

Luobitong Potentiates MTX's Anti-Rheumatoid Arthritis Activity via Targeting Multiple Inflammatory Pathways

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Background: The LuoBiTong (LBT) capsule, a novel traditional Chinese medicine formulation, is currently in Phase III clinical trials. Preliminary preclinical and Phase II clinical studies suggest its efficacy and safety in treating rheumatoid arthritis (RA). However, the underlying mechanisms of its action remain to be elucidated. This research aims to explore the effects and mechanisms of LBT in conjunction with a maintenance dose of methotrexate (M-MTX) on RA.

Methods: A Collagen-Induced Arthritis (CIA) mouse model was used to evaluate the anti-RA effects of LBT combined with M-MTX. Assessments included foot swelling, arthritis scoring, serum inflammatory factor analysis, and histopathological examination of the foot. These effects were compared with those of high-dose MTX (H-MTX). Network pharmacology was employed to construct a compound-target network for RA, based on drug composition, to predict its potential mechanism of action. Flow cytometry, Western Blot, and immunohistochemical analyses in animal models identified multiple inflammatory pathways targeted by LBT to augment the anti-RA effects of MTX.

Results: The study revealed that LBT combined with M-MTX significantly alleviated CIA-induced arthritis without adverse effects. The combination of LBT and M-MTX showed similar or superior efficacy in regulating macrophage polarization, NF- κ B, MAPK signaling pathways, and in the suppression of TH-17 expression in proinflammatory cells. These findings suggest that LBT may exert a multi-pathway therapeutic effect in RA treatment. The predicted pharmacological targets and mechanisms align well with this hypothesis.

Conclusion: LBT, when combined with MTX, enhances the anti-RA effect by targeting multiple inflammatory pathways, demonstrating significant therapeutic potential.

Keywords: rheumatoid arthritis, traditional Chinese medicine, methotrexate, network pharmacology

Introduction

Rheumatoid Arthritis (RA), one of the most frequently encountered rheumatologic conditions in clinical settings,¹ is a prevalent inflammatory autoimmune disease, affecting approximately 0.5–1% of the global population.² RA is characterized by symmetrical synovitis, which leads to the gradual degradation of articular cartilage and surfaces.^{3,4} The primary clinical manifestations of RA are pain, stiffness, and joint swelling, which can ultimately lead to deformity and functional impairment as the disease progresses.^{5,6} Additionally, RA is frequently associated with systemic complications, such as cardiovascular disease, neurological disease, and osteoporosis.^{7–9}

The pathogenesis of RA is a complex and multifactorial phenomenon that involves interactions between genetic, environmental, immunological, and other factors.¹⁰ Although the accurate pathogenesis is still elusive, RA symptomatically is a chronic inflammatory disease that causes damage to the joints and other organs.^{11,12} The inflammation is mediated by various immune cells and molecules, such as T cells, B cells, macrophages, cytokines, chemokines, and autoantibodies.^{13–16} These factors can activate the NF- κ B signaling pathway, which regulates the expression of genes involved in inflammation, cell survival, proliferation, and differentiation. Recent research has demonstrated that the development and progression of rheumatoid arthritis are influenced by multiple cytokines produced by helper T cell-17 (Th17), notably Interleukin-17A (IL-17A) and Interleukin-17F (IL-17F).^{17,18} Therefore, the management of rheumatoid arthritis currently relies on disease-modifying antirheumatic drugs (DMARDs) as soon as possible after the diagnosis.^{19–21} They work by suppressing or modifying the immune system to reduce pain, swelling, stiffness, and prevent or slow down joint damage and disability. For instance, Methotrexate (MTX), a first-line DMARD, is considered a safe and effective drug for most patients with RA when used appropriately.²² However, methotrexate also has some limitations and drawbacks in some patients,^{23,24} such as (a) Methotrexate has a narrow therapeutic window, which means that there is a fine balance between its efficacy and toxicity, and requires regular monitoring of blood counts, liver function, and kidney function.; (b) Methotrexate has a slow onset of action, which means that it may take several weeks or months to achieve its full therapeutic effect.^{25,26} Therefore, Methotrexate can also be combined with other drugs or modalities to enhance its efficacy and reduce its toxicity.^{27,28}

Additionally, combined therapy is generally considered to be more beneficial than single-agent therapy in the treatment of RA. The main reasons can be attributed to the following: RA is a complex disease with multiple inflammatory pathways involved; Combined therapy can be more effective in controlling symptoms; Combining medications can allow for lower doses of each individual medication. Traditional Chinese medicine (TCM) has emerged as a promising therapeutic alternative.^{29,30} Combining Traditional Chinese Medicine (TCM) with small molecular medicines (SMMs) is an emerging approach with significant potential for treating various diseases.³¹ For instance, the therapeutic regime of *Tripterygium wilfordii* Hook F (TwHF) combined with MTX is more effective than MTX or TwHF alone in the treatment of RA.^{32,33} Therefore, the Integrated TCM-SMM for RA treatment is promising and deserves attention. However, despite its therapeutic potential, TwHF comes with several safety concerns that require careful consideration before use.³⁴ Luobitong capsules, developed by Yiling Pharmaceutical Co., Ltd., are currently undergoing phase III clinical trials. Preclinical and phase II clinical data suggest that the drug has a therapeutic effect on RA with good safety. Luobitong capsules are composed of extracts from a variety of botanicals, including *Astragali Radix* (Huang Qi), *Gentianae Macrophyllae Radix* (Qin Jiao), *Stephaniae Tetrandrae Radix* (Fang Ji), *Radix Aconiti Praeparata* (Hei Shun Pian), *Polygoni Cuspidati Rhizoma Et Radix* (Hu Zhang), *Spatholobi Caulis* (Ji Xue Teng), *Clematidis Radix Et Rhizoma* (Wei Ling Xian), *Sinapis Semen* (Jie Zi), *Paeoniae Radix Alba* (Bai Shao), *Rehmanniae Radix* (Di Huang), *Angelicae Sinensis Radix* (Dang Gui), *Myrrha* (Mo Yao), *Cyperus Rhizoma* (Xiang Fu), *Cinnamomi Ramulus* (Gui Zhi), and *Cyathulae Radix* (Chuan Niu Xi). LuoBiTong capsules have anti-inflammatory, analgesic, and immunomodulatory effects.

In this study, to gain more insight into the therapeutic targets and pathways, we employed a “disease-drug” network pharmacological analysis to delve into the therapeutic efficacy of LBT and unravel the potential of LBT in anti-inflammation.^{35,36} Subsequently, synthetic effect of combined LBT and the maintenance dose of MTX (M-MTX) was evaluated in a CIA mouse model.

