Total glucosides of paeony improve complete Freund’s adjuvant -induced rheumatoid arthritis in rats by inhibiting toll-like receptor 2-mediated tumor necrosis factor receptor-associated factor 6/ nuclear factor-kappa B pathway activation

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Abstract

OBJECTIVE: To investigate the mechanism underlying anti-inflammatory and immunoregulatory effect of total glucosides of paeony (TGP) based on toll-like receptor 2 (TLR2) mediated tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6)/nuclear factor-kappa B (NF-kB) pathway activation in rats with rheumatoid arthritis.

METHODS: Adjuvant arthritis (AA) model was developed by complete Freund’s adjuvant (CFA) immunization. TGP (100, 50, 25 mg/kg) and celecoxib (2.8 mg/kg) were administered by intragastric administration for 21 d. Right hind paw swelling was assessed every 2 d. After 21 d, synovial changes of the ankle were detected by histopathology. CD4+ and CD8+ T cell amounts in peripheral blood were measured by flow-cytometrically. Gene and protein levels of toll-like receptor (TLR)2, TRAF6, tumor necrosis factor ligand superfamily member 6 (FASLG) in the spleen were assessed by RT-qPCR and Western Bolt, respectively. Nuclear expression of NF-kB p65 was detected by NF-kB p65 Assay Kit.

RESULTS: Paw swelling and synovium lesions were obviously aggravated in AA rats. These symptoms were significantly relieved by TGP. The ratio of CD4+/CD8+ T cell was increased in AA rats, while TGP reduced this increased ratio. Gene and protein levels of splenic TLR2, TRAF6 and FASLG, and nuclear NF-kB p65 in AA rats were significantly increased, but overtly inhibited by TGP.

CONCLUSION: These findings suggest that TGP’s anti-inflammatory effect on AA may be related to the downregulation of TLR2/TRAF6/NF-kB pathway and the regulation of T cell subsets.

Keywords: Arthritis, rheumatoid; Arthritis, experimental; Toll-like receptor 2; TNF receptor-associated factor 6; Transcription factor RelA; Fas ligand protein; CD4-CD8 ratio; Total glucosides of paeony
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, progressive autoimmune disease, which frequently involves contralateral joints and even causes systemic diseases. The pathological features of RA include synovial cell proliferation and inflammatory cell infiltration, resulting in irreversible articular cartilage and bone disruption, and thus loss of behavioral capacity. Nearly 1% of the world population suffer from RA, which preferentially affects women. While the mechanism involved in RA pathogenesis has not been thoroughly illuminated, increasing evidence suggests that it may be related to disorders of innate and adaptive immunity, as well as infection.

As pattern recognition receptors, toll-like receptors (TLRs) are widely expressed on the membrane of various immune cells, and involved in the pathology of RA. TLR2, a member of the TLR family, is highly expressed in the synovium and splenic antigen-presenting cells (APCs), indicating that TLR2 participates in the pathological process of RA. Synovial cells in RA exhibit abnormal proliferation characteristics, whereas TLR2 activation induces the migration of pathological synovial cells as well as bone and cartilage invasion. Studies have shown that peripheral TLR2 regulates the amounts of activated T cell subsets to alter the immune balance, e.g. the equilibrium of CD4+ and CD8+ T lymphocytes. CD4+ and CD8+ T lymphocytes, as important T cell subsets, promote synovitis and cartilage destruction by activating immune cells and releasing pro-inflammatory cytokines.

Moreover, activated TLR2 recruits Myd88 and IL-1 receptor-associated kinase (IRAK)-1, 4, which binds to tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) to form a complex that triggers the self-polylubiquitination of TRAF6, resulting in nuclear factor-kappa B (NF-κB) activation. Meanwhile, NF-κB mediates the release of multiple inflammatory cytokines and further expands the inflammatory response.

Considering the side-effects and high costs of existing drugs used clinically for RA treatment, Traditional Chinese Medicine preparations and their components at drugs used clinically for RA treatment, Traditional Chinese Medicine preparations and their components at drugs used clinically for RA treatment, Traditional Chinese Medicine preparations and their components at drugs used clinically for RA treatment.

METHODS

Materials and reagents
TGP was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Complete Freund’s adjuvant (CFA) was from Sigma (Saint Louis, USA). Anti-rat FITC-CD3/PE-CD4 and anti-rat FITC-CD3/PE-CD8 double-labeled monoclonal antibodies were purchased from Becton Dickinson (BD, USA). NF-κB p65 Assay Kit was obtained from Shanghai Kang Cheng Biological Engineering Co., Ltd. (Shanghai, China). Real-time PCR core kit, SYBR® Premix Ex Taq™, RNAiso Reagent and RT Reagents (for the real-time RT-PCR kit) were purchased from TaKaRa (Shiga, Japan). The 3S Spin Agarose Gel DNA Purification kit was obtained from Shenneng Boci Inc. (Shanghai, China).

