MW-9, a chalcones derivative bearing heterocyclic moieties, attenuates experimental autoimmune encephalomyelitis via suppressing pathogenic T_H17 cells

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Abstract. Previous studies have indicated that MW-9, a chalcones derivative bearing heterocyclic moieties, has considerable anti-inflammatory activity *in vitro*. Whether MW-9 may be used to treat inflammation-based diseases, such as multiple sclerosis, remains unknown. The present study was designed to determine the effect and underlying mechanism of MW-9 in experimental autoimmune encephalomyelitis (EAE). Female C57BL/6 mice immunized with MOG_{35-55} were treated with or without MW-9, then the clinical scores and other relevant parameters were investigated. Production of cytokines and specific antibodies were monitored by ELISA assays. Surface marker, Treg cell, and intracellular cytokines (IL-17A and IFN-γ) were detected by flow cytometry, and mRNA expression in the helper-T (T_H)17 cell-related signaling pathway was examined by reverse transcription-quantitative (RT-q) PCR analysis. T_H17 cell differentiation assay was performed. Herein, the present results demonstrated that oral administration of MW-9 reduced the severity of disease in EAE mice through slowing down infiltration process, inhibiting the demyelination, blocking anti-MOG_{35-55} IgG antibody production (IgG, IgG_{2a} and IgG_3), and decreasing accumulation of $CD11b^+Gr-1^+$ neutrophils from EAE mice. MW-9 treatments also led to significantly decreased IL-17A production and IL-17 expression in $CD4^+$ T-cells, but had no detectable influence on development of T_H1 and T-regulatory cells *ex vivo*. RT-qPCR analysis showed that within the spinal cords of the mice, MW-9 blocked transcriptional expression of T_H17-associated genes, including *Il17a*, *Il17f*, *Il6* and *Ccr6*. In T_H17 cell differentiation assay, MW-9 inhibited differentiation of 'naïve' $CD4^+$ T-cells into T_H17 cells and reduced the IL-17A production. The data demonstrated that MW-9 could attenuate EAE in part through suppressing the formation and activities of pathogenic T_H17 cells.

Introduction

Multiple sclerosis (MS) is a chronic auto-inflammatory disease of the central nervous system (CNS) (1). As a chronic autoimmune disease, MS has a higher incidence in women than men, and it often occurs in young adults. Abnormal inflammatory cells infiltrate into the CNS to initiate lesion formation and finally lead to demyelination of the neuronal axon (2). While, the exact etiological mechanisms remain unclear. Accumulated evidence has revealed that chronic demyelinating disorders of the CNS are primarily induced by self-reactive $CD4^+$ helper-T (T_H) cells. While mice lacking T_H1 cells can develop this pathology (3), and it has been considered that interferon (IFN)-γ produced by T_H1 cells had a key role in the pathogenesis of MS (4). Mice deficient in T_H1 cells-associated molecules, such as IFN-γ or IL-12p35 were more susceptible to EAE, while IL-12p40 deficient mice were resistant to disease. With the discoveries that p40 is also a subunit of IL-23 and IL-23 plays a pivotal role in mediating the development of disease. It was revealed that IL23-p19-deficient mice could abrogate the pathogenesis of experimental autoimmune encephalomyelitis (EAE) and downregulate the expression of T_H17 cell-associated proteins (5-7). These results highlighted the importance of IL-17-producing T_H17 cells in the pathogenesis of EAE.

Chalcones are aromatic ketones that form the central core of a variety of biological compounds. Derivatives of these agents exhibit a broad range of biological activities, in great part due to the presence of their αβ-unsaturated carbonyl system. As such, chalcones could be broadly used in drug research through

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the use of structural modifications. In previous studies, it was reported that *N*-heterocyclic-substituted chalcone compounds display a variety of biological activities in tumor cell lines and macrophages (such as A549, HeLa and RAW264.7 cell lines) (8-10). Among these, MW-9 (Fig. 1) appeared to impart a significant inhibitory effect on the generation of nitric oxide and markedly suppressed the production of tumor necrosis factor α in the RAW264.7 murine monocyte/macrophage cell line.

Nonetheless, the therapeutic potential of MW-9 in treating immune-based pathologies remains unknown. In order to explore the biological effects of MW-9 on autoimmune-related pathologies, the present study presented examined the effect of this agent on murine EAE. In the context of EAE, it remains unclear whether MW-9 may affect the levels of IL-17 and T_H17 cells. It was expected that information obtained in these studies about potential immunomodulating mechanisms of MW-9 could then be used to assess if the agent could be used for other autoimmune-based pathologies, including MS.

Materials and methods

Experimental animals. A total of 12 C57BL/6 mice (weight 18±2 g; female; 8-10 weeks-old) were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Upon arrival, all mice were housed under specific pathogen-free conditions (12-h light/dark cycles, room temperature of 22±1°C, and a 55±5% relative humidity). All mice had *ad libitum* access to standard rodent chow and filtered tap water. All mice were allowed to acclimatize for 4 weeks before initiation of any experiment. All experiments were carried out according to the institutional ethical guidelines on animal care, and were approved (approval no. S2019-001) by the Institute Animal Care and Usage Committee of The First Hospital Affiliated Yunnan University of Traditional Chinese Medicine (Kunming, China).

