Abstract. Regulatory effect of *Toll-like receptor 4* (*TLR4*) gene on periodontitis in mice was investigated to explore its possible mechanism. Thirty C57/BL6 mice were randomly divided into the blank control group (N group, n=10), the periodontitis group (P group, n=10) and the periodontitis + TAK-242 group (PT group, n=10). The mice in P and PT group were ligatured with silk threads dipped with *Porphyromonas gingivalis* (*P. gingivalis*) in the logarithmic phase to induce experimental periodontitis, and TAK-242 was intraperitoneally injected on the day when the periodontitis model was established. After fasting for 8 h, the expression levels of high-sensitivity C-reactive protein and inflammatory cytokines were measured in each group of mice. Their alveolar bones were isolated and changes were detected. Quantitative polymerase chain reaction was used to detect the expression levels of TLR4. After the mice were given TAK-242, the levels of hs-CPR, MCP-1, IL-6 and IL-1β in the PT group evidently increased (P<0.01) compared with those in the N group. After the mice were administered TAK-242, the alveolar bone density, the percentage of bone volume and the number of bone trabeculae in PT group were significantly reduced, and the bone trabecular space and structural model index were evidently decreased (P<0.01). In addition, the expression levels of and T-bet/GATA3 messenger ribonucleic acids (mRNAs) in peria of mice in the P group were significantly higher than those in the N group (P<0.01), whereas the expression level of Foxp3 mRNA was notably decreased (P<0.01). The involvement of *TLR4* gene in the inflammatory response of periodontitis results in periodontitis, and its mechanism may be that it activates TLR4, so as to affect the expression of T-bet, GATA3 and Foxp3.

Introduction

Various chronic infectious diseases can be triggered by numerous dental plaque microorganisms in the oral cavity, and periodontitis is the most common one among the diseases (1). Periodontitis is a vital disease resulting in anodontia, and its incidence rate is as high as over 80% in China (2). According to the existing studies, the main factors of periodontium destruction resulted from periodontitis include the host’s inappropriate immune response to microorganisms and the toxicity of the microorganism itself, but the former is the main factor (3). Alveolar bone resorption is the main pathological change of periodontitis. Substantial research evidence has indicated that the secretion levels of tumor necrosis factors, interleukins and other cytokines will be increased under the direct or indirect action of bacteria and inflammatory cells in patients with periodontitis, thus, further resulting in changes in bone structure and ultimately aggravating periodontitis (4). In addition to surgical treatment and basic maintenance therapy, the extremely crucial treatments for periodontitis at present include the application of effective drugs to control the inflammatory response in peria and to inhibit the alveolar bone resorption (5).

As a specific I-type transmembrane receptor and a pathogen pattern recognition receptor in the natural immune system, Toll-like receptor (TLR) exerts key effects on acute inflammatory response, cell signal transduction and apoptosis (6). At present 13 kinds of TLR family members have been identified in mammals. TLR4-related studies are in the majority among inflammatory disease-related reports (7). In addition to binding to exogenous ligands, TLR can also...
influence the expression of many endogenous transcription factors so as to mediate inflammatory responses (8). Findings have shown that T-bet and GATA3 can regulate T helper 1 (Th1)/Th2-mediated immune responses and be involved in regulating cellular immunity. Moreover, Foxp3 affects cluster of differentiation (CD4+ and CD25+ T cells so as to regulate immune responses (9). The role of TLR4 in periodontitis has rarely been reported, and there is no research reports on the roles of T-bet, GATA3 and Foxp3 in the pathogenesis of periodontitis.

The aim of the study was to establish a model of periodontitis to examine the roles of TLR4 gene in the pathogenesis and progression of periodontitis in mice, thus, revealing its possible mechanism and providing a theoretical basis for the treatment of periodontitis and further drug development.

Materials and methods

Animal grouping and model establishment. Experimental grouping: Male C57/BL6 mice aged 8 weeks were purchased from the Model Animal Research Center of Nanjing University (Laboratory Animal Production License no. SCXK2011-0022), and were raised in the specific-pathogen-free (SPF) environment of Guangdong Laboratory Animal Center (Foshan City, China). The mice were free to eat and drink. A total of 30 C57/BL6 mice were randomly divided into the blank control group (N group, n=10), the periodontitis group (P group) and the periodontitis + TAK-242 group (PT group). The mice in the P group were ligated with silk threads dipped with Porphyromonas gingivalis (P. gingivalis) in the logarithmic phase to induce experimental periodontitis, and 3 mg/kg TAK-242 was intraperitoneally injected into the mice in the PT group on the day when the periodontitis model was established, which was then administered twice a day, followed by observation for 10 days in total. Both N and P group received intraperitoneal injection of normal saline.

