Effects on the expression of pro-inflammatory cytokines in the liver and spleen after oral administration of Porphyromonas gingivalis in mice.

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Abstract

Periodontitis initiated by periodontopathic bacteria is associated with several systemic diseases. Porphyromonas gingivalis (P. gingivalis) is one of the major pathogens causing periodontal diseases, and is thought to also play a critical role in possible mechanisms linking periodontitis with other systemic disorders. We explored whether the production of pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) in the liver and spleen changed as a result of oral administration of P. gingivalis in mice. mRNA expression of pro-inflammatory cytokines was analysed by Real-time Quantitative polymerase chain reaction (RT-qPCR) and cytokine protein levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA) and immunohistochemistry. In addition, histological changes of liver and spleen tissues were monitored using haematoxylin and eosin staining. The results showed that liver and spleen tissue of P. gingivalis treated mice had higher mRNA and protein levels of pro-inflammatory cytokines compared to the control group. The production of pro-inflammatory cytokines in the liver and spleen was therefore suggested to increase as a result of oral administration of P. gingivalis in mice, and may provide further understanding of the mechanisms linking periodontitis and systemic disorders.

Keywords: Periodontitis, Porphyromonas gingivalis, Pro-inflammatory cytokines, Liver, Spleen.

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Introduction

Periodontitis, a chronic inflammatory condition of the periodontium, is one of the most common oral diseases worldwide. Many reports have revealed the correlation between periodontitis and systemic diseases including cerebrovascular and cardiovascular disease, rheumatoid arthritis, diabetes mellitus, chronic obstructive pulmonary disease and preterm low birth weight [1-6]. Periodontitis is caused by gram negative microorganisms and their production of including lipopolysaccharide (LPS), peptidoglycan, DNA which can damage the connective tissues and alveolar bone, finally leading to the loss of the tooth [7]. Porphyromonas gingivalis, a gram negative anaerobe, is the putative predominant pathogenic bacteria associated with periodontitis [8]. Oral infection by P. gingivalis has indeed been demonstrated to induce periodontitis and activate an immune response [9].

Pattern recognition receptors such as Toll-like-receptors (TLRs) are key to innate immune system as they recognise microbial structural motifs known as pathogen-associated microbial patterns (PAMPs) [10]. The TLR signalling pathway is critical for the initiation of periodontitis pathology [11]. Of ten human TLRs, TLR2 and TLR4 have previously been related to periodontal disease [12]. Moreover, TLR2 and TLR4 may also contribute to the progression of metabolic disorders like insulin resistance and hepatic steatosis [13]. In addition to the local production of inflammatory mediators, periodontitis also causes a significant up-regulation in circulating inflammatory mediators such as TNF-α, IL-6 and IL-1β [14,15]. Periodontitis is often associated with systemic disorders. The underlying mechanism possibly involves PAMPs entering the systemic circulation and activating host defence cells to induce the secretion of cytokines, chemokines and other kinds of immune factors in endothelial cells and hepatocytes [16]. In addition, various locally produced pro-inflammatory stimuli such as IL-1, TNF-α and IL-6 can also invade circulation and induce a systemic response [17,18].

The release of inflammatory mediators (TNF-α) in circulation has been related to various systemic diseases including insulin resistance, diabetes, atherosclerosis as well as non-alcoholic fatty liver disease (NAFLD) [19,20]. NAFLD is the most common liver disease worldwide and has recently been integrated with the metabolic syndrome to include steatosis, steatohepatitis, liver fibrosis, cirrhosis, and carcinoma [21-23]. Human trials reported that NAFLD patients were at greater risk of P. gingivalis infection compared to healthy patients. The
study implied that endotoxins from *P. gingivalis* and inflammatory mediators released by the microorganisms can enter the blood circulation and may participate in the mechanism linking periodontitis and NAFLD [24]. To conclude, there is a lot of data supporting the hypothesis that periodontitis can trigger a systemic inflammatory response and exert an inflammatory response in distant tissues. However, whether periodontitis can induce alterations in the expression of pro-inflammatory cytokines in remote organs such as the liver and spleen has remained unproven until now. The study presented here explored the effect of oral administration of *P. gingivalis* on the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the liver and spleen in mice.

### Materials and Methods

#### Animals and study design

This study followed guidelines from the Institutional Authority for Laboratory Animal Care of Jilin University and received the ethics committee approval of Hospital of Stomatology of Jilin University No.201703020000286. Thirty female C57BL/6 mice aged between four and six weeks old were purchased from the Animal Experiment Center of Jilin University. Animals were separated into two groups (n=15 per group): *P. gingivalis* group and the control group. Both groups received the same amount of feed and were kept under the same conditions. Mice were sacrificed by deep anesthesia after five weeks of oral administration and samples were taken from the liver and spleen.