Reagents. MOG_{35-55} used to induce EAE in the mice was purchased from ProSpec-Tany TechnoGene, Ltd. Freund's complete adjuvant (CFA) containing *Mycobacterium tuberculosis* (*M. tuberculosis*) strain H37Rv was obtained from BD Biosciences. Pertussis toxin (PTX) was obtained from List Biological Labs. Mouse allophycocyanin (APC)-conjugated IL-17antibody (cat. no. 506916; cloneTC11-18H10), mouse fluorescein isothiocyanate (FITC)-conjugated interferon (IFN)-γ antibody (cat. no. 505805; clone XMG1.2), mouse PE-Cy7-conjugated CD4 antibody (cat. no. 100528; clone RM4.5), mouse Alexa Fluor® 488-conjugated Foxp3 (cat. no. 320012; clone MF23) antibody, mouse FITC-conjugated anti-mGr.1 (cat. no 108405; clone RB6-8C5, BD), phycoerythrin (PE)-anti-mCD11b (cat. no. 101255; clone M1/7), mouse FITC-anti-mCD8a (cat. no. 100705; clone 53-6.7), PE-anti-mB220 (cat. no. 103207; clone RA3-682), PE-Cy7-anti-mCD4 (clone GK.1.5), mouse APC-conjugated CD3 (cat. no 100312; clone 145-2C11) were purchased from BioLegend, Inc. ELISA kits for IL-17A (cat. no. 88-7371), IgG (cat. no. 88-50400), IgG_1 (cat. no. 88-50410), IgG_{2a} (cat. no. 88-50420) and IgG_3 (cat. no. 88-50440) were purchased eBioscience. Reverse transcription kit and SYBR Premix Ex Taq II were purchased from Takara

Biotechnology Co., Ltd. RNA extraction kit was purchased from Qiagen, Inc. All primers used were synthesized by Shanghai Bioengineering Co., Ltd.

General procedure for the preparation of MW-9. MW-9 were synthesized in house according to the method previously described (10). Briefly, 20% KOH (10 ml) was added to a solution of 4-dimethyl amino benzaldehyde (0.75 g, 5 mmol) and 4-fluoroacetophenone (0.69 g, 5 mmol) in EtOH (10 ml), and left to react overnight at room temperature. After filtration, the precipitate was washed twice with 50% $EtOH-H_2O$ and dried without further purification. The solid was dissolved in dried DMF (15 ml), then Cs_2CO_3 (3.26 g, 10 mmol) and piperazine (258 mg, 3 mmol) was added and the mixture was stirred for 12 h at 110°C. After completion of the reaction as indicated by thin layer chromatography, the reaction was quenched by the addition of DCM (30 ml) and was washed with water (3x20 ml). The organic layer was dried using anhydrous sodium sulfate, concentrated *in vacuo* and purified by column chromatography to afford product as yellow solid.

Induction of EAE. MOG_{35-55}-induced EAE in C57BL/6 mice was established as previously described (11-13). In brief, the mice were immunized on day 0 with an intracutaneous injection of 150 mg of MOG_{35-55} emulsified in CFA-bearing *M. tuberculosis* strain H37Rv. Injection volume was set at 150 μl. Thereafter, each mouse received an additional 300 ng of PTX by intraperitoneal injection in 100 ml of phosphate-buffered saline (PBS) on day 0 and again on day 2 post-immunization. Clinical assessment of EAE was performed daily and mice were scored according to the following criteria: 0, no overt signs of disease; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund state or dead.

Treatment protocols. MW-9 was dissolved in PBS containing 0.5% sodium CM-cellulose (Qiangshun Inc.). The MW-9 solution was prepared in a manner that the mice could receive a *per os* dose of 40 mg/kg in each treatment (volume=200 μl). Control mice received an equal volume of PBS containing 0.5% CM-cellulose. MW-9 and vehicle were respectively administered from day 1 post-immunization and then daily for 25 days. This particular dose of MW-9 was chosen based upon pilot study results assessing antiarthritic potential (data not shown). On the 25th day of the experiment, all mice were euthanized using 30% carbon dioxide of the cage volume per min, then used the following methods to confirm the death of animals, including lack of a heartbeat, lack of respiration, lack of corneal reflex and presence of rigor mortis. Samples (spinal columns, spleen, blood for serum) were collected for analyses as described below.

Hematoxylin-Eosin (H&E) staining. At necropsy, the spinal cord of each mouse was collected. A portion of this tissue was then fixed with 4% formalin at room temperature for 3 days. Thereafter, each underwent dehydration in alcohols and embedment in paraffin, then these were sectioned (to 5 μm) and stained with H&E at room temperature for 1 h. Each slide was evaluated using a light microscope to detect any

inflammatory cell aggregation in the myelin and the extent of any demyelination.

Production of cytokines and specific antibodies. At necropsy, lymph nodes of all the MOG_{35-55}-induced mice were collected and lymphocytes from each group were then isolated using standard protocols. After counting, the isolated cells were placed in 24-well plates ($2x10^6$ cells/well) and then stimulated by addition of MOG_{35-55} (to yield a final dose of 10 μg MOG_{35-55}/ml in each well); and parallel wells received only vehicle as 'stimulant'. Plates were then cultured at 37˚C. The supernatants from all wells were harvested after 48 h in order to assess IL-17 produced by the cells. Each sample was evaluated using specific murine ELISA IL-17 assays (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The sensitivity level of the kits was 4 pg/ml. The concentration of IL-17A and anti-mouse IgG, IgG_1, IgG_{2a} and IgG_3 in serum were isolated from the mice at the peak of the disease process.

Reverse transcription-quantitative (RT-q) PCR assay. Total RNA from remaining spinal cord tissue isolated from each mouse was isolated using RNA simple Total RNA kit (Tiangen Biotech Co., Ltd.). After confirming the purity (wavelength 260/280 nm) and concentration (wavelength 260 nm) of each isolate using a NANODrop2000 (Thermo Fisher Scientific, Inc.), 1 μg of total RNA was used to synthesize cDNA using a PrimeScript RT Master Mix Perfect Real-Time kit (Takara Biotechnology Co., Ltd.). The transcriptional conditions were 37˚C for 15 min and 85˚C for 5 sec, and then followed by maintenance at 4˚C (14,15). Synthesized cDNA was used in RT-qPCR experiments using SYBR Premix Ex Taq II kits (Takara Biotechnology Co., Ltd.). Each 20 μl of reaction contained 10 μl of SYBR Green Mix, 8.4 μl of nuclease-free H_2O, 1 μl of cDNA, 0.3 μl of forward primer and 0.3 μl of reverse primer. The thermocycling conditions for qPCR were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 62˚C for 20 sec. From this, the relative mRNA expression of several T_H17 cell-related genes (*Il17a*, *Il17f*, *Il6* and *Ccr6*) were measured. The primer sequences were synthesized by Tsingke Biotechnology Co., Ltd. All samples were assayed on a Stratagene MX3000P Real-Time PCR system (Agilent Technologies, Inc.). Relative quantitation of mRNA expression was calculated as the fold-increase in expression by using the $2^{-\Delta\Delta Cq}$ method and the β-*actin* housekeeping gene (16). The primer sequences of specific genes were as follows: Il17a forward, 5'-TTTAAC TCCCTTGGCGCAAAA-3' and reverse, 5'-CTTTCCCTC CGCATTGACAC-3'; Il17f forward, 5'-CTGTTGATGTTG GGACTTGCC-3' and reverse, 5'-TCACAGTGTTATCCTCCA GG-3'; Ccr6 forward, 5'-CCTGGGCAACATTATGGTGGT-3' and reverse, 5'-CAGAACGGTAGGGTGAGGACA-3'; Il6 forward, 5'-CGGAGAGGAGACTTCACAGAG-3' and reverse, 5'-CATTTCCACGATTTCCCAGA-3'; and β-actin forward, 5'-GGCTGTATTCCCCTCCATCG-3' and reverse, 5'-CCA GTTGGTAACAATGCCATGT-3'.

