

# Effects of leukotriene B4 on interleukin-32, interferon- $\gamma$ and chemokines in rats with rheumatoid arthritis

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**Abstract.** The objective of this study was to investigate the effects of leukotriene B4 (LTB4) on the expression of interleukin-32 (IL-32) interferon- $\gamma$  (IFN- $\gamma$ ) and chemokine monocyte chemoattractant protein (MCP-1) and macrophage inhibitory protein (MIP-1 $\alpha$ ) in rheumatoid arthritis (RA). The rat model of RA collagen induced-arthritis (CIA) was established. The levels of LTB4, interleukin-32, IFN- $\gamma$  and chemokines MCP-1 and MIP-1 $\alpha$  in CIA rats were detected by ELISA. After the rat synovial cells were isolated and treated with different concentrations of LTB4, the effect of LTB4 the expression of IL-32, IFN- $\gamma$  and chemokines MCP-1 and MIP-1 $\alpha$  mRNA in synovial cells was detected by real-time quantitative PCR, the effect of LTB4 on protein expression was detected by immunoblotting. The effects of different concentrations of LTB4 on the viability and apoptosis of synovial cells were detected by LDH and cell proliferation reagent WST-1. Compared with the control group, the levels of LTB4, IL-32, IFN- $\gamma$  and chemokines MCP-1 and MIP-1 $\alpha$  were significantly increased in the serum of the CIA group. After treatment of CIA rat synovial cells with different concentrations of LTB4, the expression of IL-32, IFN- $\gamma$  and chemokines MCP-1 and MIP-1 $\alpha$  mRNA and protein were increased with significant differences among groups. WST-1 and flow cytometry showed that LTB4 had significant toxic effects on synovial cells and promoted apoptosis. In conclusion, LTB4 promotes the expression of interleukin-32, IFN- $\gamma$  and chemokines MCP-1 and MIP-1 $\alpha$  in synovial cells and facilitates apoptosis of synovial cells.

## Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease. Pathological changes are chronic synovial membrane inflammation or proliferation and joint erosion. Clinical manifestations are mainly symmetrical polyarthritis, extra-articular injury, ultimately leading to joint deformity, disability and loss of exercise capacity (1). At present, the exact pathogenesis of RA has not been described and studies have focused on the interaction of genetic, environmental and immune factors (2,3). In RA patients and animal models, it was observed that intra-articular synovial fibroblasts proliferate and adhere to the cartilage surface. Macrophages, T cells and other inflammatory cells are recruited there, producing tumor necrosis factor (TNF) and interleukin, which act together to cause synovitis and cartilage damage (4). Interferon- $\gamma$  (IFN- $\gamma$ ) is an important cytokine in the human body that regulates the transcription and expression of immune-related genes (5). Chemokines play an important role in chronic synovitis, and macrophage inhibitory protein (MIP-1 $\alpha$ ) and monocyte chemoattractant protein-1 (MCP-1) show abnormal expression in different stages of RA (6). Leukotriene B4 (LTB4), a metabolite of arachidonic acid, is a potent chemokine, and can induce neutrophils to aggregate. It can be activated in RA and accumulate inflammatory and immune effector cells and can also act on T cells in the immune response, prompting them to release cytokines (7). At present, oral LTB4 receptor antagonists are used for long-term treatment of RA patients in clinical practice. Interleukin-32 (IL-32) is mainly produced by immune cells and plays an important role in a variety of autoimmune diseases, such as chronic obstructive pulmonary disease and RA (7,8). It has been confirmed that LTB4 is associated with expression of interleukin-1 and TNF. High concentrations of LTB4 was detected in RA patients, suggesting that LTB4 was associated with RA pathogenesis. However, currently, the effects of LTB4 on the expression of IL-32, IFN- $\gamma$  and chemokine MCP-1 and MIP-1 $\alpha$  have not been described. In response to this question, we constructed the RA rat model collagen induced-arthritis (CIA), treating the separated CIA synovial cells with different concentrations of LTB4, in order to explore the effects of LTB4 on IL-32, IFN- $\gamma$  and chemokines MCP-1 and MIP-1 $\alpha$  at a cellular level, as well as the effect of LTB4 on apoptosis.

