



Ivermectin Protects Against Experimental Autoimmune Encephalomyelitis in Mice by Modulating the Th17/Treg Balance Involved in the IL-2/STAT5 Pathway

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Abstract— Multiple sclerosis (MS), a T-cell-mediated autoimmune disease that affects the central nervous system (CNS), is characterized by white matter demyelination, axon destruction, and oligodendrocyte degeneration. Ivermectin, an anti-parasitic drug, has anti-inflammatory, anti-tumor, and antiviral properties. However, to date, there are no in-depth studies on the effect of ivermectin on the function effector of T cells in murine experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Here, we conducted *in vitro* experiments and found that ivermectin inhibited the proliferation of total T cells (CD3⁺) and their subsets (CD4⁺ and CD8⁺ T cells) as well as T cells secreting the pro-inflammatory cytokines IFN- γ and IL-17A; ivermectin also increased IL-2 production and IL-2R α (CD25) expression, which was accompanied by an increase in the frequency of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg). Importantly, ivermectin administration reduced the clinical symptoms of EAE mice by preventing the infiltration of inflammatory cells into the CNS. Additional mechanisms showed that ivermectin promoted Treg cells while inhibiting pro-inflammatory Th1 and Th17 cells and their IFN- γ and IL-17 secretion; ivermectin also upregulated IL-2 production from MOG_{35–55}-stimulated peripheral lymphocytes. Finally, ivermectin decreased IFN- γ and IL-17A production and increased IL-2 level, CD25 expression, and STAT5 phosphorylation in the CNS. These results reveal a previously unknown etiopathophysiological mechanism by which ivermectin attenuates the pathogenesis of EAE, indicating that it may be a promising option for T-cell-mediated autoimmune diseases such as MS.

KEY WORDS: ivermectin; T cells; autoimmune diseases; multiple sclerosis; CD4⁺ T cell subsets.

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) that leads to demyelination, axon destruction, and oligodendrocyte degeneration [1]. Clinical manifestations vary depending on the location of the CNS lesion. Symptoms of MS include sensory and visual disturbances as well as motor and coordination disturbance. In addition to

spasticity, fatigue, pain, and cognitive deficits [2], the following major causes have been identified for MS as follows: cigarette smoking, female genders, and viral infection [3]. However, the exact etiology remains unknown. Currently, MS is considered to be caused by shifts in the ratio of various CD4⁺ T helper (Th) cell subsets, including an increase in pro-inflammatory Th1 and Th17 cells and a decrease in anti-inflammatory Treg and Th2 cells [4]. Therefore, an immunomodulatory strategy is crucial for patients with MS. However, drugs or antibodies with immunomodulatory effect have severe adverse effects, including immunosuppression, on patients with MS. Therefore, the development of new therapies that are both effective and selectively immunosuppressive is a major challenge for the treatment of MS.

Ivermectin, a broad-spectrum anti-parasitic medicine, has anti-inflammatory, anti-cancer, and anti-viral effects [5–9]. There is evidence that ivermectin may exert its biological effects by binding to ligand-gated ion channels, various ion transporters, or the nuclear receptor farnesoid X receptor (FXR) [10, 11]. Although ivermectin has a low FXR representation in mammals, it is well tolerated and has few side effects [12]. Moreover, ivermectin has been shown to stabilize the open state of P2X4 receptor channels [13], which bind to extracellular adenosine triphosphate [14] and are involved in neurodegenerative diseases [15]. All these highlight the additional effect of ivermectin on immune cells, particularly on receptors involved in diseases.

Interestingly, Blakley reported the immunosuppressive function of ivermectin in mice and indicated that the immunostimulatory properties of ivermectin are related to Th cell subsets [16]. Most importantly, ivermectin can alleviate experimental autoimmune encephalomyelitis (EAE) by regulating microglial activity [17]. However, the effect of ivermectin on T cell function and regulation of inflammation has not yet been demonstrated. Given the importance of CD4⁺ T cells in MS, we further investigated the role of ivermectin in effector T cell function using mitogen- or autoantigen-stimulated T cells from naive or EAE mice.

MATERIALS AND METHODS

Animals

Specific pathogen-free female C57BL/6 mice, aged 6–8 weeks, were obtained from the Nanjing Biomedical Research Institution of Nanjing University (Nanjing,

China). Mice were fed unlimited food and water and kept on a 12:12 h light/dark cycle. The Institutional Animal Care and Use Committee of Huaihe Hospital at Henan University approved all animal experiments, and the animals were handled in accordance with the National Institutes of Health regulation for the care and use of laboratory animals.

EAE Induction and Ivermectin Treatment

The EAE model was induced, as described previously [18]. Briefly, mice were immunized subcutaneously in the flanks with 200 µg of the MOG_{35–55} peptide emulsified in 200 µL of Complete Freund's Adjuvant (Sigma-Aldrich, St. Louis, MO), followed by an intraperitoneal (i.p.) of 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) on days 0 and 2. Mice were administered with ivermectin (MCE LLC, Shanghai, China) (mixed with 1% carboxymethylcellulose sodium solution) at doses of 0, 2, or 10 mg/kg per os (p.o.) once every 2 days from day 0 until euthanasia after EAE induction. The following clinical scoring system was developed: 0: no signs; 0.5: partial tail paralysis; 1: limp tail or tail paralysis; 2: complete loss of tail tonicity or abnormal gait; 3: partial hind limb paralysis; 4: complete hind limb paralysis; and 5: moribund.

Cell Proliferation

Splenocytes from naive mice were isolated and labeled with 5-µM Cell Proliferation Dye eFluor™ 450 (eBiosciences, San Diego, CA) and then co-cultured for 72 h with concanavalin A (ConA, Sigma-Aldrich, St. Louis, MO) at 1.5 µg/mL concentration in the presence of ivermectin (0, 0.5, 1, and 2 µM). The cells were then collected and stained with fluorescent-conjugated anti-CD3, anti-CD4, and anti-CD8 Abs (all from eBiosciences). Cell Proliferation Dye eFluor™ 450 is a violet fluorescent dye that attaches to cellular proteins containing primary amines. With each cell division, the dye is distributed evenly among daughter cell populations. Furthermore, on day 14, lymphocytes from draining lymph nodes of mice with EAE were stimulated for 72 h with the MOG_{35–55} peptide at 50 µg/mL concentration. After that, the cells were collected, blocked, and stained with fluorescence-conjugated anti-CD4 Ab and then fixed/permeabilized using the Mouse Foxp3 Buffer Set (BD Biosciences) and stained with fluorescent-conjugated anti-Ki-67 Ab (eBiosciences). Cell proliferation was measured with a

BD FACS Canto Plus flow cytometer (San Diego, CA), and the data were analyzed with Flowjo 10.0 software (Tree Star).

Flow Cytometry Analysis

Splenocytes from naive mice were co-cultured at the density of 3×10^6 cells/mL in 24-well plates for 72 h with ConA in the presence of ivermectin. The cultured cells were collected, blocked with anti-CD16/CD32 Ab (BD Biosciences), and stained with fluorescent-conjugated anti-CD4 and anti-CD25 Abs. After that, the cells were fixed/permeabilized using the Mouse Foxp3 Buffer Set (BD Biosciences), and stained with fluorescent-conjugated anti-Foxp3 Ab (eBiosciences). Additionally, other fresh cells from EAE mice were restimulated with Cell Stimulation Cocktail plus protein transport inhibitors (eBiosciences) for 4 h. The stimulated cells were collected, blocked, and stained with fluorescent-conjugated anti-CD4 Abs. Subsequently, they were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Bioscience), and stained with fluorescence-labeled antibodies for each cytokine tested. In addition, on day 14, lymphocytes from draining lymph nodes were obtained from EAE mice and stained with different fluorescent-conjugate anti-CD4, anti-CD25, and anti-Foxp3 Abs using the Mouse Foxp3 Buffer Set or stimulated with MOG₃₅₋₅₅ for 68 h and then restimulated with Cell Stimulation Cocktail plus protein transport inhibitor (BD Biosciences). Finally, the cells were subjected to intracellular staining. The cells were then acquired with a BD FACS Canto Plus flow cytometer, and the data were analyzed using Flowjo 10.0 software.