Flow cytometry. To assess expression of various lymphocyte types in the hosts with EAE, lymphocytes were harvested (centrifugation at 300 g x at 4˚C for 5 min) at necropsy from lymph nodes of the MOG_{35-55}-treated mice using standard protocols (15). Aliquots of the cells (10^6 total) were then blocked by incubation in 100 ml of PBS containing 1 μg/ml of rat-anti-mouse CD16/CD32 (clone 2.4G2; BioLegend, Inc.) for 10 min at 4˚C. Thereafter, dedicated sets of cells from each mouse were labeled by incubation for 15 min (at 4˚C) in a solution of staining buffer containing one of the following rat anti-mouse monoclonal antibodies (1:100 dilution; all from BioLegend, Inc.): FITC-conjugated anti-mGr.1 (clone RB6-8C5), phycoerythrin (PE)-anti-mCD11b (clone M1/7), FITC-anti-mCD8a (clone 53-6.7), PE-anti-mB220 (clone RA3-682), or PE-Cy7-anti-mCD4 (cloneGK.1.5), APC-conjugated CD3 (clone 145-2C11).

Intracellular cytokine staining for IFN-γ and IL-17A was performed using Foxp3 fixation-permeabilization reagent, following a protocol from eBioscience; Thermo Fisher Scientific, Inc. In brief, aliquots of the isolated lymphocytes ($2x10^6$ total) were plated into 24-well dishes and then stimulated for 4 h with 50 ng/ml phorbol myristate acetate and 750 ng ionomycin/ml (both from Sigma-Aldrich; Merck KGaA) in the presence of Brefeldin A (BioLegend, Inc.). The cells were then collected, blocked with rat-anti-mouse CD16/CD32 (as aforementioned), stained with PE-Cy7-conjugated rat anti-mCD4 (as aforementioned), then fixed by fixation/permeabilization buffer (eBioscience; cat. no. 005123-43) for 30 min at 4˚C permeabilized, and again stained for 45 min at 4˚C with APC-conjugated rat anti-mIL-17A (clone TC11-18H10.1) or FITC-rat anti-mIFN-γ (clone XMG1.2) (1:100 dilution; both from BioLegend, Inc.). The aforementioned stimulation was not required for the staining of intracellular Foxp3 that was also performed using Alexa Fluor@ 488-conjugated rat anti-mFoxp3 (clone 150 D; BioLegend, Inc.).

In all of the above cases, samples were evaluated on a FACs Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo software v10.6.2 (FlowJo LLC) (17,18). A minimum of 10,000 events/sample was acquired.

T_H17 differentiation of $CD4^+$ cells in vitro. The methods used to evaluate T_H17 cell polarization here are those previously described in Li *et al* (11) and Hou *et al* (19). In brief, murine splenocytes from untreated 'naïve' mice (no EAE) were prepared and incubated (1:4 dilution) with Biotin-Antibody Cocktail (Miltenyi Biotec GmbH) for 10 min at 4˚C to deplete $CD11b^+$, $CD8\alpha^+$ and $CD19^+$ cells. Then, these cells were exposed to Anti-biotin Microbeads (Miltenyi Biotec GmbH) for 10 min at 4˚C, and the enriched $CD4^+$ T cells were isolated by MACS separator. Purity of the isolated cells was evaluated by staining of representative aliquots with PE-Cy7-rat anti-mCD4 as aforementioned.

For the assay, the 'naïve' CD4 T-cells from each mouse were plated into 24-well plates ($4x10^5$/well) whose wells had been pre-coated with rat anti-mCD3 (clone 154-2C11, 5 μg/ml: BioLegend, Inc.), anti-mCD28 (clone 37.51, 2.5 μg/ml: BioLegend, Inc.), and a set of four polarizing cytokines. The latter included recombinant mouse IL-6 (10 ng/ml), recombinant human TGF-β_1 (2 ng/ml), anti-mouse IL-4 (10 μg/ml), and anti-mouse IFN-γ (10 μg/ml) (all from BioLegend, Inc.). After 72 h of incubation at 37˚C, the supernatants from each well were collected to permit quantification of IL-17A levels by ELISA.

To detect IL-17A expression on cells that were induced to differentiate into T_H17 cells, other wells containing isolated 'naïve' CD4 T-cells from each mouse plated into 24-well plates (10^6 total) were stimulated with phorbol myristate acetate and ionomycin in the presence of Brefeldin A for 4 h, and then fixed (as aforementioned) for 30 min at 4˚C, permeabilized and stained for 45 min at 4˚C with fluorochrome-conjugated anti-mIL-17A (1:100 dilution). These cells then underwent flow analyses as aforementioned.

Statistical analysis. All data are expressed as the mean ± SEM. Two independent sample t-test was used to determine whether differences between two given groups were significant. One-way analysis of variance (one-way ANOVA) and Bonferroni test were used for comparison among multiple groups and pairwise comparison from multiple groups, respectively. P<0.05 was considered to indicate a statistically significant difference. All data were analyzed using SPSS 20.0 software (IBM Corp.).

