Total glucosides of paeony (TGP) inhibits the production of inflammatory cytokines in oral lichen planus by suppressing the NF-κB signaling pathway

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Abstract

Total glucosides of paeony (TGP) is a bioactive compound extracted from paeony roots and has been widely used to ameliorate inflammation in several autoimmune and inflammatory diseases. However, the anti-inflammatory effect of TGP on oral lichen planus (OLP), a chronic inflammatory oral condition characterized by T-cell infiltration and abnormal epithelial keratinization cycle remains unclear. In this study, we found that TLR4 was highly expressed and activation of the NF- κ B signaling pathway was obviously observed in the OLP tissues. Moreover, there was significant higher mRNA expression of inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in OLP keratinocytes than normal oral epithelial keratinocytes. With the help of the cell culture model by stimulating the keratinocyte HaCaT cells with lipopolysaccharides (LPS), we mimicked the local inflammatory environment of OLP. And we further confirmed that TGP could inhibit LPS-induced production of IL-6 and TNF- α in HaCaT cells via a dose-dependent manner. TGP treatment decreased the phosphorylation of I κ B α and NF- κ B p65 proteins, thus leading to less nuclear translocation of NF- κ B p65 in HaCaT cells. Therefore, our data suggested that TGP may be a new potential candidate for the therapy of OLP.

Keywords: total glucosides of paeony; oral lichen planus; TLR4; NF- κ B.

1. Introduction

As a chronic autoimmune, mucocutaneous disease, lichen planus (LP) usually affects the oral and genital mucosa, skin, scalp and nails[1]. It occurs more commonly

1 in women aged over 30 years; however, incidence in children has also been
2 reported[2, 3]. Oral mucosal lesions appear in 50 to 70% of LP patients and may be
3 exclusive in 20 to 30% of them[4]. The occurrence of oral lichen planus (OLP) ranges
4 from 0.5 to 4%, and the prevalence of malignant transformation from 0 to 5.3%[4, 5].
5 The etiology of OLP is still unclear, but immunological processes are believed to play
6 critical roles[6]. Cytotoxic CD8⁺T cells are directed against basilar keratinocytes,
7 which leads to the degeneration and lyses of basal cells[7]. Cytokines such as IFN- γ ,
8 TNF- α , GM-SF and IL-6 are released, provoking a local inflammatory response thus
9 further exacerbating the tissue damage[8].

10 OLP may present in two main types: a reticular type which requires no treatment
11 or an atrophic/erosive type which does need therapy because of its burning sensation,
12 severe pain and malignant transformation tendency[9]. So far, treatments of OLP
13 include corticosteroids drugs, immunosuppressive agents, photodynamic therapy and
14 cryosurgery *etc*[4]. However, as inflammatory condition in OLP often recurs, surgery
15 is not first recommended. And the associated side effects and complications like
16 secondary candida infection of oral mucosa and suppression of adrenal function with
17 corticosteroids drugs; local irritation, mucosal pigmentation and the risk of promoting
18 the occurrence of squamous-cell carcinoma by affecting the MAPK and p53 signaling
19 with immunosuppressive agents; nausea, dizziness, paresthesia and headache with
20 photodynamic therapy, are disadvantageous[4, 5]. Therefore, the identification of
21 novel and effective therapeutic agents for OLP is essential.

22 Recently, the products derived from natural plant with different biological effects

1 have attracted extensive attention on their treatment for numerous diseases. One of
2 them is total glucosides of paeony (TGP), the bioactive compound extracted from
3 paenoy roots. Now, TGP has been proved to be protective in several animal disease
4 models. Wu et al. reported that TGP relieved early renal injury through inhibiting the
5 secretion of inflammatory factors like intracellular adhesion molecule-1 (ICAM-1),
6 IL-1 and TNF- α in the kidneys of diabetic rats[10]. TGP could also modulate serum
7 cholesterol and triglyceride levels and suppress TNF- α , IL-6 expression thus
8 attenuated the development of atherosclerotic disease[11]. Moreover, TGP minimized
9 damages in rheumatoid arthritis by inhibiting Th1/Th17 cells differentiation and
10 fibroblast-like synoviocytes proliferation [12-14]. However, the effect of TGP in OLP
11 has not yet been elucidated.

12 In the present study, we investigated the effect of TGP on OLP keratinocytes.
13 And with a cell culture model, we further explored the underlying mechanisms of the
14 therapeutic actions of TGP.