Methods

Materials

Universal SP kit, SP-9000, Zhongshan Jinqiao Biological Engineering Co., LTD., Beijing, China

Mouse IL-6 ELISA Kit (enzyme-linked immunosorbent assay), EK206, United Biotechnology Co., LTD., Hangzhou, China

Mouse IL-17A ELISA Kit (enzyme-linked immunosorbent assay), EK217, United Biotechnology Co., LTD., Hangzhou, China

Anti-IL-17A, ab79056, Abcam (Shanghai) Trading Co., LTD., Shanghai, China

Anti-IL-6, ab290735, Abcam (Shanghai) Trading Co., LTD., Shanghai, China

Anti-p38 MAPK, 9216s, Cell Signaling Technology company, USA

Anti-SAPK/JNK, 9255s, Cell Signaling Technology company, USA

Anti-TNF alpha, ab183218, Abcam (Shanghai) Trading Co., LTD., Shanghai, China

Anti-NF-kB p65, ab32536, Abcam (Shanghai) Trading Co., LTD., Shanghai, China

Methotrexate, 036210701, 2.5mg/tablet, Shanghai medicine sym pharma co., LTD., Shanghai,

LuoBiTong, A2103001,0.44g/tablet (Equivalent to 1.42g of crude drugs) shijiazhuang ridge pharmaceutical co., LTD., Shijiazhuang, China

Animal Handling

Eight-week-old SPF-grade DBA/1J mice ($n=70$, body weight 20 ± 2 g) were purchased from Jiangsu Jicui Pharmachem Biotech Co. and its molecular companies. Seventy mice were placed in a light-dark cycle at $23\pm 3^{\circ}\text{C}$ and $50\pm 10\%$ humidity for 12 h. Food and water were available ad libitum. mice were acclimatized to these living conditions one week before the experiment. All animal experiments were performed in strict accordance with the guidelines approved by the Ethical Committee of Animal Experiments of Jiangsu Jichi Pharmaceutical Kang Biotechnology Co., LTD.

Drugs Preparation

Calculation of LBT Formulation Concentration

The low and high doses correspond to 1.42 and 5.66 g of crude drug/kg, respectively. The volume of the administered animal is 10 mL/kg. Therefore, the calculation of the formulation concentration of the drug is 0.142 g of crude drug/mL and 0.566 g of crude drug/mL, respectively. Each tablet of LBT contains 1.42 g of crude drug. The drug is formulated according to the specifications of the whole tablets, which are listed as follows:

- (1) LBT Low Dose: Prepare at a concentration of 0.142 g of crude drug/mL, where 1 tablet of LBT is equivalent to 1.42 g of crude drug. Place the tablet in a mortar and gently grind it into a fine powder. Wet the powder with a small amount of 0.5% CMC-Na grinding, add solvent for grinding suspension, and adjust the volume to 10 mL.
- (2) LBT High Dose: Prepare at a concentration of 0.566 g of crude drug/mL, where 4 tablets of LBT are equivalent to 5.68 g of crude drug. Place the tablets in a mortar and gently grind them into a fine powder. Wet the powder with a small amount of 0.5% CMC-Na grinding, add solvent for grinding and mixing, and adjust the volume to 10 mL.

MTX Formulation Method, Configured to Whole Tablet Size (2.5 Mg/Tablet)

(1) MTX Alone Group (high-dose MTX group, H-MTX): Prepare at a concentration of 0.14 mg/mL, where 1 tablet is taken and ground into powder. Moisten the powder with a small amount of 0.5% CMC-Na, grind it, suspend it with solvent, and adjust the volume to 18 mL.

(2) MTX Co-administration Group (Maintenance dose MTX group, M-MTX): Prepare at a concentration of 0.05 mg/mL, where 1 tablet is taken and ground into powder. Moisten the powder with a small amount of 0.5% CMC-Na grinding, grind it, suspend it with solvent, and adjust the volume to 50 mL.

Establishment of Collagen-Induced Arthritis Model

Ten mice were randomly selected as a Control+vehicle group, and another 60 were subjected to CIA modeling. The RA model was constructed by subcutaneous immunization of the mice at the tail root using type II bovine collagen in combination with complete Fuchsin's adjuvant as well as incomplete Fuchsin's adjuvant, respectively. Specifically, 50 μL of type II bovine collagen emulsion formulated with complete Freund's adjuvant was injected subcutaneously into the tail root of each mouse after acclimatization feeding as the first immunization. Three weeks after the first immunization, a second immunization was performed using incomplete Fuchs' adjuvant formulated type II bovine collagen emulsion.

Grouping and Treatment

Control+Vehicle group (0.5% sodium carboxymethyl cellulose) ($n=10$).

After the second immunization, 60 CIA model mice were randomly divided into four groups according to their body weight: CIA+Vehicle group (0.5% sodium carboxymethyl cellulose) (n=15); CIA + MTX (1.4 mg/kg) (n=15); CIA + MTX (0.5 mg/kg) +LBT-L(1.42g crude drugs/kg) (n=15); The CIA + MTX (0.5 mg/kg) + LBT - H (5.66 g crude drugs/kg) (n=15). All animals were administered with the corresponding agents through oral gavage. Methotrexate was administered every 2 days for a total of 14 doses. LBT was administered once daily for a total of 28 doses. Body weight was measured three times a week. Animals were sacrificed on day 51 after the first immunization.

Arthritis Score

Body weight and arthritis scores were measured weekly after the first immunization.

Body weight and arthritis scores were measured twice weekly after the second immunization.

A score of 0 represented no obvious symptoms in the limbs of the mice. A score of 1 represents slight swelling of the lesser metatarsal joints; A score of 2 represents swelling of the metatarsal joints and toes; A score of 3 represents paw swelling below the ankle; A score of 4 represents total paw swelling including within the ankle joints.

The score for each mouse was the sum of the limb scores and the severity of arthritis was assessed by visual inspection by three independent observers. Observers were unaware of the presence of the treatment group.

Serum and Tissue Collection

After CIA was established, the experimental endpoints were amenable to mice after 4 weeks of administration by drug grouping. Blood was collected from the heart and a portion of the blood was anticoagulated and then split red and used for FACS to detect macrophage percentage. The other portion of blood was separated from serum and used for ELISA assay. Blood was taken and then executed, and the skin, fascia and muscles around the ankle joint were excised. The sole of the right foot (together with the bone) was fixed in 4% paraformaldehyde for 72 hours. The left paw was frozen instantly in liquid nitrogen.

Histological Examination

Upon completion of the experiment, the soles of the mice's feet were removed, fixed with 4% paraformaldehyde, and decalcified with 10% EDTA for histopathological analysis. Afterwards, the specimens were embedded in paraffin, sectioned into 5 mm thick sections, and stained with Hematoxylin and Eosin (H&E) and Safranin O-Fast Green.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum levels of IL-6 and IL-17A were measured by ELISA. The blood samples collected were 10 in Control+Vehicle group, and 15 in each of the other four groups.

The procedure was performed as directed by the ELISA kit for the detection of the serum inflammatory factors IL-6 and IL-17A. The absorbance of the samples at 450 nm was determined and the OD values were recorded for data processing and analysis.