Results

MW-9 alleviates development of EAE in MOG_{35-55}-immunized C57BL/6 mice. To assess therapeutic potential of MW-9, mice were immunized with MOG_{35-55} peptide emulsified with CFA on day 0 and then gavaged with MW-9 (40 mg/kg) or vehicle on the day of immunization to the end of experiment. Severity of EAE due to the presence or absence of MW-9 treatment was then evaluated. As revealed in Fig. 2A, clinical scores gradually increased as EAE progressed. Scores in the MW-9 treatment group were significantly reduced as compared with those in the vehicle group. In addition, by day 19, 80% of the control mice developed severe EAE. In comparison, <20% of mice showed mild signs of disease in the MW-9-treated group (Fig. 2B). MW-9 treatment also markedly prevented a loss of body weight due to EAE (Fig. 2C). Although MW-9 exhibited delayed and ameliorated disease, the incidence in MW-9 treated group was increased after day 24 post immunization, thus the difference between these two groups decreased after day 24.

To assess effects of MW-9 on infiltration of immune cells and demyelination of the spinal cord, histologic analysis was performed after the treatments ended. The data indicated that by day 25, mice in the vehicle group had typical pathological changes, including severe infiltration of inflammatory cells and demyelination of their spinal cords. By contrast, in mice treated with MW-9, the extent of both infiltration and demyelination was markedly reduced (Fig. 2D).

MW-9 blocks anti-MOG_{35-55} IgG antibody production. To monitor effects of MW-9 on antibody responses to MOG_{35-55} peptide, serum levels of anti-MOG_{35-55} peptide IgG, IgG_1, IgG_{2a} and IgG_3 were determined by ELISA. As revealed in Fig. 3, serum levels of total IgG in the MW-9 treatment group were significantly decreased on day 25 compared with the control EAE mice (Fig. 3A). Furthermore, compared with the control group, MW-9 treatment also reduced the production of anti-MOG_{35-55} IgG_3 (Fig. 3B) and IgG_{2a} (Fig. 3C). However, MW-9 appeared to impart no effect on anti-MOG_{35-55} IgG_1 levels (Fig. 3D).
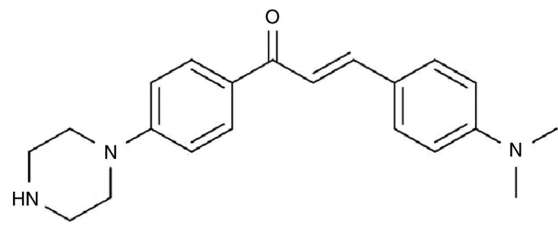

Figure 1. Structure of MW-9.

MW-9 inhibits accumulation of neutrophils. To examine the effects of MW-9 on the types of leukocytes in the spleens of mice with EAE, flow cytometric analysis was performed. On day 25, the proportion and cell number of $CD11b^+Gr-1^+$ neutrophils in the MW-9 treated mice was decreased compared with the vehicle group (Fig. 4A and B). The data indicated that MW-9 did not influence the expression of $CD3^+$, $CD8^+$, or $CD4^+$ T cells, and no effects on γδ TCR^+ T or $B220^+$ B cells were observed (Fig. 4C).

MW-9 markedly suppresses T_H17 cell responses in EAE. Since T_H17 cells and IL-17 contribute to the pathogenesis of autoimmune disorders such as EAE (20), it was further determined whether MW-9 could influence these parameters during EAE through evaluating the expression of intracellular cytokines in $CD4^+$ T cells via flow cytometry. As revealed in Fig. 5, compared with the vehicle control mice, MW-9 treatment significantly suppressed IL-17A expression in $CD4^+$ T cells (Fig. 5A and B). However, MW-9 treatment did not affect the expression of IFN-$γ^+$ and $Foxp3^+$ $CD4^+$ T cells in the hosts (Fig. 5A and B).

MW-9 suppresses production of IL-17 and expression of T_H17 cell-related genes. There is evidence that T_H17 cells play a critical pathogenic role in EAE (21) and the present data indicated that MW-9 markedly inhibited T_H17 cell responses during EAE. To assess the expression of T_H17 cell-related cytokines/proteins, spinal cords were isolated on day 25 and analyzed by RT-qPCR. The results demonstrated that MW-9 treatment significantly downregulated *Il17*a, *Il17f*, *Ccr6* and *Il6* mRNA expression (Fig. 6A-D). The data also indicated that IL-17A produced by cells from the MW-9-treated mice was lower than that from the vehicle control. These findings were consistent with the gene expression data (Fig. 6E and F).

MW-9 suppresses T_H17 cell differentiation in vitro. To ascertain whether MW-9 could affect T_H17 cell differentiation, $CD4^+$ T cells were isolated from the spleens of naive mice and then induced to differentiate into T_H17 cells under polarizing conditions for 3 days in the presence/absence of MW-9. The results indicated that MW-9 treatment significantly suppressed T_H17 cell differentiation (Fig. 7A and B). In addition, IL-17A production was also reduced in MW-9-treated cells (Fig. 7C).

