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Expression of spleen macrophages in a mouse model of alveolar bone resorption periodontitis

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ABSTRACT

It has been shown that macrophages can be endotoxin-tolerant under the stimulation of continuous endotoxin of *Porphyromonas gingivalis*. Macrophage transforms into M2-type which inhibits inflammation, and its pro-inflammatory cytokine secretion is reduced to avoid the tissue damaged by inflammation. This experiment established the corresponding animal model to explore the relative number, phenotypic proportion, and function of spleen macrophages in mice with chronic periodontitis. Twenty 16-week-old mice were randomly divided into a true ligation group (LFP group) and a pseudo-ligation group (LFC group). The periodontitis in the LFP group was induced by experimental ligation, and the LFC group was treated as a control. After 10 days of ligation, the maxilla was taken, IHC and HE staining were performed to observe the pathological changes of periodontal tissues, and IHC staining was performed to observe the RANKL/OPG ratio. Spleen mononuclear cells were isolated and counted. The ratio of M1 and M2 phenotypes was determined by fluorescence-activated cell sorting (FACS) in the spleen. The relative expression levels of macrophage-associated inflammatory cytokine TNF-a, IL-1β and anti-inflammatory cytokine IL-10 mRNA were detected by real-time PCR. Compared with the control group (LFC:M2/M110.04%), the M2 ratio among spleen mature macrophages in the periodontitis group (LFP: M2/M135.86%) was significantly increased ($P<0.01$) in the spleen. The proportion of M1 macrophages was not significantly different, and the ratio of M1/M2 was significantly decreased ($P<0.05$) in the spleen. But the expression level of M1-type macrophage inflammatory factor TNF-a mRNA was inclined. Chronic periodontitis can up-regulate the proportion of M2 macrophages, decrease the ratio of macrophage phenotype M1/M2, and incline the expression of pro-inflammatory factor TNF-a mRNA.

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Introduction

As a chronic infectious disease caused by plaque microorganisms, periodontitis is not only regulated by the immune inflammatory response but also by the release of pro-inflammatory factors into the blood circulation (1-3). Berglundh et al. (4) found that there are autoreactive B cells in the periodontal and blood circulation system of patients with severe periodontitis in 2002. The serum C-reactive protein level (CRP) is decreased in chronic periodontal patients with type 2 diabetes after a complete periodontal treatment, and CRP is an important infection phase acute protein (5).

Porphyromonas gingivalis, mainly colonized on the surface of the periodontal pocket epithelium and subgingival plaque, and is one of the main pathogens of periodontitis. P.gingivalis can adhere to or invade the gingival epithelial cells, grow and multiply in the cells, release toxins, and escape the host's defense mechanism (6,7). In severe periodontitis, the periodontal tissue basement membrane is destroyed. P.gingivalis, lipopolysaccharide, and other toxic products can penetrate the gingival epithelial barrier into the bloodstream, continue to stimulate the systemic host response, activate inflammation and immune cells, and produce cytokines (8,9).

The balance of bone metabolism mainly includes the synthesis of bone matrix by osteoblasts and resorption by osteoclasts after bacterial infection and subsequent inflammatory reaction this balance will be broken, eventually leading to bone destruction, which can be observed in periodontitis (10,11). Periodontal bone destruction usually includes two stages. The first stage is caused by matrix metalloproteinases (MMPs), in which the osteogenic ability is weakened, the newly formed bone is obviously reduced, the bone matrix is obviously destroyed, and osteoclasts are active. The second stage is mainly caused by osteoclast activation, in which osteoclast cells can be infiltrated around the alveolar bone, and the osteoclast ability is obviously enhanced (12,13). Osteoclastogenesis is the process of osteoclast differentiation which involves a molecular triad consisting of the Receptor Activator of nF-κB (RANK), Receptor Activator of NF-κB Ligand (RANKL), and osteoprotegerin (OPG) (10,11). Under normal conditions, bone remodeling activities are controlled by a balanced RANKL/OPG ratio. Osteoblasts can either enhance or inhibit osteoclast differentiation through RANKL/OPG ratio, simultaneously decreasing or increasing the rate of bone formation, respectively. Changes that occur in the

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RANKL/OPG ratio affect bone resorption or bone formation (14,15).