15

16 **2. Materials and methods**

17 **2.1. Immunohistochemistry assay**

18 Human OLP specimens were obtained from patients diagnosed with OLP at the
19 Department of Oral Medicine, Shanghai Ninth People's Hospital. Normal oral
20 mucosal tissues were collected from the Department of Oral and Maxillofacial
21 Surgery from 2014 to 2015. Signed informed consent forms were acquired before
22 biopsy and pathological examination. All experimental procedures were ethically

1 approved by the Independent Ethics Committee of Shanghai Ninth People's Hospital
2 (No. 2014069). Specimens were fixed and embedded with paraffin. Sections of the
3 specimens were blocked with 5% BSA and incubated with anti-TLR4 (1:100, Abcam,
4 MA, USA) and anti-NF- κ B p65 (1:100, Cell Signaling, MA, USA) antibodies
5 overnight at 4°C. Next, slides were washed and incubated with second antibodies
6 conjugated with peroxidase (Dako, CA, USA) for 1 h. Then, slides were developed
7 with 3, 3'-Diaminobenzidine (Dako, CA, USA) substrate and counter-stained with
8 hematoxylin. After dehydration, slides were visualized under Axio Imager (Zeiss,
9 Oberkonchen, Germany).

10

11 **2.2. Quantitative real-time PCR (qRT-PCR)**

12 Human OLP specimens (n=27) and normal oral mucosal tissues (n=14) were
13 digested using 0.25% dispase II (Stemcell, CAN) at 4°C for 12 h. Characteristics of
14 the patients with OLP were shown in Table 1. The epithelium and subepithelial layers
15 were separated by sterile muscle forceps. The isolated epithelium was digested in
16 0.25% trypsin for 8 min to obtain a single-cell suspension of keratinocytes. TRIzol
17 reagent (Invitrogen, CA, USA) was used to extract total RNA from keratinocytes.
18 cDNA was reverse transcribed from up to 1 μ g total RNA using a PrimeScriptTM RT
19 reagent kit (Takara, Kyoto, Japan). mRNA expression was determined by qRT-PCR
20 assay using the Applied Biosystems 7500 Real-Time PCR System (Life technologies,
21 CA, USA). The following PCR conditions were used: 95°C for 5 min, followed by 40
22 cycles of 95°C for 15 s and 60°C for 1 min, completed with a melt curve analysis.

1 Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method and was calculated
2 using β -actin as the reference gene. Sequences of PCR primer pairs were given in
3 Table 2.

4 5 **2.3. Cell line and culture**

6 The HaCaT cell line was provided by China Infrastructure of Cell Line
7 Resources (Beijing, China). HaCaT cells were maintained in Dulbecco's modified
8 Eagle's medium (DMEM) (Gibco, CA, USA) supplemented with 10% FBS (Gibco,
9 CA, USA) and penicillin-streptomycin (Gibco, CA, USA) in a humidified 5% CO₂
10 incubator at 37°C. To simulate a local immune microenvironment of OLP, the culture
11 medium was replaced with keratinocyte serum free medium (KSFM) (Gibco, CA,
12 USA) in which HaCaT cells were cultured overnight and then stimulated with LPS
13 (Sigma Aldrich, MO, USA) at a concentration of 10 μ g/ml.

14 15 **2.4. Enzyme-linked immunosorbent assay (ELISA)**

16 HaCaT cells were seeded in a 96-well plate (5×10^4 cells per well) and cultured
17 in KSFM for 24 h. Then cells were stimulated by TGP (Liwah Plant Extract
18 Technology, Ningbo, China) at serial concentrations (1, 2.5, 5, and 10 μ g/mL) in
19 KSFM for 2 h and then treated by LPS (10 μ g/mL) for 48 h. Supernatants were
20 collected to measure the concentration of IL-6 and TNF- α by ELISA (eBioscience,
21 CA, USA) assays according to the manufacturer's instructions.

22

2.5. Cell viability assay

To investigate the toxicity of TGP, CCK-8 (Dojindo, Kumamoto, Japan) assay was applied to assess cell viability. HaCaT cells were seeded in a 96-well plate (5000 cells per well) and treated with increasing concentrations of TGP in DMEM for 24 h or 48 h. Then, 10 µl of CCK-8 reagent were added to each well. Wells containing incubation media without cells were incubated in parallel with test samples to measure the absorbance background. After incubation for 1.5 h, absorbance values were measured at 450 nm.