Discussion

MS is an autoimmune disease affecting both adults and children. It is driven by T_H cells and white blood cells that result in inflammatory attacks on the brain and spinal cord, and ultimately the degradation of the protective myelin sheath on nerve fibers.
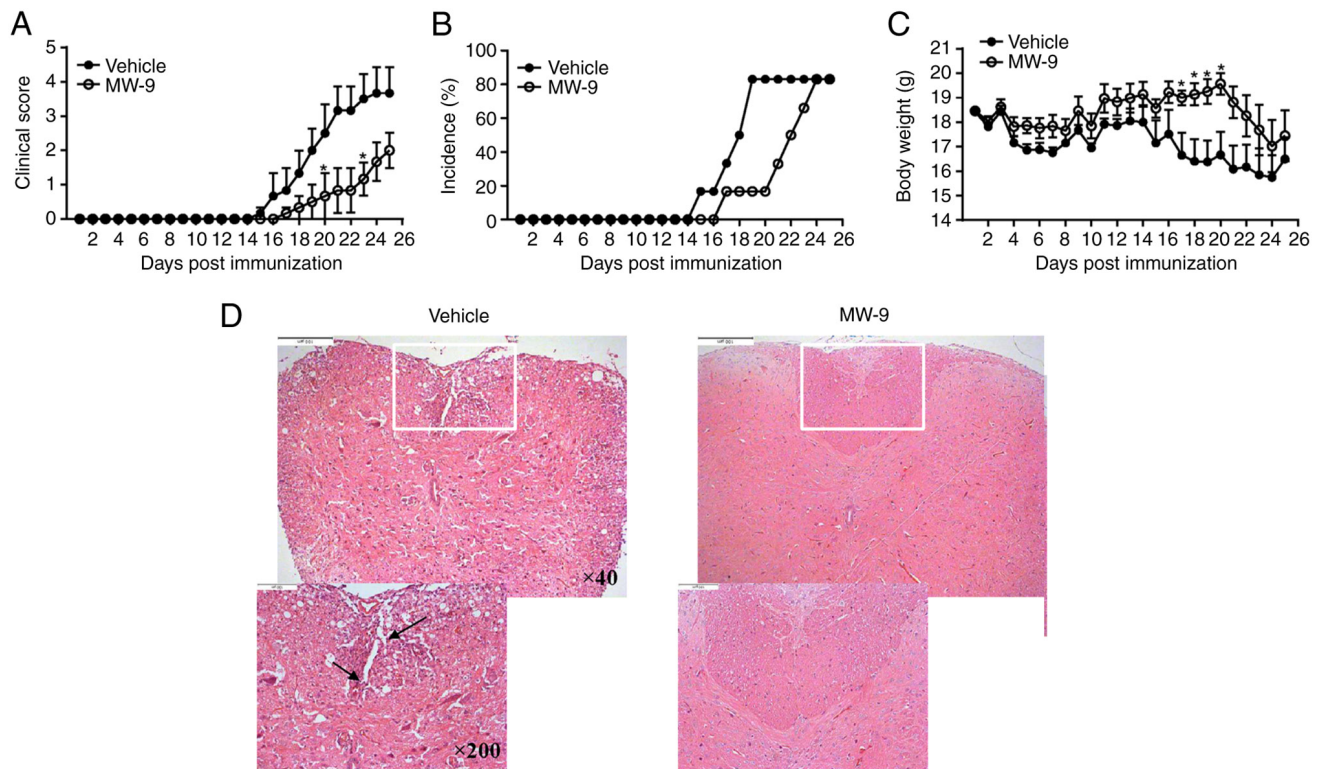
Figure 2. MW-9 effects on development of EAE in MOG$_{35-55}$-immunized C57BL/6 mice. EAE was induced in female C57BL/6 mice by immunization with MOG$_{35-55}$ in CFA followed by Pertussis toxin injection. The mice were then orally treated with vehicle or MW-9 (40 mg/kg) from day 1 post-immunization. (A) The clinical score, (B) incidence of disease, (C) body weight and (D) pathological changes to the spinal cord were investigated at indicated days (magnification, x40 or x200). Arrows indicate inflammatory cells infiltration. All data are presented as the mean ± SEM (n=6). *P<0.05 vs. the vehicle group. Three experiments were performed with similar results. EAE, experimental autoimmune encephalomyelitis.

There are numerous different kinds of T$_H$ cells, but which ones are the main pathogenic forms in MS remain unknown. EAE, a classical animal model for MS, is characterized by abnormal inflammatory cells that infiltrate into the CNS, initiating lesion formation and demyelination of neuronal axons (2). In the present study, the therapeutic potential of the chalcone MW-9 on autoimmune disorders such as MS was evaluated *in vivo* in murine hosts with EAE. Notably, oral administration of MW-9 for a period of 19 days ameliorated the severity of the induced EAE. This effect was accompanied by significant reduction in leukocyte infiltration and demyelination of the spinal columns. It was reported that T-cells played a pivotal role in the pathogenesis of numerous autoimmune diseases and chronic inflammatory disorders, including EAE (22). CD4$^+$ T$_H$ cells are important mediators in immune responses, as they regulate other cellular components of the immune system. Upon activation by antigens or cellular signals, naïve CD4 cells can differentiate into various subtypes, including T$_H$1, T$_H$2, T$_H$17 and T$_{reg}$, which in turn produce their own arrays of cytokines and perform specific immune regulatory functions (22). Inflammatory T$_H$17 cells have been identified as a distinct T$_H$ cell lineage that can mediate tissue specificity during autoimmunity (23,24), including pathologies such as EAE (25).

The therapeutic potentials of MW-9 on treating immune-based pathologies remains unknown. In order to explore the biological effects of MW-9 on autoimmune-related pathologies, the effect of this agent on murine EAE was examined in the present study. In the context of EAE, it remains unclear what effects MW-9 may have on IL-17 levels, T$_H$17 cells, and on key cytokines in hosts treated with this
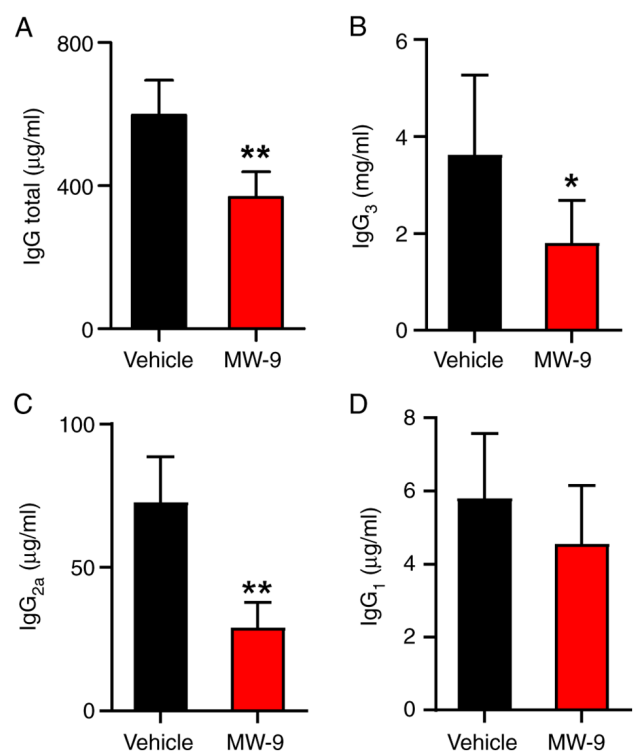