#### Oral administration of *P. gingivalis*

*P. gingivalis* ATCC33277 was cultured in Columbia Blood Agar (BIO-KONT, Wenzhou, China). The density of *P. gingivalis* in an overnight culture was examined by spectrophotometry at 550 nm. 100 μl phosphate buffered saline with 2% carboxymethyl cellulose (Aladdin Industrial Corporation, Shanghai, China) containing 109 colony forming units of live *P. gingivalis* was given to the mice in the *P. gingivalis* group with a feeding needle. The suspension was given every other day for five weeks. Similarly, the control group was given the control solution of phosphate buffered saline with 2% carboxymethyl cellulose.

#### Quantitative Real-time PCR for gene expression of IL-1β, IL-6 and TNF-α in the liver and spleen

TRIZol reagent (Invitrogen Corp, Carlsbad, CA) was used to extract total RNA of liver and spleen samples according to manufacturer’s instructions. The isolated RNA was used as the template with the PrimeScript RT reagent Kit and gDNA Eraser (DRRO47A, TaKaRa, China) to obtain cDNA. RT-qPCR was performed using a 25 μl reaction with SYBR Premix Ex Taq II (RR420Q, TaKaRa, China) containing cDNA from 25 ng of total RNA per sample (Mx3005P Real-Time QPCR System; Agilent Technologies, USA). The housekeeping gene mouse β-actin was used as a loading control. Gene expression was calculated using the 2^ΔΔCt method. The following primers were used for RT-qPCR:

- IL-1β: (F5’-TCCAGGATGGACATGAC-3’) and (R5’-GAACGTCCACACCAAGAGTTA-3’);
- IL-6: (F5’-CCACTTCAACAGTCGAGCTTA-3’) and (R5’-CCAGTTGTTGACCATCATTACT-3’);
- TNF-α: (F5’-ACTCCAGGCCTGCTATGT-3’) and (R5’-GTGAGGCTTTGGGCCATAGA-3’);
- β-actin: (F5’-CATCCGGTAAGACGCTTACGCAAC-3’) and (R5’-ATGGAGCCACCGATCCACA-3’).

#### ELISA assay

Protein levels of IL-6, IL-1β and TNF-α in the liver and spleen were measured using Mouse IL-6, IL-1β and TNF-α ELISA kits (Lengton Bioscience Co, Shang Hai, China) in accordance with respective manufacturer’s protocols. In brief, the monoclonal antibody specific for either mouse TNF-α, IL-6 or IL-1β was coated on the 96-well plates. Then, specimens and biotin-labelled antibodies against mouse TNF-α, IL-6, IL-1β and specimens were added. After adding the HRP-conjugated antibody into each well, the plates were incubated at 37°C for 1 h and rinsed five times. Chromogen solutions A and B were used as substrate and the reaction was terminated with Stop reagent after colour formation. The absorbance of each well was read at 450 nm.

#### Immunohistochemistry

Tissue samples taken from the liver and spleen were fixed overnight in 10% formalin. The sections from the liver and spleen were embedded in paraffin and cut to make 5 μm slides. After hydrating and treatment with 0.3% H2O2 in methanol, the sections were incubated with primary rabbit polyclonal TNF-α (1:100, Abcam, ab66761), IL-6 (1:100, Bioss, bs-0379R, China), IL-1β (1:100, Bioss, bs-0812R, China) antibodies in a humidified chamber for 1 h at 37°C. The sections were washed with phosphate buffered saline, followed by incubation with polymer helper and secondary antibodies (goat anti-rabbit IgG HRP) from the Polink-2 plus kit (ZSGB-BIO, PV-9001). Finally, colour was developed with 3, 3-diaminobenzidine (ZSGB-BIO, ZLI-9018) and counterstained with hematoxylin. Positive expression was evident as brown staining. Images were captured with an Olympus BX 51 (Japan) operated with the micro imaging software cellSens (Olympus, Japan). Five fields were randomly selected from each section and assessed at magnification X200. Quantification of positive expression in each field was quantified as the mean optical density (MOD; integral optical density/total area) with Image Pro Plus 6.0.

#### Histopathology

After fixing overnight in 10% formalin, sections of liver and spleen tissue were cut as described in immunohistochemistry and stained with haematoxylin and eosin for histological examination. Five randomly selected fields from each section were analysed under a light microscope (Olympus BX 51,
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Japan) at magnification X200. The histological changes of liver and spleen tissue were evaluated by a specialist blind to the grouping.

