

Immunomodulatory effects of Longdan Xiegan Tang on CD4⁺/CD8⁺ T cells and associated inflammatory cytokines in rats with experimental autoimmune uveitis

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Abstract. Longdan Xiegan Tang (LXT) is a mixture of herbal extracts commonly used in traditional Chinese medicine that may exert immunomodulatory effects for the treatment of autoimmune diseases. However, the detailed mechanisms that mediate the actions of LXT are unclear. The present study induced an experimental autoimmune uveitis (EAU) model in Lewis rats via injection of IRBP1177-1191 emulsion. The model was used to investigate the effects of LXT on EAU rats and assess the efficacy of LXT by measuring clinical manifestations and histopathological changes caused by EAU. Additionally, alterations in the ratio of CD4⁺/CD8⁺-T cells were determined by flow cytometry, and the expression of interferon (IFN)- γ , interleukin (IL)-17, IL-10 and tumor necrosis factor (TNF)- α were measured using reverse transcription-quantitative polymerase chain reaction and enzyme-linked immunosorbent assay analysis. The results of the present study demonstrate that LXT can efficiently alleviate the symptoms of EAU, inhibit the differentiation of uveitogenic CD4⁺ T cells and reduce the expression of proinflammatory cytokines, including IFN- γ , IL-17 and TNF- α . Furthermore, LXT promotes the production of IL-10 and accelerates the recovery of EAU, indicating that

the immunomodulatory effects of LXT may potentially be used for the treatment of uveitis.

Introduction

Autoimmune and inflammatory uveitis are potentially blinding intraocular diseases that are often associated with immunological responses to unique retinal proteins (1). Experimental autoimmune uveitis (EAU) animal models of ocular autoimmunity targeting retinal proteins are useful to investigate the underlying causes of human uveitis and have improved the understanding of the basic immunological mechanisms of uveitis. EAU models act as templates for the development of novel therapeutic strategies for autoimmune and inflammatory uveitis (1,2). Previous investigations have used proteomic profiling to measure changes in EAU rat plasma compared with control samples, indicating that the pathogenesis of EAU is associated with the aberrant expression of proteins involved in multiple signaling pathways (3). Additionally, the development of EAU is also associated with the regulation of mRNAs by microRNAs (4).

Previous studies have investigated the changes of uveitogenic CD4⁺ T cell populations, particularly Th1 and Th17, during the development of autoimmune uveitis (5,6). Further studies indicate that alterations to the levels of cytokines secreted by T cells are correlated with the occurrence and development of uveitis. Elevated levels of interferon (IFN)- γ , (interleukin) IL-17 and tumor necrosis factor (TNF)- α are associated with the exacerbation of uveitis, indicating that these cytokines are uveitogenic (7). Furthermore, IL-10 has been demonstrated to ameliorate the clinical disease scores of EAU and has a protective function in uveitis (7). Additionally, measuring changes to the CD4⁺/CD8⁺ ratio in aqueous humor (8) or vitreous fluid (9,10) may be useful during the diagnosis of uveitis.

Longdan Xiegan Tang (LXT) is a commonly prescribed herbal formula in traditional Chinese medicine. It has been

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widely used in clinical practice for its anti-inflammatory, anti-infection, antibacterial, anti-allergy, hepatoprotectant, cholagogic and immunostimulatory activities (11-14). Traditionally, LXT is composed of 10 plant extracts: *Radix Gentianae*, *Radix Scutellariae*, *Fructus Gardeniae*, *Rhizoma Alismatis*, *Caulis Clematidis Armandii*, *Semen Plantaginis*, *Radix Angelicae Sinensis*, *Radix Rehmanniae*, *Radix Bupleuri* and *Radix Glycyrrhizae*. Lee and Chang (14) reported that LXT exerts immunomodulatory effects and regulates the immune function of mice with systemic autoimmune lupus erythematosus. Based on the theories of traditional Chinese medicine, LXT has been used in clinical trials for the treatment of uveitis and the syndrome of burning-heat in the liver and gallbladder (15). However, the detailed mechanisms of how LXT exerts its beneficial effects remain unclear. To investigate the effects of LXT on the development of uveitis, the present study established an EAU model in Lewis rats via immunization with interphotoreceptor retinoid-binding protein (IRBP) in complete Freund's adjuvant (CFA) solution supplemented with *Mycobacterium tuberculosis* H37Ra strain. Furthermore, the efficacy of LXT on EAU rats was determined by evaluation of clinical manifestations and histopathology. Changes to CD4⁺ and CD8⁺ T cell populations, and the CD4⁺/CD8⁺ ratio in EAU and LXT-treated rats were measured by flow cytometry. Additionally, the expression levels of IFN- γ , IL-17, TNF- α and IL-10 were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA) analysis. The findings of the present study provide insight into the molecular mechanisms that mediate the effects of LXT on uveitis.