2.6. Western blot

HaCaT cells were treated with increasing concentrations of TGP (0, 1, 2.5, 5, and 10 µg/ml) for 2 h, followed by LPS incubation (10 µg/mL) in the continued presence of TGP for another 2 h. The cells were lysed with RIPA buffer (Beyotime, Shanghai, China) containing phosphatase inhibitor cocktail (Roche, Germany) and phenylmethanesulfonylfluoride (PMSF). The total cell lysates were centrifuged and the supernatants were used for western blotting. Equal quantities of protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 5% nonfat milk for 1 h at room temperature and incubated overnight at 4°C with appropriate primary antibodies (1:1000): anti-phospho-IκBα, anti-IκBα, anti-phospho-NF-κB p65, and anti-NF-κB p65 (Cell Signaling, MA, USA); anti-β-actin (Sigma-Aldrich, Mo, USA). After washed by

TBST, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000) (Cell Signaling, MA, USA). Labeled proteins were visualized using ECL reagent (Merck Millipore, USA). The protein levels were quantified by Image-Pro Plus 6.0 (Media Cybernetics, CA, USA).

2.7. Immunofluorescence assay

HaCaT cells deposited on glass slides were fixed with 4% paraformaldehyde for 20 min. After permeabilized with 0.2% Triton X-100 for 6 min and blocked with 1% bovine serum albumin for 40 min, the slides were treated with anti-NF- κ B p65 (1:400) antibody at 4°C overnight. Anti-rabbit IgG labeled with Alexa Fluor 488 (1:1000) served as the secondary antibody. After washing three times, sections were stained with DAPI for 3 min to visualize the nuclei. Slides were examined using an inverted microscope equipped with fluorescence optics (Olympus, Osaka, Japan).

2.8. Statistical analysis

Each experiment was repeated at least three times. The Student's *t* test was used to analyze the statistical significance between two groups. Statistical differences were considered significant when value of $p < 0.05$.

3. Results

3.1. Expression of TLR4 and NF- κ B p65 in OLP keratinocytes

Since OLP is a long-lasting inflammatory process, some studies recently reported

that keratinocytes play a critical role in maintaining the inflammatory environment of OLP through TLR4/NF- κ B activation [15-17]. To verify the local inflammatory environment of OLP, immunohistochemical staining was performed to analyze the expression of TLR4 and NF- κ B p65 in OLP specimens. As shown in Fig. 1, both TLR4 and NF- κ B p65 were highly expressed in keratinocytes of OLP lesions compared with normal oral mucosal tissues, which confirmed the significant role of TLR4/NF- κ B signaling pathway in pathogenesis of OLP.

3.2. Expression of IL-6 and TNF- α in OLP keratinocytes

The production of inflammatory cytokines should be increased when the NF- κ B inflammation signaling pathway is activated. To observe the levels of inflammatory cytokines in OLP, 27 OLP specimens and 14 normal control mucosal tissues were digested with dispase II and trypsin to release keratinocytes. The mRNA expression of IL-6 and TNF- α in keratinocytes was measured by qRT-PCR assay. As shown in Fig. 2, expression of IL-6 and TNF- α mRNA was increased in OLP keratinocytes compared to keratinocytes obtained from normal mucosal tissues. These results, together with the data shown in Fig.1, suggested that TLR4 was highly expressed and NF- κ B inflammatory signaling pathway was activated in OLP keratinocytes.

3.3. TGP inhibition of IL-6 and TNF- α production in LPS-treated HaCaT cells

Following stimulation by exogenous factors such as TLR4 ligand LPS, keratinocytes actively secrete a large amount of cytokines. HaCaT cells, the

1 immortalized human skin keratinocytes, are useful substitutes for oral keratinocytes
2 [15]. Therefore experiments were performed on HaCaT cells with LPS stimulation to
3 mimic the immune microenvironment of OLP lesions [15]. To determine if LPS
4 stimulation of HaCaT cells simulates the inflammatory environment in OLP, the
5 levels of NF- κ B-dependent inflammatory cytokines were measured by ELISA. After
6 stimulating HaCaT cells with LPS (10 μ g/ml) in KSFM for 48 h, we found that
7 production of IL-6 and TNF- α was markedly increased (Fig. 3). The data suggested
8 that the mimic inflammatory responses could to some extent reflect the immune
9 environment in OLP.