Establishment of a model of experimental periodontitis (10): Mice were intraperitoneally injected with 4% chloral hydrate, and 5-0 sterile non-absorbable silk threads dipped with P. gingivalis in the logarithmic phase of the inoculum was intraperitoneally injected to the mice in the PT group on the day when the periodontitis model was established, which was then administered twice a day, followed by observation for 10 days in total. Both N and P group received intraperitoneal injection of normal saline.

Detection via ELISA. Hs-CRP, MCP-1, IL-6 and IL-1β test kits were employed to detect changes in these factors in each group of mice strictly according to the instructions of the kits: The standard solution was diluted with diluent until the corresponding concentration, followed by the plotting of a standard curve. After 10 days, serum samples were taken from each group of mice, diluted 10 times and placed on ice for standby application. The antibody-coated 96-well plates were taken out, and then standard wells, sample wells and blank control wells were set. Each well was added with 100 µl standard solution or sample solution, respectively, and the plates were sealed with plate sealers, followed by incubation in a thermostat at 37°C for 60 min. Subsequently, the liquid in the plates was discarded, the plates were patted dry, the corresponding 100 µl, rabbit anti-mouse polyclonal antibodies (insulin, hs-CRP, MCP-1, IL-6 and IL-1; cat. nos. 15848-1-AP, 24175-1-AP, 25542-1-AP, 21865-1-AP and 16765-1-AP; dil, 1:300; Wuhan Sanying Biotechnology) were added, and the plates were sealed with plate sealers, followed by incubation in a thermostat at 37°C for 45 min. Subsequently, each well was washed with 200 µl washing solution to wash plates for a total of 3 times with 1 min each time. After that, 100 µl secondary goat anti-rabbit polyclonal antibody (insulin, hs-CRP, MCP-1, IL-6 and IL-1; cat. no. SA00001-2; dil, 1:500; Wuhan Sanying Biotechnology) was added, and the plates were sealed with plate sealers, followed by incubation in a thermostat at 37°C for 30 min. A total of 90 µl 3,3',5,5'-tetramethylbenzidine (TMB) coloring solution was added to each sample well, and the plates were sealed with the plate sealers, followed by incubation in a thermostat at 37°C for 30 min in the dark. A total of 100 µl TMB stop solution was added to each sample well to terminate the reaction. The optical density was measured at 450 nm using a microplate reader, and the concentrations of hs-CRP, MCP-1, IL-6 and IL-1β in each sample were calculated.

Detection of changes in alveolar bones via micro-computed tomography (CT). After the mice in each group were sacrificed, their right mandibles were completely peeled, and the soft tissues were carefully removed (from the lowest point of the middle tooth enamel to the point where the shortest tooth root of the middle tooth disappears). The right mandibles were fixed in 10% neutral formaldehyde solution for 2 days, washed with water overnight, and stored in 75% alcohol. After desiccation, the conditions of the Micro CT instrument (1172 micro-CT system; Bruker Co.) were adjusted as follows: X-ray
source voltage: 50 KV, filter disc: 0.5 mm aluminum, exposure time: 970 ms and scanning rotation step: 0.3°. The alveolar bone density, the percentage of bone volume, the number of bone trabeculae, the thickness of bone trabeculae, trabecular space and the structural model index were measured in each group of mice. The peria of 3 mice in each group were taken, respectively, and embedded with paraffin for H&E staining subsequently.

