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ORIGINAL ARTICLE

In vivo expression of Toll-like receptor 2, Toll-like receptor 4, CSF2 and LY64 in Chinese chronic periodontitis patients

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OBJECTIVE: Toll-like receptors (TLRs) are the essential components in the innate and adaptive immune systems. Colony stimulating factor 2 (CSF2) is a cytokine that may prevent endotoxin tolerance, and LY64 has the ability to interfere with the recognition of bacteria via TLR4. The aim of this study was to explore the *in vivo* expressions of TLR2, TLR4, CSF2 and LY64 in Chinese chronic periodontitis patients.

METHODS: Gingival biopsies were collected from 24 chronic periodontitis patients and 19 healthy controls. The gene expression profiles of TLR2, TLR4, CSF2 and LY64 were investigated by real-time polymerase chain reaction, and the protein expressions of TLR2 and TLR4 were detected by immunohistochemistry. In addition, the levels of CSF2 in gingival crevicular fluid (GCF) were determined by ELISA.

RESULTS: The higher mRNA expressions of TLR2, TLR4 and CSF2, and the lower mRNA expression of LY64 were detected in chronic periodontitis patients. And the increased protein expressions of TLR2 and TLR4 were confirmed by immunohistochemistry. In addition, the increase of total amount of CSF2 in GCF was observed in chronic periodontitis patients.

CONCLUSIONS: Our results suggest that TLR2 and TLR4 may play a role in periodontal pathogenesis. In addition, CSF2 and LY64 may contribute to the regulation of inflammatory response and maintaining periodontal homeostasis.

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Keywords: Toll-like receptor 2; Toll-like receptor 4; colony-stimulating factor 2; LY64; chronic periodontitis

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Introduction

Periodontitis is one of the most common oral diseases in humans, and is characterized by gingival inflammation and alveolar bone resorption. The most common form of this disease is chronic periodontitis, which is frequently observed in adult populations. According to the recent data, 39.53% Chinese individuals aged between 35 and 74 years have suffered from periodontitis (Shen *et al*, 2007). It is well known that bacteria-induced inflammatory responses are involved in the damage of periodontal tissues, and the severity of chronic periodontitis is somewhat dependent on a dynamic equilibrium of interactions between the microbial challenge and the host immuno-inflammatory responses (Page *et al*, 1997). However, the exact mechanisms of the molecular recognition and signaling transduction of host immuno-inflammatory responses in chronic periodontitis still remain obscure.

The recently described Toll-like receptors (TLRs) recognize a set of structurally conserved pathogen-associated molecular patterns (PAMPs) and play an essential role in detecting microorganisms and initiating inflammatory responses (Medzhitov *et al*, 1997). To date, at least 10 different TLRs in humans have been described. Among them, TLR2 and TLR4 are the most extensively studied members of the TLRs family. These receptors recognize a variety of microbial components, such as peptidoglycan and lipoteichoic acid from gram-positive bacteria, and lipopolysaccharide (LPS) from gram-negative bacteria. Importantly, cell-wall components of periodontopathic bacteria, including LPS and fimbriae from *Porphyromonas gingivalis*, and LPS from *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*, have been demonstrated to be recognized by TLR2 and TLR4, respectively (Asai *et al*, 2001; Hirschfeld *et al*, 2001; Yoshimura *et al*, 2002; Mochizuki *et al*, 2004; Suzuki *et al*, 2007), suggesting that TLR2 and TLR4 might play a potential role in the progress of chronic periodontitis. However, studies concerning the *in vivo* expression of TLR2 and TLR4 in chronic periodontitis are limited, and the results of

those studies are inconsistent (Muthukuru *et al*, 2005; Ren *et al*, 2005).

In our previous studies, the gene expression profile in human periodontal ligament cells (HPDLCs) in response to TLR4 ligand, LPS treatment was analysed by microarray analysis. It was observed that LPS treatment could induce TLR4 activation, increase the gene expression of colony stimulating factor 2 (CSF2), and decrease the gene expression of LY64 *in vitro* (Sun *et al*, 2008). These results suggested the possible involvement of CSF2 and LY64 in TLR4-mediated periodontal inflammation responses.