Macrophages, as an important innate immune cell, play an important role in the regulation of the pro-inflammatory and anti-inflammatory immune balance (13,16). Its function of phagocytosis and antigen presentation is a key link in causing periodontitis and related immune inflammatory reactions (12). Macrophages have plasticity and pluripotency, which can differentiate into M1 phenotype and M2 phenotype according to the microenvironment (17). The former is pro-inflammatory, mainly secreting pro-inflammatory cytokines IL-6, IL-1b, and TNFa. The latter is an anti-inflammatory type, mainly secreting cytokines, such as IL-10. The proportion change of M1 and M2 types reflects the immune state to a certain extent and has become a classic paradigm in immunopathology, such as adipose tissue inflammation (18,19).

The spleen is one of the main colonies of macrophages in the body, containing a large number of lymphocytes and macrophages. It is the center of cellular immunity and humoral immunity (20). Spleen macrophage is one of the important cells regulating the immune response, and plays an important role in maintaining immune tolerance and antigen presentation (21-23). In recent years, the relationship between periodontitis and diabetes, cardiovascular diseases, and other systemic diseases has become a hot topic. Periodontal disease is also considered to be an important potential risk factor for systemic diseases such as atherosclerosis (2,3,8). Although the role of innate immune response and adaptive immune response in the development of periodontitis has been widely recognized, there are few reports on how periodontal pathogen infection affects systemic immune function. In order to investigate the relationship between periodontitis and systemic immune function, this study explored the effect of periodontitis on the M1/M2 phenotypic polarization and function of spleen macrophages.

Materials and Methods

Experimental animals

SPF-level experimental mice were provided by the Guangdong Medical Laboratory Animal Center and raised in the animal center. This study was approved by the Animal Ethics Committee of the Center.

Ethical approval

The research related to animal use has complied with all the relevant national regulations and institutional policies for the care and use of animals. The Animal Experimental Committee of Southern Medical University approved all experimental protocols. The study protocol followed all recommendations of the National Institutes of Health Guide for the care and use of laboratory animals (24).

Main reagents and instruments

Conventional surgical instruments, stereo microscope, 10% neutral formaldehyde solution, liquid nitrogen, 75% alcohol, RNA iso plus lysate, PrimeScript RT reagent kit, SYBR Premix ExTaq PCR kit (TAKARA), RNase-free water, chloroform, absolute alcohol, isopropanol, cryogenic high-speed centrifuge, vortex shaker (Bio-Rad), 200 μL octagonal tube. Applied Biosystems Vii™ 7A real-time quantitative PCR instrument, DMEM, fetal bovine serum, and PBS (Gibico) were purchased from Life Technologies Invitrogen. FITC-murine anti-F4/80 antibody, APC-mouse anti-CD206 antibody, and PE-mouse anti-CD11c antibody were purchased from BioLegend. BD Calibur flow cytometer.

Methods

Periodontitis animal model establishment

Male 16-week-old C57 BL/6J mice with normal mental and coat color were used for the experiment after 3 days of routine animal quarantine. Twenty mice were randomly divided into the true ligation group (LFP group) and pseudo ligation group (LFC group). Group LFP was treated with porphyrin sclerotium ligation to induce experimental periodontitis while group LFC was used as control. *Porphyromonas gingivalis* (*P.g*) was cultured as previously studied in our group (9,18). The periodontal ligation period was 10 days.

IHC and HE, andTRAP staining

After 10 days of ligation, the blood in C57 BL/6J mice model was collected by cardiac puncture. The bilateral sides of the second molar wire retention in the true ligation group were recorded. The maxilla was separated and immersed in a 10% neutral formaldehyde solution for fixation. One side was used for immunohistochemistry (IHC) the other side was used for HE staining and TRAP staining. Fixed for 2 days, the jaw was decalcified in decalcifying solution (10% EDTA, 0.2 M Tris) at room temperature for 3 weeks. Next, the tissue was treated with alcohol and xylene and then embedded for a section. The primary antibodies used were mouse monoclonal RANKL (AB45039) (Abcam, 1:400), and rabbit polyclonal osteoprotegerin (OPG) (AB9986) (Abcam,1:100). At last, the section was photographed under × 200 magnifications to qualitatively analyze bone loss in the jaw and× 400 magnifications to qualitatively analyze RANKL and OPG expression. TRAP staining solution (Sigma-Aldrich, 387A-1KT) prepared according to the reagent instructions was preheated in the oven at 37°C. Dye the sections and keep them out of light for 30 minutes. Then, wash the sections with PBS and shake them for 3 minutes. Re-dye the nucleus with methyl green for 2 minutes, wash again, absorb excess water, and seal with glycerin.