**Detection of the expression level of mRNA via RT-qPCR.** The peria of each group of mice were collected and placed in a centrifuge tube. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added at a volume ratio of 100 mg/1 ml. After incubation on ice for 5 min, the tissues were crushed with an ultrasonic cell disruptor, followed by centrifugation at 10,000 x g and 4°C for 10 min. After the supernatant was taken out, the tissues were added and mixed well with 200 µl chloroform, and incubated on ice for 10 min. The tissues were then centrifuged at 12,000 x g and 4°C for 10 min, after which the supernatant was removed. Subsequently, the tissues were added with pre-cooling isopropanol at equal volume and mixed well through inversion, followed by centrifugation at 12,000 x g and 4°C for 10 min. Afterwards, the supernatant was discarded (it should be guaranteed that the bottom sediment would not be poured out), and the tissues were added with 75% 1 ml freshly prepared ethanol for washing, followed by centrifugation at 12,000 x g and 4°C for 10 min. After the supernatant was discarded, the tissues were let dry naturally at room temperature and added with 50 µl diethyl pyrocarbonate (DEPC)-treated water to dissolve RNAs, followed by preservation at -20°C for standby application. Reverse transcription (RT) was performed using PrimeScript RT reagent kit (Takara Bio, Inc.), and the reaction system was configured according to the instructions. Primers, Taq polymerase, Taq buffer, deoxyribonucleoside triphosphate (dNTP) mixture and double distilled H2O (ddH2O) were added for reverse transcription, after which the RT-qPCR reaction system was configured, and then placed on a fluorescence RT-qPCR machine for PCR amplification. Amplification conditions were at 95°C for 2 min, 95°C for 15 sec and 58°C for 60 sec for a total of 30 cycles. Primers were produced by Sangon Biotech Co., Ltd., and the sequences are shown in Table I. The results were calculated using the 2-ΔΔCq method (10). The expression levels of the corresponding mRNAs are presented as TLR4/glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Foxp3/GAPDH, T-bet/GAPDH and GATA3/GAPDH, respectively.

**Statistical analysis.** Data in the present study are expressed as the mean ± standard deviation (SD) and processed using Statistical Product and Service Solutions (SPSS 19.0) software (IBM Corp.). One-way analysis of variance (ANOVA) was used for the comparison between groups. The homogeneity test of variance revealed that if the variance was homogenous, the Bonferroni method was applied. P<0.05 represented a statistically significant difference.

**Results**

**Changes in hs-CRP in peripheral blood of mice in each group.** The level of hs-CRP in peripheral blood of each group of mice was measured using the ELISA kit at 10 days after modeling. Compared with that of mice in the N group, the level of hs-CRP in peripheral blood of mice in the P group was notably increased (P<0.01), but after administration of TAK-242, the level in the PT group significantly declined (P<0.01) (Fig. 1). Changes in inflammatory cytokines in each group of mice. The levels of inflammatory cytokines in peripheral blood of mice in each group were measured using the ELISA kit at 10 days after modeling. Compared with those in the N group, the levels of MCP-1, IL-6 and IL-1β in peripheral blood of mice in the P group were obviously increased, but the levels were significantly increased in the PT group after administration of TAK-242 (P<0.01) (Fig. 2).

**Detection results of alveolar bones in each group of mice.** Changes in alveolar bones can markedly reflect the severity of periodontitis, and Micro CT instrument was used to detect changes in alveolar bones. At 10 days after the model was established, the mice in each group were sacrificed, and changes in alveolar bones were detected. Compared with those

| Genes  | Sequence                                                                 |
|--------|--------------------------------------------------------------------------|
| TLR4   | F: 5'-ATGGCATGGCTTACACCAC-3' R: 5'-GAGGCCAATTTTGCTCCACA-3'              |
| Foxp3  | F: 5'-CTGCCCTACTGTCATGGTG-3' R: 5'-CCGTGCTTTAAGCAACTC-3'                |
| T-bet  | F: 5'-CTGGAGGAGTGCTGATAGTG-3' R: 5'-TCTGGCTCCTCCGTGTTCA-3'              |
| GATA3  | F: 5'-TCACAAAAATGACCGACAGAC-3' R: 5'-GGTGTGTCTGACAGTTCCGAC-3'           |
| GAPDH  | F: 5'-TGTTGGGATCAATGGATTTGG-3' R: 5'-ACACCATGTATTCCGGGTCAAT-3'           |

F, forward; R, reverse.
in the N group, the alveolar bone density, the percentage of bone volume, the number of bone trabeculae and the thickness of bone trabeculae in the P group were notably reduced compared with those in the N group (P<0.01), while the trabecular space and the structural model index were significantly increased (P<0.01). After administration of TAK-242, the alveolar bone density, the percentage of bone volume and the number of bone trabeculae in mice of the PT group significantly increased (P<0.01), whereas the trabecular space and the structural model index were significantly decreased (P<0.01) (Fig. 3).

Expression of TLR4 pathway-related cytokines. The expression levels of TLR4, Foxp3, T-bet and GATA3 mRNAs in peria of each group of mice were detected via RT-qPCR. The expression levels of TLR4 and T-bet/GATA3 mRNA in peria of mice in the P group were significantly higher than those in the N group (P<0.01), while the expression level of Foxp3 mRNA was notably lower than that in the N group (P<0.01). After administration of TAK-242, the expression levels of TLR4 and T-bet/GATA3 mRNAs in peria of mice in the PT group were obviously decreased (P<0.01), whereas the expression level of Foxp3 mRNA significantly increased (P<0.01) (Fig. 4).