Colony stimulating factor 2 (CSF2), which is also called granulocyte-macrophage colony-stimulating factor (GM-CSF), is produced by a variety of cell types, including T cells, macrophages, endothelial cells and fibroblasts, upon receiving immune stimuli (Shi *et al*, 2006). It is a kind of immunostimulatory cytokine, and could enhance the functions of neutrophils, monocytes and lymphocytes in host defense. Previous researches indicated that a slight increase of TLR4 mRNA expression and prevention of endotoxin tolerance could be observed in monocyte after CSF2 pretreatment (Adib-Conquy and Cavaillon, 2002; Lendemans *et al*, 2006). Chronic periodontitis is a kind of long-standing inflammation, and periodontal tissues are afflicted by a persistent irritation from pathogenic microorganisms. There is a trend to develop endotoxin tolerance to suppress inflammation (Muthukuru *et al*, 2005). For these reasons, we presume that CSF2 might be produced as an important mediator in a paracrine fashion contributing to the regulation of inflammatory response in periodontal tissues.

LY64, also known as RP105, was originally cloned as a B-cell-specific molecule to drive B-cell proliferation (Miura *et al*, 1998). As a specific homolog of TLR4, LY64 could interfere with the recognition of bacteria and the activation of innate immunity via TLR4 (Divanovic *et al*, 2005). However, the involvement of LY64 in chronic periodontitis has not yet been established.

It is hypothesized that 1) TLR2 and TLR4 might be activated in periodontal tissues in response to the microbial challenge; and 2) CSF2 and LY64 might also be involved in periodontal inflammation responses. Therefore, in this study, we further investigated the *in vivo* expression of TLR2, TLR4, CSF2, and LY64 in Chinese chronic periodontitis patients and clinical healthy controls.

Materials and methods

Subjects

The study population consisted of 43 patients (24 chronic periodontitis patients and 19 healthy controls) who had been referred to Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China, during the period between January 2007 and February 2008.

Twenty-four non-smoking chronic periodontitis patients (aged between 29 and 67 years, mean 49.88 years; 12 male and 12 female patients) took part in this study. Inclusion criteria were: 1) presentation of untreated chronic periodontitis, with probing depth (PD) ≥5.0 mm, clinical attachment loss (CAL) ≥3.0 mm, and radiographic evidence of alveolar bone loss exceeding 1/3 of the root on at least two teeth per quadrant, excluding third molars; 2) healthy systemic condition; 3) no prior periodontal treatment; 4) no use of any immunosuppressive agents; 5) no antibiotics or anti-inflammatory drugs taken within 6 months. After a course of non-surgical periodontal therapy, all the subjects exhibited unresolved chronic periodontitis with remaining PD ≥5.0 mm and bleeding on probing (BOP) (+) in at least one quadrant of their dentition, and would scheduled periodontal surgery. Nineteen systemically and periodontally healthy non-smoking subjects (aged between 16 and 29 years, mean 21.74 years; 10 male and nine female patients) who required tooth extraction for orthodontic treatment were selected as healthy controls. Inclusion criteria were: 1) no site with PD >3 mm or CAL >1 mm, and no radiographic evidence of alveolar bone loss in the whole dentition; 2) a full-mouth score of BOP <15% of sites; 3) gingival index (GI) of the tooth that would be extracted ≤1; 4) healthy systemic condition; 5) no use of any immunosuppressive agents; 6) no antibiotics or anti-inflammatory drugs taken during the previous 6 months.

This study was approved by the Ethical Committee of Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University. The purposes and procedures of the study were explained and informed consents were obtained from all recruits.

Clinical periodontal measurements

Prior to sampling, clinical periodontal characters, including plaque index (PLI), GI, PD, CAL and BOP, were recorded as shown in Table 1. All measurements were performed by a calibrated examiner.

Collection of samples

Gingival samples from chronic periodontitis patients were collected during periodontal surgery, and gingival tissues from healthy controls were obtained at the time of tooth extraction. Each sample was divided into two parts. One part was immediately transferred to 1 ml Sample Protector (Takara, China) to avoid RNA degradation, then stored at −40°C for RNA extraction. The other part was transferred to 10% buffered formalin, processed and blocked in paraffin wax within 2 days for immunohistochemistry.

