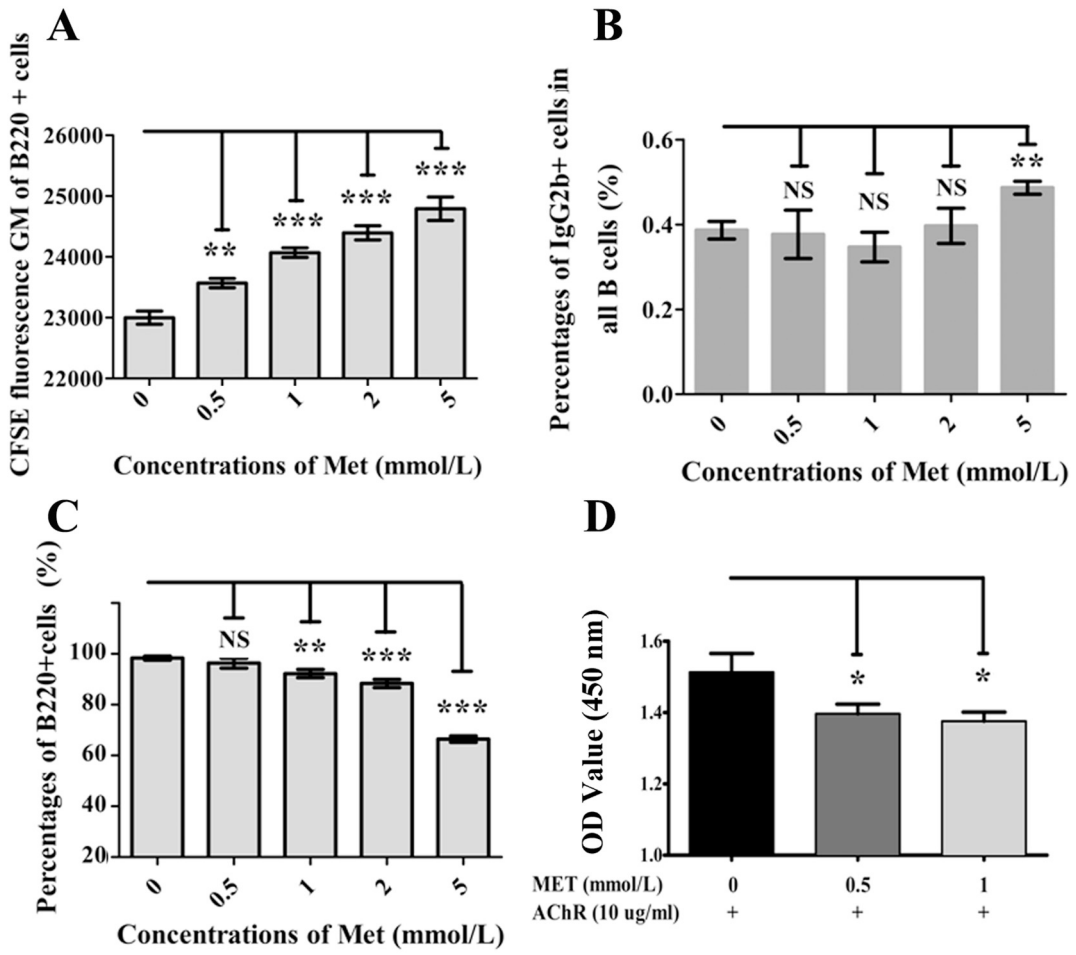

Fig. 7. Metformin restrains the antibody production by B lymphocytes. (A) The effect of metformin on B cell proliferation was measured by the CFSE assay. The graph represents the mean ± SD of the geomean fluorescence intensity (GM) of CFSE. (B–C) Flow cytometry analysis results of the percentages of $IgG2b^+$ B cells and $B220^+$ cells under various concentrations of metformin are shown on representative histograms. (D) Metformin inhibited AChR-specific antibody production by B cells *in vitro*. Data are shown as the mean ± SD from three independent experiments, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, NS, no significant difference.

of B cells ($CD45R^+$) were reduced by metformin (1 to 5 mM) in a dose-dependent manner (Fig. 7C, **, $P < 0.01$, ***, $P < 0.001$, NS, no significant difference), and a reduction of the total level of AChR-specific antibody secretion by B cells *in vitro* was also observed (Fig. 7D, *, $P < 0.05$).

4. Discussion

Metformin is the first line drug for newly diagnosed type 2 diabetic patients that has been shown to have novel pleiotropic actions on various regulatory properties, including cardio- and nephro-protection, as well as antiproliferative, antifibrotic, and antioxidant effects. Emerging evidence has demonstrated its ability to prolong healthspan and lifespan in mice, providing the basis for defining metformin as a potential anti-aging molecule. Moreover, the novel hypothesis that metformin can exhibit immune-modulatory features has also been supported *via* T cell regulation, revealing the candidate potential of metformin for immune-mediated diseases in clinic [33,37]. However, whether metformin has therapeutic roles in B cell-mediated disorders has not been verified thus far. Here, we demonstrated the contribution of metformin in preventing EAMG disease, a B cell-mediated autoimmune disease model of human MG. Our study observed a significantly relieved clinical severity and decreased mortality of EAMG disease. A reversal of the Th17/Treg imbalance by the activation of the AMPK pathway, accompanied by a significant inhibition of AChR-specific B cell function, which is connected with a favorable prognosis for MG patients, was observed in this article.