Constructing the Network Pharmacology of Diseases and Drugs

We explore the molecular mechanism of LBT for the treatment of RA using a network pharmacological approach combining chemical and therapeutic properties. Using "rheumatoid arthritis" as the search term, the RA gene was retrieved from six sources, including DisGeNET,³⁷ Open Target Platform,³⁸ MalaCards,³⁹ CTD,⁴⁰ GeneCards and text mining.⁴¹ Using The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2021 (<https://david.ncicrf.gov/>) for standardization gene name.⁴² To ensure the reliability of the data, only genes that appear in more than three databases are retained as the core gene set for RA. The gene sets related to RA and the potential target of LBT were analyzed to find the functional target of LBT for preventing and treating RA. Then the target data were submitted to STRING Version 12.0 (<https://string-db.org/>) protein interaction background network for the construction of PPI network (confidence 0.7).⁴³ The obtained PPI network is visualized in Cytoscape v3.9.0.⁴⁴ The comprehensive target spectrum of LBT is essential for the study of its substantive basis and mechanism of action in the treatment of RA. We collected targets from DrugBank,⁴⁵ TTD,⁴⁶ ChEMBL,⁴⁷ PubChem and standardized their name through UniProt.⁴⁸⁻⁵⁰ In order to

further explore LBT key active compounds in their treatment of RA and targets, we collected the literature of targets and therapeutic targets from PHARMACODIA database (<https://www.pharmacodia.com/>). To explain the mechanisms of LBT against RA from a systematic perspective, we performed gene ontological (GO) and KEGG pathway enrichment analyses using Metascape (<https://metascape.org>) and ClueGO plugin in Cytoscape.⁵¹

Flow Cytometry Analysis of Blood Cells

Macrophage proportions were detected by FACS and stained with various anti-mouse antibodies coupled with different fluorescent markers. Indicators included CD45, CD11b, F4/80, MHC II, and CD206. After gating the live cells according to the scatter characteristics according to the manufacturer's protocols, acquisition and analysis were performed using BD Aril3 and FlowJO software.

Western Blot Analysis

Paw tissue specimens were ground into powder and used for Western blot analysis. After lysis and centrifugation, sample supernatants were quantified using the BCA protein assay kit. The protein samples were then separated by 10% sodium polyacrylamide gel electrophoresis (SDS-PAGE). These proteins were transferred to PVDF membranes and blocked with 5% skim milk to prevent non-specific binding. Membranes were then incubated with appropriate anti-IL-6 (1:1000), TNF- α (1:1000), IL-17A (1:1000), P-NF κ B p65 (1:1000), P-p38 (1:1000), P-SAPK/JNK(1:1000), and GAPDH. The primary antibody (Abcam) was incubated overnight at 4°C. After incubation, membranes were washed with PBST and then incubated with appropriate peroxidase-labeled secondary antibodies. Mean area density was expressed for all protein blots relative to GAPDH expression. Results for all target bands were performed at least 3 times.

Immunohistochemistry

For immunohistochemical (IHC) staining, tissue sections were rehydrated and microwave radiation antigen repair method was used. Specifically, the slices were exposed to 10 mM citrate buffer in a microwave oven for 3 minutes and then left for 1 minute. Low fire for 3 min, let stand for 1 min, and this operation was repeated three times. The plates were allowed to drop to room temperature, and flushed 3 times with PBS buffer. An appropriate amount of endogenous peroxidase blocker was added, and the cells were incubated at room temperature for 10 minutes, followed by three flushes with PBS buffer. An appropriate amount of normal goat serum working solution for blocking was added and incubated at 37 ° C for 15 min. Rabbit anti-IL-17A antibody (1:200 concentration) was added to the serum and incubated at 4 ° C overnight. The next day, the cells were flushed with PBS buffer three times. Biotin-labeled goat anti-mouse IgG was added dropfold and incubated at 37 ° C for 15 min followed by three rinses with PBS buffer. The cells were incubated with the working solution of horseradish enzyme-labeled streptavidin for 15 min at 37 ° C and then rinsed with PBS buffer three times. Sections were incubated with DAB chromogenic reagent (Zhongshan Jinqiao Biological Engineering Co., LTD.) for 5 min at room temperature. These sections were then viewed with a light microscope.

Statistical Analysis

The experimental data are expressed as Mean \pm SEM. Independent sample *T*-Test was used for comparison between Two groups of samples, One-way ANOVA test or two-way ANOVA was used for comparison between multiple groups. The data analysis and mapping software was Graphpad prism. $p < 0.05$ was considered as significant difference.

Results

LBT Alleviates Arthritis Symptoms in CIA Mouse Models

The experiment was terminated on the 51st day after the construction of the CIA model, as shown in [Figure 1A](#). Each mouse was weighed three times a week for 49 days during the experiment ([Figure 1B](#)). Compared with CIA+Vehicle group, body weight in CIA+Vehicle group decreased from day 23 to 49. Compared with CIA+Vehicle, the weight of mice after drug treatment increased, but the difference was not statistically significant ($p > 0.05$).