10 To investigate the anti-inflammatory effect of TGP, HaCaT cells were
11 pre-incubated with different concentration of TGP for 2 h, followed by stimulating
12 with LPS for an additional 48 h. The levels of IL-6 and TNF- α in cell culture
13 supernatants were measured by ELISA assay. As shown in Fig. 3, secretion of IL-6
14 and TNF- α was significantly decreased in a dose-related fashion with the increase in
15 TGP concentrations. This result suggested that TGP could reduce the LPS-induced
16 HaCaT cell inflammation response by inhibiting the production of inflammatory
17 cytokines.

18

19 **3.4. TGP did not affect the proliferation of HaCaT cells**

20 To further study the effect of TGP on keratinocytes, we first investigated if TGP
21 affected HaCaT cell proliferation using the CCK-8 assay. HaCaT cells were cultured
22 with increasing concentrations of TGP for 24 or 48 h. CCK-8 was then added to the

1 culture medium for 1.5 h, followed by measurement of absorbance values at 450 nm
2 as an indicator of HaCaT cell viability. We found no obvious change in the
3 proliferation of HaCaT cells, which suggested that TGP was non-toxic at doses of
4 1–10 µg/ml under culture conditions (Fig. 4).

5

6 **3.5. TGP suppression of LPS-induced activation of the NF-κB inflammatory** 7 **signaling pathway in HaCaT cells**

8 NF-κB activation of keratinocytes is critically involved in the progression of
9 OLP and involves the production of inflammatory cytokines. To study the
10 anti-inflammatory mechanism of TGP, we investigated its effect on LPS-induced
11 NF-κB activation in HaCaT cells. After treating HaCaT cells with increasing amounts
12 of TGP, LPS (10 µg/ml) was added to the incubation medium and cellular protein was
13 extracted for western blot assay. As shown in Figs. 5A and 5B, expression of
14 phosphorylated-IκB and phosphorylated-NF-κB p65 proteins was significantly
15 decreased in a dose-related fashion with elevating concentrations of TGP, with no
16 change in total IκBα, total NF-κB p65 and β-actin. These results indicated that the
17 phosphorylation of IκBα and NF-κB p65 was suppressed by TGP.

18 Immunofluorescence assays were then conducted to examine the effect of TGP
19 on nuclear translocation of NF-κB p65. As shown in Fig. 5C, LPS-stimulated HaCaT
20 cells displayed greater amounts of nuclear NF-κB p65 than control cells. Treatment of
21 HaCaT cells with 10µg/ml TGP reduced the intensity of NF-κB p65 nuclear staining,
22 indicating that TGP had suppressed nuclear translocation of NF-κB p65. The above

1 results shown also suggested that TGP could inhibit the activation of NF- κ B
2 inflammatory signaling pathway in a mimic OLP local immune environment *in vitro*.

4 **4. Discussion**

5 Our findings demonstrated that activation of TLR4/NF- κ B pathway was detected
6 in OLP tissue. Moreover, the expression of inflammatory cytokines IL-6 and TNF- α
7 was significantly up-regulated in OLP keratinocytes. In this study, we found that TGP
8 markedly inhibited the inflammatory response of the mimic OLP by suppressing the
9 secretion of inflammatory cytokine IL-6 and TNF- α . The inhibiting effect of TGP may
10 be attributed to its suppression of NF- κ B signaling pathway.

11 OLP is a common autoimmune mucocutaneous disease of unknown etiology,
12 characterized by a T-cell-mediated immune process against keratinocytes leading to
13 keratinocytes damage and subepithelial band-like infiltration of T lymphocytes[18-20].
14 In the early stages of disease development, the keratinocytes are target cells to suffer
15 apoptosis. Once active, however, those keratinocytes have the ability to amplify an
16 inflammatory signal by producing inflammatory mediators and recruiting
17 inflammatory cells that can mediate the development of OLP, as well as favor the
18 disease chronicity[21, 22]. These findings confirmed the critical role of keratinocytes
19 in the pathological process in OLP lesions.