Figure 3. MW-9 effects on production of anti-MOG$_{35-55}$ IgG antibodies. MOG$_{35-55}$- immunized C57BL/6 mice were treated with MW-9. On day 25 post-immunization, levels of anti-MOG$_{35-55}$ (A) IgG, (B) IgG$_3$, (C) IgG$_{2a}$ and (D) IgG$_1$ in serum were measured by ELISA. All data are presented as the mean ± SEM (n=6). *P<0.05 and **P<0.01 vs. the vehicle group. Two experiments were performed with similar results.
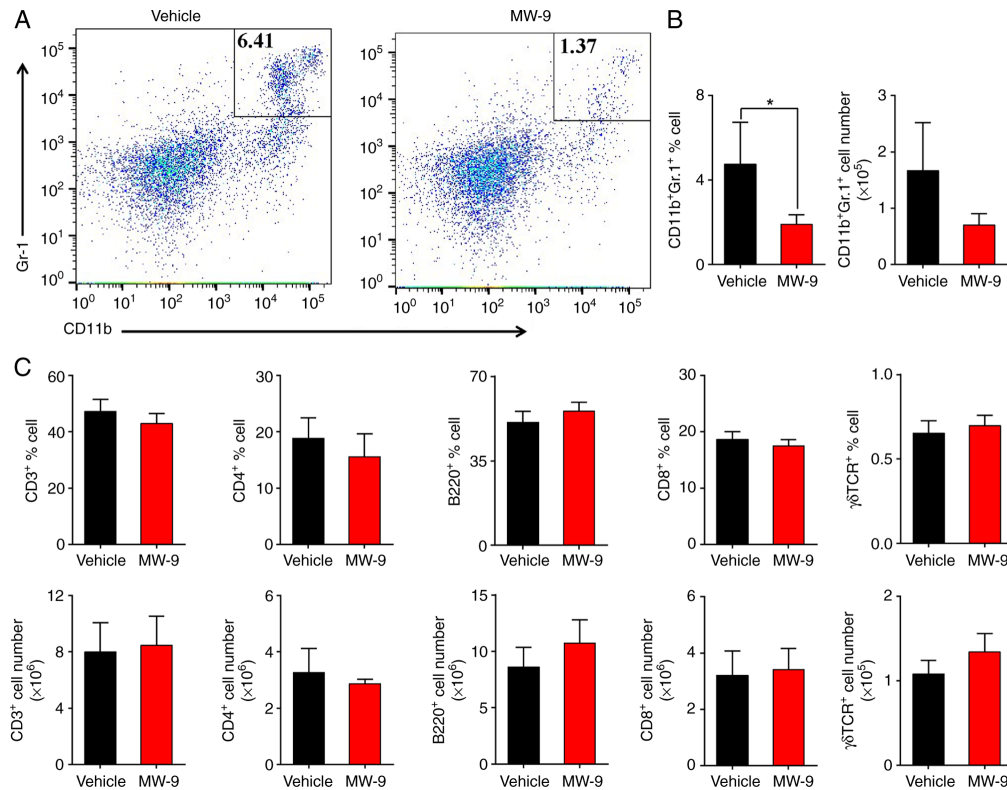
Figure 4. MW-9 effects on frequency of CD11b+Gr-1+ neutrophils in EAE. Lymphocytes from EAE mice were harvested at necropsy from LN at day 25 post-immunization and then labeled with the indicated antibody. Samples were then evaluated by flow cytometric expression of CD11b+Gr-1+ neutrophils from lymphocytes of MOG-immunized mice. (A) Representative plots and mean frequencies and (B) absolute cell numbers for LN at indicated days. (C) Expression of surface markers CD3+, CD8+, CD4+ T cells, γδ TCR+ T cells or B220+ B cells from lymphocytes of EAE mice (Upper panel). Mean frequencies and (lower panel) absolute cell numbers from LN at indicated days. Data are representative of two experiments, all data are presented as the mean ± SEM (n=4). *P<0.05 vs. the vehicle group. EAE, experimental autoimmune encephalomyelitis; LN, lymph nodes.
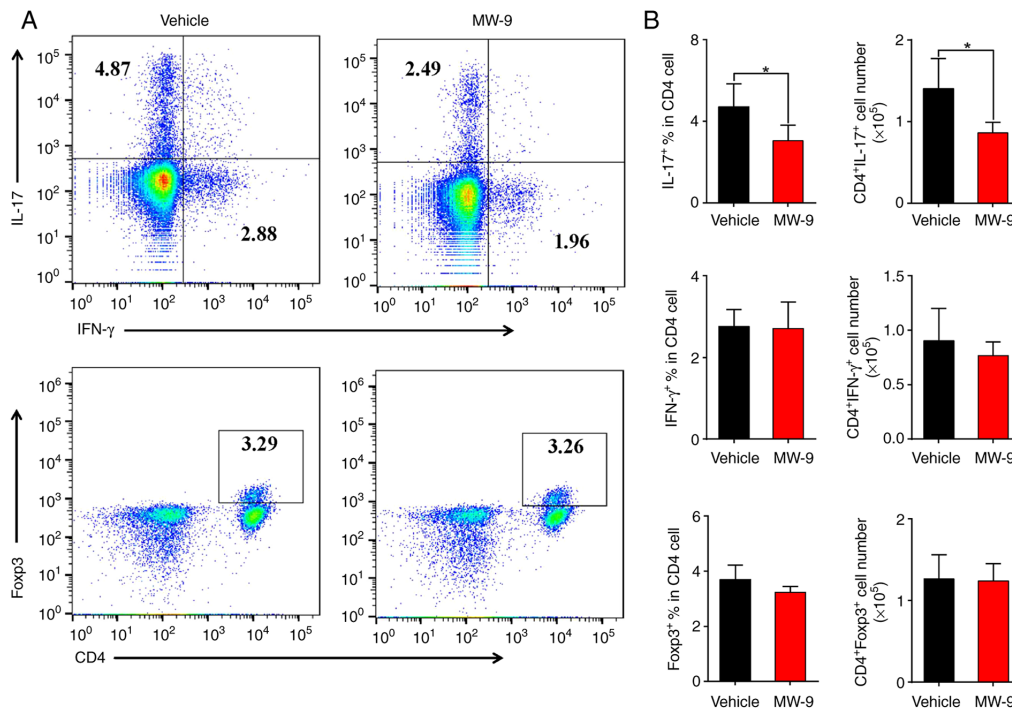