Cytokine Production

Splenocytes from naive mice were cocultured with ConA in 24-well plates for 72 h in the presence of ivermectin at the indicated doses. Cell-free supernatants were then collected to measure the levels of IL-2, IFN- γ and IL-17A by using their corresponding ELISA kits (IL-2 and IFN- γ kits: BD Biosciences; IL-17A kit: eBiosciences). Additionally, on day 14, lymphocytes from draining lymph nodes of mice with EAE were stimulated for 72 h with the MOG₃₅₋₅₅ peptide and then the cell-free supernatants were collected to measure the levels of IL-2, IFN- γ , and IL-17A. Lastly, on day 14, the brain and spinal cord of EAE mice were collected and lysed to measure IL-2, IFN- γ , and IL-17A levels, as described previously [19].

Histopathological and IHC Analysis

After 30 days of EAE induction, mice were euthanized with CO₂ and then perfused with an intracardiac infusion of 10% formalin. The spinal cord was removed and fixed with 10% paraformaldehyde. The fixed spinal cord was embedded in paraffin, and cross-sections were stained with hematoxylin and eosin (H&E) to assess infiltration of inflammatory cells and stained with Luxol fast blue (LFB) to assess CNS demyelination. The levels of inflammatory cell infiltration were categorized as follows: no cells were assigned a severity level of 0; a few scattered cells were assigned a severity level of 1; organization of infiltrates near blood vessels were assigned a severity level of 2 and substantial perivascular cuffing with expansion into surrounding parenchyma or parenchymal infiltration without evident cuffing were assigned a severity level of 3 [20]. The degree of demyelination was graded as 1 for traces of subpial demyelination, 2 for marked subpial and perivascular demyelination, 3 for confluent perivascular or subpial demyelination, 4 for massive perivascular and subpial demyelination involving one half of the spinal cord with the presence of cellular infiltrates into CNS parenchyma, and 5 for extensive perivascular and subpial demyelination involving the entire section of the cord with the presence of cellular infiltration into CNS parenchyma [21].

Immunohistochemical single stainings using 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) were routinely conducted on formalin-fixed paraffin-embedded tissue sections that had been deparaffinized and rehydrated. To retrieve antigens for immunohistochemistry, the tissue sections were steamed in either 10-mM citrate buffer (pH6.0) or 1-mM EDTA in 10-mM Tris buffer (pH8.6) for 1 h. Primary antibodies for MBP (1:300), Iba1 (1:500), CD45 (1:300), and CD68 (1:250, all from Servicebio®, Wuhan, China) were allowed to incubate overnight at 4 °C. HRP-conjugated secondary antibodies were each applied at room temperature for 1 h. Finally, DAB development was carried out. Under a microscope observation, the inflammatory cell count (Iba1, CD45, and CD68) and demyelinated white matter percentage were determined using the Image-Pro Plus software.

Western Blotting Assay

On day 14, EAE mice were euthanized with CO₂ overdose; their spinal cord was collected and lysed with a RIPA lysis buffer containing PMSF and phosphatase inhibitors.

The lysates were centrifuged, and the supernatants were collected for determination of concentration protein using a BCA assay. The same amount of protein was separated on 10% SDS-PAGE gels and then transferred to the nitrocellulose membrane. The membrane was blocked before incubation with the following primary antibodies: phosphor-STAT5 (1:1000) (Cell Signaling Technologies, Danvers, MA) and β -actin (1:5000) (Sigma-Aldrich). The membrane was then incubated with HRP-conjugated secondary antibodies. Detection was performed using ECL reagents. The β -actin staining of each lane was used as a loading control and as a normalization factor for the optical density of the band.

Quantitative Real-time PCR

Total RNA was extracted from the brain and spinal cord by using a modified chloroform/phenol method (TRIzol™, Invitrogen, Carlsbad, CA), and the first strand of cDNA was generated using the Universal RT-PCR Kit (M-MLV) (Solarbio, Beijing, China) in accordance with the manufacturer's protocol. The obtained cDNA was then amplified by PCR in triplicate by using the Universal SYBR Green Fast qPCR Mix (Abclonal, Wuhan, China) and the following specific primers: CD25-forward: 5'-GAAGTGTGGGAAAACGGGGT-3', CD25-reverse: 5'-GGCAGGAAGTCTCACTCTCG-3'; CD45-Reverse: 5'-GTGCCTCTCCAGATTTCCCC-3', CD45-reverse: 5'-TGTCCACATGACTCCTTTCC-3'; CD68-forward: 5'-TGATCTTGCTAGGACCGCTTA-3'; CD68-reverse: 5'-TAACGGCCTTTTTGTGAGGA-3'; Iba1-forward: 5'-GGATTTGCAGGGAGGAAAA-3', Iba1-reverse: 5'-TGGGATCATCGAGGAATTG-3'; myelin basic protein (MBP)-forward: 5'-GCTTCTGGAGGGTGATGCC-3'; MBP-reverse: 5'-CCAAGAGTCGTCAGGTCATAG-3'; β -actin-forward: 5'-CTTTCCAGCCTTCCTTCTTGG-3', β -actin-reverse: 5'-CAGCAC TGTGTTGGCATAGAGG-3'. The $2^{-\Delta\Delta CT}$ method was used to determine the expression of CD25 mRNA normalized to β -actin mRNA, as described previously [22].

Statistical Analysis

All data were expressed as mean \pm SEM. Graph-Pad Prism 8.0 was used for statistical analysis. Statistical differences were determined by using one-way ANOVA, followed by Tukey's test for multiple comparisons, or a non-paired Student's *t*-test. Statistical significance was defined as a *P*-value < 0.05 .

RESULTS

Ivermectin Inhibited T Cell Proliferation *In Vitro*

To investigate the role of ivermectin in T cell proliferation, we cultured naive splenocytes labeled with the cell proliferation dye eFlour™ 450 in the presence of ivermectin and ConA. Similar with the findings of Ventre [23], we observed that ivermectin did not affect cell viability at the indicated doses (Fig. 1a), but it significantly inhibited the proliferation of total T cells (Fig. 1b, c), CD4⁺ T cells (Fig. 1b, d), and CD8⁺ T cells (Fig. 1b, e) at 2.0 μ M, but not at 0.5 and 1.0 μ M.

Ivermectin Inhibited IFN- γ and IL-17A Production While Increasing IL-2 Production in ConA-Stimulated Splenocytes

To determine the effect of ivermectin on the secretion of pro-inflammatory cytokines by T cells, we cultivated naive splenocytes in the presence of ivermectin and ConA. As shown in Fig. 2, ivermectin dose-dependently suppressed secretion of IFN- γ and IL-17A, from Th1 and Th17 cells, respectively, in ConA-treated splenocytes from naive mice. Surprisingly, ivermectin treatment at 2.0 μ M increased the production of IL-2 by ConA-treated naive splenocytes. All these findings suggest that ivermectin may selectively affect Th cells that selectively secrete cytokines.

Ivermectin Promoted CD25 Expression and Treg Cell Proportions

IL-2 is an important growth factor for maintaining T cell activation and clonal expansion by binding its receptors. To better understand the mechanisms underlying the effect of ivermectin, we measured IL-2 receptor (IL-2R) α -chain (CD25) and CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) in ConA-stimulated splenocytes. We found that ivermectin at 2.0 μ M, but not at 0.5 and 1.0 μ M, increased the expression of CD25, a middle/late activation marker of T cells, in total T cells (Fig. 3a), CD4⁺ T cells (Fig. 3b), and CD8⁺ T cells (Fig. 3c) following activation of naive splenocytes, indicating that ivermectin may promote middle/late T cell activation. Furthermore, an interesting finding was that ivermectin administration increased the proportion of Treg in splenocytes treated with ConA (Fig. 3d).