Materials and methods

Animals. Female Lewis rats (6-8 weeks old; 160-180 g; Grade II; certificate number of the breeder, SCXK Jing 2012-0001) were purchased from Beijing Vital River Laboratory Animal, Co., Ltd. (Beijing, China) and bred at The Eye Institute of Shandong University of Traditional Chinese Medicine (Jinan, China). Rats were housed at room temperature (25 \pm 1°C) with relative humidity 50 \pm 10%. The animal facility was under a 12 h light/dark cycle. Prior to the experimental procedures, all rats were acclimatized to the housing room and experimental handling for 1 week. Animal care and use strictly followed the National Institutes of Health guidelines (16), and all experiments were approved by the Laboratory Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine.

Reagents. IRBP peptide (amino acids 1177-1191; sequence, ADGSSWEGVGVVDPDV) and primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). CFA and 2-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Mycobacterium tuberculosis* (strain H37RA) was purchased from Difco; BD Biosciences (Franklin Lakes, NJ, USA). Recombinant rat IL-2, fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (11-0040) and phycoerythrin (PE)-conjugated anti-CD8 antibody (12-0084) were purchased from eBioscience, Inc. (San Diego, CA, USA). IFN- γ (DKW12-3000-096), IL-17A (DKW12-3170-096),

TNF- α (DKW12-3720-096) and IL-10 (DKW12-3100-096) ELISA kits were purchased from Dakewe Biotech Co., Ltd. (Shenzhen, China). Phosphate-buffered saline (PBS), formaldehyde, paraffin, hematoxylin and eosin (HE) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). RPMI 1640 medium was purchased from Gibco; Thermo Fisher Scientific, Ltd. (Waltham, MA, USA). All experimental procedures adhered to the Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and vision research (17).

Induction of EAU and intervention of LXT. Lewis rats were randomly divided into three groups (normal control, EAU and LXT group) with 15 rats in each group. On day 0, each rat in the EAU and LXT groups were subcutaneously immunized with 300 μ l IRBP (100 μ g) emulsion containing 100 μ l CFA (2.5 mg/ml) and 100 μ g *Mycobacterium tuberculosis*, distributed at five sites, including one footpad, two flanks and the backside. Rats in the normal control group were treated with 300 μ l emulsion containing 100 μ l of CFA (2.5 mg/ml), 100 μ g of *Mycobacterium tuberculosis* and sterilized PBS.

Rats in the LXT group received LXT decoctions on day 5 following EAU induction. The decoctions included 10 types of boil-free granules of aforementioned traditional Chinese medicine extracts. All boil-free granules were purchased from China Resources Sanjiu Medical & Pharmaceutical Co., Ltd. (Shenzhen, China). The identification for each granule was determined by thin layer chromatography (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China) according to Chinese Pharmacopeia (edition 2010), and the quality of all products met the requirements of Chinese Pharmacopeia (18). The administration of LXT was performed by oral gavage (200 mg/kg/day) over the experimental period. Rats in normal and EAU groups received equal volumes of sterilized distilled water.

Clinical and histopathological assessment. A hand-held retinal camera (Genesis-D; Kowa Company, Ltd., Aichi, Japan) was used to record the inflammatory response of the anterior segment of rats each day following immunization until the end of the experimental protocol. At the desired intervals following immunization (days 4, 8, 12, 16 and 20), rats were euthanized using excess phenobarbital and the eyes were extracted. The harvested eyes were fixed in 4% formaldehyde for 24 h, embedded in paraffin blocks and serially sectioned (5 μ m) in the transverse plane. All sections were stained with HE and were observed under a light microscope (Ti; Nikon Corporation, Tokyo, Japan). The score of eye inflammation was evaluated using previously described criteria (15) and the severity of EAU was scored on a scale of 0 (no inflammation) to 4 (maximum inflammation).

Flow cytometry analysis. Briefly, 3 rats in each group were randomly sacrificed on days 4, 8, 12, 16 and 20 following immunization. T cells were isolated from lymph node and spleen by passage through a nylon wool column (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Following collection, 1 \times 10⁷ cells were cultured in 6-well plates and stimulated with 10 μ l IRBP (10 mg/ml) and 1 μ l recombinant rat IL-2 (10 ng/ml) for 48 h in the presence of 1 \times 10⁷ irradiated syngeneic spleen antigen presenting cells in 2 ml RPMI 1640 medium

