



Research paper

Curcumin ameliorates experimental autoimmune myasthenia gravis by diverse immune cells



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HIGHLIGHTS

- Investigate the effects of curcumin on experimental autoimmune myasthenia gravis (EAMG).
- Curcumin treatment alters CD4⁺ Th subset distribution in EAMG rats.
- Curcumin increases the numbers of NK and NKT cells from lymph nodes in EAMG rats.
- Curcumin increases the numbers of B10 cells in MNCs in EAMG rats.
- Curcumin increases the levels of anti-R97-116 peptide IgG1 and decreases the relative affinity indexes of anti-R97-116 peptide IgG in EAMG rats.

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ABSTRACT

Curcumin is a traditional Asian medicine with diverse immunomodulatory properties used therapeutically in the treatment of many autoimmune diseases. However, the effects of curcumin on myasthenia gravis (MG) remain undefined. Here we investigated the effects and potential mechanisms of curcumin in experimental autoimmune myasthenia gravis (EAMG). Our results demonstrated that curcumin ameliorated the clinical scores of EAMG, suppressed the expression of T cell co-stimulatory molecules (CD80 and CD86) and MHC class II, down-regulated the levels of pro-inflammatory cytokines (IL-17, IFN- γ and TNF- α) and up-regulated the levels of the anti-inflammatory cytokine IL-10, shifted the balance from Th1/Th17 toward Th2/Treg, and increased the numbers of NKR-P1⁺ cells (natural killer cell receptor protein 1 positive cells, including NK and NKT cells). Moreover, the administration of curcumin promoted the differentiation of B cells into a subset of B10 cells, increased the anti-R97-166 peptide IgG1 levels and decreased the relative affinity indexes of anti-R97-116 peptide IgG. In summary, curcumin effectively ameliorate EAMG, indicating that curcumin may be a potential candidate therapeutic agent for MG.

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1. Introduction

Myasthenia gravis (MG) and its animal model experimental autoimmune myasthenia gravis (EAMG), are antibody-mediated, T-cell dependent autoimmune diseases [1]. Both cellular and humoral immunity are thought to contribute to the pathogenesis of MG/EAMG [2].

Curcumin is derived from the rhizomes of the plant *Curcuma longa*, which has anti-tumor and anti-inflammatory properties [3]. Previous studies have demonstrated curcumin inhibits inflammation in many autoimmune and inflammatory diseases (such as atherosclerosis, arthritis, experimental autoimmune neuritis (EAN), and experimental autoimmune encephalomyelitis (EAE)) [4]. The exact anti-inflammatory mechanisms of curcumin remain unknown, and many experiments suggest that it may associate with its ability to reduce pro-inflammatory cytokines, such as IL-1, tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) through deactivation of JAK-STAT and NF- κ B signaling pathways in immune cells [5]. Recent reports also indicate that curcumin attenuate autoimmune diseases by inhibiting Th1 differentiation [6] and

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down-regulating Th17 production [7], shifting immune responses from Th1 to Th2 type responses [8].

However, the immunomodulatory effects of curcumin in MG and EAMG have not been reported. Therefore, we investigated the effects and potential mechanism of curcumin on the pathogenesis of EAMG.

2. Materials and methods

2.1. Animals

Female Lewis rats (6–8 weeks, 160–180 g; Vital River, Beijing, China) were housed under a 12 h light–12 h dark cycle with free access to standard food and water. All the experimental protocols were approved by the institutional ethics committee. All efforts were made to minimize the number of animals and their suffering. We obtained these rats and performed the following experiments under protocols approved by the Medical Ethics Committee of Shandong University.

2.2. Establishment of EAMG rat model and evaluation of EAMG clinical symptoms

EAMG was induced by subcutaneous injection into both hind foot pads with the R97-116 peptide (50 g/rat; synthesized by CL Bio-scientific, Inc., Xi'an, China) emulsified in incomplete Freund's adjuvant (IFA; Sigma-Aldrich, St. Louis, MO, USA) combined with 1 mg of *M. tuberculosis* (day 0). A boost immunization was performed on day 11 with the same dose of R97-116 peptide in IFA with 1 mg *M. tuberculosis*. Rats were examined before immunization and thereafter every second day until day 45 post-immunization (p.i.). The severity of clinical signs is scored by observational assessment of muscular weakness. Disease severity was graded as follows: 0, normal; 1, mildly decreased activity, weak grip, fatigable; 2, weakness, hunched posture at rest, decreased body weight, tremor; 3, severe generalized weakness, marked decrease in body weight, moribund; 4, death [9]. Rats with intermediate signs were assigned grades of 0.5, 1.5, 2.5 or 3.5, respectively. Results were expressed as the mean score of each group at each time point.

2.3. Curcumin administration

EAMG rats were randomly divided into three groups ($n=6$): control group, low-dose group and high-dose group. For treatment with curcumin (Sigma), EAMG rats were injected intraperitoneally (i.p.) with 50 mg/kg (low-dose group) or 100 mg/kg curcumin (high-dose group) dissolved in (Polyethylene glycol PEG) every other day from day 11 to day 45 p.i. Rats in the control group received the same volume of PEG. The severity of clinical symptoms of EAMG was recorded from day 0 to day 45 p.i.