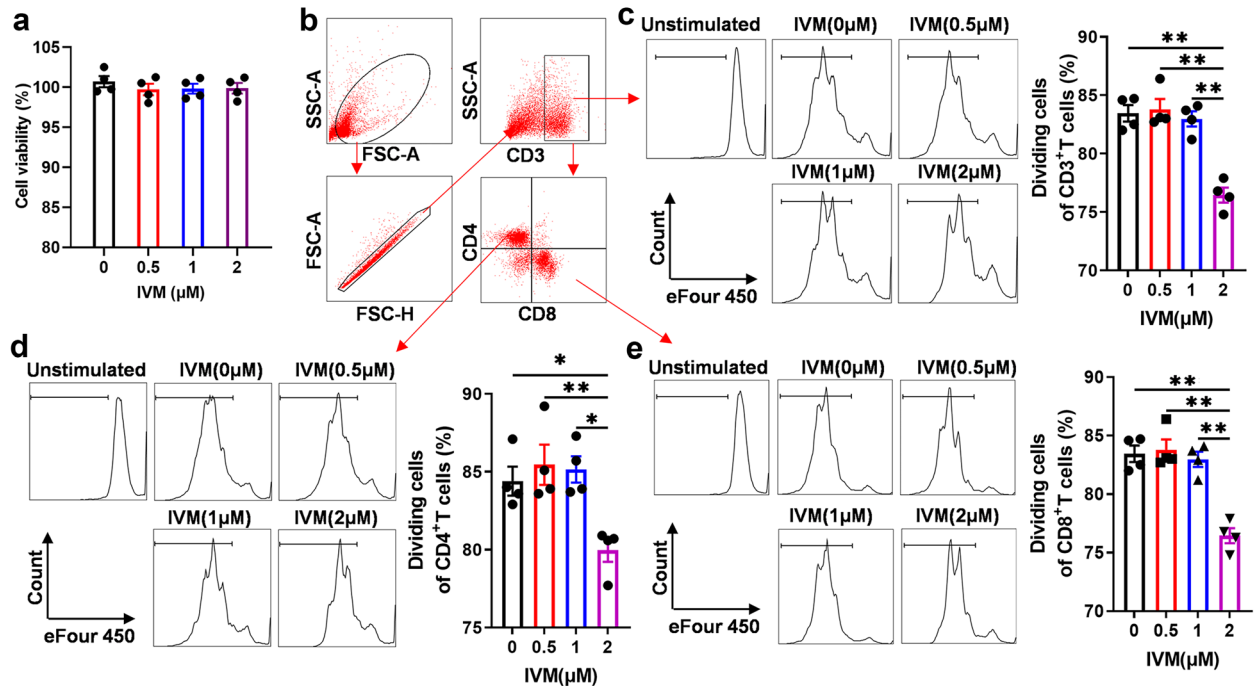


Fig. 1 Ivermectin inhibits T cell proliferation. **a** Naive mice splenocytes were incubated with ivermectin at 0, 0.5, 1, and 2 μM for 24 h, and cell viability was determined using a CCK8 kit. **b–e** Naive mice splenocytes were stained with the dye eFlour™ 450 and then co-incubated with ConA in the presence of ivermectin (0, 0.5, 1, and 2 μM) for 72 h. Cells were collected and then co-stained with fluorescent-conjugated anti-CD3, anti-CD4, and anti-CD8 Abs. Flow cytometry was used to classify (**b**) and obtain the proliferation of total T cells (**c**), CD4⁺ T cells (**d**), and CD8⁺ T cells (**e**) gated on CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ cells, respectively. Cell proliferation was assessed, as described in the “Materials and Methods” section. The values are expressed as mean \pm SEM, $n=4$. * $P<0.05$, ** $P<0.01$. IVM, ivermectin.

Ivermectin Alleviated Both Clinical Sign and CNS Destruction in EAE Mice

Next, we used a T-cell-mediated autoimmune disease model, the mice EAE, to determine the effect of ivermectin on effector T cell responses. As observed in a previous study [23], we noted that ivermectin at a dose of 10 mg/kg body weight attenuated the clinical signs of EAE (Fig. 4a, b). Histopathological analysis showed that administration of ivermectin at the same dose reduced infiltration of inflammatory cells (Fig. 4c) and demyelination of the spinal cord in EAE mice (Fig. 4d). Then, we assessed how 10 mg/kg ivermectin affected demyelination and infiltrating cells of CNS in EAE mice. Compared to those with EAE alone, the mice treated with ivermectin showed higher intensity of the MBP marker as demonstrated by immunostaining for MBP (Fig. 4e, f). This was confirmed through evaluation of MBP mRNA expression (Fig. 4h). Additionally, the ivermectin-treated

EAE mice showed a reduction in microglia, lymphocytes, and macrophages, as indicated by immunostaining for Iba1, CD45, and CD68 (Fig. 4e, g). This was further substantiated by evaluating the mRNA expression of these markers (Fig. 4h). These results indicated that ivermectin prevented CNS demyelination and infiltration of inflammatory cells.

Ivermectin Inhibited Autoantigen Myelin-Induced Specific T Cell Proliferation in EAE Mice

To assess whether ivermectin alleviates the clinical symptoms of EAE through its effect on autoantigen reactive T cell response, we examined antigen-specific T cell proliferation in draining lymphocytes from EAE mice by flow cytometry. As shown in Fig. 5a, administration of 10 mg/kg ivermectin reduced MOG-induced CD4⁺ T cell proliferation *ex vivo*.

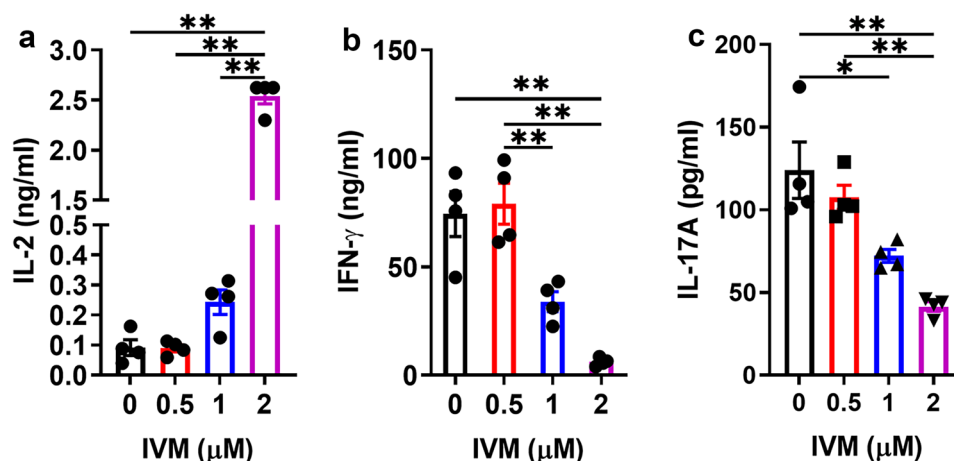


Fig. 2 Effect of ivermectin on cytokine production in response to ConA stimulation. Cell-free supernatants were obtained from naive mice splenocytes co-incubated with ConA for 72 h in the presence of the indicated concentrations of ivermectin. IL-2 (a), IFN-γ (b), and IL-17A (c) levels were then measured in the cell-free supernatants by using ELISA. The values are expressed as mean ± SEM, n=4. *P<0.05, **P<0.01. IVM, ivermectin.