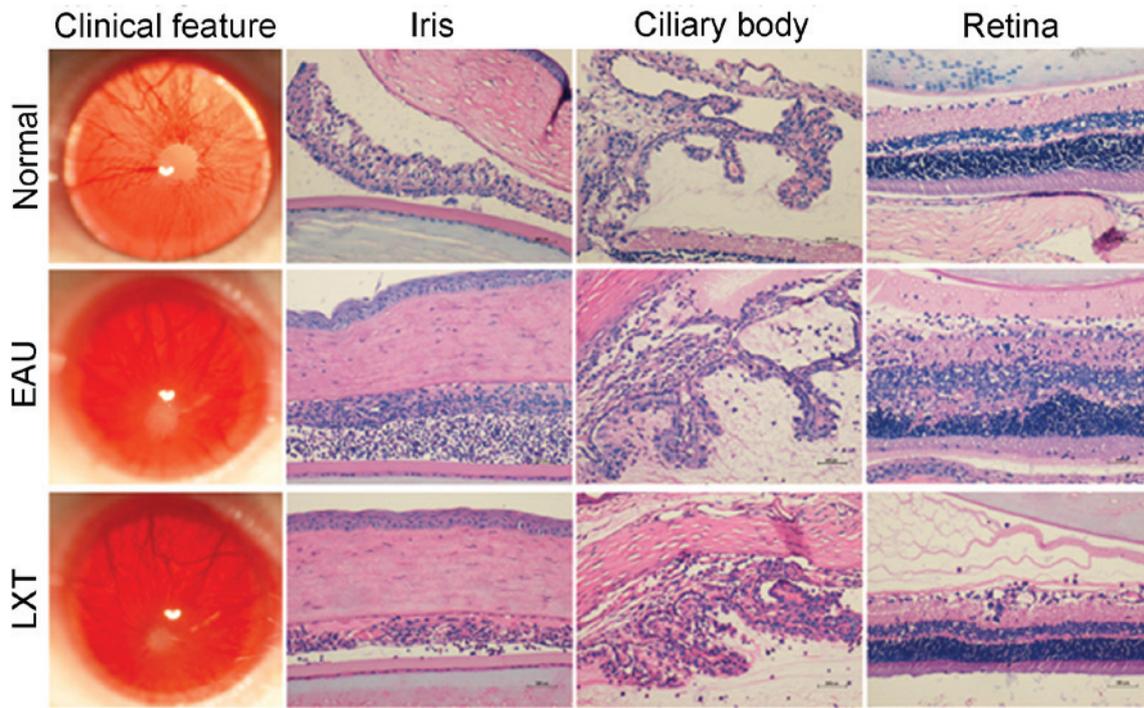


Figure 1. Clinical and histopathological features of rat eyes from the normal, EAU and LXT groups on day 12 post-immunization. Retinal images were captured with a hand-held retinal camera and hematoxylin and eosin staining was performed on eye sections (magnification, x200). EAU; experimental autoimmune uveitis; LXT, Longdan Xiegan Tang.

supplemented with 2-mercaptoethanol (final concentration, 5×10^{-5} mol/l). Subsequently, the activated T cells were isolated by Ficoll gradient (GE Healthcare Life Sciences, Uppsala, Sweden) at $600 \times g$. T cells were then stained with FITC-CD4 and PE-CD8 antibodies (1:100) in the dark at 4°C for 25 min. The stained cells were washed with PBS twice and analyzed using a BD FACSVers flow cytometer and BD FACSuite, version 1.0 (BD Biosciences, Franklin Lakes, NJ, USA).

RT-qPCR. To determine the alterations to IFN- γ , IL-17, TNF- α and IL-10 mRNA levels, RT-qPCR was performed using samples of blood, lymph node and spleen from rats at days 4, 8, 12, 16 and 20 post-immunization. The relevant tissues and blood from normal control rats were used as negative control. Briefly, rats were humanely euthanized at the indicated intervals and the lymph nodes, spleen and blood in each group were isolated and stored in liquid nitrogen. Following homogenization, total RNA was extracted from these tissues using TRIzol reagent (Thermo Fisher Scientific, Inc.) and the RNA purity and concentration were determined with a K5600 spectrophotometer (Beijing Kaiiao Technology Development Co., Ltd., Beijing, China). Synthesis of first-strand cDNA was performed using $1 \mu\text{g}$ total RNA and Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. The RT-qPCR reaction was performed in a $20 \mu\text{l}$ reaction using 2X SYBR Green qPCR Mix (Aidlab Biotechnologies Co., Ltd., Beijing, China). The PCR reaction was performed using a Realtime PCR System (Agilent Technologies, Inc., Santa Clara, CA, USA) with an initial denaturation step of 95°C for 5 min, followed by 45 cycles of 95°C for 20 sec, 58°C for 25 sec and 72°C for 25 sec. Relative gene expression

levels were quantified using the $\Delta\Delta\text{C}_q$ method. The $\Delta\Delta\text{C}_q$ values were calculated as the fold change in expression over tissues cells in mean \pm standard error following normalization to the β -actin reference gene (19). The primers used for RT-qPCR were as follows: β -actin, forward 5'-CAC CCGCGAGTACAACCTTC-3', reverse 5'-CCCATACCC ACCATCACACC-3'; IFN- γ , forward 5'-GGATATCTG GAGGAACTGGCA-3', reverse 5'-GCTAGATTCTGGTGA CAGCTG-3'; IL-17, forward 5'-TTGCTGCTACTGAAC CTGGAG-3', reverse 5'-GCATGGCGGACAATAGAG-3'; IL-10, forward 5'-TTCCATCCGGGGTGACAATAA-3', reverse 5'-TTCTGGGCCATGGTTCTCTGC-3'; TNF- α , forward 5'-TACTGAACTTCGGGGTGATTGGTCC-3', reverse 5'-CAGCCTTGCCCTTGAAGAGAACC-3'.