For collecting the gingival crevicular fluid (GCF) from chronic periodontitis patients, we first selected the tooth with remaining PD ≥5.0 mm and BOP (+) by probing six sites per tooth. The teeth with at least one site bleeding on probing were considered as BOP (+). Then, the GCF sample was collected from the sites of the same tooth without bleeding on probing by using paper strips (Periopaper, Amityville, NY, USA) before periodontal surgery. Cotton rolls and saliva ejector were used to isolate the tooth from saliva contamination. The paper strips were consecutively inserted into the crevice

Table 1 Clinical characteristics of chronic periodontitis patients and healthy controls

Parameters	*Chronic periodontitis patients*	*Healthy controls*
PLI	1.50 ± 0.46	0.34 ± 0.20*
GI	1.53 ± 0.42	0.32 ± 0.21*
PD (mm)	5.74 ± 0.53	1.62 ± 0.36*
CAL (mm)	4.87 ± 1.46	0*
BOP (%)	100	0*

Clinical periodontal measurements, including plaque index (PLI), gingival index (GI), probing depth (PD), clinical attachment loss (CAL) and bleeding on probing (BOP), were recorded at the sampling teeth. The data points for PLI, GI, PD and CAL represented the mean ± standard deviation (s.d.).
*Significantly lower than chronic periodontitis patients, $P < 0.05$.

until mild resistance was felt, then left in place for 30 s. Strips contaminated with blood were discarded. The absorbed GCF volume was estimated by a calibrated instrument (Periotron 8000, Oraflow Inc., Plainview, NY, USA). Four strips of each patient were immediately placed in an eppendorf tube containing 500 μl phosphate-buffered saline (PBS) and transported to the laboratory. To retrieve the sample from the paper strip, the GCF was eluted by centrifugal filtration at 15,000 *g* for 5 min, then the samples were stored at −70°C for subsequent assays (Balducci *et al*, 2007). GCF from the controls was collected in the same way before tooth extraction.

RNA extraction and real-time PCR

Total RNA was prepared from gingival tissues, which were obtained from chronic periodontitis patients and healthy controls. cDNA was synthesized using a RT Reagent kit (Takara, China). Levels of β-actin mRNA served as internal controls. Primer sequences of each gene (TLR2, TLR4, CSF2, LY64 and β-actin) were shown in Table 2.

Real-time PCR analysis was performed in duplicates in an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, USA) with SYBR Premix EX Taq (Takara, China). The reaction product was quantified by the standard curve method. Briefly, standard curves were constructed by using serial diluted respective PCR amplification products with predetermined concentrations on the basis of the linear relationship between the Ct and the logarithm of the starting gene amount. The relative gene expression level for each sample was expressed as the ratios between the amount of the selected gene and β-actin for the same sample.

Immunohistochemistry

Immunohistochemical staining was performed to demonstrate the protein expression of TLR2 and TLR4 as previously described (Yu *et al*, 2003). Deparaffinized sections were hydrated through graded ethanol. The endogenous peroxidase signal was quenched by incubation in 1.5% H_2O_2 for 15 min. After 20 min of blocking with normal goat serum, the sections were incubated with the primary antibodies against TLR2 (eBioscience, USA) or TLR4 (Abcam, UK) at a dilution of 1:50 at 4°C overnight. The sections then were washed three times with PBS and incubated with biotinylated IgG antibodies at 37°C for 20 min. Finally, the reaction was incubated with the chromogen 3–3-diaminobenzidine (Dako, Denmark) and counterstained with hematoxylin. Negative controls were performed by incubation of the sections with PBS instead of the primary antibodies.

Five views per section were taken under microscope. KS400 image analysis system (Carl Zeiss, Germany) was used to quantify the expression of TLR2 and TLR4. Briefly, image noise was reduced by a median filter first. A threshold level was then established for the color tone area of the TLR2- or TLR4-positive regions. All the pixels in the region thus selected were represented in white, and all other pixels were shown in black. In this way, a binary image was created that served as a measurement mask for the quantitative image analysis. The binary image could be improved by further steps in the procedure, for example, by deleting disturbing pixels. Finally, percentage of positive area was measured, and was used to show the levels of TLR2 or TLR4.