Metformin could inhibit the development of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis [41]. According to the Food and Drug Administration guidelines, the common dose of metformin ranges from 500 mg/day to 2500 mg/day in diabetic patients [42]. Thus, the oral administration dose of metformin we selected in our study (100 mg/kg/rat) was similar to that in humans, suggesting the clinical application of our study for MG. Metformin administration to R-$AChR_{97\text{-}116}$ peptide-induced EAMG rats significantly alleviated the clinical scores and body weight loss, prolonged the survival time and decreased anti-AChR antibody levels in serum, indicating the therapeutic effects of metformin on EAMG disease.

Regarding mechanism insight, we found that both 0.5 and 1 mM metformin treatment displayed immunoregulatory properties without nonspecific lymphocyte toxicity. Consistent data show that mitochondria are the main subcellular targets of metformin due to the selectively high drug accumulation in mitochondria, resulting in activation of the energy sensor 5′-AMP-activated protein kinase (AMPK) [43].

The pro-inflammatory cell subset, *i.e.*, neutrophils, M1 macrophages, and effector T cells, preferentially produce ATP through glycolysis, whereas cells with an anti-inflammatory lineage, *i.e.*, memory and regulatory T cells (Tregs) and M2 macrophages, favor mitochondrial ATP generation [44]. Studies have also confirmed that metformin inhibits Th17 differentiation by activating AMPK. T cells are key master players in the delicate equilibrium, and the imbalance of T cells leads to immune tolerance breakdown and autoimmunity [45]. In this study,

similar results were also observed wherein metformin inhibited TGF-β- and IL-6-dependent Th17 generation, followed by the downregulation of IL-17 expression. Only the treatment of iTreg throughout the induction with 1 mM metformin resulted in a downregulation of Foxp3. Of note, 0.5 mM metformin throughout the experiments did not result in the downregulation of Foxp3.

Reactive oxygen species (ROS) are byproducts of mitochondrial metabolism. Increased cellular metabolism is accompanied by an increase in ROS production. Mitochondria-derived ROS, as important second messengers in cell signaling, play an important role in regulating cell proliferation and cell differentiation. AMPK could be directly activated by mitochondrial ROS through the glutathionization of AMPKα and β subunits [29]. Mitochondrial ROS could also activate the transcription factor nuclear factor of activated T-cells (NFAT), promoting IL-2 production, which is essential for T cell activation [30].

In the ROS analysis in mitochondria of $CD4^+$ and $CD4^+CD25^+$ cells that have experienced the Th17 and iTreg induction processes, respectively, we observed that increased ROS is a hallmark of metformin treatment throughout the experiments. These results are consistent with a previous study showing that iTregs are more capable of handling ROS in cells [39,40]. The results also suggest that the effects observed *in vitro* may be caused by AMPK activation. The effect of metformin treatment in light of the main outcome of metformin treatment is AMPK activation triggered by increased mitochondrial production of ROS [29]. The present study further reinforces the idea that altering T helper cell metabolism mediates the metformin-induced anti-inflammatory effect.

Antigen-specific autoantibody production by B cells in EAMG rats is dependent on aid from $CD4^+$ T helper cells [46]. Antigen-encountered and activated B cells interact with T cells in the secondary lymphoid organs and form germinal centers (GCs), followed by differentiation into high-affinity IgG antibody-producing plasma cells [47]. Th17 cells are indicated to be a distinct $CD4^+$ T cell subset responsible for the differentiation of autoreactive GC B cells and the humoral response *in vivo* [48]. We found that metformin attenuated antigen-induced Ig-G2b antibody production in AChR-specific recall responses in lymphocytes of EAMG rats, further supporting its anti-inflammatory properties. This result is consistent with previous research suggesting that metformin inhibits IgG production in B cells [33].

In summary, we observed significant amelioration of the severity of EAMG disease by metformin administration, and the relative mechanisms were that the ROS formation in mitochondria that was promoted by metformin inhibited the development of Th17 cells without limiting that of iTreg cells, resulting in a decrease in anti-AChR antibody levels. From another standpoint, metformin could also affect B cell antibody secretion directly. Metformin has already been known to be safe and minimally toxic in treating patients with various disorders. Our study supported that metformin might have novel therapeutic potential in the clinical treatment of MG and other autoantibody-mediated diseases.

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Declaration of competing interest

All authors claim that there are no conflicts of interest.

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