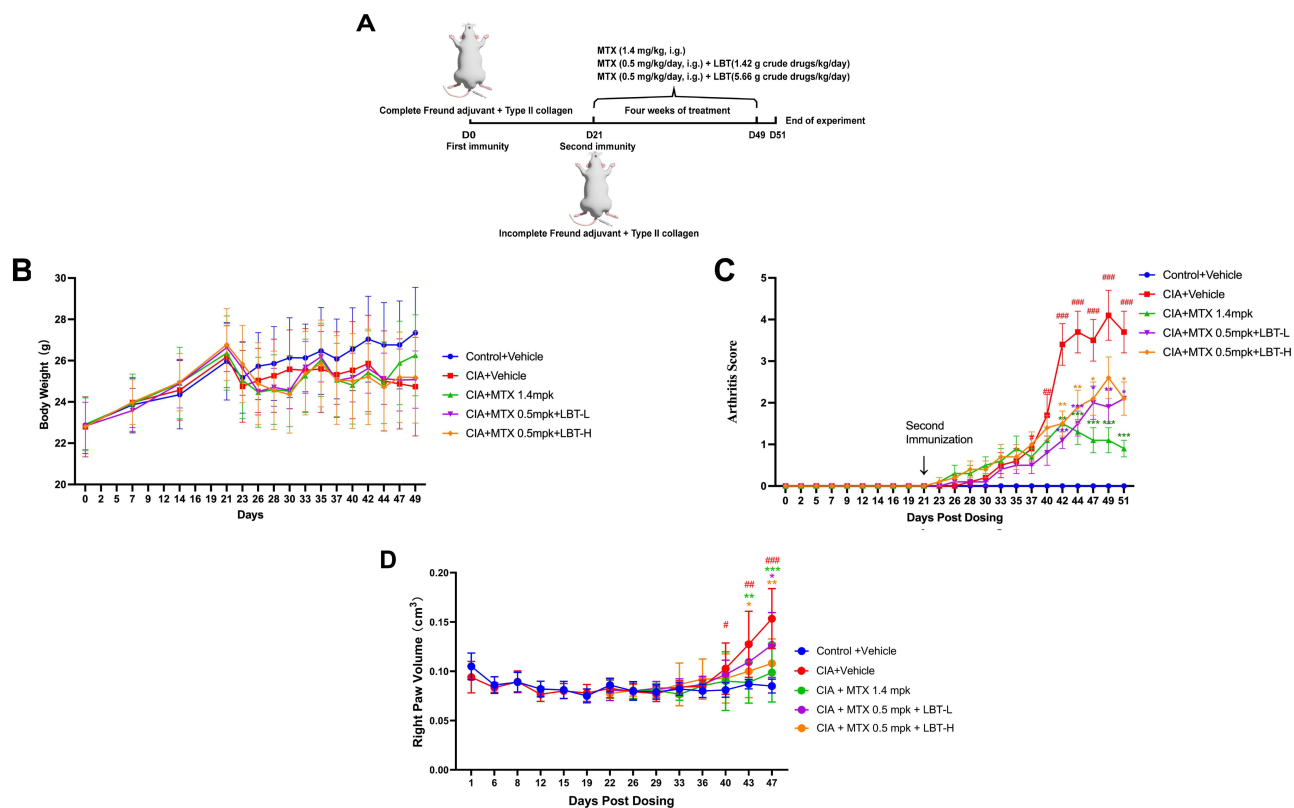


Figure 1 LBT alleviated the signs and symptoms of CIA in mice. **(A)** Flow chart of animal experiments. **(B)** Changes in body weight of mice. **(C)** Effect of LBT and MTX on arthritis score. **(D)** Effects of LBT and MTX on claw swelling score. # $P < 0.05$, compare with Control +Vehicle; ### $P < 0.01$, compare with Control +Vehicle; #### $P < 0.001$, compare with Control +Vehicle; * $P < 0.05$, compare with CIA+Vehicle; ** $P < 0.01$, compare with CIA+Vehicle; *** $P < 0.001$, compare with CIA+Vehicle.

To assess the severity of arthritis in CIA mice, arthritis scores were measured from day 0 to day 51 (Figure 1C). In the CIA+Vehicle group, arthritis scores increased significantly from day 28 to day 51. Compared with CIA+Vehicle mice, both MTX and MTX combined with LBT reduced the severity of arthritis in CIA mice.

Interestingly, we found the same trend in paw volume scores (Figure 1D). Compared with the Control +Vehicle group, we found that the degree of toe swelling in the CIA+Vehicle group was statistically significant on the 40th day after modeling ($P < 0.05$). At the end of the experiment, the volume was significantly higher than that in the Control +Vehicle group ($P < 0.001$). Again, the medication group alleviated this phenomenon. CIA+MTX 1.4mpk group, CIA +MTX 0.5mp+ LBT-L group and CIA+MTX 0.5mp+ LBT-H group reduced the paw swelling to different degrees ($P < 0.05$, $P < 0.01$, $P < 0.001$).

MTX and M-MTX Combined with LBT Can Alleviate Collagen-Induced Arthritis Inflammation and Cartilage Damage

To investigate the anti-inflammatory effects of LBT on RA in vivo, we conducted histological analyses on CIA DBA/1J mice (Figure 2). H&E staining revealed prominent histomorphological changes in the arthritis of the CIA model group, characterized by extensive infiltration of inflammatory cells and fibrosis in the interdigital joints and surrounding tissues of the mouse paws. Additionally, joint synovial hyperplasia and erosion of the joint cartilage were observed. In comparison to the CIA+Vehicle group, the high dose of MTX (H-MTX) group, as well as the high and low dose LBT combined with M-MTX groups, exhibited significant improvement. These treatment groups showed reduced infiltration of inflammatory cells, slight synovial membrane hyperplasia, and no evident bone destruction. Analysis of Safranin O-Fast Green staining revealed that the articular cartilage matrix between the hind paws and toes of the CIA+Vehicle group was visibly diminished, with multiple cracks present. However, treatment with H-MTX or M-MTX combined with

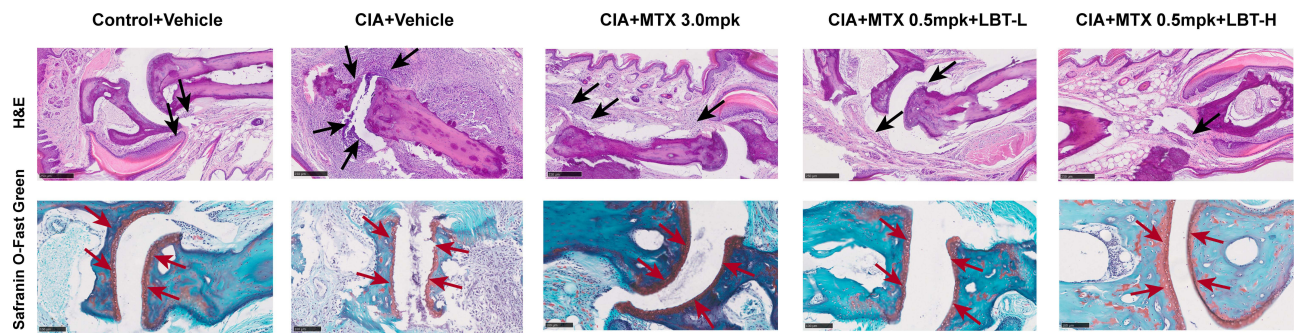


Figure 2 H&E staining (Scale bar = 250 μ m) and Safranin O-Fast Green staining (Scale bar = 100 μ m) results (n = 3), (The black arrows are inflammatory cells and the red silk arrows are cartilaginous surfaces).

LBT resulted in reduced cartilage injury in CIA mice, with no significant loss of cartilage matrix observed. This was evident from the smooth surface of the articular cartilage and the absence of cartilage fractures.

LBT Effectively Suppressed the Production of Inflammatory Factors in the Serum

To assess the impact of LBT on inflammatory factors, we conducted enzyme-linked immunosorbent assay (ELISA) tests to measure the levels of IL-6 and IL-17A in the serum. The concentrations of IL-6 and IL-17A in the serum of CIA +Vehicle group mice were significantly elevated compared to the control+Vehicle group ($P < 0.05$) (Figure 3A and B). However, the H-MTX group and the M-MTX combined with LBT treatment group significantly reduced the serum levels of these inflammatory cytokines. Notably, the combined treatment with a high dose of LBT exhibited the most pronounced effect.

Establishment of Network Pharmacology and Target Analysis of LBT and RA

In view of the efficacy of LBT, to further understand its mechanism of action, we constructed a network pharmacology of rheumatoid arthritis and LBT. Based on the pathogenic genes reported in the RA literature and the therapeutic effect targets of approved drugs, the PPI molecular network of RA-specific pathogenesis was constructed and mechanism were explored. There are 309 targets that can be regulated by LBT, and 31 key targets were selected through network parameters, such as IL6, TNF, IL1B, IL10, STAT1, CXCL8, JAK2, IL17A, TLR2, MMP9, etc. (threshold: degree > 11, between centrality > 0.011, closeness centrality > 0.265) (Figure 4A). In addition, key biological processes regulated by LBT include inflammatory response, cytokine-mediated signaling pathway, leukocyte migration, etc. (Figure 4B). IL-17