Statistical analysis

All data are present as mean ± standard deviation (SD). The significance of the differences between the *P. gingivalis* group and the control group was analysed using the Student’s t-test in SPSS version 17.0.0. P<0.05 was considered as significant difference.

Results

Gene and protein expression of pro-inflammatory cytokines in liver tissue

Elevated mRNA levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were found in the *P. gingivalis* group (Figure 1A and Table 1).

Gene expression was calculated using the 2-ΔΔCt method. Data is expressed as mean ± SD. "*" indicates statistical significant difference (P<0.05) between *P. gingivalis* infected mice and the corresponding control group.

In addition, oral administration of *P. gingivalis* increased protein levels of TNF-α, IL-6 as well as IL-1β (Figure 1B). Immunohistochemical staining of liver tissues also indicated up-regulation of protein expression compared with the control group. There was a significant difference in MOD between the *P. gingivalis* and the control group (p<0.01) (Figures 1C and 1D).

Gene and protein expression of the pro-inflammatory cytokines in spleen tissue

Production of pro-inflammatory genes TNF-α, IL-6 and IL-1β were significantly up-regulated in the *P. gingivalis* group as compared to the control group (p<0.01) (Figure 2A and Table 2).

Correspondingly, protein levels of TNF-α, IL-6 and IL-1β were also higher (Figure 2B). Furthermore, the immunohistochemical staining of spleen tissue from the *P. gingivalis* group indicated elevated levels of MOD which implied positive expression of TNF-α, IL-6 and IL-1β proteins (Figures 2C and 2D).

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<th>Table 1. Analysis of TNF-α, IL-6, IL-1β mRNA expression levels in mice liver tissues by qRT-PCR.</th>
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Histological analysis of liver tissue

Scattered microvesicular steatosis was observed in hepatocytes of the \( P. \) gingivalis group. A majority of hepatocytes had “foamy” changes where the nuclei were typically centrally located. Furthermore, a few inflammatory cells had infiltrated the tissue (Figure 3). In contrast, liver sections from the control group showed no obvious alterations in the hepatic cord structure or hepatic cells (Figures 3).

![Figure 3](image-url) Histological analysis of liver tissue. (A, B) No evidence of inflammation was observed in the control group; (C, D) Widespread microvesicular steatosis was observed in liver tissue from the \( P. \) gingivalis group, as characterised by the “foamy” changes. (A, C) Scale bar=100 μm; (B, D) Scale bar=50 μm.

Histological analysis of spleen tissue

Several visible histological changes were found in the spleen tissue of \( P. \) gingivalis administered mice. We observed obvious activation of the white pulp and reduction of the red pulp compared with the control group. In addition, neutrophil infiltration was detected in the spleen tissue of the \( P. \) gingivalis group (Figure 4). In contrast, no histological changes of inflammation were detected in spleen tissue obtained from the control group (Figure 4).

Discussion

To our knowledge, this is one of the first animal model studies revealing the effects of periodontitis on the expression of pro-inflammatory cytokines in liver and spleen by oral administration of \( P. \) gingivalis. \( P. \) gingivalis can initiate periodontitis; LPS from \( P. \) gingivalis can enter gingival tissues and elicit an inflammatory response leading to an increase in pro-inflammatory cytokines [25]. The proposed pathways linking periodontitis and its systemic effects include the direct impact of bacterial products from the oral cavity and the dissemination of inflammatory mediators and immune complexes, including cytokines and chemotactic factors, generated by periodontitis lesions [26,27]. Recent studies reported that elevated levels of inflammatory cytokines such as IL-6 and IL-1β were secreted by the host in response to \( P. \) gingivalis stimulation [28,29]. Although the precise mechanisms remain unclear, the accumulation of inflammatory mediators in the circulation is thought to trigger the inflammatory response in remote tissues.