Ivermectin Inhibited Th1/Th17 Cells While Promoting Treg Cells and IL-2 Production in EAE Mice

We then investigated whether the protective role of 10 mg/kg ivermectin in EAE development was mediated by changes in CD4⁺ T cell subsets. Draining lymphocytes from

EAE mice were obtained on day 14 post-immunization and restimulated with the MOG₃₅₋₅₅ peptide, followed by flow cytometry evaluation of Th1, Th17, and Treg cells and CD25 expression as well as ELISA-based evaluation of the secretion of the cytokines IFN-γ, IL-17A, and IL-2 by T cells. Ivermectin treatment increased the

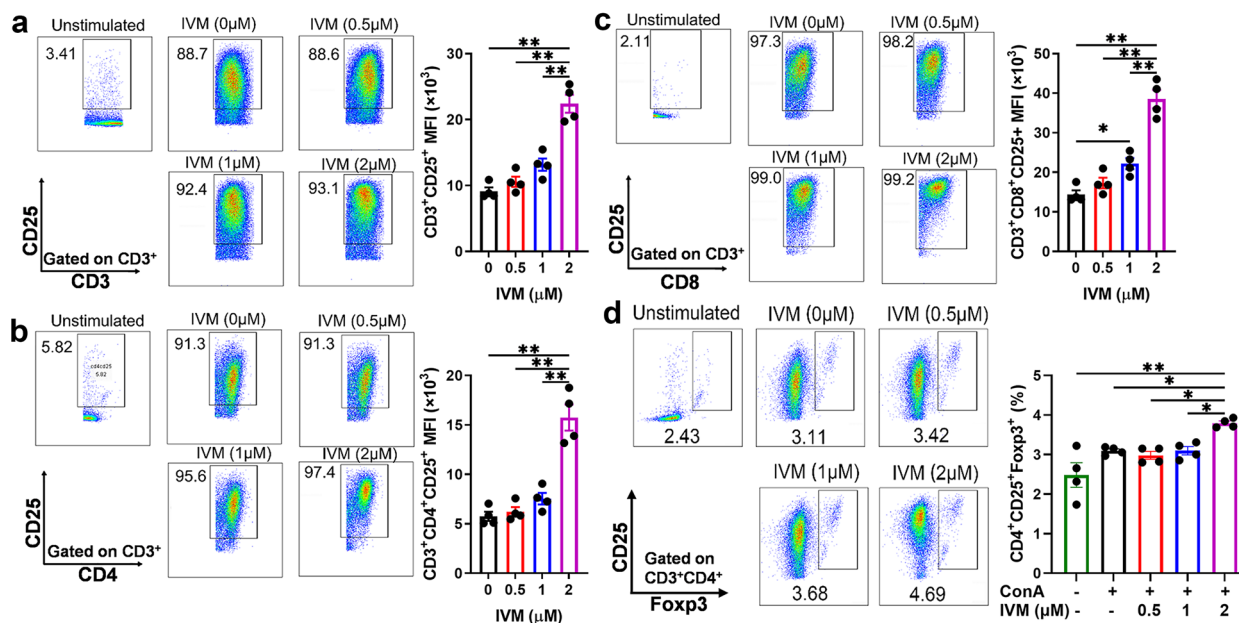


Fig. 3 Effect of ivermectin on CD25 expression and Treg cells in response to ConA stimulation. Naive mice splenocytes were co-incubated with ConA in the presence of the indicated concentrations of ivermectin for 48 h. The cells were collected to measure CD25 expression on CD3⁺ (a), CD4⁺ (b), CD8⁺ (c) T cells, and CD4⁺CD25⁺Foxp3⁺Treg cells (d) gated on CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, and CD3⁺CD4⁺CD25⁺ cells. The values are expressed as mean ± SEM, n=4. *P<0.05, **P<0.01. IVM, ivermectin; MFI, mean fluorescence intensity.

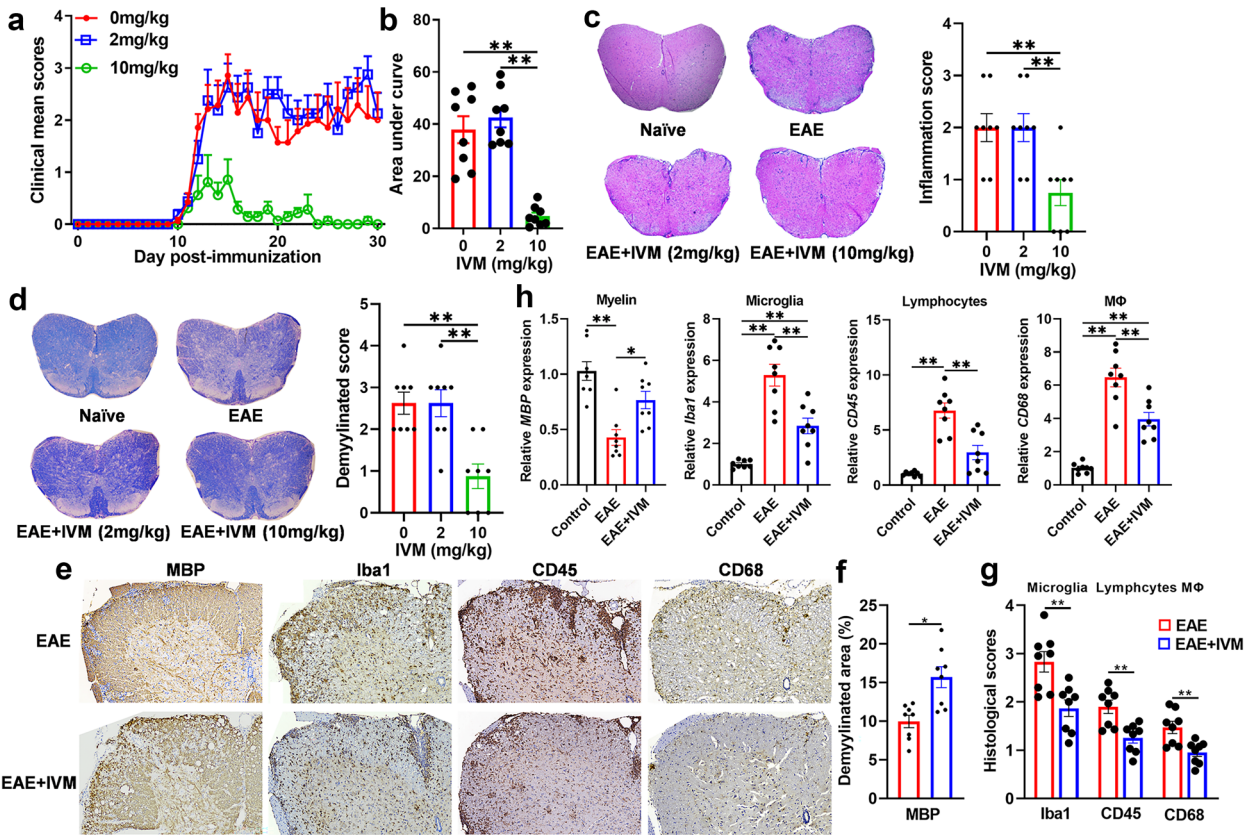


Fig. 4 Ivermectin alleviates the clinical symptoms of EAE. Female C57BL/6 mice were administered ivermectin at the doses of 0, 2, or 10 mg/kg p.o. once every 2 days from day 0 until euthanasia after EAE induction. **a** EAE clinical symptoms were monitored daily until the end of the experiment. **b** GraphPad Prism 8.0 software was used to calculate the area under the curve for clinical mean scores from (a). **c** and **d** On day 30 post-immunization, the spinal cord of EAE mice was collected and stained with H&E and LFB for the evaluation of inflammation and demyelination, respectively. Representative sections and statistical analysis for inflammation and demyelination score are shown in (c) and (d), respectively. **e–g** Representative images of MBP, Iba1, CD45, and CD68 immunostaining in spinal cords at day 30 from control EAE and IVM-treated EAE mice at day 30, and quantification of MBP, Iba1, CD45, and CD68 positively stained areas in spinal cords of IVM-treated and control group. **h** On day 14 post-immunization, the spinal cord of EAE mice was collected and lysed to MBP (myelin), Iba1 (microglia), CD45 (lymphocytes), and CD68 (macrophage, MΦ) mRNA expression by real-time PCR. Data are displayed as mean \pm SEM ($n = 8/\text{group}$). * $P < 0.05$, ** $P < 0.01$.

proportion of CD4⁺CD25⁺ T cells (Fig. 5b) and Treg cells (Fig. 5c) but decreased the proportion of pathogenic Th1 (Fig. 6a, b) and Th17 (Fig. 6a, c) cells as well as their IFN- γ (Fig. 6e) and IL-17A (Fig. 6f) secretion. However, a significant increase of IL-2 was observed in EAE mice treated with 10 mg/kg ivermectin.