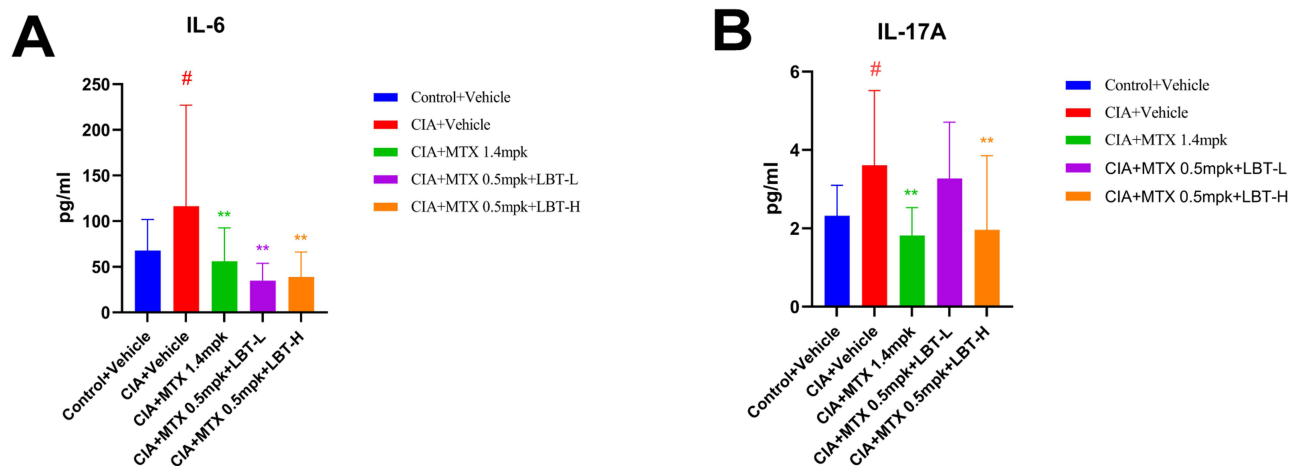


Figure 3 The Effects of Methotrexate or Methotrexate Combined with LBT on Serum Inflammation in DBA/1J Mice with Collagen-Induced Arthritis (Control+Vehicle group n = 10, other groups n = 15). (A) Serum IL-6 Levels; (B) Serum IL-17 Levels. ** $P < 0.01$, compare with CIA+Vehicle. # $P < 0.05$, compare with Control +Vehicle.

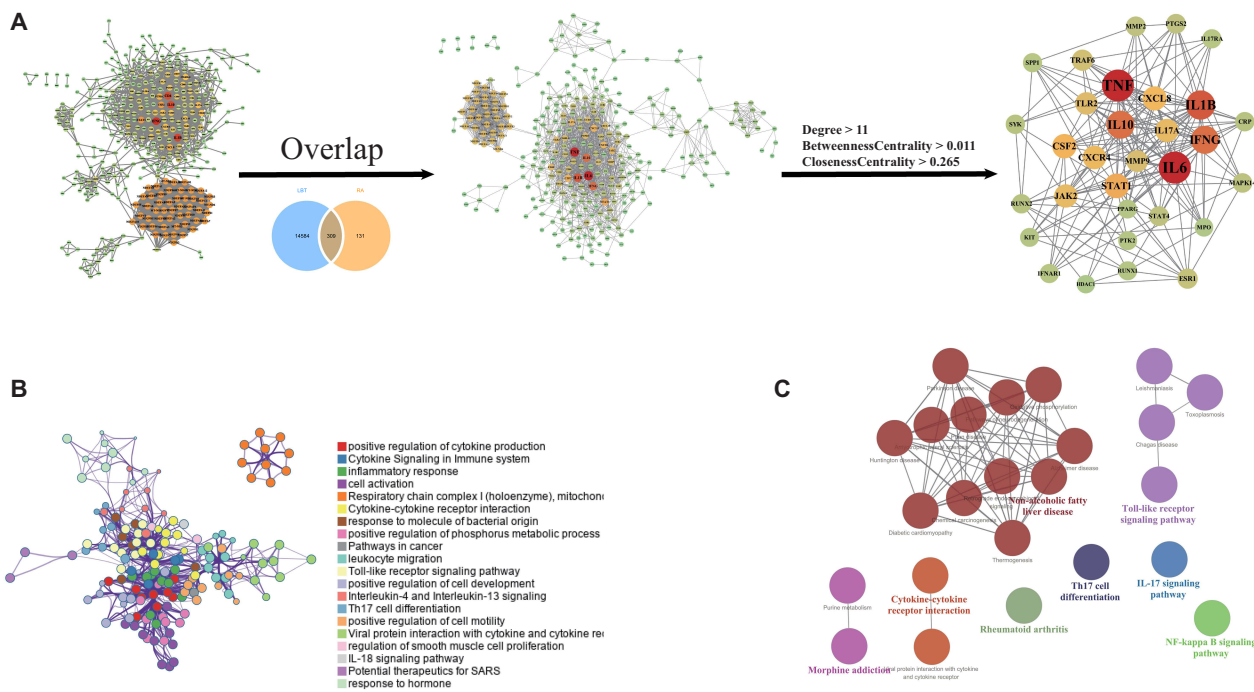


Figure 4 RA PPI network and mechanism of LuoBiTong exerting RA effects. **(A)** PPI network of RA and key targets for LBT anti-RA effects. **(B and C)** Enrichment analysis of biological processes and KEGG pathway for targets regulated by LBT exerting anti-RA effects.

signaling pathway, Th17 cell differentiation, Toll-like receptor signaling pathway, NF-kappa B signaling pathway, and Rheumatoid arthritis, etc. are the potential key signaling pathways regulated by LBT exerting RA effects (Figure 4C).

Effect of LBT Combined with MTX Treatment on Macrophages

Based on the network pharmacology results, we found that LBT targets multiple inflammatory pathways, so we first performed flow cytometry analysis of macrophages in serum. Macrophages were screened from serum samples of each group, following the general procedure shown (Figure 5A-F). Under inflammatory conditions, macrophages undergo activation and differentiation into two distinct subtypes: classically activated macrophages (M1) and alternatively activated macrophages (M2). Thus, further differentiation between M1/M2 macrophages was conducted in each group (Figure 5F-J), and macrophages in each group were quantified (Figure 5K). Our findings revealed a dysregulated expression pattern of macrophage subtypes in the CIA+Vehicle group, characterized by a high proportion of M1 macrophages (Figure 5L) and a low proportion of M2 macrophages (Figure 5M), leading to an imbalanced M1/M2 ratio (Figure 5N). However, treatment with H-MTX or M-MTX combined with LBT resulted in a shift in macrophage polarization, with an increase in the proportion of M2 macrophages, which possess anti-inflammatory properties, and a suppression of M1 macrophages, which have pro-inflammatory characteristics.