Figure 5. MW-9 effects on T$_H$17 cells during EAE. IL-17+, IFN-γ+ and Foxp3+ in CD4 T cells at onset of EAE. LN cells from EAE mice were stimulated with phorbol myristate acetate and ionomycin in the presence of Brefeldin A for 4 h. The cells were then collected, blocked, stained with PE-Cy7-anti-mCD4 antibody, fixed, permeabilized and stained intracellularly with fluorochrome-conjugated anti-mIL-17A or anti-mIFN-g. The aforementioned stimulation was not required for the staining of intracellular Foxp3 that was also performed using fluorochrome-conjugated anti-mFoxp3, samples were analyzed by FACS. (A) Representative plots and mean frequencies and (B) absolute cell numbers. Data are representative of two experiments, all data are presented as the mean ± SEM (n=4). *P<0.05 vs. the vehicle group. EAE, experimental autoimmune encephalomyelitis.
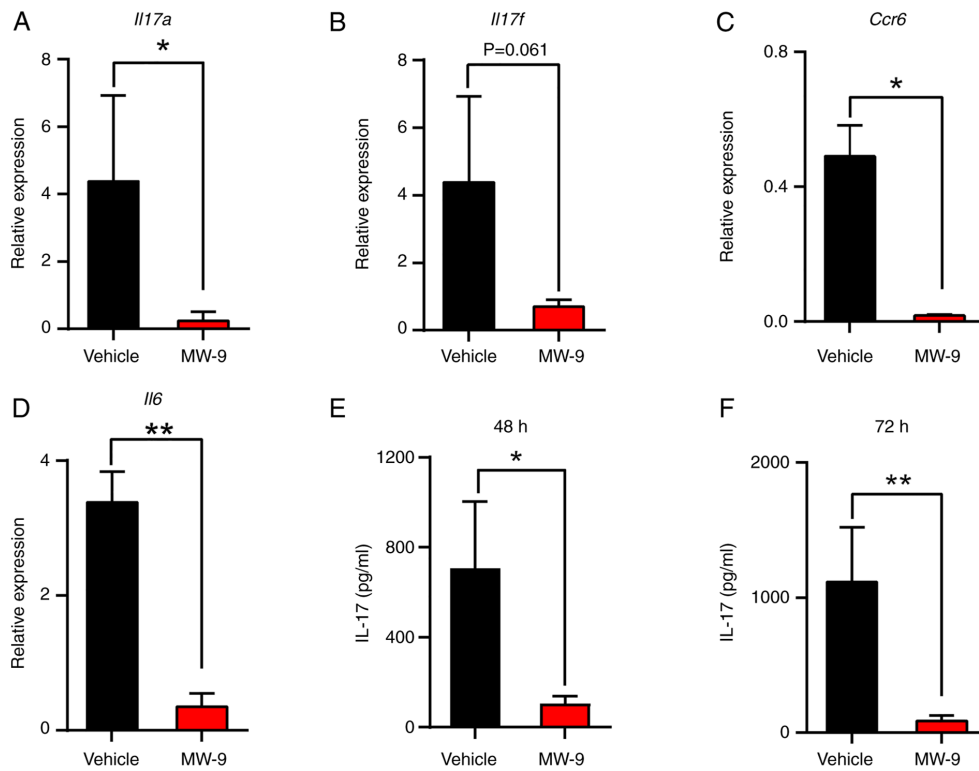
Figure 6. MW-9 effects on production of IL-17 and expression of T-helper 17-related genes. Spinal cords were isolated and analyzed by reverse transcription-quantitative PCR for mRNA expression of (A) *Il17a*, (B) *Il17f*, (C) *Ccr6* and (D) *Il6*. Separately, lymphocytes from LN were obtained from each mouse and cultured at 37˚C in 24-well plates in the co-presence of 10 µg/ml MOG$_{35-55}$ stimulant. Supernatants were harvested at (E) 48 and (F) 72 h to permit measures of IL-17A (ELISA). Data are representative of two experiments, all data are presented as the mean ± SEM (n=4). *P<0.05 and **P<0.01 vs. the vehicle group.
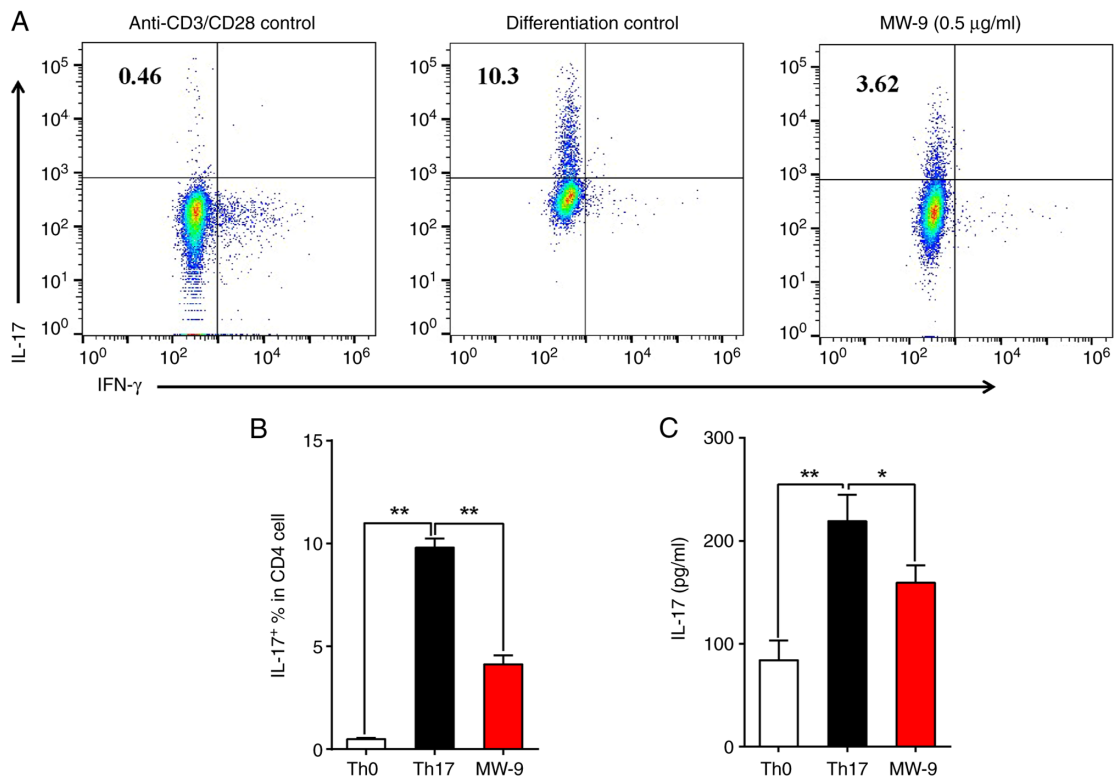