2.4. Preparation of lymph node mononuclear cells (MNCs)

Rats were sacrificed by cervical vertebra dislocation on day 45 p.i. and inguinal lymph nodes were collected under aseptic conditions. Single-cell suspensions of MNCs were obtained by grinding the inguinal lymph nodes through cell strainers in serum-free medium. Then, cells were washed three times and diluted to 2×10^6 cells/ml in RPMI 1640 (containing 2.05 mM glutamine; HyClone, Logan, UT, USA) supplemented with 1% (v/v) penicillin-streptomycin (containing 10,000 IU/ml penicillin and 10,000 μ g/ml streptomycin; HyClone) and 10% (v/v) fetal bovine serum (FBS; Gibco, Grand Land, NY, USA).

2.5. Flow cytometric immunophenotyping

Briefly, MNCs suspensions in PBS containing 0.5% bovine serum albumin (BSA; Sigma) were incubated with PE-labeled anti-rat CD80 (Biolegend, San Diego, CA, USA), FITC-labeled anti-rat CD86 (BioLegend), FITC-labeled anti-rat MHC class II (eBioscience, San Diego, CA, USA), FITC-labeled anti-rat CD4 (BioLegend), PE-labeled anti-rat CD161a (PharMingen, San Diego, CA, USA), FITC-labeled anti-rat CD3 (eBioscience), and PE-labeled anti-rat CD19 (eBioscience) for 30 min at 4 °C in the dark. For intracellular cytokines analysis, MNCs were fixed with 2% paraformaldehyde for 20 min at 4 °C, permeabilized with 0.5% saponin in PBS containing 0.5% BSA, and then incubated with the FITC-labeled anti-rat IFN- γ (BioLegend), PE-labeled anti-rat IL-10 (PharMingen), PE-labeled anti-rat TNF- α (BioLegend), and FITC-labeled anti-rat IL-17A (eBioscience), for 30 min at 4 °C in the dark respectively. For analysis of Treg cells, fixation and permeabilization of lymph node MNCs were performed using the eBioscience Foxp3 Staining Buffer Set (eBioscience). The antibodies of FITC-conjugated anti-rat CD4 (eBioscience) and PE-conjugated anti-mouse/rat Foxp3 (eBioscience) were used for staining according to the protocol recommended by eBioscience. Samples were analyzed within 24 h with a BD FACScan (BD Biosciences, San Jose, CA, USA) and using Cell Quest software (BD Biosciences).

2.6. Detection of levels of anti-R97-116 peptide antibodies and their isotypes by ELISA

Levels and isotypes of anti-R97-116 peptide antibodies were measured by ELISA. Briefly, 96-well flat-bottom microtiter plates were coated with 100 μ l R97-116 peptide (2 μ g/ml) at 4 °C overnight. After washing with PBS-T (PBS 0.05% Tween 20), the plates were incubated with 10% FBS at room temperature for 2 h to block the uncoated sites. Then, serum samples, collected on days 45 p.i. and diluted 1:100, were added and incubated for 2 h at 37 °C. After washing, biotinylated rabbit anti-rat IgG antibody (1:1000, 100 μ l; Biolegend) or mouse anti-rat IgG1 (1:500, 100 μ l; Biolegend), IgG2a (1:500, 100 μ l; Biolegend) and IgG2b (1:500, 100 μ l; Biolegend) were added into corresponding wells and incubated at 37 °C for 1 h. Then, streptavidin horseradish peroxidase (HRP; 1:1000, 100 μ l; Biosynthesis Biotechnology, Beijing, China) was added and incubated at 37 °C for 30 min. Plates were washed with PBST and followed by development with Tetramethylbenzidine (TMB) substrate (Tiangen Biotechnology, Beijing, China) in the dark. Finally, plates were measured using a microplate reader (Epoch Biotek, USA) at 450/630 nm. Results were expressed as the difference between the two OD values, which was OD value = OD450–OD630. Each serum sample was tested in triplicate.

2.7. Detection of relative affinity of serum anti-R97-116 peptide IgG antibody by ELISA

The relative affinity of anti-R97-116 antibodies was determined by ELISA using thiocyanate elution. Microplates were coated with R97-116 peptide (10 μ g/ml) and uncoated sites were blocked with 10% FBS. Diluted serum with a predetermined amount of anti-R97-116 antibodies was added and incubated. 200 μ l of appropriate quantities of potassium thiocyanate (KSCN) were added in duplicate and incubated at room temperature for 15 min, followed by biotinylated rabbit anti-rat IgG and streptavidin horseradish peroxidase. The color was developed with TMB and expressed as OD at 450 nm subtracted from 630 nm. The relative affinity is expressed as affinity index, equal to the molarity of KSCN resulting in 50% of the absorbance obtained in the absence of KSCN.

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