Cytokine detection

Levels of CSF2 in GCF were determined by using an appropriate commercial ELISA kit (R&D, Minneapolis, MN, USA), and 100 μl of eluted sample was assayed according to the manufacturer's instructions. The amount of CSF2 was determined using a standard curve (7.8–1000 pg) obtained with a standard recombinant cytokine (R&D). Cytokine values lower than the detection limit (< 7.8 pg) were considered undetectable, and taken as 7.8 pg in statistical analysis. Cytokine concentration (pg ml^{-1}) was calculated from the volume of GCF estimated from the Periotron 8000 reading, according to the following formula: cytokine concentration (pg ml^{-1}) = total cytokine (pg)/volume (ml) (Gamonal *et al*, 2003).

Statistical analysis

Statistical analysis of the clinical parameters, the real-time PCR data, and the total amount and concentration

Table 2 Primer sequences of target genes for real-time PCR

Target gene	*Primer sequence forward/reverse*	*Size of amplified products (bp)*
TLR2	GCCAAAGTCTTGATTGATTGG/TTGAAGTTCTCCAGCTCCTG	368
TLR4	AGGATGAGGACTGGGTAAGGA/CTGGATGAAGTGCTGGGACA	182
CSF2	CCGGAAACTCCTGTGCAACCC/TCCATTCTTCTGCCATGCCTGTAT	268
LY64	CATCTCATCCACCACATTTCA/AGCAAGTTCAGACCCTTCATC	105
β-actin	CCTGTACGCCAACACAGTGC/ATACTCCTGCTTGCTGATCC	211

of CSF2 in ELISA was performed using Student's *t*-test. The differences in detection frequency of CSF2 between chronic periodontitis patients and healthy controls were analysed using the chi-squared test. The image analysis data were analysed using two-tail Mann–Whitney test. The level of significance was set at $P < 0.05$.

Results

Clinical analysis

Clinical periodontal measurements were recorded at the sampling teeth. The clinical characteristics of the study population are outlined in Table 1. The healthy control group exhibited significantly lower values in all clinical periodontal measurements ($P < 0.05$).

Expression of TLR2 and TLR4

We first performed real-time PCR to determine the mRNA expression levels of TLR2 and TLR4 as well as those of CSF2 and LY64 in gingival samples. Although the mRNA expressions of TLR2 and TLR4 were detectable in gingival tissues irrespective of their disease entities, they were significantly higher in chronic periodontitis patients than in healthy controls ($P < 0.05$) (Figure 1a,b).

Then, using immunohistochemical staining, we found that the protein expression of TLR2 and TLR4 in gingival tissues was consistent with the result of real-time PCR (Figure 2a–d). In contrast, gingival tissues were not stained with PBS as a substitute for anti-TLR2 and anti-TLR4 antibodies in negative controls (Figure 2e). Furthermore, we found that TLR2 and TLR4 expressed in both pocket epithelia and underling connective tissues.

To quantify the protein expression levels of TLR2 and TLR4, image analysis was done and the results were statistically summarized. Our results indicated that mean percentage of positive area for TLR2 in chronic periodontitis patients and healthy controls was 60.46% and 6.17% respectively, while the value for TLR4 in these two groups was 49.45% and 2.93% respectively. Mean percentages of positive area for TLR2 and TLR4 in chronic periodontitis patients were all significantly higher than the ones in healthy controls ($P < 0.05$) (Figure 3).

Expression of CSF2 and LY64

Our previous *in vitro* research showed the increased expression of CSF2 and the decreased expression of LY64 in HPDLCs by LPS treatment (Sun *et al*, 2008). To further explore the involvement of CSF2 and LY64 in chronic periodontitis, in this study, we performed real-time PCR to confirm the gene expression levels of CSF2 and LY64 *in vivo* (Figure 1c,d). The increased CSF2 mRNA expression and decreased LY64 mRNA expression were found in chronic periodontitis patients, in comparison with those in healthy controls ($P < 0.05$). These data were consistent with our previous *in vitro* observations.