LBT Combined with M-MTX Inhibited the Expression of IL-6, TNF- α and IL-17A Proteins

To investigate the impact on local inflammation, we conducted Western Blot analysis on paw tissue samples, focusing on the corresponding inflammatory factors. As depicted in Figure 6A-F, the expression levels of IL-6, TNF- α , and IL-17A were significantly lower in the Control + Vehicle group compared to the CIA + Vehicle group. Remarkably, the CIA + MTX 1.4 mpk group and the CIA + MTX 0.5 mpk + LBT-H group exhibited a substantial downregulation of IL-6 and IL-17A expressions ($p < 0.01$). Additionally, TNF- α showed a moderate decrease ($p < 0.05$). These observations highlight the significant reduction in IL-6, IL-17A, and TNF- α levels in CIA mice following treatment with MTX 0.5 mpk + LBT-H, achieving a therapeutic efficacy comparable to that of high-dose MTX. However, it is important to note

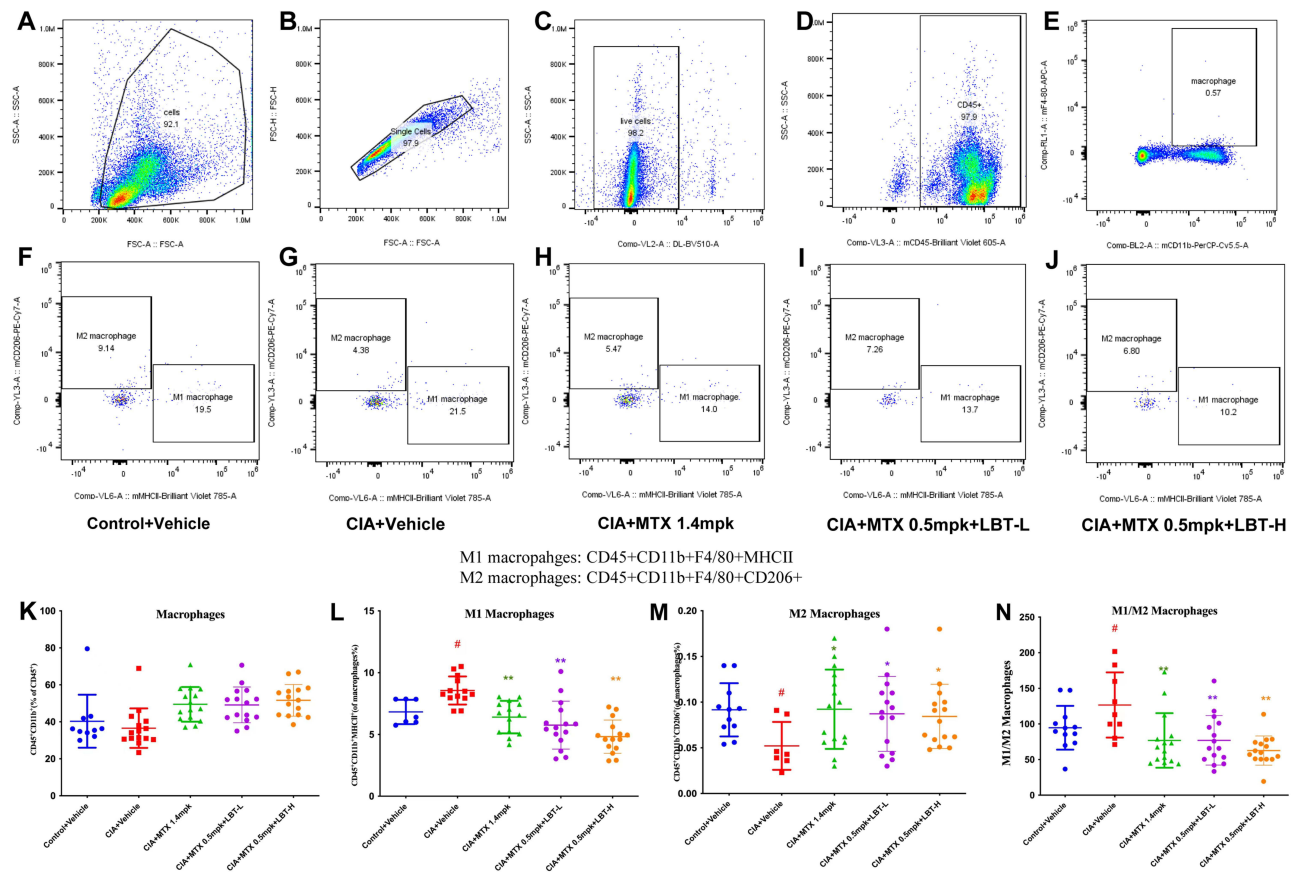


Figure 5 Effect of LBT combined with MTX treatment on macrophages. (A) Flow Cytometry Sample Charts Depicting All Cells Detected in a Single Sample; (B) Diagram of a Single Cell in a Single Sample; (C) Diagram of Living Cells in a Single Sample; (D) Examples of White Blood Cells in a Single Sample; (E) Samples of Macrophages in a Single Sample; (F) Macrophage Polarization Levels in a Single Sample of the Control + Vehicle Group; (G) Macrophage Polarization Levels in a Single Sample of the CIA + Vehicle Group; (H) Macrophage Polarization Levels in the CIA + MTX 1.4mpk Group; (I) Macrophage Polarization Levels in the CIA + MTX 0.5mpk + LBT-L Group; (J) Macrophage Polarization Levels in the CIA + MTX 0.5mpk + LBT-H Group; (K) Macrophage Levels in the Blood of Each Group; (L) The Number of M2 Macrophages in Each Group; (M) The Number of M1 Macrophages in Each Group; (N) The M1/M2 Ratio of Macrophages in Each Group. #P < 0.05, compare with Control +Vehicle; *P < 0.05, compare with CIA+Vehicle; ** P < 0.01, compare with CIA+Vehicle.

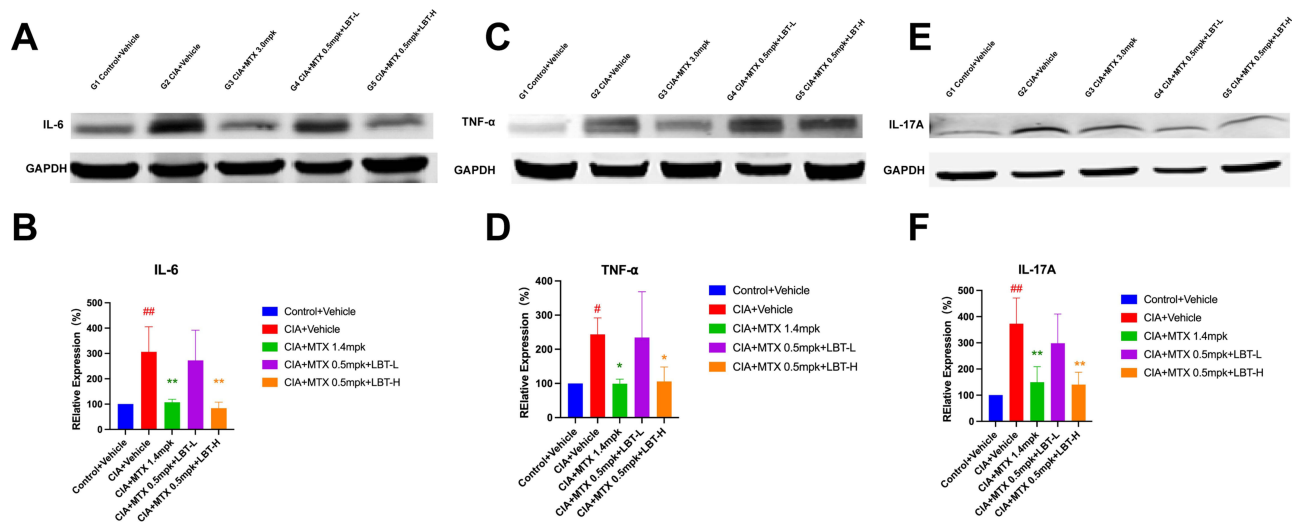


Figure 6 Effects of MTX combined with LBT on inflammation-related markers (n = 3). (A, C, E) representative Western blot bands of IL-6, TNF- α and IL-17A. (B, D, F) Quantitative analysis of the relative expression levels of IL-6, TNF- α and IL-17A. # p < 0.05, compared with Control +Vehicle; ## p < 0.01, compared with Control +Vehicle; * P < 0.05, compare with CIA+ Vehicle; ** P < 0.01, compare with CIA+ Vehicle.