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## Materials and methods

**Experimental materials and main instruments.** The experimental rats were purchased from the Nanjing model animal center and grown for one month; cattle type II collagen (CII) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); LTB<sub>4</sub>, IL-32, IFN- $\gamma$  and chemokine MIP-1 $\alpha$ , MCP-1 enzyme-linked immunosorbent assay (ELISA) kits all from Wuhan Boster Biological Technology Ltd. (Wuhan, China). Primary rabbit polyclonal LTB<sub>4</sub> antibody (dilution, 1:1,000; cat. no. ab133040); rabbit polyclonal IL-32 antibody (dilution, 1:1,000; cat. no. ab37158); rabbit polyclonal IFN- $\gamma$  antibody (dilution, 1:1,000; cat. no. ab77246); rabbit polyclonal MIP-1 $\alpha$  antibody (dilution, 1:1,000; cat. no. ab171336); rabbit polyclonal MCP-1 antibody (dilution, 1:1,000; cat. no. ab30512) secondary goat anti-rabbit (HRP) IgG antibody (dilution, 1:2,000; cat. no. ab6721) were all purchased from Abcam Co. Ltd. (Cambridge, MA, USA). RNA-extraction reagents, reverse transcription kits and PCR enzymes were from Takara Co. Ltd. (Los Angeles, CA, USA); RT-PCR primers were forward, ATGTATTGCTAATCTTGATGTCTCTCGA and reverse, CTTTCAGAGAACTTCTTGAGGCTTGTCCTAAAGTG GAG, synthesized by Nanjing Genscript Co. Ltd. (Nanjing, China); RT-PCR instrument was from Applied Biosystems (Foster City, CA, USA); flow cytometry was from Thermo Fisher Scientific (Attune NxT; Grand Island, NY, USA).

**Experimental animal selection and model construction.** Forty male Wistar rats of 8 weeks weighing 180 $\pm$ 10 g were randomly assigned to the normal control group (n=20) and CIA group (n=20). Experimental rats were healthy and free of disease, feeding conditions were at SPF level. We used CII to induce the CIA group. Incomplete Freund's adjuvant (FICA) was made by mixing 1:2 of anhydrous lanolin and paraffin after sterilization; 2 mg/ml live Bacillus Calmette-Guerin was added dropwise to FICA and complete Freund's adjuvant was obtained after emulsification. CII was dissolved in 0.1 mol/l acetic acid, to a final concentration of 2 mg/ml. CII and FCA were fully emulsified in 1:1 ratio and reserved. Each rat in the CIA group was injected subcutaneously with 0.2 ml of the mixture at the foot, tail and back and a second injection was performed at the same site seven days later. After 28 days, rats with joint swelling and movement disorders were set as the standard of experimental materials. The control group rats were injected with equal volume of saline at the same site and time point. This study was approved by the Animal Ethics Committee of the Animal Center of the First People's Hospital of Yunnan.

**Determination of serum LTB<sub>4</sub>, IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$ , MCP.** Twenty-eight days after modeling, rat femoral artery blood was drawn and centrifuged at 4°C and once the supernatant was discarded, stored at -20°C. Serum LTB<sub>4</sub>, IL-32, IFN- $\gamma$  and chemokine MIP-1 $\alpha$ , MCP-1 were measured by ELISA. The experimental procedure strictly followed the kit operation guidelines.

**Synovial tissue extraction and cell culture modeling.** After 28 days, rats with dyskinesia were selected and the knee was exposed after anesthesia. An incision was made along the patella up to the femur and then along the patella to the

tibia, to open the knee joint cavity and the synovial tissue was completely stripped. Stripped synovial tissue was cut into small pieces, a proper amount of trypsin added, centrifuged after complete digestion and the supernatant with dead and broken cells was discarded. Isolated cells were added to an appropriate amount of complete cell culture medium (DMEM added to 10% fetal bovine serum) and placed in a 5% CO<sub>2</sub> 37°C incubator after separation.

**RT-PCR detection of mRNA expression.** Isolated synovial tissue cells were treated with different concentrations of LTB<sub>4</sub> (0.1 and 1  $\mu$ M) and the control group was treated with an equal volume of DMSO and the cells were collected after 24 h. RT-PCR detection of mRNA expression: proper amount of TRIzol was added to the collected cells, after which they were placed in room temperature for 5 min and then centrifuged for 5 min at 10,500 x g. The supernatant was aspirated, a mixture of chloroform and TRIzol added, mixed and placed at room temperature for 15 min.