Spleen cells isolation, counting, and flow cytometry analysis

The spleen was taken out under aseptic conditions and placed in a petri dish containing 10 mL of 4 °C DMEN complete medium. The tissue was gently crushed and filtered through a 400 mesh cell strainer to a 50 mL test tube. The plate was repeatedly washed with 10 mL of DMEN complete medium and centrifuged at 1000 g and 4 °C for 10 min. After removing the supernatant, the sample was added with 2 mL of red blood cell lysate for 2 min. The isolated spleen mononuclear cells were incubated in FITC-anti F4/80, PE-anti CD206, and FITC-anti CD11c antibodies and analyzed by flow cytometry to identify M1 and M2 macrophages.

Quantitative PCR analysis

The spleen was added with 1 mL of Takara RNAiso plus lysate to isolate total RNA. Next, the RNA was quan-

Table 1. Primer sequences.

Gene name	GenBank Accession	PrimerBank ID		Primer sequence (5' to 3')
Gadph	NM_008085	126012538C1	forward	AGGTCGGTGTGAACGGATTTG
			reverse	GGGGTCGTTGATGGCAACA
Tnf-a	NM_013693	133892368c1	forward	CAGGCGGTGCCTATGTCTC
			reverse	CGATCACCCCGAAGTTCAGTAG
IL-1β	NM_008361	118130747c1	forward	GAAATGCCACCTTTTGACAG
			reverse	TGGATGCTCTCATCAGGACAG
IL-10	NM_010548	291575143c1	forward	CTTACTGACTGGCATGAGGATCA
			reverse	GCAGCTCTAGGAGCATGTGG

Primer Bank website: http://pga.mgh.harvard.edu/primerbank/

tified using a Biodrop micro-quantitative meter and reverse transcribed using the Takara Primescript RT reagent kit. The PCR reaction was performed using a Takara SYBR Premix Ex Taq PCR kit on the Applied Biosystems Vii 7 PCR amplifier. The internal reference gene was Gadph, while the inflammatory genes were TNF-a, IL-1β, and IL-10. The primer was designed by the online website PrimeBank (Table 1).

Statistical analysis

All data analyses were performed on SPSS 19.0 software. The test data were expressed as mean ± standard deviation and compared by one-way ANOVA and LSD test. $P<0.05$ was considered as statistical significance.

Results

Analysis of HE, TRAP and IHC staining in mouse jaw

HE staining showed gingival papilla atrophy, gingival epithelial root displacement, and alveolar crest absorption significantly in the LFP group (Figure 1: BD). The gingival nipple and epithelium were intact, and the alveolar dome was normal in the control group (LFC) (Figure 1A AC). Periodontitis was successfully modeled.

According to the IHC staining results, the expression of RANKL was significantly increased in the LFP group compared with those in the LFC group (Figure 1B AC), and the expression of OPG was decreased in the LFP group (Figure 2: CD). The ratio of RANKL/OPG was effectively increased, indicating the activation of RANKL/OPG.

Trap staining showed that in the control (LFC) group, the alveolar bone was in order, and there was no obvious osteoclast infiltration on the alveolar bone surface (Figure 3 AC), while in the LFP group, the alveolar bone absorption increased, and the bone mass decreased obviously. More importantly, a large number of osteoclasts infiltrated the absorbed alveolar bone surface and gathered on the bone absorption surface (Figure 3, BD), suggesting that

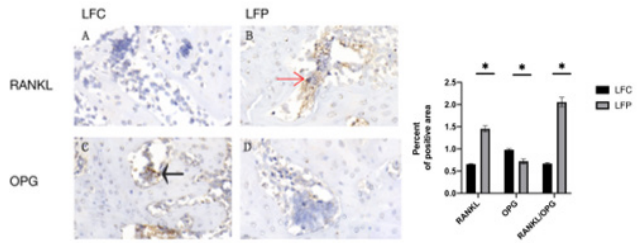

Figure 2. Representative images of OPG and RANKL expression in periodontal ligation modelofC57 BL/6Jmiceas shown by IHCstaining (A, B, C, D, 400×). A, B: Compared with the LFC group, the positive area of Rankl expression increased significantly in the LFP group. C, D: Compared with the LFC group, the positive area of OPGepression-decreasedinLFPgroup.