that the regulatory effect on inflammatory factors in the paw tissues of the CIA + MTX 0.5 mpk + LBT-L group was suboptimal.

LBT Combined with M-MTX Regulates IL-17A-Related Signaling Pathways

In light of the aforementioned experimental findings, we were particularly intrigued by the expression of IL-17A, as recent studies have implicated its pivotal role in the pathogenesis of RA.^{52–54} Consequently, we proceeded to investigate the pharmacological mechanism underlying the downstream signaling pathway of IL-17. To this end, we employed Western Blot analysis to assess the levels of downstream inflammation-related proteins, namely P-NFκB p65, P-p38, and P-SAPK/JNK. As depicted in Figure 7A and B, the expression of P-NFκB p65 in the paw tissue of CIA + Vehicle mice was significantly upregulated compared to the Control + Vehicle group ($p < 0.05$). However, treatment with MTX 1.4 mpk or MTX 0.5 mpk + LBT-H effectively attenuated the levels of P-NFκB p65 in the paw tissue. Moreover, the expression levels of P-p38 and P-SAPK/JNK were notably elevated in the paw tissue of CIA + Vehicle mice relative to the Control + Vehicle group (Figure 7C-F). Remarkably, treatment with MTX or MTX combined with LBT resulted in a significant reduction in the expression of P-p38 and P-SAPK/JNK in the paw tissue of CIA mice (Figure 7C-F). Collectively, these findings strongly suggest that LBT exerts regulatory effects on the IL-17 inflammatory signaling pathway. Additionally, it is worth noting that while low-dose LBT combined with MTX also exhibited an effect, it was not as pronounced as that observed with high-dose LBT.

LBT Regulates the Production of IL17-Related Inflammatory Factors by Inhibiting the Proliferation of TH17 Cells

Based on the aforementioned findings, it has become evident that the primary mechanism underlying LBT therapy in CIA mice involves the regulation of IL-17 and its downstream inflammatory signaling pathways. Extensive research has demonstrated that IL-17 is primarily secreted by TH17 cells.^{55,56} Furthermore, the proliferation of TH17 cells has been identified as a key pathogenic factor in RA.^{57,58} In light of these observations, we conducted immunohistochemistry analysis to assess the expression of TH17 cells (Figure 8A). Our results revealed a significant increase in the expression of TH17 cells in the model group, particularly around the joints, compared to the Control + Vehicle group (Figure 8B). However, this increase was mitigated by LBT intervention. Consequently, we can infer that LBT primarily exerts its

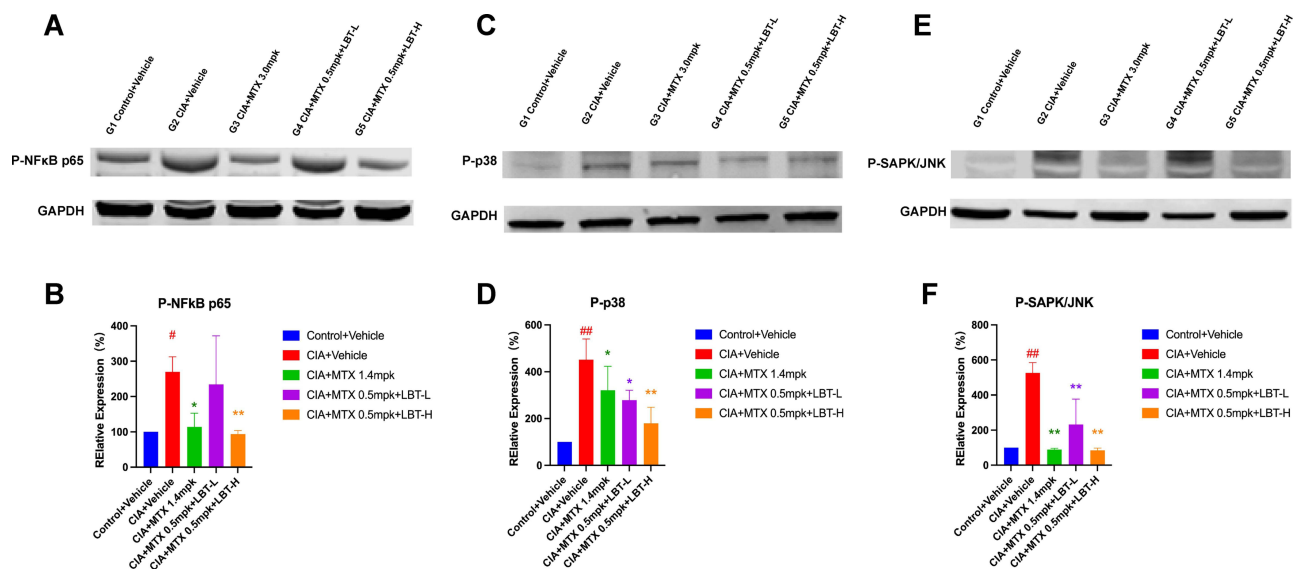


Figure 7 The impact of MTX or MTX combine LBT treatment on IL-17 signaling ($n = 3$). (A, C, E) Representative Western blot band. (B, D, F) Quantification of the relative expression of P-NFκB p65, P-p38 and P-SAPK/JNK. # $p < 0.05$, compared with Control + Vehicle; ## $p < 0.01$, compared with Control + Vehicle; * $p < 0.05$, compare with CIA + Vehicle; ** $p < 0.01$, compare with CIA + Vehicle.