The mixture was then centrifuged for 15 min at 12,000 rpm at 4°C and the supernatant was aspirated. Isopropanol and TRIzol mixture was added, placed at room temperature for 10 min, centrifuged for 10 min at 10,500 x g at 4°C and the supernatant discarded. A 75% ethanol TRIzol mixture was added to wash the RNA precipitate, and centrifuged for 5 min at 7,050 x g at 4°C, the supernatant discarded and dried at room temperature. After adding 5% SDS to dissolve the RNA, the product was stored at -80°C.

The extracted RNA was prepared into cDNA using a reverse transcription kit. The cDNA was mixed with RT-PCR reaction reagent and placed into an RT-PCR instrument for polymerase chain reaction.  $\beta$ -actin was used as the internal reference. The PCR reaction procedure was set as follows: 97°C pre-denaturation 5 min, denaturation at 70°C for 40 sec, annealing at 63°C for 40 sec, extension at 72°C for 2 min, extension at 72°C for 5 min, for 30 cycles. The reagents for the annealing reaction were purchased from Takara Co. Ltd. and the primer was supplied by Nanjing Genscript Biotechnology Co. Ltd. (Nanjing, China) The PCR products were electrophoresed on 1% agarose gel and the results were collected by a multifunctional imaging system, gray values were collected for further analysis.

**Immunoblotting detection of protein expression levels.** Treated cells were collected and split with the proper amount of RIPA, centrifuged for 20 min at 10,500 x g at 4°C, and the supernatant collected and placed at -20°C. Protein samples from each group were added to SDS gel and after 2 h of 80 V constant pressure vertical electrophoresis, membrane transfer was performed under 150 mA constant current overnight. After blocking for 1 h in 5% milk powder, the primary antibody was diluted and incubated according to the dilution specified at room temperature for 4 h. After the membrane was washed three times with Tris-buffered saline with Tween-20 (TBST), it was incubated with the secondary antibody at room temperature for 1 h. After washing three times with TBST, the membrane was developed in a dark room, the results were scanned and the gray value was analyzed using ImageJ software and the data saved for analysis. Antibodies for immunoblotting were purchased from Abcam Co. Ltd.

Table I. Changes in indicators in CIA serum.

Groups	LTB4 (ng/ml)	IL-32 (ng/ml)	IFN- $\gamma$ (pg/ml)	MIP-1 $\alpha$ (pg/ml)	MCP-1 (pg/ml)
Control group (n=18)	29.32 $\pm$ 4.24	24.91 $\pm$ 3.72	214.62 $\pm$ 26.38	17.22 $\pm$ 2.31	0.26 $\pm$ 0.04
CIA group (n=16)	96.21 $\pm$ 10.36	82.64 $\pm$ 7.66	342.88 $\pm$ 40.09	24.36 $\pm$ 3.36	1.14 $\pm$ 0.24
P-value	0.0008	0.0008	0.006	0.009	0.0009