**Discussion**

Dental plaques in peria interact with the body during periodontitis, which results in enhanced humoral and cellular immunity in the body and destroys peria around the gums, thus increasing alveolar bone resorption (11). The mechanism of alveolar bone resorption is that osteoclasts and precursor cells further differentiate, and osteoclasts become mature, so that the organic and inorganic components in the bone tissues are degraded, resulting in bone resorption, which is an important pathological feature of periodontitis (12). Research evidence has identified that inflammation upregulates the levels of TLR4 in various cells and tissues and enhances cellular immunity, whereas inhibiting or knocking out TLR4 receptors in mice can effectively reduce the inflammatory response and cellular immunity in mice (13). TAK-242, as a cyclohexene derivative,
is a specific antagonist of TLR4, with extremely low affinity with other TLRs. According to research evidence, TAK-242 can efficaciously suppress the intracellular signal transduction pathway of TLR4, thus further impeding the production of multiple inflammatory mediators and cytokines (14).

It has been found from research data that the progression of a variety of in vivo inflammatory responses can be regulated by TLR4. The increased expression level of TLR4 gene can rapidly activate mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) through the myeloid differentiation primary response 88 (MYD88) pathway, which increases the release of pro-inflammatory cytokines (15). Abed El-Gaphar et al (16) found that the expression level of TLR4 gene in synovial macrophages in patients with rheumatoid arthritis is evidently increased. The increased expression of TLR4 in vivo results in the increased binding of TLR4 to its specific ligands, thus inducing cellular and humoral immunity. The transcription factors, T-bet and GATA3, can specifically suppress the production of the cytokine IL-4 by affecting the production of the cytokine interferon-gamma (IFN-γ) and mediate cellular immune responses by stimulating the differentiation of Th1 cells and inhibiting the differentiation of Th2 cells (17). Foxp3 is a specific marker protein of CD4+CD25+ T cells, whose expression level is closely associated with the immune function of cells. A study by Takada et al (18) manifested that the reduced expression level of Foxp3 gives rise to the loss of regulatory T cells in the body, and ordinary T cells lose cells and tissues, thus causing a train of immune responses. It was found in this study that the increased expression level of TLR4 gene in peria resulted in a significant increase in the expression of T-bet but an obvious decrease in the expression level of Foxp3 mRNA, while inhibiting TLR4 gene significantly reduced the expression level T-bet in peria but increased the expression level of Foxp3. The above results suggest that suppressing the expression of TLR4 gene can influence the expression of T-bet, GATA3 and Foxp3 in periodontitis tissues, so as to regulate cellular and humoral immunity in vivo and alleviate periodontitis.

Kondo et al (19) took lymphocytes from peripheral blood of patients with periodontitis and established the immunized nude mouse model of periodontitis under the action of P. gingivalis. They found that obvious alveolar bone resorption appears in nude mice of the P group, while the inhibition of cellular immunity in vivo by administration of baicalin can markedly impede bone resorption and improve the symptoms of periodontitis. A study of Tani et al (20) revealed that obvious alveolar bone resorption occurs in T cell immunized nude mice under the action of endotoxins, and a large number of inflammatory cells are infiltrated into peria. Research evidence has demonstrated that cellular and humoral immunity exerts a crucial effect in the pathogenesis of periodontitis, and can markedly affect the alveolar bone absorption (21). This study illustrated that the expression levels of inflammatory cytokines in peripheral blood of mice with periodontitis and the alveolar bone resorption in peria were obviously increased. Inhibiting TLR4 gene by administration of TAK-242 could significantly improve the alveolar bone resorption, thus further improving the inflammatory response and symptoms of periodontitis in mice. The results of the present study are consistent with those expected, and TLR4 plays key roles in the pathogenesis and progression of periodontitis.

In conclusion, TLR4 gene participates in the pathogenesis of periodontitis, and it is highly expressed with periodontitis, which influences the expression of T-bet, GATA3 and Foxp3, increases the inflammatory responses in vivo and inhibits TLR4 in vivo, thus remarkably improving periodontitis symptoms in mice with periodontitis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

WQ wrote the manuscript. WQ and XY conceived and designed the study, and were responsible for model establishment. NY, SL and QH helped with the sample collection and ELISA. JH and BW helped with PCR and statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Stomatological Hospital, Southern Medical University (Guangzhou, China). The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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