20 Recently, the role of keratinocytes in sustaining the inflammatory environment of
21 OLP has attracted extensive attention, one critical possible pathogenesis of which is
22 NF- κ B activation. Previous studies demonstrated that NF- κ B was highly expressed on

1 basal and suprabasal keratinocytes of the OLP lesions [17, 23], which was in positive
2 correlation with the degree of cytotoxic T cells infiltration [17]. As an inflammatory
3 regulator, NF- κ B could be activated by multiple inflammatory signals, one crucial
4 pathway was triggering of toll-like receptors (TLRs). Recently, Janardhanam SB et
5 al.[24] and Siponen M et al.[16] demonstrated the high expression of TLR4 in OLP
6 epithelium. In accordance with previous studies, our data indicated that both TLR4
7 and NF- κ B p65 were highly expressed in keratinocytes of OLP lesions, verifying the
8 critical role of TLR4/NF- κ B activation in OLP pathogenesis. Moreover, some articles
9 reported that NF- κ B associated cytokines played important roles in the exacerbation
10 and perpetuation of OLP. Recently, the elevated levels of NF- κ B-dependent
11 inflammatory cytokines such as IL-1 β , IL-6, IL-8, and TNF- α were detected in both
12 salivary and serum of OLP patients [25, 26]. In our study, we found that mRNA levels
13 of IL-6 and TNF- α in keratinocytes of OLP lesions were significantly increased,
14 suggesting that keratinocytes might maintain the inflammatory response of OLP
15 through activating TLR4/NF- κ B signaling pathway and secreting IL-6 and TNF- α .

16 However, due to the pathological condition of OLP keratinocytes and severe oral
17 fungal infection of OLP patients, the primary culture and its passage always turn out
18 failed. So far, there is no appropriate model to study the pathogenesis of OLP. HaCaT
19 cells are immortalized human skin keratinocytes and frequently substituted for oral
20 keratinocytes in many studies due to their easy growth and infinite proliferation
21 [27-29]. LPS, the ligand of TLR4, has long been used as a mediator to induce
22 inflammatory response. Therefore, we used LPS-induced HaCaT cell inflammatory

1 responses to simulate the local immune environment of OLP in the current study.
2 After stimulating with LPS, the production of inflammatory cytokines IL-6 and
3 TNF- α was markedly increased, this demonstrated that our cell model was proved to
4 well reflect the inflammatory environment of OLP.

5 TGP is extracted from the roots of *Paeonia lactiflora* and has been widely used in
6 the therapy for autoimmune disease in Chinese medicine. It is reported that TGP has
7 anti-inflammatory, hepatoprotective [30, 31] and immuno-regulatory activities.
8 Recently, the anti-inflammatory effect of TGP has been attracting increasing attention.
9 In our study, we demonstrated that TGP has marked therapeutic effect in the mimic
10 OLP local immune environment by reducing the production of IL-6 and TNF- α from
11 LPS-treated HaCaT cells. Based on our results and others, IL-6 and TNF- α released
12 by keratinocyte from OLP lesions are likely contribute to the pathogenesis of OLP. As
13 primary cytokines, IL-6 and TNF- α can induce proliferation and differentiation of T
14 cells, meanwhile, they also upregulate adhesion molecules expression and increase T
15 cell adhesion which may contribute to T cell infiltration in OLP[32]. Furthermore,
16 previous studies reported the high increase of inflammatory cytokines in salivary from
17 OLP patients, and the change of which could be used for monitoring the therapeutic
18 response[33]. Therefore, the anti-inflammatory effect of TGP in mimic OLP offered
19 the possibilities of a therapeutic drug for OLP through suppressing secretion of IL-6
20 and TNF- α from keratinocyte.

21 Subsequently, we further investigated the mechanisms by which TGP suppress
22 inflammatory responses in the mimic OLP. NF- κ B is an important inflammatory

1 mediators which plays critical roles in regulating inflammatory signaling pathway
2 [34]. It has been confirmed that in the NF- κ B inflammatory signal pathway, NF- κ B
3 p50/p65 and I κ B tripolymer is inactive in the cytoplasm. Upon activation, I κ B is
4 phosphorylated and degraded, freeing NF- κ B p50/p65 complexes. Then active NF- κ B
5 p65 translocate to the nucleus, either alone or in combination with other transcription
6 factors, where it induces target gene expression including genes encoding
7 proinflammatory cytokines[35]. In our experiment, we demonstrated that NF- κ B
8 signaling pathway was activated in mimic OLP by using the model of LPS-induced
9 HaCaT cell inflammatory responses. Therefore, we speculated whether
10 anti-inflammatory effect of TGP was related to the inhibition of NF- κ B signaling
11 pathway. As a result, TGP could obviously decrease the phosphorylation of I κ B α and
12 NF- κ B p65, thus leading to less nuclear translocation of NF- κ B p65. Our results
13 suggested that the anti-inflammatory mechanism of TGP in a mimic OLP local
14 immune environment is mediated by suppressing the NF- κ B signaling pathway.