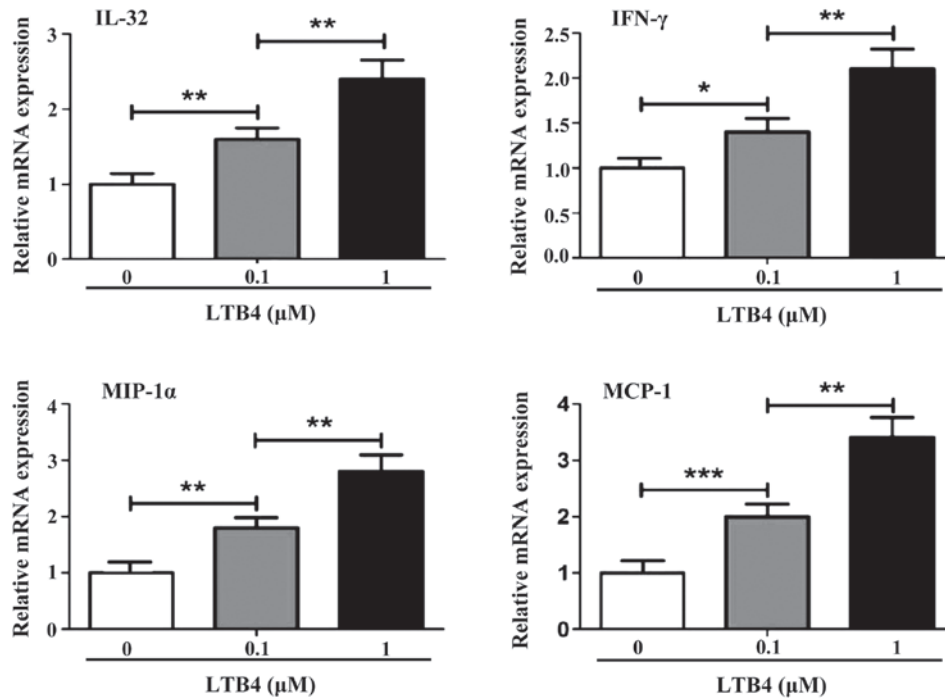


Figure 1. Changes in IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$ , MCP-1 mRNA expression. IL-32, interleukin-32; IFN- $\gamma$ , interferon- $\gamma$ ; MIP-1 $\alpha$ , macrophage inhibitory protein; MCP-1 chemokines monocyte chemoattractant protein; LTB4, leukotriene B4. \*\*P<0.01; \*\*\*P<0.001.

**WST-1 cell viability detection after LTB4 treatment.** Cell viability was measured using a WST-1 cell activity assay kit after treating with different LTB4 concentrations for 24 h. The experimental procedure was conducted according to the kit instructions. The kit was purchased from Roche Ltd. (New York, NY, USA).

**Cell apoptosis detection by flow cytometry.** The cells treated with LTB4 were digested with trypsin and prepared into a single cell suspension. The cells ( $2 \times 10^5$ ) were added to a 100  $\mu$ l 1X binding buffer, then 5  $\mu$ l of Annexin V-FITC and 5  $\mu$ l PI reagent were added and incubated for 15 min at room temperature in the dark, then resuspended by adding 400  $\mu$ l of 1X binding buffer, after which cell apoptosis rate was measured by flow cytometry.

**Statistical analysis.** Late time data were analyzed using SPSS 17.0 statistical analysis package (IBM, Armonk, NY, USA), downloaded from <http://www.spss.com.cn/>, and experimental data were expressed as mean  $\pm$  standard deviation (mean  $\pm$  SD). Variance analysis was adopted while analyzing data between the groups and P<0.05 was considered as statistically significant.

## Results

**Changes in LTB4, IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$ , and MCP-1 expression in serum of CIA group.** ELISA was used to detect the expression of cytokines in the control and CIA groups. The results showed that the levels of LTB4, IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1 in the serum of the CIA group were significantly higher than those in the control group and the difference was statistically significant (Table I).

**Effects of LTB4 on IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$ , and MCP-1 mRNA expression.** The expression of IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1 mRNA was detected by RT-PCR. The results showed that IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1 mRNA were increased and the expression of cytokine mRNA in the 1  $\mu$ M LTB4 treated group was significantly higher than that in the 0.1  $\mu$ M LTB4 treated group (Fig. 1).

**Effects of LTB4 on protein expression of IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1.** We examined the changes of cytokine protein expression in CIA synovial cells treated with different concentrations of LTB4 (0.1 and 1  $\mu$ M) by