ELISA. To measure the alterations of cytokines in the blood on days 4, 8, 12, 16 and 20 post-immunization, 3 rats in each group were humanely euthanized at the indicated time-point. Blood samples were then collected and plasma was isolated by centrifugation at $2,000 \times g$ for 10 min at 4°C . The levels of IFN- γ , IL-17A, TNF- α and IL-10 were measured using the relevant ELISA kit. All procedures were conducted in accordance with the manufacturer's instructions.

Statistical analysis. The results are expressed as the mean \pm standard deviation. Each experiment was performed in triplicate and repeated 3 times. SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. Statistical analysis was performed using one-way analysis of variance followed by the least significant difference multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Alterations of CD4⁺ and CD8⁺ T cells in lymph nodes.

Lymph node	CD4 ⁺ T cell population		CD8 ⁺ T cell population		CD4 ⁺ /CD8 ⁺ ratio	
	EAU group	LXT group	EAU group	LXT group	EAU group	LXT group
Normal	77.99±1.03		20.85±0.84		3.74±0.20	
Day 4	71.44±4.47	69.14±4.62	28.36±2.84	29.53±3.13	2.55±0.42 ^b	2.40±0.39 ^b
Day 8	73.11±4.29	60.83±6.16	26.80±5.19	36.35±5.39	2.82±0.75 ^{a,c}	1.71±0.39 ^{b,c}
Day 12	84.19±1.36	77.47±1.68	14.20±0.97	17.26±1.79	5.95±0.51 ^{b,d}	4.52±0.54 ^d
Day 16	81.29±3.43	78.66±0.69	19.39±2.70	18.65±0.42	4.26±0.76	4.21±0.13
Day 20	78.46±3.04	77.62±1.65	18.06±1.71	20.59±0.84	4.36±0.50	3.78±0.24

^aP<0.05, ^bP<0.01 vs. normal rats. ^cP<0.05, ^dP<0.01 vs. EAU or LXT group at equivalent time point. CD, cluster of differentiation; EAU, experimental autoimmune uveitis; LXT, Longdan Xiegan Tang.

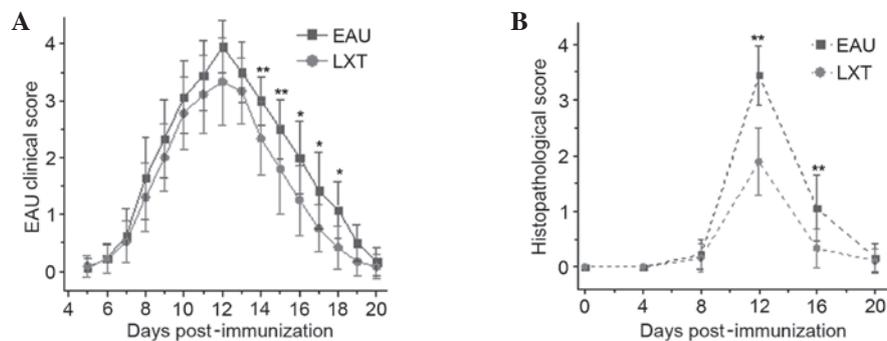


Figure 2. The effect of LXT on EAU clinical score and histopathology. (A) Clinical and (B) histopathological scores in EAU and LXT groups. *P<0.05, **P<0.01 vs. EAU group at the equivalent time point. EAU, experimental autoimmune uveitis; LXT, Longdan Xiegan Tang.

Results

Efficacy of LXT on EAU rats. The inflammatory response of rats in each group was observed each day until day 20. Additionally, histopathological examination was performed following euthanization of 3 rats on days 4, 8, 12, 16 and 20. Clinical signs of dilated blood vessels in the iris of rats were first observed on day 5 post-immunization and thus, LXT treatment was administered to rats in the LXT group. On day 12 post-immunization, rats in the EAU group exhibited opaque anterior chambers with severe fibrin exudation, obscured pupil, absence of red reflex and proptosis (Fig. 1). The intensity of uveitis was scored 4. However, the majority of rats in the LXT group exhibited relatively lighter symptoms, including moderately opaque anterior chambers and visible pupils (Fig. 1). Furthermore, in the EAU group, histopathological examination demonstrated a large number of inflammatory cells and fibrin exudation in anterior chamber, synechia of the iris and ciliary body structure disorder, and full-thickness retinal damage on day 12 post-immunization. However, compared with the EAU group, the LXT group demonstrated decreased inflammatory infiltration and fibrin exudation in anterior chamber, reduced synechia of the iris and ciliary body structure disorder, mild to moderate inflammation of the retina, and photoreceptor outer segment damage or lesions extending to the outer nuclear layer (Fig. 1).

Although the EAU and LXT groups developed severe inflammation from day 5 until day 12 post-immunization, rats

in the LXT group exhibited a faster recovery compared with EAU group, particularly between day 14 until 18 (P<0.001; Fig. 2A). Additionally, the histopathological scores of LXT mice were significantly reduced compared with EAU mice at days 12 and 16 (P<0.01), indicating that LXT has a protective effect on the retina of rats with EAU (Fig. 2B).

Alterations in the CD4/CD8 ratio in lymph node and spleen.