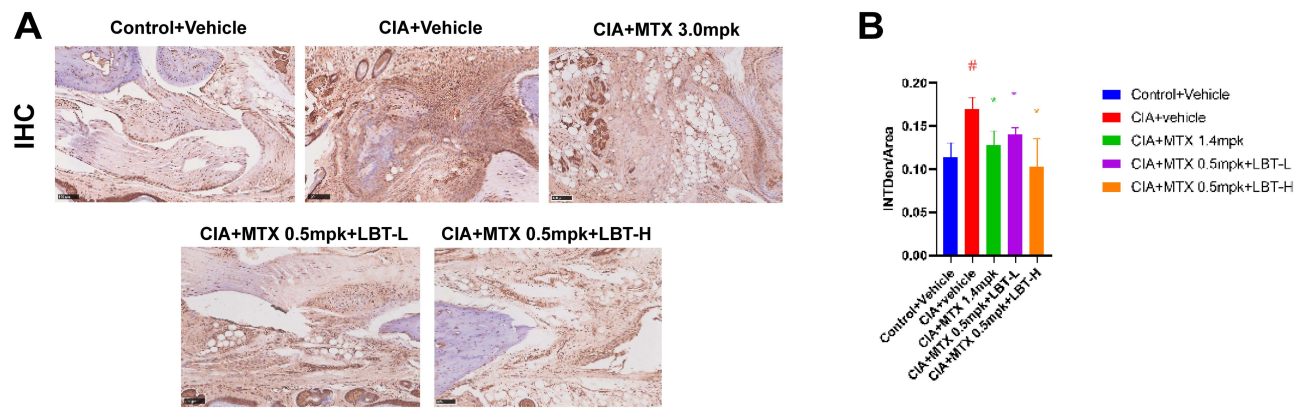


Figure 8 Results of immunohistochemical staining of th17 cells. **(A)** Immunohistochemistry of TH17 cells in each group (Scale bar = 100 μ m, n = 3). **(B)** AOD analysis. **(B)** # $p < 0.05$, compared with Control +Vehicle; * $P < 0.05$, compare with CIA+ Vehicle.

effects by inhibiting TH17 cell proliferation, thereby reducing the levels of inflammatory factors such as IL-17 and its downstream inflammatory pathways.

Discussion

In this study, we demonstrated that combined therapy with varying doses of LBT and M-MTX is effective in treating RA. Significantly, high-dose LBT combined with M-MTX matched the efficacy of the H-MTX dose. This combination notably reduced hallmark symptoms of CIA in mice, including synovial hyperplasia, paw swelling, arthritis scores, and cartilage degradation, and also alleviated local and systemic inflammation. Pharmacological network analysis and in vivo validation revealed that LBT's effect in RA might be due to modulation of multiple inflammatory pathways, including the inhibition of Th17 cell proliferation and related signaling pathways.

While MTX remains a mainstay in treating inflammatory arthritis due to the lack of definitive cures,⁵⁹ its side effect profile, heavily influenced by dosage and regimen, remains a concern. Highlighting this dose-dependent variability, we employed a murine model of CIA to explore the efficacy of a novel combination therapy: LBT with a low MTX maintenance dose (0.5mg/kg every other day, mirroring the clinically recommended 7.5mg/week). Notably, despite this significantly lower MTX dose compared to the monotherapy group receiving the high dose (20mg/week, equivalent to 1.4mg/kg every other day), this LBT-MTX combination demonstrated comparable, if not superior, efficacy in certain parameters, particularly reducing arthritis activity and paw swelling (Figure 1). These findings suggest that LBT-MTX combination therapy could not only significantly enhance the therapeutic effectiveness of MTX but also hold promise for mitigating safety concerns associated with long-term use of high-dose MTX.

The intricate nature of Traditional Chinese Medicine (TCM) compounds often involves targeting multiple components and pathways, making their therapeutic mechanisms challenging to elucidate.⁶⁰ To address this complexity, we employed network pharmacology analysis, a method merging systems biology and network theory,^{61,62} to elucidate the mechanisms of LBT in RA treatment. This approach excels at revealing intricate drug-disease-pathway interactions, particularly in the context of TCM.⁶³ Our analysis revealed that LBT directly targets key inflammatory molecules like IL-6, TNF- α , and IL-17A (Figure 7), suggesting its potent anti-inflammatory effects. Abundant in the synovial fluid and tissue of RA patients, M1 macrophages, or classically activated macrophages, play a crucial role in RA pathogenesis.^{64,65} They are major producers of pro-inflammatory cytokines, including IL-6 and TNF- α , which drive crucial disease processes.^{66,67} These macrophage-derived cytokines not only contribute to pannus formation but also stimulate osteoclastogenesis, leading to bone erosion, a hallmark of RA severity.^{68,69} Additionally, emerging evidence highlights the key role of Th17 cells in RA pathogenesis.⁷⁰ Their elevated presence in RA patients and their ability to secrete pro-inflammatory cytokines like IL-17A further illustrate their pathogenic contribution.^{71–73} Furthermore, clinical studies report high levels of IL-17A in RA patients, and anti-IL-17 therapy has demonstrated efficacy in reducing arthritis

severity in animal models.^{74,75} These findings collectively support the potential of LBT in modulating both M1 macrophage and Th17 cell signaling pathways, thereby targeting critical pathogenic factors in RA.

To ascertain if the observed synergistic effect stemmed from inflammation suppression, we examined the impact of LBT in combination with M-MTX on M1 macrophage polarization. Flow cytometry analysis showed that this combination therapy led to a decrease in pro-inflammatory M1 macrophages and an increase in anti-inflammatory M2 macrophages, effectively altering the M1/M2 ratio. Notably, LBT combined with M-MTX outperformed the H-MTX in modulating macrophage polarization (Figure 3M-P). Immunohistochemical analysis showed that the combination of LBT and M-MTX more effectively diminished Th17 cell expression compared to the H-MTX (Figure 6A-B). Additionally, we assessed the levels of inflammatory cytokines, including IL-17 and IL-6, in the serum and paw tissues. The results demonstrated that high-dose LBT with M-MTX was as effective as H-MTX in diminishing inflammatory cytokine expression (Figures 3A and 4A-F). A subsequent focus on IL-17's downstream signaling pathways, such as NF- κ B, MAPK, ERK, and JNK, revealed that the LBT and M-MTX combination notably inhibited these inflammation-related pathways, showing greater efficacy than H-MTX alone (Figure 5A-F). All these results indicated that high-dose LBT in combination with M-MTX exhibits a clear advantage in anti-inflammatory effects compared to M-MTX.

Conclusions

In conclusion, our findings demonstrate that LBT significantly enhances the anti-inflammatory effects of MTX at a lower dose, potentially offering a safer and more effective treatment option for RA patients. This synergy likely involves targeting multiple pathways, including modulation of macrophage polarization, Th17 cell activation, and downstream inflammatory signaling.

Abbreviations

LBT, LuoBiTong; MTX, Methotrexate; RA, Rheumatoid arthritis; DMARDs disease-modifying anti-rheumatic drugs; TCM, Traditional Chinese medicine; TwHF, *Tripterygium wilfordii* Hook F; SMMs, small molecular medicines; H&E, hematoxylin-eosin staining; ELISA, enzyme-linked immunosorbent assays; Th17, helper T cell-17; IL-17A, Interleukin-17A; IL-17F, Interleukin-17F; IL-6, Interleukin 6; TNF- α , tumor necrosis factor-alpha; NF κ B, nuclear factor kappa B; IHC, immunohistochemical.

Ethics Statements

This study was approved by the Ethics Committee of Jiangsu Jicuiyaokang Institutional Animal Care and Use Committee, and all animals received humanitarian care. The institution is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and holds a license for animal use.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. All authors read and approved the final manuscript.

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Disclosure

The authors report no conflicts of interest in this work.

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