Figure 7. MW-9 effects on T$_H$17 differentiation *in vitro*. 'Naïve' CD4 T cells (4x10^5/well) in 24-well plates pre-coated with anti-CD3, anti-CD28 as well as with IL-6, TGF-β and anti-mouse IL-4, anti-mouse IFN-γ were cultured for 72 h in the presence of MW-9 (0.5 µg/ml). The cells were then re-stimulated for an additional 4 h with phorbol myristate acetate and ionomycin in the presence/absence of Brefeldin A. Thereafter, the cells were harvested and stained for evaluation using flow cytometry. (A) Representative plots and (B) statistical analysis. (C) The supernatants of the now-differentiated cells were harvested at 72 h to quantify IL-17 production. Data are representative of two experiments, Results are presented as the mean ± SEM of three wells (n=3). *P<0.05 and **P<0.01 vs. the vehicle group.

chalcone. The results showed that MW-9 treatment (under the specified paradigm) significantly reduced the production of T_H17-related cytokine IL-17A in the MOG_{35-55}-induced immune responses in the mice. Intracellular cytokine staining revealed that MW-9 suppressed IL-17A expression in $CD4^+$ T-cells without affecting the proportions of IFN-γ^+ and $Foxp3^+$ $CD4^+$ T-cells. This suggested that MW-9 could selectively inhibit T_H17-mediated tissue inflammatory responses, but may exert no influence on T_H1 and T_{reg} cell levels/activities, at least in the context of an ongoing EAE response.

It has been reported that T_H17 differentiation is initiated by TGF-b and IL-6, and is further reinforced by IL-23 (26). T_H17 cells produce IL-17A, IL-17F and IL-22, that regulate inflammatory responses (21,27), but the present study did not directly examine the effect of MW-9 on these cytokines. Thus, the changes in T_H17 levels/activities observed could be a result of either changes in T-cell responsivity to one or more of the aforenoted activating cytokines or in the host ability to form these agents. The results from the differentiation assay using naïve T-cells revealed that MW-9 treatment markedly inhibited T_H17 differentiation. Thus, it holds true that TH17 responses are altered in the EAE mice. Ongoing studies to re-analyze the mice sera for levels of TGF-b, IL-6, IL-23 and IL-22 will help to clarify the underlying mechanisms in an improved way.

Analogous to STAT4 and STAT1 in Th1 and STAT6 in Th2 cell, STAT3 was found to selectively mediate T_H17 differentiation (28). Overexpression of a hyperactive STAT3 enhanced Th17 differentiation, whereas STAT3 deficiency impaired Th17 differentiation *in vitro*. Th17-specific transcription factor RORγt was recently shown to be selectively expressed in Th17 cells and regulated by STAT3 (29). Overexpression of RORγt promotes Th17 differentiation when Th1 and Th2 development is inhibited. Conversely, ROR$\gamma\tau$ deficiency results in profound Th17 deficiency. *In vivo*, oral administration of MW-9 significantly reduced the expression of IL-17 producing T cells compared with the vehicle group. Consistent with *in vivo* results, MW-9 also impaired Th17 cell differentiation and IL-17-production *in vitro*, confirming that the inhibitory effect of MW-9 on Th17 cell differentiation is responsible for the effect of MW-9 on EAE. In the present study, oral administration of MW-9 significantly decreased the IL-17 production in MOG_{35-55} induced-specific immune response and in serum of mice. Importantly, MW-9 treatment significantly inhibited IL-17 expression in $CD4^+$ T-cells in EAE model, accompanied by the reduced production of MOG_{35-55} peptide-specific antibodies (IgG, IgG2a and IgG3), which may contribute to the protective effect of MW-9 on EAE.

Overall, the data demonstrated that oral administration of MW-9 could attenuate EAE in mice by inhibiting pathogenic T_H17 cells. Importantly, MW-9 treatment significantly inhibited IL-17 expression in $CD4^+$ T-cells and TH17 cell differentiation *in vitro*. Both of these changes induced by MW-9 may help to explain why under the current experimental paradigm, MW-9 may provide a protective effect against EAE.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CW and ZM designed the present study; BL, SY and NY performed biological research, analyzed data and wrote the manuscript; and QG, YQ, XL, HY, ZW and NZ participated in data analysis. CW reviewed and edited the manuscript. CW, BL and SY confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments were carried out according to the institutional ethical guidelines on animal care and were approved (approval no. S2019-001) by the Institute Animal Care and Usage Committee of the No. 1 Hospital Affiliated Yunnan University of Traditional Chinese Medicine (Kunming, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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