Ivermectin Ameliorates Neuroinflammation in EAE Mice

Secretion of IFN- γ and IL-17 by CNS-infiltrating T cells is critical for EAE development [24, 25]. To assess the protective effect of 10 mg/kg ivermectin on EAE development, we collected brain and spinal cord from

EAE mice at day 14 after MOG immunization to measure IFN- γ and IL-17A levels (Fig. 7). In addition to lowering IFN- γ and IL-17A levels in the brain, ivermectin inhibited IFN- γ , but not IL-17A, in the spinal cord.

Ivermectin Exerts Its Protective Effect Through the STAT5 Pathway

Signal transducers and activators of transcription 5 (STAT5) play a key role in mediating cell response following the stimulation with cytokines, interferons, and growth factors through various mechanisms [26]. STAT5 can promote Treg cell development while inhibiting Th17

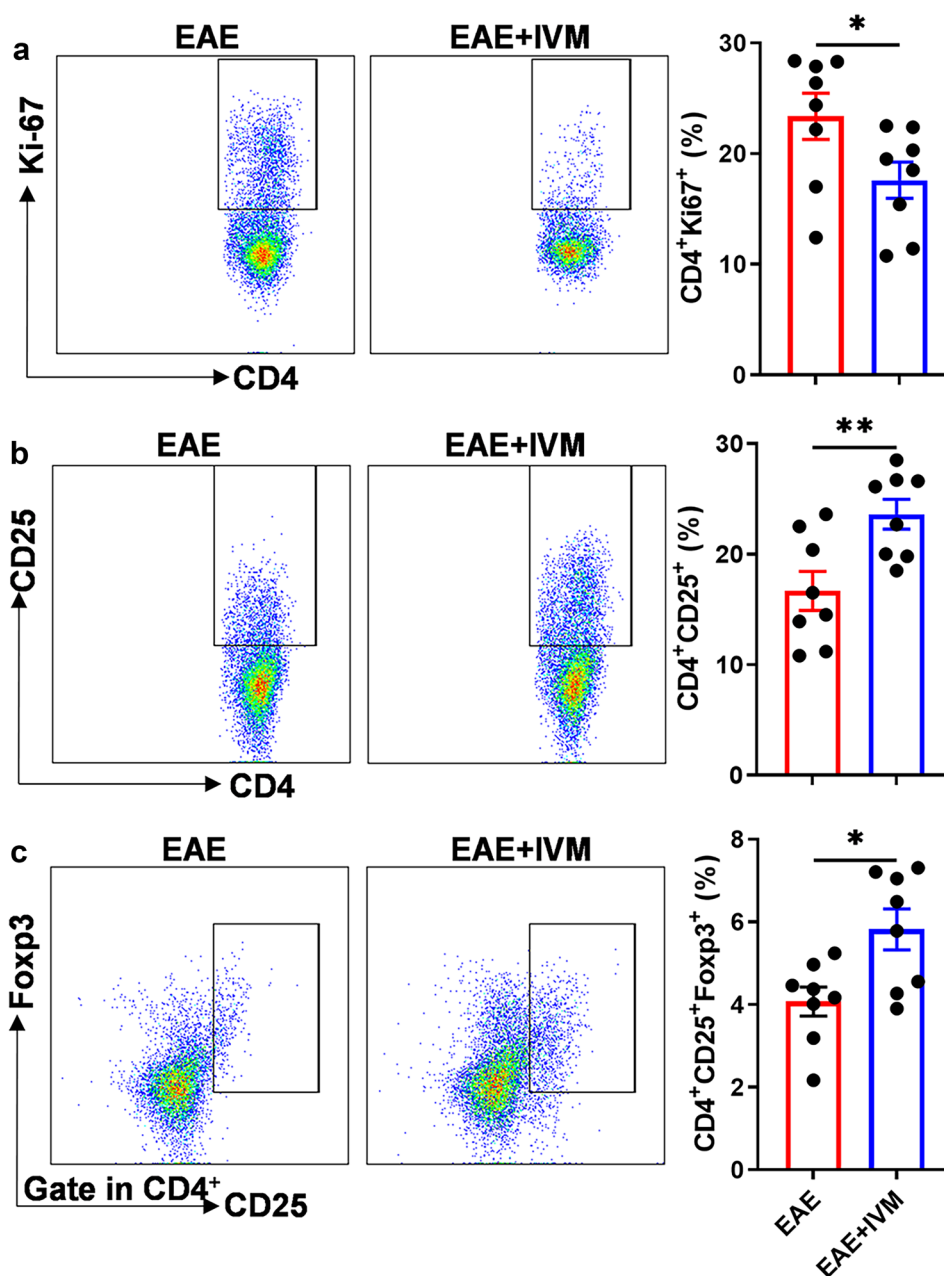


Fig. 5 Ivermectin inhibits autoantigen-specific CD4⁺ T cell proliferation while increasing CD25 expression and number of Treg cells. **a** Draining lymphocytes from EAE mice were collected on day 14 post-immunization and restimulated with the MOG₃₅₋₅₅ peptide for 72 h to determine T cell proliferation by using co-staining with fluorescent-conjugated anti-CD4 and anti-Ki-67 antibodies, as described in the “Materials and Methods” section. Representative flow cytometry data showed the percentage of Ki-67 gated on CD4⁺ T cells of two groups (a, left panel). Summary of the CD4⁺Ki-67⁺ population (a, right panel). **b** and **c** Freshly isolated lymphocytes were multi-stained with fluorescent-conjugated anti-CD4, anti-CD25, and anti-Foxp3 Abs to determine CD25 expression (b) in CD4⁺ T cells and Treg cells (c). Representative flow cytometry data showed the percentage of CD25 (b, left panel) and Treg (c, left panel) gated on CD4⁺ and CD4⁺ CD25⁺ cells of two groups. Summary of the CD4⁺CD25⁺ (b, right panel) and Treg population (c, right panel). Data are expressed as mean ± SEM (n = 8/group). A significant difference was determined by the unpaired *t*-test. **P* < 0.05, ***P* < 0.01. IVM, ivermectin.