The results of flow cytometry analysis demonstrated that in the lymph nodes of normal control rats, the levels of the CD4⁺ and CD8⁺ T cell populations were 77.99±1.03% and 20.85±0.84%, respectively, producing a CD4⁺/CD8⁺ ratio of 3.74±0.20 (Table I). However, following treatment with IRBP emulsion, the ratio of CD4⁺/CD8⁺ in the lymph nodes of EAU rats was significantly decreased compared with normal controls at day 4 (P<0.001), and then subsequently elevated reaching a peak on day 12 (P<0.001). However, following treatment with LXT, the ratio of CD4⁺/CD8⁺ returned to normal levels on day 12, and were significantly reduced compared with EAU rats at the equivalent time point (P=0.002). These results demonstrated that LXT regulates the immune response and maintains the normal physiological functions following EAU (Table I).

In the spleen in normal control rats, the percentage of the CD4⁺ and CD8⁺ T cell populations were 52.36±8.40% and 37.95±6.36%, respectively, producing a CD4⁺/CD8⁺ ratio of 1.43±0.45 (Table II). The CD4⁺/CD8⁺ ratio in the spleen was increased continuously during the development of inflammation

Table II. Alterations of CD4⁺ and CD8⁺ T cells in spleens

Spleen	CD4 ⁺ T cell population		CD8 ⁺ T cell population		CD4 ⁺ /CD8 ⁺ ratio	
	EAU group	LXT group	EAU group	LXT group	EAU group	LXT group
Normal	52.36±8.40		37.95±6.36		1.43±0.45	
Day 4	59.88±5.88	58.77±4.73	32.60±3.03	30.92±2.67	1.86±0.34	1.90±0.21
Day 8	73.59±4.72	62.75±1.63	25.13±6.49	30.65±2.90	3.13±1.15 ^a	2.06±0.24
Day 12	77.17±5.25	70.38±3.50	17.73±5.02	24.02±3.09	4.71±1.92 ^{b,c}	2.98±0.54 ^{b,c}
Day 16	73.59±4.08	52.54±2.03	23.34±2.03	36.15±6.00	3.18±0.43 ^{b,c}	1.48±0.27 ^c
Day 20	65.68±6.42	52.04±2.74	27.99±6.34	40.99±3.83	2.45±0.71	1.28±0.19

^aP<0.05, ^bP<0.01 vs. normal rats. ^cP<0.05 vs. EAU or LXT group at equivalent time point. CD, cluster of differentiation; EAU, experimental autoimmune uveitis; LXT, Longdan Xiegan Tang.

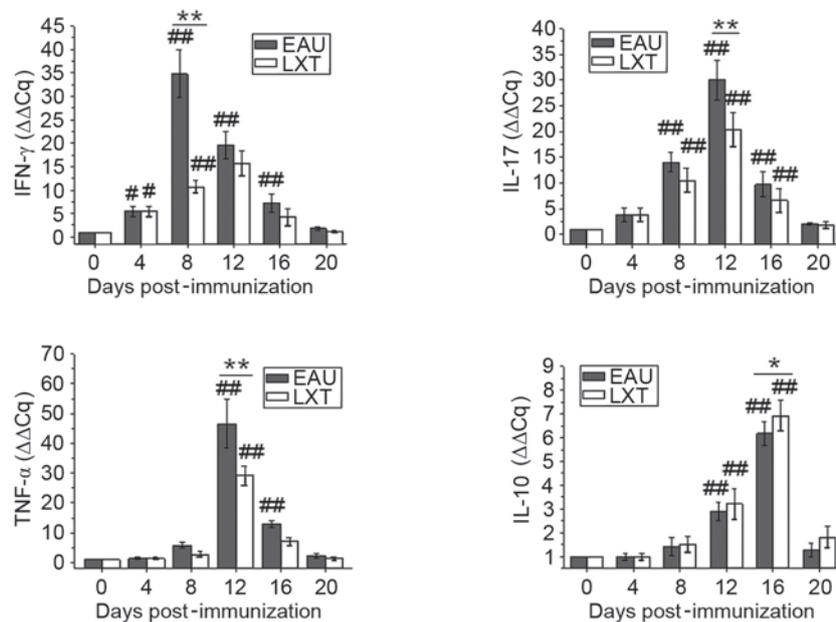


Figure 3. mRNA levels of IFN- γ , IL-17, TNF- α and IL-10 in blood. [#]P<0.05, ^{##}P<0.01 vs. normal control samples. ^{*}P<0.05, ^{**}P<0.01 vs. LXT or EAU group at the equivalent time point. IFN- γ , interferon- γ ; IL, interleukin; TNF- α ; tumor necrosis factor- α ; EAU, experimental autoimmune uveitis; LXT, Longdan Xiegan Tang.

reaching a peak day 12 in the EAU and LXT groups. The ratios differed from those measured in the lymph nodes. On day 12, the CD4⁺/CD8⁺ ratio in the LXT group (2.98±0.54) was significantly reduced compared with the EAU group (4.71±1.92; P=0.016). By contrast, the CD4⁺/CD8⁺ ratio in the LXT group was significantly increased compared with the normal group at day 12 only (P=0.028; Table II).