Next, the levels of CSF2 protein in GCF were measured by ELISA. Most sites analysed in chronic periodontitis patients had detectable levels of CSF2 (70.83%, 17/24), whereas only 36.84% (seven out of 19) had detectable CSF2 in healthy controls. This difference between two groups was statistically significant ($P < 0.05$). The total amount and concentration of CSF2 are shown in Table 3. The total amount of CSF2 in GCF from chronic periodontitis patients was higher than that from healthy controls ($P < 0.05$); however, there was no significant difference in CSF2 concentration between the two groups ($P > 0.05$).

Discussion

Our study evaluated the *in vivo* expression of TLR2 and TLR4 in a Chinese population with chronic periodontitis, indicating the role of TLR2 and TLR4 in triggering periodontal infection. In addition, we investigated the *in vivo* expression of LY64 and CSF2 in the same population, and their potential association with the inflammation responses in chronic periodontitis for the first time.

In this study, the expressions of TLR2 and TLR4 were detected in both gingival epithelia and underling connective tissues from all gingival samples, and the increased expressions of TLR2 and TLR4 were observed in chronic periodontitis patients. In the early researches, Ren also found the weaker expression density of TLR2 in healthy gingival tissues compared with that in periodontal pocket tissues. Interestingly, in contrast to our results, he reported that the expression of TLR2 was

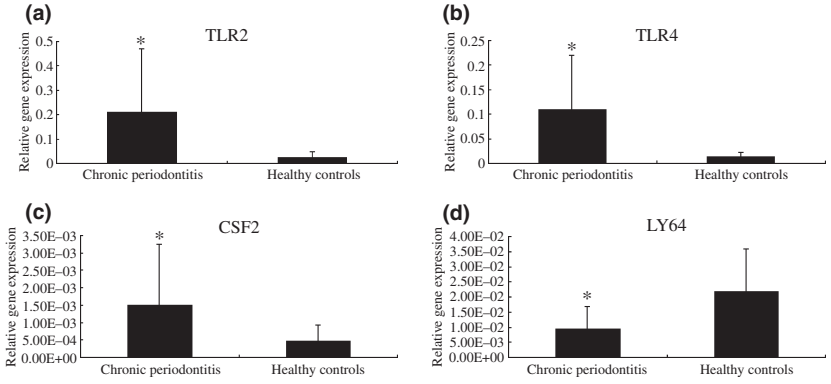

Figure 1 Expression of TLR2, TLR4, CSF2 and LY64 mRNA in chronic periodontitis patients and healthy controls. Real-time PCR was used to quantify TLR2 (**a**), TLR4 (**b**), CSF2 (**c**), and LY64 (**d**) mRNA expression levels. *$P < 0.05$ *vs* healthy controls