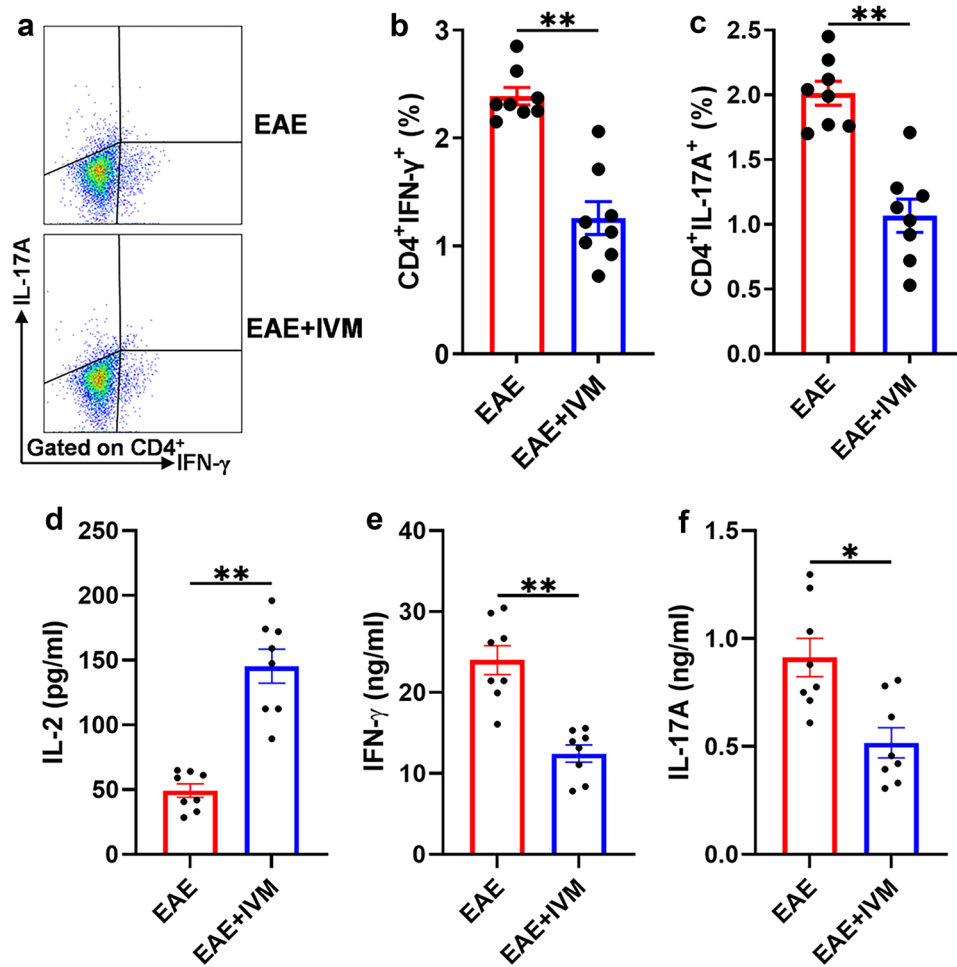


Fig. 6 Ivermectin suppresses autoantigen-specific Th1 and Th17 cells while increasing IL-2 levels. Lymphocytes from draining lymph nodes of EAE mice were collected on day 14 post-immunization and restimulated with Cell Stimulation Cocktail plus protein transport inhibitors₅₋₅₅ for 4 h to determine Th1 (IFN- γ -producing CD4⁺ T cells) and Th17 (IL-17A-producing CD4⁺ T cells) cells by flow cytometry. Representative flow cytometry data showed Th1 and Th17 cells gated on CD4⁺ T cells of two groups (a). Summary of the Th1 (b, right panel) and Th17 population (c, right panel). d–f ELISA was used to determine the IL-2 (d), IFN- γ (e), and IL-17A (f) level in the cell-free supernatants from lymphocytes of mice with EAE that had been stimulated for 72 h with the MOG₃₅₋₅₅ peptide. The values are expressed as mean \pm SEM ($n=8$ /groups). * $P<0.01$, ** $P<0.001$. IVM, ivermectin.

cell differentiation through the transmission of the IL-2/IL-2R signals [27]. Because 10 mg/kg ivermectin promotes IL-2 production and CD25 expression in T cells, we hypothesized that STAT5 phosphorylation is involved in the protective effect of ivermectin in EAE development. To confirm this hypothesis, we first examined the IL-2 level and CD25 mRNA expression level in the brains of EAE mice and found that ivermectin increased IL-2 production (Fig. 8a) and CD25 expression (Fig. 8b) in the brains of EAE mice. Subsequently, the expression of phosphorylated STAT5 expression in mouse brain was

measured using a Western blotting assay. As shown in Fig. 8c, EAE mice exhibited lower p-STAT5 expression in their brain than naive mice; however, this effect was reversed by ivermectin administration.

DISCUSSION

Ivermectin has recently attracted considerable attention following its promising results against SARS-CoV-2, such as the alleviation of the symptoms of COVID-19

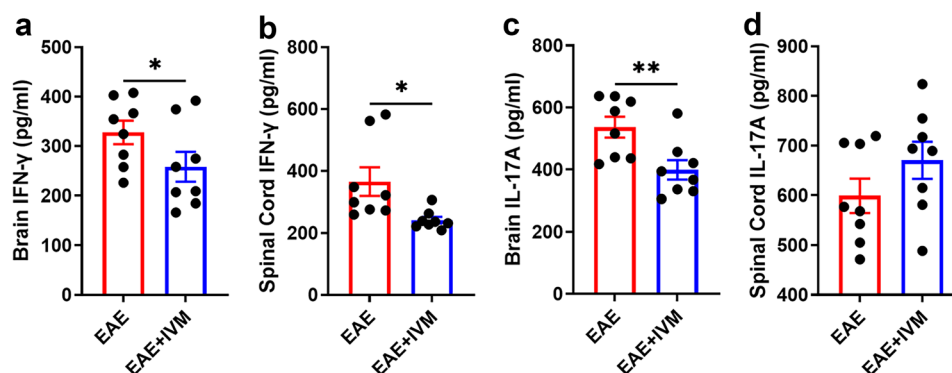


Fig. 7 Effect of ivermectin on IFN- γ and IL-17A levels in the brain and spinal cord. The brain and spinal cord were collected from EAE mice on day 14 post-immunization and lysed, as described in the “Materials and Methods” section to evaluate the levels of IFN- γ (a and b) and IL-17A (c and d) by conducting an ELISA assay. The values are expressed as mean \pm SEM ($n = 8/\text{group}$). * $P < 0.05$, ** $P < 0.01$. IVM, ivermectin.

patients and lowering of the inflammatory response in animal models [28, 29]. In addition, ivermectin can improve the clinical symptoms of EAE in part by inhibiting the activation of microglia [17]. Ivermectin may also reduce allergic skin inflammation by inhibiting antigen-specific T-cell proliferation and activation and cytokine production [23]. However, it remains unclear whether ivermectin regulates pathological T-cell responses in EAE mice. In the present study, we found that ivermectin inhibited T-cell proliferation and IFN- γ and IL-17A secreted by Th1 and Th17 cells *in vitro*, which might be supported by the protective effect of ivermectin on the progression of EAE by inhibiting the proliferation of pathological T cells and reducing Th1 and Th17 cells. Interestingly, ivermectin treatment increased IL-2 production, CD25 expression, and the number of Treg cells both *in vitro* and *in vivo*. Finally, we discovered that this effect could be mediated possibly through an increase in the phosphorylation of STAT5, the IL-2/IL2R transducer. Taken together, these results suggest that ivermectin has a broad suppressive effect on T cell-mediated immune function, including alteration of CD4⁺ T cell subsets.

IL-2 is a multi-functional cytokine that regulates T cell growth and proliferation, increases cytolytic activity, promotes Treg development, and triggers activation-induced cell death [30]. Therefore, IL-2 serves as an important immunomodulator for the balance between regulatory and effector T cells [31]. Blocking IL-2 can trigger and exacerbate autoimmune diseases [32]. STAT5, an important IL-2/IL-2R signal transducer, is required for Treg development. Thus, IL-2 promotes and maintains Treg cell development and function by increasing

STAT5 phosphorylation [33, 34]. IL-2 and phosphorylated STAT5, however, can inhibit the differentiation of Th17 cells [35]. The development of Th17 cells can be enhanced by the deficiency of IL-2 or the disruption of its transcription factor STAT5 [36], while the administration of IL-2 can directly inhibit Th17 cell differentiation [37]. In the present study, ivermectin treatment resulted in a decrease of Th17 cells and concomitant increase in the secretion of IL-2 levels by T cells, CD25 expression level, and the proportion of Treg cells in peripheral lymphocytes both *in vitro* and *in vivo*. Ivermectin also inhibited pro-inflammatory cytokines such as IFN- γ and IL-17A and increased the level of IL-2 and CD25 in the CNS. A noteworthy observation is that ivermectin treatment increased STAT5 phosphorylation in the brain of EAE mice. These results suggest that ivermectin exerts its anti-inflammatory effects by maintaining Treg/Th17 cell balance, which may be mediated partly by the IL-2/STAT5 signaling pathway.