Animals
Forty-eight Sprague-Dawley rats (all male, 160-180 g) were obtained from the Institute of Laboratory Animals Sichuan Academy of Medical Sciences (China), and maintained under standard experimental conditions. They were regularly provided drinking water and feed in an environment at 20-22 °C. The animal protocol was approved by the institutional Animal Experimental Ethics Review Committee.

Induction of AA and treatment
Rats were randomly divided into six groups, consisting of control (Ctrl), adjuvant arthritis (AA), celecoxib (2.8 mg/kg), TGP high dose (100 mg/kg), TGP medium dose (50 mg/kg) and TGP low dose (25 mg/kg) groups. CFA was prepared by suspending heat-inactivated Bacillus Calmette-Guerin (BCG) vaccine in liquid paraflin at a concentration of 10 mg/mL. Except for the control group, the remaining five groups were injected with 0.1 mL of CFA in the right hind metatarSal foot pad. After CFA administration, the celecoxib and TGP groups were treated with the corresponding drugs from days 1 to 21 per os. Meanwhile, the control and AA groups were given equal amounts of sodium carboxymethyl cellulose (CMC-Na) solution.

Evaluation of arthritis
Paw swelling in rats was measured by water displacement plethysmography (Murom chi Kikai Co., Japan) on days 0 (basic value), 3, 6, 9, 12, 15, 18 and 21, respectively. Paw swelling data for each rat were determined by averaging two measurements. The results were expressed as change in paw swelling, specifically \( \Delta V = V_t - V_n \), where \( V_t \) and \( V_n \) are paw volume after CFA injection and normal paw volume before CFA administration, respectively.

Histopathological examination
After the final treatment, the immunized ankle was obtained and fixed with 10% neutral formalin solution overnight. Then, the samples were rinsed with tap wa-
ter overnight, submitted to automatic dehydration and embedded in paraffin. 5μm sections were stained with hematoxylin and eosin (HE), and mounted in Canada balsam for microscopic examination. Synovial cell hyperplasia and inflammatory cell infiltration, proliferation of the fibrous tissue and macrophage proliferation in the ankle were evaluated with a common scoring system. For inflammatory cell infiltration: grade 0, no inflammatory cell infiltration; grade 1, 1 to 5/HP; grade 2, 5 to 10/HP; grade 3, 11 to 15/HP; grade 4, formation of small abscesses. For synovial cell hyperplasia: grade 0, no infiltration; grade 1, swelling hyperplasia (poly); grade 2, high connection; grade 3, high bilayer level; grade 4, 3 or more layers. For fibrous tissue proliferation: grade 0, no hyperplasia; grade 1, hyperplasia in 1/3 HP; grade 2, hyperplasia in 1/2 HP; grade 3, hyperplasia in 1HP; grade 4, hyperplasia in >1HP.

**CD3+, CD4+ and CD8+ T cell detection by flow cytometry**

Blood samples from rats with or without TGP treatment were collected for analysis. After addition of 10 μL of anti-rat FITC-CD3/PE-CD8 and anti-rat FITC-CD3/PE-CD4 double-labeled monoclonal antibodies, respectively, into 100 μL anticoagulated blood, the samples were incubated at room temperature in the dark for 30 min. Then, hemolysin (1 mL) was added for 10 min incubation. A total of 3×10³ events were assessed per sample with the CellQuest software. In negative controls, the appropriate FITC-IgG1 and PE-IgG2 antibodies were added, respectively, for measurements. The FACScan (Becton Dickinson, USA) flow cytometer was used, and data were analyzed with the CellQuest software (Becton Dickinson, USA).

**Quantitative PCR**

Total RNA was extracted as previously described. Briefly, total RNA from spleen tissue samples (10 mg) was extracted using RNAiso Reagent (TaKaRa, Japan) and transcribed to cDNA with RT Reagent kit (TaKaRa, Japan), according to the manufacturer’s protocol. Then, cDNA was amplified with specific primers: TLR2, 5'- CTTACAGGACACTGGGAGAAAC-3' (forward) and 5'-AAGTTACGTTGAGAG-3' (reverse); TRAF6, 5'-CAGGAATGATGATGTGGAGT-3' (forward) and 5'-TACCGTACGGGAAAGAT-3' (reverse); 18S rRNA (reference gene), 5'-GACTCAACGGGAAACCTCA-3' (forward) and 5'-GAGACAAATCGCTCACCAC-3' (reverse). PCR products were electrophoresed on 2% agarose gels, stained with Gold view, and detected on a UV gel imaging system.