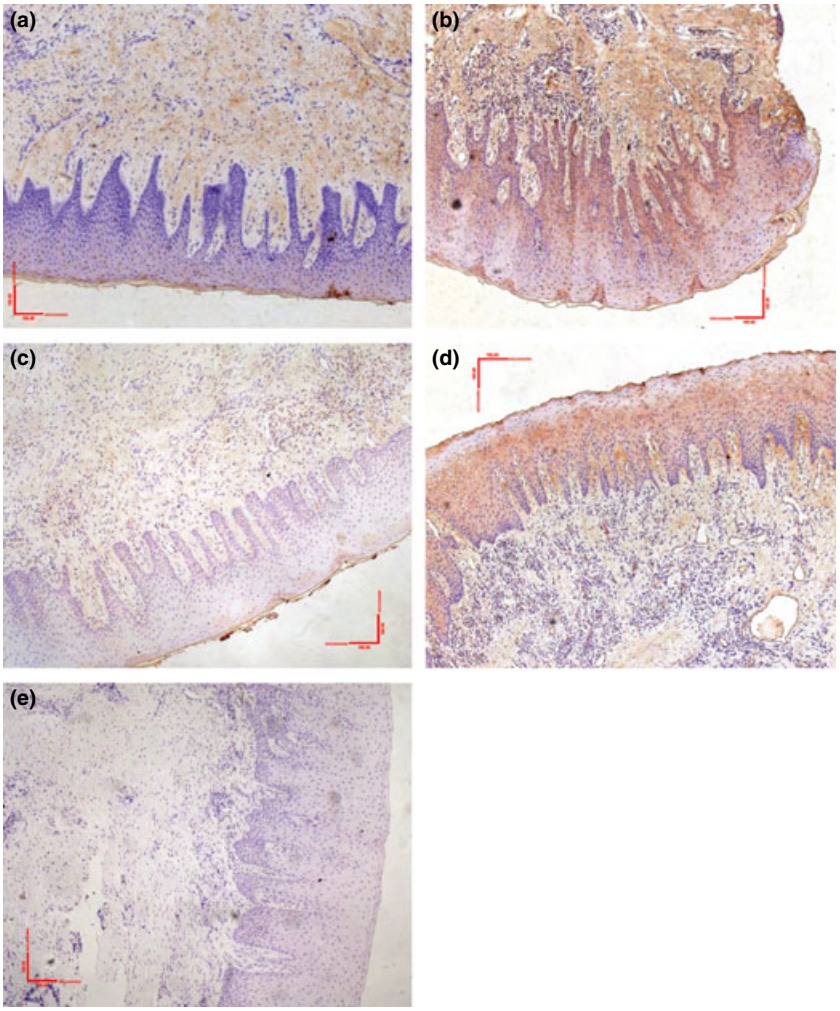

Figure 2 Expression of TLR2 and TLR4 protein in chronic periodontitis patients and healthy controls. (**a**) expression of TLR2 in healthy controls, (**b**) expression of TLR2 in chronic priodontitis patients, (**c**) expression of TLR4 in healthy controls, (**d**) expression of TLR4 in chronic priodontitis patients, and (**e**) negative controls. The sections were counterstained blue with hematoxylin (a–e). Scale bars = 100 μm

detected in pocket epithelia in both chronic periodontitis patients and healthy controls, whereas TLR4 was only predominantly detected in connective tissues from periodontitis patients (Ren *et al*, 2005). In another related study, the prominent mRNA and protein expressions of TLR2 and TLR4 were detected in gingival epithelia cell lines, primary gingival epithelial cells, and gingival tissue samples from chronic periodontitis patients and healthy controls. In addition, in inflamed oral epithelia, cell-surface localizations of TLR2 and TLR4 were more clearly observed than in healthy tissues (Sugawara *et al*, 2006). Gingival epithelia are the outmost covering and exposed to a high density and diversity of gram-positive and gram-negative bacteria. As a physically protective organ and a part of the innate branch of immune defense, epithelium seems to allow the host immunity to respond to the invading pathogens by recognition via TLR2 and TLR4, as well as other pattern recognition receptors (PRRs). Therefore, it has been speculated that gingival epithelia actively participate in inflammation responses associated with chronic periodontitis. A possible explanation for the discrepancies between reported studies might be the variation in the samples attributable to the health condition of the participants. In response to the microbial challenge, activation of the epithelial cells might also vary from patient to patient. Moreover, different antibodies to TLR2 and TLR4 used in different studies might also explain the contradictory results between the studies.

Subjects in our study, both chronic periodontitis patients and healthy controls, were all non-smokers. Tobacco smoking is associated with an increased incidence of bacterial infections. It could suppress the ability of the host to develop the immune response to infection, as evidenced by suppression of NK cytotoxicity, inhibition of B-cell proliferation and antibody production, and impaired antigen- and mitogen-mediated T-cell responses (Chen *et al*, 2007). There is a lot of evidence concerning higher risk of periodontal disease in smokers than in non-smokers (Tomar and Asma, 2000; Johnson and Slach, 2001; Bergström, 2004). However, the effects of smoking on the expression of TLR2, TLR4, CSF2 and LY64 remain unclear and to some extent, remain controversial (Sato *et al*, 1999; Hellermann *et al*, 2002; Droemann *et al*, 2005; Pace *et al*, 2008). Therefore, to avoid the possible influence of smoking on the expression of the target molecules, we excluded the smoking subjects in this study.