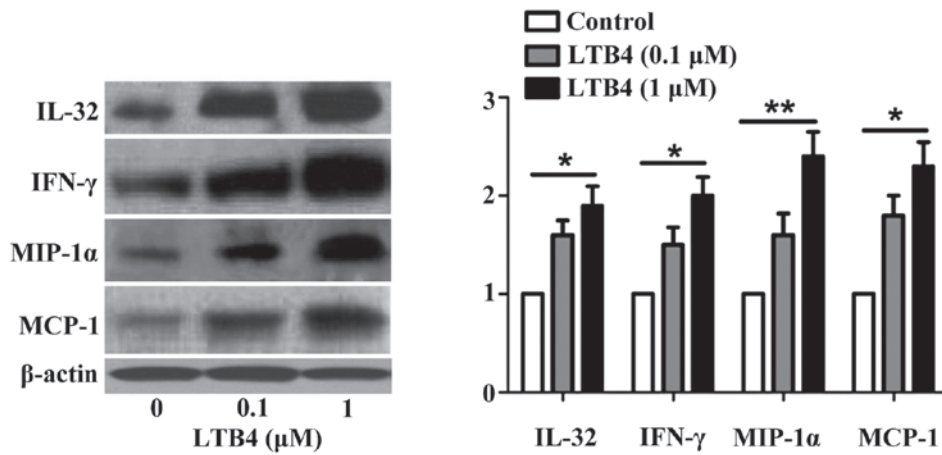


Figure 2. Changes in protein expression of IL-32, IFN-γ and chemokines MIP-1α and MCP-1. IL-32, interleukin-32; IFN-γ, interferon-γ; MIP-1α, macrophage inhibitory protein; MCP-1 chemokines monocyte chemoattractant protein; LTB4, leukotriene B4. \*P<0.05; \*\*P<0.01.

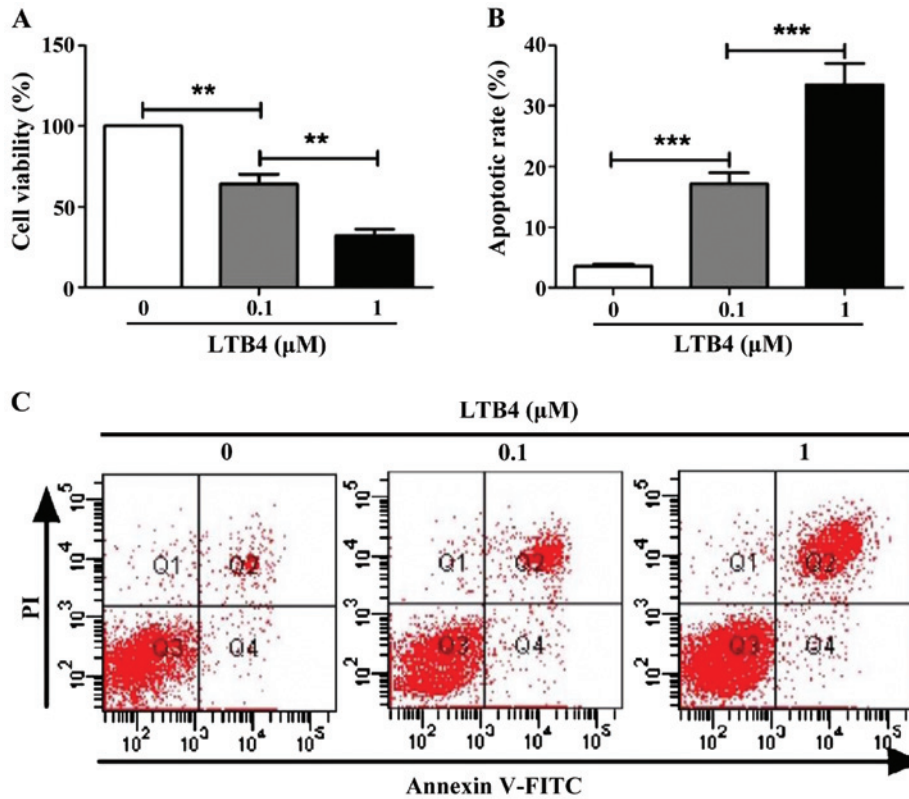


Figure 3. Effects of LTB4 on cell viability and apoptotic rate of synovial cells. LTB4, leukotriene B4. \*\*P<0.01; \*\*\*P<0.001.

immunoblotting. The results showed that IL-32, IFN-γ and chemokines MIP-1α and MCP-1 were significantly increased in the 0.1 and 1 μM LTB4 treated groups compared with the control group. In the group treated with 1 μM LTB4, these four proteins had a significantly higher expression than in the 0.1 μM LTB4 group (Fig. 2).

*Effects of different concentrations of LTB4 on cell viability and apoptotic rate of synovial cells.* The effects of different concentrations of LTB4 on synovial cell activity were examined by LDH. The results showed that the activity of synovial cells decreased after LTB4 treatment compared with the control group and the higher concentration of LTB4 had a

greater effect on the activity of synovial cells (Fig. 3A). The results of flow cytometry showed that the rate of apoptosis in the LTB4 group was significantly higher than that of the control group (Fig. 3B).