**Measurement of NF-κB p65 protein levels**

According to the instructions of NF-κB p65 Assay Kit (BioRad, USA), 100g spleen tissue was obtained for the extraction of nuclear proteins. The protein content was corrected, and NF-κB p65 content per milliliter was assessed.

**Statistical analysis**

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Data were expressed as mean ± standard deviation (x ± s). Except for the non-parametric test used for pathological data, the remaining results were assessed by t-test. A P < 0.05 was considered statistically significant.

**RESULTS**

**Anti-inflammation effects of TGP**

TGP was continuously administered intragastrically for 21 d after immunization. Right paw swelling was assessed at 0, 3, 6, 9, 12, 15, 18 and 21 d, respectively. As shown in Table 1, after CFA injection, right paw volumes in AA rats increased rapidly from 0 to 5 d, and remained relatively stable from 5 to 21 d. The effect of CFA on the joints is symmetrical, so whether the animals have contralateral paw swelling is also an evaluation index in the AA model. Secondary swelling of the left hind paw was assessed at 0, 9, 12, 15, 18 and 21 d, respectively. As shown in Table 2, left paw swelling in AA rats from 12 to 21 d showed a persistent increase. Meanwhile, TGP at 100, 50 and 25 mg/kg, respectively, significantly blunted right hind paw swelling at 3, 6, 9, 12, 15, 18 and 21 d, and left hind paw swelling at 12, 15, 18 and 21 d, respectively. The pathological features of RA include synovial cell hyperplasia, inflammatory cell infiltration, and cartilage destruction. HE staining of ankle sections from rats showed disease severity of the AA group, and the improved effect of TGP on synovial pathology (Figure 1). No inflammatory cell infiltration or cartilage destruction was found in control rats (Figure 1A). In contrast, rats with AA showed synovial cell proliferation, inflammatory cell infiltration, cartilage destruction and synovial fibrosis (Figure 1B). However, compared with rats with AA, TGP treatment resulted in significantly ameliorated symptoms (Figure 1). Specifically, the middle dose TGP group (50 mg/kg) showed mildest inflammatory infiltration, synovial fibrosis, and cartilage destruction; there was no dose-dependent relationship among the three TGP groups (Figure 1G-I).

**Effects of TGP on CD4+ and CD8+ T cell levels**

T cells are closely related to RA pathology. As shown in Figure 2, there was no significant variation in CD3+ and CD4+ T cell amounts in various group groups (Figure 2A, B). However, the amounts of CD8+ T cells in AA rats were significantly reduced compared with control values (Figure 2C). After treatment with TGP, CD8+ T cell amounts were significantly improved. CD4+ to CD8+ T cell ratios in the AA group were lower than those of the control group; compared with the
Effects of TGP on right hind paw swelling in rats with AA

The effects of TGP on right hind paw swelling in rats with AA were measured. As shown in Table 1, TGP treatment significantly decreased paw swelling compared with the AA group. The Paw swelling index (WSI) was calculated as follows:

\[ WSI = \frac{\text{Paw swelling in treated group}}{\text{Paw swelling in control group}} \times 100\% \]

The WSI values for the control group, AA group, and the TGP-treated groups (25, 50, and 100 mg/kg) were 121.0 ± 8.2, 121.0 ± 9.2, and 116.0 ± 5.8, respectively. The Paw swelling index was significantly reduced in the TGP-treated groups compared with the AA group.

Table 1: Effects of TGP on right hind paw swelling in rats with AA

<table>
<thead>
<tr>
<th>Group</th>
<th>WSI</th>
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<tbody>
<tr>
<td>Control</td>
<td>121.0 ± 8.2</td>
</tr>
<tr>
<td>AA</td>
<td>121.0 ± 9.2</td>
</tr>
<tr>
<td>TGP 25</td>
<td>116.0 ± 5.8</td>
</tr>
<tr>
<td>TGP 50</td>
<td>117.0 ± 5.6</td>
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<tr>
<td>TGP 100</td>
<td>120.0 ± 6.8</td>
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DISCUSSION