15 In summary, we utilized LPS-induced HaCaT cell inflammatory responses to
16 simulate the local OLP immune environment and found TGP has a potentially
17 therapeutic effect on OLP. Furthermore, the anti-inflammatory mechanism of TGP is
18 associated with inhibition of NF- κ B signaling pathway leading to reduced secretion of
19 inflammatory cytokines. The results of our study suggest new opportunities to use
20 TGP in treatment of OLP.

21

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Conflict of interest

The authors declare no financial or commercial conflicts of interest.

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Figure Legends

Fig. 1. Expression of TLR4 and NF-κB p65 analyzed by immunohistochemical staining in keratinocytes of OLP and healthy control tissue. TLR4 were stained brown and were detected in the membrane and cytoplasm of HC (A) and OLP (C) keratinocytes; NF-κB p65 were stained dark brown and were detected in cytoplasm of HC (B) and OLP (D) keratinocytes. Ten sections were observed and pictures were representative of enlarged images (200×).

1 Fig. 2. Levels of IL-6 and TNF- α evaluated by qRT-PCR in keratinocytes from 27
2 OLP patients and 14 healthy control subjects. (A) mRNA expression levels of IL-6. (B)
3 mRNA expression levels of TNF- α . Data are presented as mean $\pm$ SD. * $p < 0.05$.

4
5 Fig. 3. TGP inhibition of LPS-induced IL-6 and TNF- α secretion in HaCaT cells.
6 HaCaT cells were treated with TGP (0, 1, 2.5, 5, and 10 $\mu\text{g/ml}$) for 2 h, followed by
7 addition of LPS (10 $\mu\text{g/ml}$) for 48 h. Culture medium was analyzed for cytokine
8 levels by ELISA. (A) level of IL-6 in cell culture supernatant. (B) level of TNF- α in
9 cell culture supernatant. Results are representative of three independent experiments
10 and are presented as mean $\pm$ SD. ## $p < 0.01$ (# VS cells without LPS treatment); * p
11 < 0.05 , ** $p < 0.01$ (* VS LPS-stimulated cells without TGP treatment).

12
13 Fig. 4. CCK-8 assay of potential cytotoxic effects of TGP (1–10 $\mu\text{g/ml}$) at 24 h or 48 h
14 in HaCaT cells. Data are presented as mean $\pm$ SD.

15
16 Fig. 5. TGP suppression of the NF- κB inflammatory signaling pathway in LPS-treated
17 HaCaT cells. (A) western blot assay was performed to investigate expression of
18 NF- κB inflammatory signaling proteins. β -actin was monitored as a control for
19 protein loading. (B) quantitative analysis of phospho-I $\kappa\text{B}\alpha$ (left panel) and
20 phospho-NF- κB p65 (right panel) protein expression. Data are presented as mean $\pm$
21 SD. # $p < 0.05$, ## $p < 0.01$ (# VS cells without LPS treatment); * $p < 0.05$, ** $p < 0.01$,
22 *** $p < 0.001$ (* VS LPS-stimulated cells without TGP treatment). (C) nuclear

- 1 translocation of NF- κ B p65 was visualized under fluorescence microscopy. (a), (d),
- 2 and (g), staining of nuclei (blue); (b), (e), and (h), detection of NF- κ B p65 (green); (c),
- 3 a merge of (a) and (b); (f), a merge of (d) and (e); (i), a merge of (g) and (h).

Table 1

Characteristics of the patients with OLP and HC

	Number of cases	Age range (years)	Average age (years)	Number of females	Number of males
HC	14	19-49	38.2±7.62	9	5
OLP	27	21-61	40.3±10.57	18	9

Table 2

The primers for qRT-PCR analysis.

Gene	Primer sequence 5'–3'
IL-6	F-CCTGAACCTTCCAAAGATGGC R-TTCACCAGGC AAGTCTCCTCA
TNF- α	F-CCTCTCTCTAATCAGCCCT CTG R-GAGGACCTGGGAGTAGAT GAG
β -actin	F-CCTGGCACCCAGCACAAT R-GGGCCGGACTCGTCATACT

Figure. 1

HC

OLP

TLR4

NF- κ B p65

Figure. 2

Figure. 3

Figure. 4

Figure. 5

A

B

C