The purinergic P2X4 receptor (P2X4R) plays an important role in neurological diseases such as MS and amyotrophic lateral sclerosis, as well as in immune cell function [17, 38–40]. The expression of P2X4R is increased in the optic nerve of patients with MS and in the spinal cord of EAE mice [39]. On the other hand, blockade or deficiency of P2X4R may exacerbate clinical symptoms in EAE mice by activating microglia and macrophages and stopping remyelination but not by impairing immune priming [17]. Interestingly, ivermectin, as a positive allosteric modulator of P2X4R, alleviated clinical EAE symptoms in mice injected with 1 mg/kg ivermectin after disease onset (14 days post-immunization [dpi]).

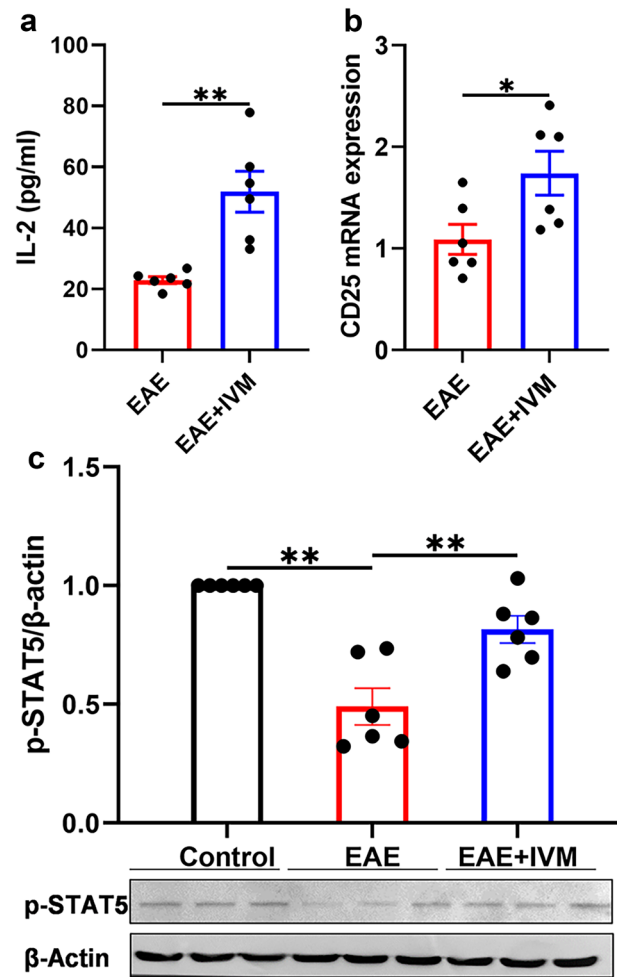


Fig. 8 Effect of ivermectin on the IL-2/IL-2R α /STAT5 signaling in the brains of EAE mice. **a** and **b** On day 14 post-immunization, the brains of EAE mice were collected and lysed to determine IL-2 level (**a**) and CD25 mRNA expression (**b**) by ELISA and real-time PCR, respectively. **c** The expression of p-STAT5 and β -actin proteins in the brains was measured by a Western blotting assay. The values are expressed as mean \pm SEM ($n=6$ /group). * $P < 0.05$, ** $P < 0.01$. Control: naive mice; IVM, ivermectin.

However, mice treated with ivermectin before EAE induction or preonset (5 dpi) were not protected from the subsequent EAE. These results suggest that ivermectin treatment might have different effects on disease progression at different stages of EAE. Indeed, we found no evidence of protection by ivermectin in EAE mice administered 2 mg/kg ivermectin orally at preonset (0 dpi), whereas this protective effect was observed in EAE mice administered 10 mg/kg ivermectin orally. Furthermore, using *in vitro* microglial polarization and myelin phagocytosis, Zabala *et al.* showed that ivermectin promotes a switch of microglia to an anti-inflammatory phenotype, which

then promotes remyelination [17]. However, it remains unknown whether ivermectin affects T cell responses in EAE mice. In the present study, we confirmed and extend a previously unknown mechanism by which ivermectin protects against EAE.

Regrettably, only a few research studies have concentrated on the impact of ivermectin on T-cell-mediated autoimmune illnesses, which include MS, rheumatoid arthritis, type 1 diabetes, inflammatory bowel diseases (IBD), Sjogren's syndrome, systemic lupus erythematosus, and psoriasis. While case reports have displayed the protective effect of ivermectin on systemic lupus

erythematosus [41–43] and psoriasis [44], there is still insufficient evidence to demonstrate its effectiveness on rheumatoid arthritis, type 1 diabetes, and Sjogren's syndrome. Moreover, the few investigations carried out on IBD and MS have solely centered on animal models [17, 45]. Hence, it is crucial to conduct more human studies to comprehensively examine the therapeutic potential of ivermectin in several autoimmune diseases.

In summary, the presented study showed that ivermectin can directly attenuate EAE clinical symptoms by altering T-cell responses and CD4⁺ T cell subsets. To our knowledge, this is the first study to show that the protection is related to its effect on T-cell responses, particularly by the reduction of pathogenic Th1 and Th17 cells and enhancement of Treg cells; all these processes may be related to the ivermectin-induced IL-2/STAT5 pathway. These new findings contribute to a better understanding of the mechanisms underlying the protective effects of ivermectin against T-cell-mediated autoimmune diseases. Further studies are required to further extend this study by establishing a causal relationship between the events involved to determine whether ivermectin directly affects the differentiation of naive CD4⁺ T cells into different CD4⁺ T cell subsets and to provide direct evidence for the role of ivermectin in controlling the IL-2/STAT5 pathway.

AUTHOR CONTRIBUTION

Junpeng Wang and Yu Xie designed this study; Yu Xie, Chaolei Jin, Hongzhen Sang, and Wenhua Liu analyzed the data; Yu Xie drafted the manuscript; and Junpeng Wang and Chaolei Jin critically revised the manuscript. All the authors have read and approved the final manuscript to be published.

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AVAILABILITY OF DATA AND MATERIALS

All the data supporting the findings of the study were presented in this paper and are available upon reasonable request.

DECLARATIONS

Conflict of Interest The authors declare no competing interests.