The liver is pivotal in protecting the host against microorganisms and microbial components as it can trigger an immunological response against endogenic and exogenic toxins present in the portal blood [30]. To date, an increasing number of studies have evidenced a correlation between periodontal diseases and the pathogenesis of liver diseases such as NAFLD, cirrhosis and hepatocellular carcinoma [31]. However, there is no direct evidence implicating the oral administration of \( P. \) gingivalis with the induction of pro-inflammatory cytokines expression in the liver. In this study, we observed that livers from mice in the \( P. \) gingivalis group exhibited increased levels of pro-inflammatory cytokines TNF-α, IL-6, IL-1β compared with the control group. Numerous studies demonstrated a close correlation between hepatic inflammation and chronic liver disease [32,33]. Cytokines produced by hepatic and inflammatory cells significantly contributed to the development of liver disease. TLRs are capable of recognising endotoxins such as LPS and induce the secretion of pro-inflammatory cytokines like IL-1, IL-6 and TNF-α from macrophages and adipocytes; thus promoting the liver disease [34]. A number of studies have shown that Kupffer cells play a pivotal role in the development of NAFLD involving TLR4 response to LPS and the consequent activation of Kupffer cell to release inflammatory cytokines [35-37]. In agreement, mice lacking TLR4 were less likely to develop NAFLD and were insulin resistance [38-40]. In the present study, we observed a significant up-regulation of TNF-α, IL-6, IL-1β in the liver of \( P. \) gingivalis administered mice. TNF-α is a key inflammatory mediator and can be secreted directly by hepatic cells and Kupffer cells [41]. Both human and animal studies have confirmed the role of TNF-α in the development of NAFLD and non-alcoholic steatohepatitis [42]. Moreover, a
study using the rat periodontitis model found that after chronic administration of LPS from Escherichia coli and Streptomyces griseus proteases, rats developed periodontitis and the liver manifested steatosis, with inflammation and fibrosis following the production of TNF-α in the liver [43]. As a powerful mediator in the acute phase response of hepatocytes, IL-6 is generated by a number of cells including activated macrophages and lymphocytes [44]. The secretion of IL-6 is induced by IL-1 and TNF-α. In turn, IL-6 is able to regulate the production of IL-1, by activating the secretion of the IL-1 receptor antagonist and TNF-α directly [45]. IL-6 is therefore a key to local pathological processes as well as systemic inflammation. Mas et al. stated that IL-6-deficient mice showed a reduction in diet-induced non-alcoholic steatohepatitis in comparison with controls [46]. Further, IL-6 accumulated in the livers of patients with non-alcoholic steatohepatitis compared to control patients, suggesting a positive relationship between IL-6 expression in the liver and the degree of NAFLD [47]. In the case of IL-1β, an in vivo study has shown that IL-1β deficient mice had a marked reduction in the development of steatosis to steatohepatitis and liver fibrosis [48]. Thereby demonstrating the important role for IL-1β in the promotion of liver disease. Apart from the up-regulation of pro-inflammatory cytokines, we also observed the presence of diffuse micro vesicular steatosis in the majority of hepatocytes in the P. gingivalis group. Micro vesicular steatosis has been implicated in the advanced histology of NAFLD. A clinical trial has demonstrated the association between micro vesicular steatosis and hepatocyte injury including the presence of steatosis, ballooning cell injury and fibrosis [49]. Based on our results, we propose that P. gingivalis participates in the development of liver disease by activating the production of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 in the liver. However, the precise mechanism of P. gingivalis mediated pathogenesis of liver disease is still unknown. As for the liver, the levels of TNF-α, IL-1β and IL-6 were significantly up-regulated in the spleen of mice following oral administration of P. gingivalis. Also histological changes were observed in this tissue. The spleen is the largest peripheral lymphoid organ, composed of two distinct components: the white and red pulp. The white pulp is made up of lymphoid tissue, mostly lymphocytes (T cells and B cells) and macrophages, while the red pulp is composed of parenchyma and lots of vascular sinuses and sinusoids [50]. The white pulp is thus regarded as the main site of immunological function in the spleen. The spleen interacts with the circulatory, reticuloendothelial, and immune systems and is key to developing an immune response to antigens. As the first line of defence, the spleen is capable of recognising specific PAMPS such as LPS. Moreover, the spleen is considered a reservoir for inflammatory monocytes [51,52]. A study in vivo showed that LPS from Escherichia coli could activate dendritic cells of the spleen and elevate the expression of pro-inflammatory cytokines including IL-6, interleukin 12 p40, and TNF-α compared to the control group [53]. Another study reported that pro-inflammatory cytokines IL-6 and TNF-α and the anti-inflammatory cytokine IL-10 were secreted locally in the spleen after LPS injection [54]. In addition, Semaeva et al. found that during LPS-induced inflammation, the spleen showed a significant efflux of lymphocytes and up-regulation of pro-inflammatory cytokines released into the systemic circulation through the isolation of spleen lymph [55]; thus confirming the central role of the spleen in a systemic immune reaction. There are some limitations to our study. Histological changes in the liver were for example examined by haematoxylin and eosin staining which may have underestimated the appearance of micro vesicular steatosis. Compared to haematoxylin and eosin stain, fat-specific oil red O stain would have been better for the purpose of our study. Future work is needed to determine whether the oral administration of P. gingivalis changes the expression of hepatic injury markers including aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase.

**Acknowledgement**

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