RT-qPCR. Following different treatments, the levels of IFN- γ , IL-17, TNF- α and IL-10 were determined by RT-qPCR analysis of rat blood, lymph node and spleen on days 4, 8, 12, 16 and 20. The relative expression of the normal rats was set as 1.00, and the relative gene expression levels were quantified using the $\Delta\Delta Cq$ method.

In blood (Fig. 3), the changes to the mRNA levels of IFN- γ , IL-17 and TNF- α demonstrated that the alterations in cytokine levels are associated with the exacerbation and alleviation of

inflammation in the EAU and LXT groups, respectively. The mRNA levels of IFN- γ increased from day 4 and peaked on day 8 post-immunization, and both IL-17 and TNF- α levels peaked on day 12 in the EAU group. However, the levels of IL-10 increased from day 12 and peaked on day 16 in the EAU and LXT groups. Furthermore, the peak mRNA levels of IFN- γ , IL-17 and TNF- α were significantly decreased (P<0.001), and IL-10 levels significantly increased (P=0.042), in the LXT group compared with those in the EAU group (Fig. 3).

Additionally, following immunization, the mRNA levels of IFN- γ , IL-17 and TNF- α were elevated from day 8 and peaked on day 12 in the EAU and LXT groups in lymph node (Fig. 4). By contrast, following LXT treatment, the mRNA levels of IFN- γ and IL-17 were reduced compared with the EAU group on days 12 (both P<0.001), 16 (both P<0.001) and 20 (P=0.032 and P=0.046) post-immunization. The levels of IL-10 in the EAU group increased on day 4 (P=0.011) and decreased between

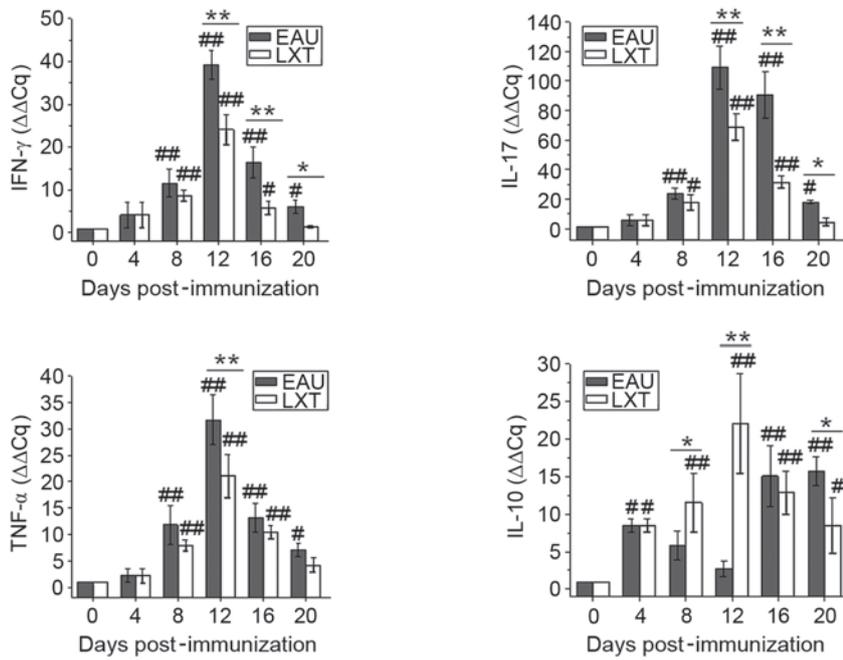


Figure 4. mRNA levels of IFN- γ , IL-17, TNF- α and IL-10 in lymph node tissue. #P<0.05, ##P<0.01 vs. normal control samples. *P<0.05, **P<0.01 vs. LXT or EAU group at the equivalent time point. IFN- γ , interferon- γ ; IL, interleukin; TNF- α ; tumor necrosis factor- α ; EAU, experimental autoimmune uveitis; LXT, Longdan Xiegan Tang.