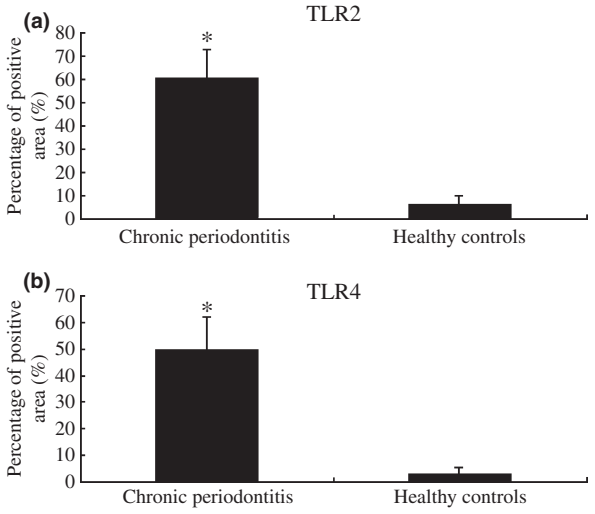

Figure 3 Image analysis for expression of TLR2 and TLR4 in chronic periodontitis patients and healthy controls. KS400 image analysis system was used to quantify the expression of TLR2 (**a**) and TLR4 (**b**). $^*P < 0.05$ *vs* healthy controls

Table 3 Levels of CSF2 in GCF from chronic periodontitis patients and healthy controls

	Chronic periodontitis patients	*Healthy controls*
Subjects with detectable levels	70.83% (17/24)*	36.84% (7/19)
Total amount (pg)	58.52 ± 20.03*	33.61 ± 23.37
GCF volume (μl)	8.37 ± 3.17*	3.2 ± 1.79
Concentration (pg ml^{-1})	7.96 ± 1.76	8.41 ± 2.54

Levels of CSF2 in GCF were measured by ELISA. The values represented the mean ± standard deviation (s.d.).
**vs* healthy.controls, $P < 0.05$.

In our experiment, healthy controls were much younger than chronic periodontitis patients (21.74 years on average for healthy controls compared to 49.88 years on average for chronic periodontitis patients). From ethical considerations, it was not possible to conceive collecting gingival tissues from systemically and periodontally healthy subjects who do not need any oral operations, except from young healthy subjects at the time of tooth extraction for orthodontic treatment. In Murciano's researches, no significant differences between aged and young donors were observed on cell surface TLR2, TLR4 and TLR6 expression on lymphocytes, monocytes and granulocytes (Murciano *et al*, 2007). Similar finding was also reported in paper concerning TLR4 expression on macrophages from older and younger mice (Boehmer *et al*, 2004). These made it reasonable to observe and compare the expression levels of TLRs between inflammatory and healthy periodontal tissues from subjects with different ages, as in the present and some previous studies (Jin *et al*, 2004; Ren *et al*, 2005). On the other hand, dysfunction of T cells and macrophages by aging might lead to the decrease of CSF2 production (Yoneda *et al*, 1995; Saitoh *et al*, 1999). In our experiment, an increase of CSF2 mRNA and protein expression was observed in chronic periodontitis patients who were significantly older than healthy controls, suggesting that the increased expression of CSF2 might result from inflammation, but not aging. LY64 is a molecule discovered recently, and the effect of aging on human LY64 expression remains unknown. Further studies should be conducted to ascertain whether inflammation was the only reason for the decrease of LY64 expression in chronic periodontitis patients.

In a previous study, we demonstrated that LPS treatment could up-regulate the mRNA expression of TLR4 in HPDLCs and lead to the secretion of proinflammatory cytokine, IL-6. In addition, suppression of TLR4 expression could down-regulate the secretion of IL-6 (Sun *et al*, 2008). And in this study, the significantly higher expression of TLR2 and TLR4 was observed in chronic periodontitis patients compared with that in healthy controls. It is now known that the immune response applies TLRs as tools to trigger an inflammatory response to microbial invasion (Takeda and Akira, 2005). Gingiva is consistently exposed to a variety of bacteria, and TLRs sensing and signaling in periodontal tissue might lead to the secretion of proinflammatory cytokines from bacteria activated gingival epithelial cells, fibroblasts and various kinds of immune cells. Cytokine production could promote periodontal inflammation, activate osteoclasts, cause bone resorption and eventually contribute to the initiation and development of periodontitis (Wang *et al*, 2000; Asai *et al*, 2001; Yoshimura *et al*, 2002; Mahanonda and Pichyangkul, 2007). Our findings from *in vitro* and *in vivo* observations, further suggest that up-regulated expression of TLR2 and TLR4 in response to the bacteria and their products, might be involved in the triggering of inflammation in chronic periodontitis. Although the host responses to foreign pathogens are intended to eliminate the microbial challenge, they may often cause further tissue damage (Madianos *et al*, 2005). Therefore, appropriate expression levels of TLR2 and TLR4 are critical to maintain homeostasis in periodontal tissues during the course of controlling periodontal infection.