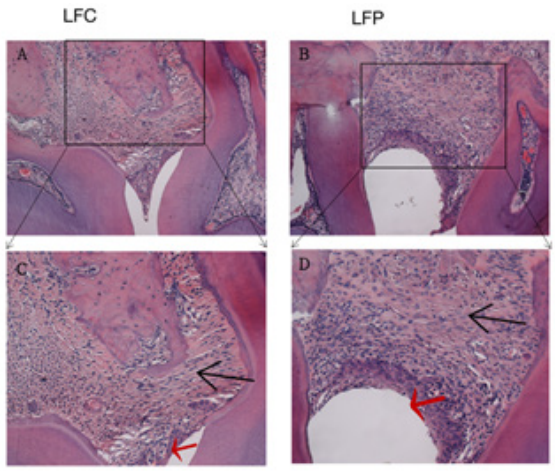

Figure 1. Loss of gingival attachment (red arrow) and bone volume (black arrow) at day 10 for periodontal ligation in C57 BL/6Jmiceas shown by H&E staining. A, C: In the control group (LFC), the alveolar bone was 2mm below the mucogingival junction. B, D: in the periodontitis group (LFP), the mucogingival junction retreated to the root, and no alveolar bone was found around it.

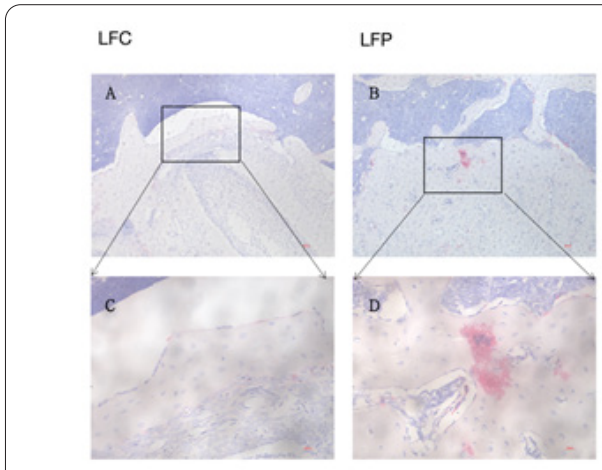

Figure 3. TRAP staining images of periodontal ligation model of C57 BL/6J mice with 100 /400 magnification. A, C: In the control group, the alveolar bone was not absorbed and arranged orderly, and there was no obvious osteoclast infiltration around the alveolar bone. B, D: In the LFP group, the alveolar bone was damaged and disordered, and osteoclasts infiltrated the surface of the alveolar bone. (A, B, 100×; C, D, 200×)

osteoclasts were found on the bone absorption surface during the progress of periodontitis.

The impact of periodontitis on the number and function of spleen macrophages

The effect of periodontitis on the proportion of spleen macrophages: Compared with the control group, the percentage of macrophage phenotype in the periodontitis group decreased5.86% (P>0.05) (Figure 4A). The proportion of M2 macrophages in macrophages was significantlyincreased LFP35.86%>LFC=10.04%, p<0.01) (Figure 4 B) while M1/M2 ratio was obviously decrease (LFC0.935>LFC=0.340, P <0.05) (Figure 4C). It indicated that compared with the control group, the total amount of macrophages decreased, the proportion of M2 macrophages inclined, and the proportion of M1/M2 markedly declined in periodontitis (p<0.05) (Figure 4C).