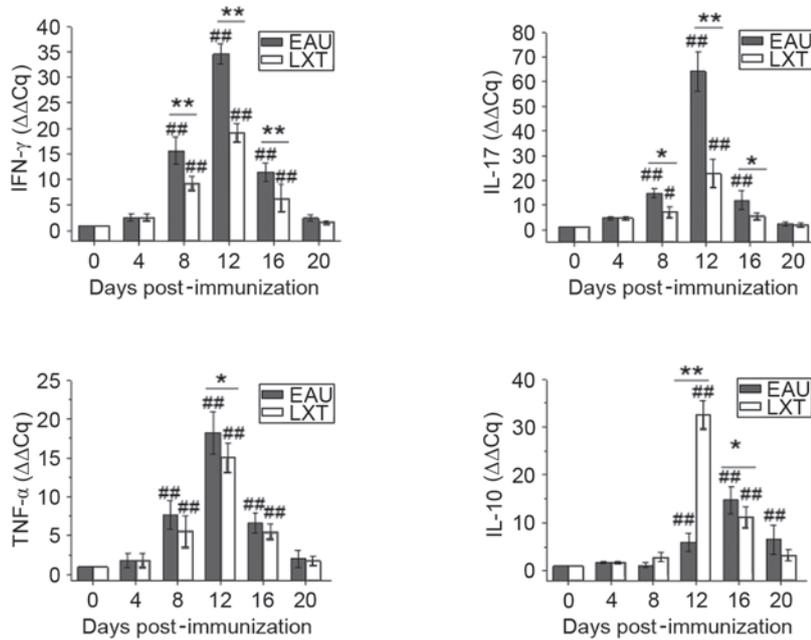


Figure 5. mRNA levels of IFN- γ , IL-17, TNF- α and IL-10 in spleen tissue. #P<0.05, ##P<0.01 vs. normal control samples. *P<0.05, **P<0.01 vs. LXT or EAU group at the equivalent time point. IFN- γ , interferon- γ ; IL, interleukin; TNF- α ; tumor necrosis factor- α ; EAU, experimental autoimmune uveitis; LXT, Longdan Xiegan Tang.

day 8 and 12 compared with day 0. Whereas compared with the levels at day 0, the IL-10 expression levels increased from day 4 (P=0.098) and peaked on day 12 (P<0.001) in the LXT group, then were maintained at an increased level throughout the experiment (Fig. 4).

Correspondingly, compared with the levels at day 0, the mRNA levels of IFN- γ , IL-17 and TNF- α in the spleen were

increased from day 8 and peaked on day 12 in the EAU and LXT groups (Fig. 5). However, following LXT treatment, the mRNA levels of IFN- γ and IL-17 were reduced compared with the EAU group on days 8 (P<0.001 and P=0.017, respectively), 12 (both P<0.001) and 16 (P=0.001 and P=0.035, respectively) post-immunization. By contrast, compared with the levels at day 0, the mRNA level of IL-10 was significantly increased

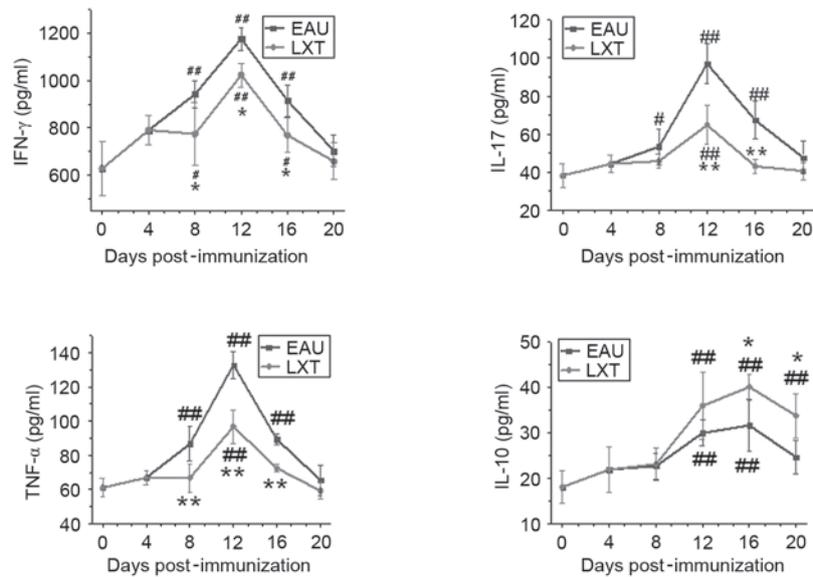


Figure 6. Levels of IFN- γ , IL-17, TNF- α and IL-10 in blood serum. #P<0.05, ##P<0.01 vs. normal control samples. *P<0.05, **P<0.01 vs. LXT or EAU group at the equivalent time point. IFN- γ , interferon- γ ; IL, interleukin; TNF- α ; tumor necrosis factor- α ; EAU, experimental autoimmune uveitis; LXT, Longdan Xiegan Tang.

from day 12 (P=0.005) and peaked on day 16 (P<0.001) in the EAU group. Additionally, compared with the EAU group, the mRNA level of IL-10 was significantly increased on day 12 in LXT group (P<0.001).

ELISA. Serum samples from EAU at LXT rats were used to determine the levels of IFN- γ , IL-17 and TNF- α and IL-10. The results indicated that the levels of IFN- γ , IL-17 and TNF- α in the EAU group exhibited similar expression patterns associated with the exacerbation and decrease of inflammation post-immunization (Fig. 6). The levels of IFN- γ , IL-17 and TNF- α increased from day 8 and peaked on day 12 post-immunization in the EAU group. However, following LXT treatment, the peak values of IFN- γ , IL-17 and TNF- α were significantly reduced compared with the levels of the EAU group (P=0.028, P<0.001 and P<0.001, respectively). By contrast, IL-10 expression increased from day 12 and peaked on day 16 in the EAU and LXT groups, and the levels of IL-10 were significantly increased in the LXT group compared with the EAU group on days 16 and 20 (P=0.031 and P=0.021; Fig. 6).