**Discussion**

RA is a common autoimmune disease. Pathological features of affected joints include synovial hyperplasia, accumulation of inflammatory cells and cartilage and bone tissue damage, which eventually lead to joint deformity and disability. Studies have shown that RA development is associated with a variety of antigens that are known to activate T cells and release a

series of immune mediators that cause cell-cell interactions, leading to excessive activation of plasma B cells (4). The abnormal performance of these two types of cells produces a large amount of immunoglobulin and rheumatoid factor (RF), gathering immune complexes in the synovial tissue and generating a variety of chemokines (C5a, C3a) (9). In addition, IL-1, TNF- $\alpha$  and other related molecules further induce leukocytes to enter the joint tissue (10,11). Other factors related to dilation of blood vessels (e.g. prostaglandin E2) also facilitate inflammatory cells to enter the inflammatory site. These factors together lead to damage to the synovial surface and articular cartilage (12). In this study, we found that the expression of LTB4, IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1 were significantly higher in the blood of CIA model rats, compared with healthy controls, which was consistent with previous studies (6,7,9), suggesting that LTB4, IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1 play an important role in the development and progression of RA.

LTB4 is a very strong chemokine produced by leukocytes. Both serum and synovial fluid of RA patients contain high concentrations of LTB4 (8). Current research suggests that LTB4 has an important relationship with neutrophils in RA patients. Neutrophils of RA patients have an enhanced ability to release LTB4 and the inflammation caused by migration of neutrophils, inducing aggregation of other cells at the joints, is also associated with LTB4 (5,13). In a CIA model, inhibition of 5-LOX activity inhibited neutrophil aggregation and exudation in the joints, slowing the development of the disease. This suggested that neutrophils play a key role in the pathogenesis of RA (14). LTB4 has been shown to be associated with the expression of TNF- $\alpha$  and IL-1 $\beta$  and high concentrations of exogenous LTB4 resulted in increased levels of TNF- $\alpha$  and IL-1 $\beta$  mRNA and protein in the cells. Suppressing LTB4 reduced the expression of these two factors, indicating that LTB4 can regulate the expression of TNF- $\alpha$  and IL-1 $\beta$  (10,12). Related studies have shown that interleukin plays an important role in the joint inflammation in RA. IL-1 $\beta$  causes local inflammation of the joint, resulting in increased articular cartilage and bone resorption and impaired cartilage reconstruction (11). IL-32 is a proinflammatory cytokine that can induce the production of TNF- $\alpha$  and other inflammatory factors, leading to RA lesions (15). IFN- $\gamma$  is synthesized in the cellular immune response process and is a strong immune regulator, promoting inflammation through the induction of expression of a variety of cytokines, including TNF- $\alpha$ , IL-2 and IL-10 (16). The development of RA is also due to the interaction of a variety of chemokines, including MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-1 (17). Studies have found that MIP-1 $\alpha$  overexpression can recruit inflammatory cells including T cells to the RA lesion, therefore facilitating the production of inflammation (18,19). MCP-1 is also an inflammatory chemokine involved in the regulation and recruitment of effector T cells and plays an important regulatory role in the immune response at the lesion site (20). In order to detect whether LTB4 overexpression affects the expression of IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1 in RA and whether excessive LTB4 affects synovial cell activity, we used a CIA rat model of RA. Expression of IL-32, IFN- $\gamma$  and chemokine MIP-1 $\alpha$  and MCP-1 mRNA were increased upon treatment with LTB4. In addition, LTB4 treatment led to an increase in IL-32, IFN- $\gamma$

and chemokine MIP-1 $\alpha$  and MCP-1 protein expression. LTB4 treatment resulted in decreased activity of synovial tissue cells and increased apoptosis. These results suggested that LTB4 may cause apoptosis by upregulating the expression of IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1. LTB4 may promote inflammation by regulating the level of inflammatory factors, eventually leading to joint damage and soft tissue damage.

In conclusion, this study found that increased expression of LTB4 in the CIA group rats resulted in the increased expression of IL-32, IFN- $\gamma$  and chemokines MIP-1 $\alpha$  and MCP-1, leading to apoptosis. This study further elucidates the pathogenesis of RA, and suggests that LTB4 can be used as a clinical indicator for RA patients and that LTB4 antagonists may be an effective strategy for the treatment of RA.

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