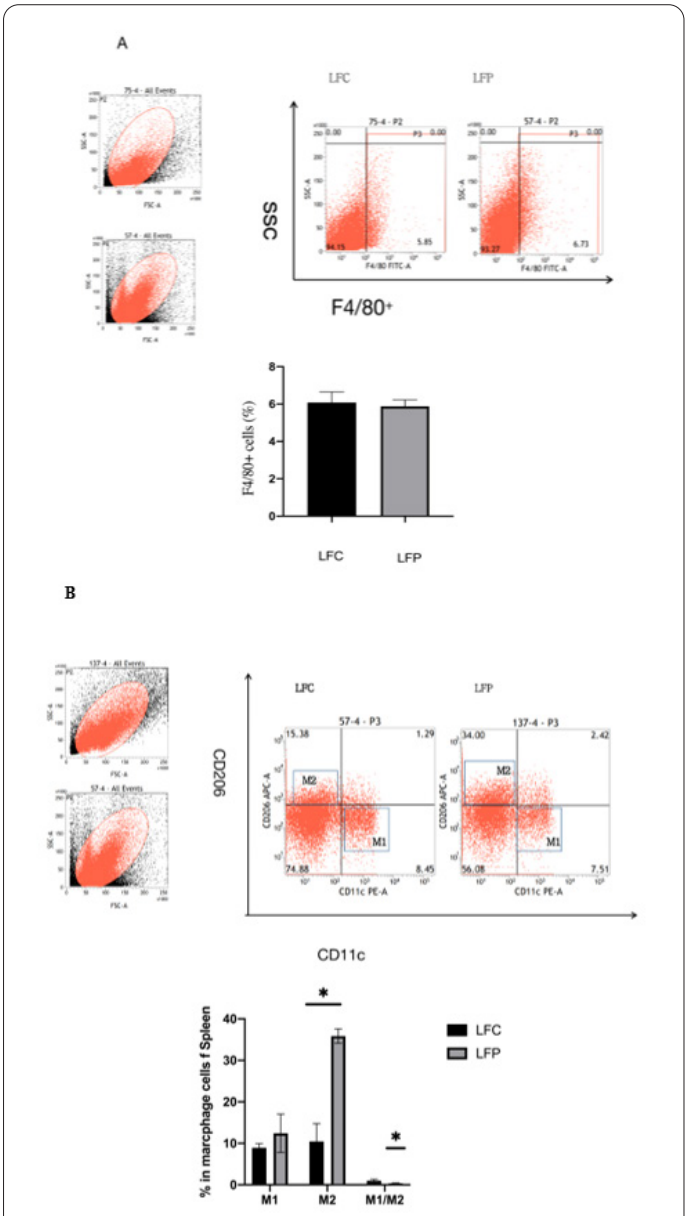

Figure 4. Flow cytometry analysis of the cell complexity (SSC) in the spleen. A: The percentage of macrophage phenotype in the periodontitis group is higher than in the control group (6.08% >5.87% P>0.05). B: The proportion of M2 macrophages in macrophages was significantly increased in the periodontitis group. (LFP=35.86% >LFC=10.04%, p<0.01). C: M1/M2 ratio was obviously reduced (LFC=0.9437> LFP=0.3413, p<0.05). B: M1 and M2 contents, and M1/M2 ratio ($\bar{x}\pm s$, n=4-8). * P < 0.05, compared with LFC group; ** P < 0.01, compared with LFC group.

The changes in macrophage-associated inflammatory factor mRNA expressions

Compared with the control group, the expression of M1 macrophage inflammatory factor TNF-amRNA was significantly increased (p<0.05), whereas M2 macrophage inflammatory factor IL-10 mRNA exhibited no statistical difference in group LFP (p>0.05). In summary, the expression of pro-inflammatory factors related to M1 macrophages in the body infected with periodontal pathogens was markedly increased compared with the control group (Figure 5). The primer sequences of primers used forRT-PCR (Sangon, Shanghai, China) are listed in Table 1.

Discussion

Periodontitis is a chronic infectious disease caused by plaque microorganisms and is interact with the immune system. In the periodontitis host immune response, the autoimmune response is involved in the chronic inflammatory destruction process of periodontal tissue caused by microbial infection (25). The chronic immune inflammatory response in the periodontal may also stimulate the function of the immune system. Abhijit N. Gurav pointed out that an autoimmune response triggered by periodontitis accumulates a large number of immune cells in the periodontal area (26). The cytokines released by these immune cells will circulate through the blood vessels to various systems of the body (9).