Discussion

LXT has been widely used in traditional Chinese medicine clinical trials to treat patients with anti-inflammatory, hepatoprotectant and immunoregulatory symptoms (13,14). Additionally, it has been used to treat the syndrome of burning-heat in the liver and gallbladder as described in traditional Chinese medicine theory. In the present study, an EAU rat model was induced using IRBP emulsion. Notably, in the EAU model fibrin exudation occurred in the anterior chamber of the eye, similar to the symptom of rising of yellow fluid in the eyes, which is a clinical manifestation of the syndrome of burning-heat in the liver and gallbladder (20). Therefore, LXT may be useful for the treatment of uveitis. The present study

observed that the majority rats in the LXT group exhibited reduced fibrin exudation in the anterior chamber compared with EAU rats, although there was no statistical difference in the clinical scores of the EAU and LXT groups on day 12. Furthermore, the results of histopathological examination revealed that LXT exerts protective effects on various tissues including the iris, ciliary body and retina in EAU rats. Based on the histopathological grading of the retina of EAU in Lewis rats (21), mild to moderate inflammatory infiltration and photoreceptor outer segment damage or lesions extending to the outer nuclear layer of retina were observed in the LXT group, which was less severe compared with the changes observed in the EAU group (Fig. 1).

EAU is initiated by the activation of CD4⁺ T cells that respond to ocular antigens located within or around photoreceptor segments (22). Kerr *et al* (23) demonstrated that the retinal infiltration of CD4⁺ T cells peaked on day 13 post-immunization and indicated EAU in the B10 mouse model. The induction of EAU in RIII mice induced by IRBP-3 161-180 peptides is predominantly mediated by CD4⁺ T cells. Thus, the characterization of different T-lymphocyte populations (CD4⁺ and 8⁺) during an ocular inflammatory episode may be useful for the diagnosis of uveitis (8). Kojima *et al* (9) reported the diagnostic value of measuring the CD4⁺/CD8⁺ ratio in vitreous fluid in uveitis with ocular sarcoidosis, and further revealed that a vitreum CD4⁺/CD8⁺ ratio >3.5 is a useful diagnostic parameter with 100% sensitivity and 96.3% specificity. The ratio of CD4⁺/CD8⁺ in aqueous humor can also be measured in patients with sarcoid uveitis (8). The present study demonstrated that the percentage of CD4⁺ T cell population and the CD4⁺/CD8⁺ ratio reached maximal values on day 12, which was also the time point of peak inflammation in both lymph nodes and spleen. However, following LXT treatment, the percentage of the CD4⁺ T cell population and CD4⁺/CD8⁺ ratio were reduced in the LXT group compared with the EAU group at the same time point.

Previous studies have indicated that IFN- γ , which is highly secreted by CD4⁺ Th1 cells, is pivotal in the development of EAU (24,25). Previous studies have demonstrated that IL-17, which is secreted by CD4⁺ Th17 cells and $\gamma\delta$ T cells, is important during the pathogenesis of autoimmune diseases (26-30). Peng *et al* (31) demonstrated that IL-17⁺ and IFN- γ ⁺ IRBP-specific T cells are uveitogenic. The present study observed that alterations of IFN- γ and IL-17 levels were closely associated with the exacerbation and subsequent reduction of inflammation in blood, lymph nodes and spleen in EAU rats, despite variations between levels in the types of sample. A previous study noted that differentiation of Th1 cells occurred earlier than Th17 cells in the spleen (32). The present study demonstrated that the mRNA expression levels of IFN- γ in the blood increased from day 4 post-immunization and peaked on day 8. These findings indicate that IFN- γ may be associated with the acute onset of inflammation. However, following LXT treatment, the relative expression of IFN- γ and IL-17 at the mRNA level were significantly reduced in the blood, lymph node and spleen. TNF- α is another important pathogenic cytokine and the blockade of TNF- α has been used as a successful immunotherapy in preclinical models of uveitis and in human disease (22). The current study observed that the expression of TNF- α exhibited the same trend as those of IFN- γ or IL-17 during the development of EAU, and that treatment with LXT reduced the expression of TNF- α at the peak stage of inflammation.

IL-10 is predominantly secreted by Th2 and T regulatory cells. It can alleviate the development of EAU by suppressing *de novo* priming of Ag-specific T cells and inhibiting the recruitment and function of inflammatory leukocytes (33). Under the negative regulatory effect of IL-10, the expression of IFN- γ and IL-17 decrease rapidly and exert additional regulatory effects on EAU (34). The present study observed that the expression of IL-10 peaked around day 16 post-immunization in blood, lymph nodes and spleen of the EAU group. However, following LXT treatment, the IL-10 mRNA expression was elevated in the LXT group compared with the EAU group at the same time point. Furthermore, the mRNA expression of IL-10 in the LXT group peaked on day 12 in the lymph nodes and spleen. These findings indicate that the elevation of IL-10 expression may mediate the alleviation of the inflammatory symptoms in EAU rats following LXT treatment.

In conclusion, LXT effectively alleviates the clinical manifestation of EAU in rats, suppresses the differentiation of uveitogenic CD4⁺ T cells and inhibits the expression of Th1 and Th17 signature cytokines, including IFN- γ and IL-17. Furthermore, LXT also promotes the secretion of IL-10, restores the immune balance, and thus, accelerates the recovery of autoimmune uveitis, indicating that LXT may be useful for the treatment of uveitis via regulation of the immune response.

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