Paonia lactiflora Pall is a common traditional Chinese medicinal plant used for RA treatment. Its main active ingredient, TGP, has good anti-inflammatory effects in both clinical and animal studies of RA. Meanwhile, studies have shown that TGP improves RA by regulating the proliferation of fibroblastic synoviocytes at joints, peripheral bone loss, and dendritic cell activation. However, the effect of TGP on peripheral immunity in AA rats remains undetermined. This study showed that TGP reduced TLR2 activity, inhibited TLR2-mediated TLR6/NF-κB p65 inflammation signaling, maintained the CD4/CD8+ T cell balance, and improved paw swelling and synovial pathology, in AA rats. These findings indicated that the therapeutic effect of TGP may be related to improved peripheral immune activity.
In recent years, reports have suggested that TLR2 plays a crucial role in the pathogenesis of RA. Studies have shown that TLR2 activation promotes the proliferation and differentiation of CD4+ and CD8+ T cells in infected mice. However, only CD8+ T cells were significantly reduced in AA rats as shown above, while there was no significant change in CD4+ T cells. To explore the underlying mechanism, the expression of the apoptosis-associated protein FASLG in the spleen was assessed; we found that FASLG levels were significantly increased, which may be one of the factors explaining CD8+ T cell apoptosis. Besides, TLR2 is involved in the migration of CD8+ T cells from periphery to RA synovial membrane. However, whether TLR2 associated increased migration of CD8+ T cells is responsible for the decrease of peripheral CD8+ T cells remains unclear. TGP significantly increased the amounts of
CD8+ T cells and maintained the immune balance, which may be related to TLR2 and FASLG downregulation in the spleen.

NF-κB signaling is one of the classical pathways of inflammation, and closely related to the occurrence and development of RA. The NF-κB signaling pathway mediates the release of inflammatory cytokines from immune cells, e.g. IFN-γ, TNF-α, IL-1β, IL-8, IL-17, IL-6, IL-12 and iNOS, which further aggravate RA. When TLR2 is activated, the downstream MyD88 and IRAK1/4 complexes are recruited, and TRAF6 is activated to form the MyD88-IRAKs-TRAF6 complex, which eventually leads to NF-κB entering the nucleus and mediating the release of inflammatory cytokines. Studies have shown that TGP can effectively reduce TNF-α and IL-1 levels in CIA rats, improving the inflammatory response. In addition, TGP inhibits the release of the inflammatory cytokines IL-6 and TNF-α from oral lichen planus by inhibiting the NF-κB signaling pathway. In AA rats, TGP inhibited the production of IL-1, TNF-α and IL-6 in synovial cells. The current experimental results showed that TGP indeed inhibited the activation of NF-κB p65 mediated by TLR2, preventing the release of inflammatory factors. A limitation of this experimental study is that inflammatory cytokines such as IL-1, TNF-α and IL-6 were not assessed in serum samples. However, through paw swelling and synovial pathology data, it was demonstrated that TGP improved the inflammatory response at the joints, which may be related to the regulation of the TLR2/TRAF6/NF-κB p65 signaling pathway, directly inhibiting the release of inflammatory cytokines.

In summary, TGP’s protective effects in AA rats were suggested by the paw swelling rate, histopathology, the expression levels of TLR2, TRAF6 and NF-κB p65, and the amounts of T cell subsets. The mechanism behind its effects might involve the regulation of TLR2/ TRAF6/NF-κB signaling pathway, and immune balance maintenance by controlling the CD4+ to CD8+ T cell ratio.

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The express of NF-κB p65 protein -actin mRNA expression

Figure 3 Effects of TGP on TLR2, TRAF6 and FASLG mRNA levels
Ctrl: control group; AA: adjuvant arthritis group; Cel: celecoxib (positive control) group; TGP: total glucosides of paeony; TLR2: toll-like receptor 2; FASLG: tumor necrosis factor ligand superfamily member 6; TRAF6: tumor necrosis factor receptor-associated factor 6; NF-κB: nuclear factor-kappa B. Data are mean ± standard deviation (n = 8). *p < 0.01, **p < 0.05, ***p < 0.001, compared with the adjuvant arthritis group.

Figure 4 Effects of TGP on TLR2, FASLG, TRAF6 and NF-κB p65 protein expression levels
A: representative examples of FASLG, TLR2 and TRAF6 protein bands in the spleen from various groups; B: NF-κB p65 Assay Kit was used to detect NF-κB p65 protein levels in the spleen from various groups; C: semi-quantitative statistical graph of TLR2, Faslg, and TRAF6 protein levels in various groups, β-actin serves as the house-keeping protein; D: the correlation analysis between TLR2 protein and NF-κB p65 protein; E: the correlation analysis between TLR2 protein and the ratio of CD4+/CD8+ T cell. Ctrl: control group; AA: adjuvant arthritis group; Cel: celecoxib (positive control) group; TGP: total glucosides of paeony; TLR2: toll-like receptor 2; FASLG: tumor necrosis factor ligand superfamily member 6; TRAF6: tumor necrosis factor receptor-associated factor 6; NF-κB: nuclear factor-kappa B. Data are mean ± standard deviation (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, compared with the adjuvant arthritis group.
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