Based on these understandings, we attempted to further explore the participation and expression patterns of various accessory molecules in association with the TLR2 and TLR4 regulations in chronic periodontitis. We found that the mRNA expression levels of CSF2 in gingival tissues and the total amount of CSF2 in GCF were up-regulated in chronic periodontitis patients compared with those in healthy controls. There was no significant increase of CSF2 concentration in periodontitis sites, which might be related to the increase of total volume of GCF. GCF is an inflammatory exudate. As mentioned previously, minor mechanical irritation, represented by periodontal probing and intrasulcular placement of paper strips, could increase the production of fluid (Bulkacz and Carranza, 2002). But in our experiment, the effects of mechanical stimuli on the amount of GCF did exist in both chronic periodontitis patients and healthy controls. On the other hand, the

amount of GCF is also greater when inflammation is present and is sometimes proportional to the severity of inflammation (Garnick *et al*, 1979; Shapiro *et al*, 1979). Therefore, this might be the reason for the insignificant increase of CSF2 concentration in periodontitis sites. Previous studies indicated that CSF2 treatment could up-regulate TLR2 and CD14 mRNA levels in neutrophils, and enhance the IL-8 secretion and superoxide-priming responses of neutrophils to stimulation with TLR2 ligands (Kurt-Jones *et al*, 2002). CSF2 also might prevent endotoxin tolerance induced by low but not by high doses of LPS. After a second LPS stimulation, CSF2 could up-regulate TLR4 mRNA expression, inhibit IL-1 receptor-associated kinase (IRAK) degradation and promote its association with myeloid differentiation 88 (MyD88), which in turn leads to nuclear factor-$_k$B (NF-$_k$B) activation and TNF-α production (Adib-Conquy and Cavaillon, 2002; Lendemans *et al*, 2006). Therefore, the increase of CSF2 in chronic periodontitis patients might contribute to preventing endotoxin tolerance induced by persistent irritation of bacteria and their products, and maintaining homeostasis in periodontal tissues.

LY64 is the other molecule associated with expression of TLR4. It has a conserved extracellular leucine-rich repeat domain and a TLR-like pattern of juxtamembrane cysteines. However, unlike TLRs, LY64 lacks a Toll-IL-1 receptor domain, containing a mere 6–11 intracytoplasmic amino acid. It could interact directly with TLR4, and inhibit the LPS-binding activity of TLR4. The down-regulated expression of LY64 in inflamed gingival tissues in this study might be the self-adjustment of the host to regulate TLR4 expression and to maintain the appropriate inflammation in periodontal tissues.

It is clear that controlling inflammation and maintaining homeostasis are required for clinical therapy of periodontitis. The up-regulated expression of CSF2 and the down-regulated expression of LY64 might be effective in facilitating inflammation and eliminating periodontopathic bacteria. But the inappropriate expression of these molecules might lead to persistent inflammation and tissue damage at last.

In summary, this study showed the *in vivo* expressions of TLR2, TLR4, CSF2 and LY64 in a Chinese population with chronic periodontitis. The up-regulated expressions of TLR2 and TLR4 in inflamed gingival tissues suggest that these receptors might play a role in periodontal pathogenesis. And the altered expression profiles of CSF2 and LY64 in chronic periodontitits patients imply that these molecules may contribute to maintaining periodontal homeostasis. Further investigations of CSF2 and LY64 are necessary for a better understanding of their molecular mechanisms in periodontal pathogenesis.

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Author contributions

Ying Sun designed the study, performed part of the experiment, analyzed part of the data and drafted the paper. Qiu-Man Guo performed part of the experiment and analyzed part of the data. Da-Li Liu critically revised the manuscript. Ming-Zhu Zhang provided her expertise and knowledge about real-time PCR and immunohistochemistry technology. Rong Shu provided expertise in designing the study and critically revised the manuscript.

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