REFERENCES

- Olek, M. 2021. Multiple sclerosis. *Annals of internal medicine* 174: ITC81–ITC96.
- Dendrou, C., L. Fugger, and M. Friese. 2015. Immunopathology of multiple sclerosis. *Nature Reviews Immunology* 15: 545–558.
- Olsson, T., L. Barcellos, and L. Alfredsson. 2017. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nature Reviews Neurology* 13: 25–36.
- Baecher-Allan, C., B. Kaskow, and H. Weiner. 2018. Multiple sclerosis: Mechanisms and immunotherapy. *Neuron* 97: 742–768.
- Chen, W.-J. 2016. Honoring antiparasitics: The 2015 Nobel Prize in Physiology or Medicine. *Biomedical Journal* 39: 93–97.
- Tang, M., X. Hu, Y. Wang, X. Yao, W. Zhang, C. Yu, et al. 2021. Ivermectin, a potential anticancer drug derived from an antiparasitic drug. *Pharmacological Research* 163: 105207.
- Omura, S., and A. Crump. 2014. Ivermectin: Panacea for resource-poor communities? *Trends in Parasitology* 30: 445–455.
- Formiga, F., R. Leblanc, Rebouças J. de Souza, L. Farias, R. de Oliveira, and L. Pena. 2021. Ivermectin: An award-winning drug with expected antiviral activity against COVID-19. *Journal of Controlled Release: Official journal of the Controlled Release Society* 329: 758–761.
- Kotze, A.C., B.M. Hines, and A.P. Ruffell. 2012. A reappraisal of the relative sensitivity of nematode pharyngeal and somatic musculature to macrocyclic lactone drugs. *International Journal for Parasitology: Drugs and Drug Resistance* 2: 29–35.
- Lynagh, T., and J.W. Lynch. 2012. Ivermectin binding sites in human and invertebrate Cys-loop receptors. *Trends in Pharmacological Sciences* 33: 432–441.
- Jin, L., X. Feng, H. Rong, Z. Pan, Y. Inaba, L. Qiu, et al. 2013. The antiparasitic drug ivermectin is a novel FXR ligand that regulates metabolism. *Nature Communications* 4: 1937.
- Ashour, D.S. 2019. Ivermectin: From theory to clinical application. *International Journal of Antimicrobial Agents* 54: 134–142.
- Silberberg, S.D., M. Li, and K.J. Swartz. 2007. Ivermectin Interaction with transmembrane helices reveals widespread rearrangements during opening of P2X receptor channels. *Neuron* 54: 263–274.
- Khakh, B.S., and R.A. North. 2006. P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 442: 527–532.
- Inoue, K., and M. Tsuda. 2018. Microglia in neuropathic pain: Cellular and molecular mechanisms and therapeutic potential. *Nature Reviews Neuroscience* 19: 138–152.
- Blakley, B.R., and C.G. Rousseaux. 1991. Effect of ivermectin on the immune response in mice. *American Journal of Veterinary Research* 52: 593–595.
- Zabala, A., N. Vazquez-Villoldo, B. Rissiek, J. Gejo, A. Martin, A. Palomino, et al. 2018. P2X4 receptor controls microglia activation and favors remyelination in autoimmune encephalitis. *EMBO Molecular Medicine* 10: e8743.
- Niu, X., H. Sang, and J. Wang. 2021. Naringenin attenuates experimental autoimmune encephalomyelitis by protecting the intact of blood-brain barrier and controlling inflammatory cell migration. *The Journal of Nutritional Biochemistry* 89: 108560.

19. Xie, Y., Z. Li, Y. Wang, X. Xue, W. Ma, Y. Zhang, et al. 2019. Effects of moderate- versus high- intensity swimming training on inflammatory and CD4(+) T cell subset profiles in experimental autoimmune encephalomyelitis mice. *Journal of Neuroimmunology* 328: 60–67.
20. O'Neill, E.J., M.J. Day, and D.C. Wraith. 2006. IL-10 is essential for disease protection following intranasal peptide administration in the C57BL/6 model of EAE. *Journal of Neuroimmunology* 178: 1–8.
21. Kuerten, S., D.A. Kostova-Bales, L.P. Frenzel, J.T. Tigno, M. Tary-Lehmann, D.N. Angelov, et al. 2007. MP4- and MOG:35–55-induced EAE in C57BL/6 mice differentially targets brain, spinal cord and cerebellum. *Journal of Neuroimmunology* 189: 31–40.
22. Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 25: 402–408.
23. Ventre, E., A. Rozières, V. Lenief, F. Albert, P. Rossio, L. Laoubi, et al. 2017. Topical ivermectin improves allergic skin inflammation. *Allergy* 72: 1212–1221.
24. Goverman, J. 2009. Autoimmune T cell responses in the central nervous system. *Nature Reviews Immunology* 9: 393–407.
25. Constantinescu, C., N. Farooqi, K. O'Brien, and B. Gran. 2011. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British journal of pharmacology* 164: 1079–1106.
26. Lin, J., and W. Leonard. 2019. Fine-tuning cytokine signals. *Annual Review of Immunology* 37: 295–324.
27. Jones, D.M., K.A. Read, and K.J. Oestreich. 2020. Dynamic roles for IL-2-STAT5 signaling in effector and regulatory CD4(+) T cell populations. *The Journal of Immunology* 205: 1721–1730.
28. de Melo, G., F. Lazarini, F. Larrous, L. Feige, E. Kornobis, S. Levallois, et al. 2021. Attenuation of clinical and immunological outcomes during SARS-CoV-2 infection by ivermectin. *EMBO molecular medicine* 13: e14122.
29. Babalola, O.E., C.O. Bode, A.A. Ajayi, F.M. Alakaloko, I.E. Akase, E. Otrfanowei, et al. 2022. Ivermectin shows clinical benefits in mild to moderate COVID19: A randomized controlled double-blind, dose-response study in Lagos. *QJM* 114: 780–788.
30. Liao, W., J.-X. Lin, and W.J. Leonard. 2011. IL-2 family cytokines: New insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Current Opinion in Immunology* 23: 598–604.
31. Nelson, B.H. 2004. IL-2, regulatory T cells, and tolerance. *The Journal of Immunology* 172: 3983–3988.
32. Willerford, D.M., J. Chen, J.A. Ferry, L. Davidson, A. Ma, and F.W. Alt. 1995. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity* 3: 521–530.
33. Yao, Z., Y. Kanno, M. Kerenyi, G. Stephens, L. Durant, W.T. Watford, et al. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 109: 4368–4375.
34. Antov, A., L. Yang, M. Vig, D. Baltimore, and L. Van Parijs. 2003. Essential role for STAT5 signaling in CD25+CD4+ regulatory T cell homeostasis and the maintenance of self-tolerance. *The Journal of Immunology* 171: 3435–3441.
35. Kim, H.S., S.W. Jang, W. Lee, K. Kim, H. Sohn, S.S. Hwang, et al. 2017. PTEN drives Th17 cell differentiation by preventing IL-2 production. *The Journal of Experimental Medicine* 214: 3381–3398.
36. Laurence, A., C.M. Tato, T.S. Davidson, Y. Kanno, Z. Chen, Z. Yao, et al. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26: 371–381.
37. Liao, W., J.X. Lin, L. Wang, P. Li, and W.J. Leonard. 2011. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nature Immunology* 12: 551–559.
38. Volonté, C., and S. Amadio. 2022. Rethinking purinergic concepts and updating the emerging role of P2X7 and P2X4 in amyotrophic lateral sclerosis. *Neuropharmacology* 221: 109278.
39. Vázquez-Villoldo, N., M. Domercq, A. Martín, J. Llop, V. Gómez-Vallejo, and C. Matute. 2014. P2X4 receptors control the fate and survival of activated microglia. *Glia* 62: 171–184.
40. Di Virgilio, F., and A.C. Sarti. 2018. Microglia P2X4 receptors as pharmacological targets for demyelinating diseases. *EMBO Molecular Medicine* 10: e9369.
41. Yoshida, H., H. Endo, S. Tanaka, A. Ishikawa, H. Kondo, and T. Nakamura. 2006. Recurrent paralytic ileus associated with strongyloidiasis in a patient with systemic lupus erythematosus. *Modern Rheumatology* 16: 44–47.
42. Ashida, C., K. Kinoshita, Y. Nozaki, and M. Funachi. 2020. Fatal outcome in a patient under immunosuppressant therapy infected with human T-lymphotropic virus type 1 (HTLV-1), cytomegalovirus (CMV) and Strongyloides stercoralis: A case report. *BMC Infectious Diseases* 20: 470.
43. Werbel, T., B.R. Hinds, and P.R. Cohen. 2018. Scabies presenting as cutaneous nodules or malar erythema: reports of patients with scabies surreptitious masquerading as prurigo nodularis or systemic lupus erythematosus. *Dermatology Online Journal* 24.
44. Pereyra-Rodríguez, J.J., J. Bernabeu-Wittel, M.D. Conejo-Mir, M. Ruiz-Perez de Pipaon, and J. Conejo-Mir. 2010. Treatment of cutaneous myiasis associated with scalp psoriasis in a 13-year-old girl with oral ivermectin. *Journal of the American Academy of Dermatology* 63: 908–909.
45. Aryannejad, A., M. Tabary, N. Noroozi, B. Mashinchi, S. Iranshahi, S.M. Tavangar, et al. 2022. Anti-inflammatory effects of ivermectin in the treatment of acetic acid-induced colitis in rats: involvement of GABA(B) receptors. *Digestive Diseases and Sciences* 67: 3672–3682.

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