As a low-level infection, periodontal local pro-inflammatory factors may trigger a systemic immune response after entering the systemic circulation. The levels of inflammatory factors and C-reactive protein in the body are elevated, and the systemic immune function of the body is regulated (1,5). As an important pathological basis of periodontitis, the abnormal innate immune response of the body represented by macrophages plays an important role in the occurrence and development of periodontitis. In periodontal inflammatory tissues, macrophages, as one of the main inflammatory infiltrating cells, regulate the immune response by antigen presentation and secretion of cytokines to trigger a series of immune-inflammatory reactions, leading to the destruction of periodontal tissues (27,16,28).

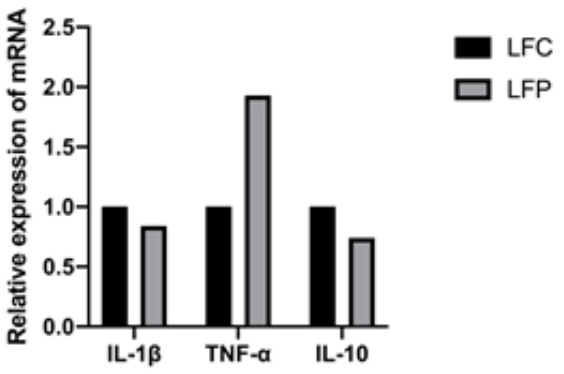

Figure 5. The mRNA expressions of a macrophage-associated inflammatory factor in the spleen. Compared to the LFC group, TNF-α mRNA expressions were significantly increased (p<0.05) in the LFP group. IL-10 and IL-1β mRNA expressions exhibited no statistical difference in the two groups (p>0.05).

Macrophage is an immune-assisted cell that differentiates and matures from mononuclear cells with plasticity and pluripotency (17). When tissue damage changes the microenvironment, activated macrophages can polarize into classical inducible macrophages (M1 type) that promote inflammation and conditionally activated macrophages (M2 type) that inhibit inflammation (29,30). M1 macrophages mainly trigger the Th1 response by secreting proinflammatory factors and NO, thus activating the immune response to kill the pathogens. M2 macrophages mainly express ornithine and polyamines, which have immunomodulatory effects and high phagocytic activity, and thus have a certain inhibitory effect on the inflammatory response (31,13,17). The proportional changes of M1 and M2 reflect the immune status to a certain extent, which has become a classic paradigm for studying the immune function of macrophages (32,33).

IL-1β is mainly produced by activated macrophages and is an important mediator of inflammatory response. At local low concentrations, it synergistically stimulates APC and T cell activation, promotes B cell proliferation and secretion of antibodies, and regulates immunity (4,34,35). Continue increased IL-1β may induce autoimmune disease and promote inflammation.IL10, also known as human cytokine synthesis inhibitory factor, is an anti-inflammatory factor that regulates inflammation and antagonizes inflammatory mediators to reduce immune damage to the body (20,36).

Our experiment showed that compared with the control group, the proportion of spleen M2 macrophages increased, and the secretion of M1-type macrophage-associated proinflammatory factors TNF-a was significantly attenuated in the periodontal pathogen-infected group. It is suggested that *P. gingivalis* infection for 10 days inhibits the polarization of mouse spleen macrophages to M1 type. At the same time, the pro-inflammatory cytokine IL-1β mRNA level was downregulated, which may be related to endotoxin immune tolerance caused by pg lps (20). Excessive activation of the immune system-induced severe inflammatory response can cause immune injury (32). As a protective regulatory mechanism, the body produces an adaptive response to long-term endotoxin stimulation, while the immune cells such as macrophages declined reactivity to subsequent stimulation. Periodontitis as a chronic infectious disease, the immune cells in periodontitis reduce or even negatively regulate the response to further stimulation after being stimulated by early endotoxin (30). Foey et al. (34) found in 2013 that macrophage colonies declined pathogen-associated molecular patterns (PAMPs) pathway-related TNF-α, IL-6, and IL-10 secretions and inhibited NFκB activation in vitro after pretreatment with *P. gingivalis* endotoxin and heat killing protein.

In 2005, Muthukuru et al. (5) discovered that the expression of TNF-αdecreased nearly 10-fold in monocytes after repeated stimulation with *P. gingivalis*, and the expressions of IL-1b, IL-8, and IL-10 were also reduced, indicating that endotoxin tolerance may exist in macrophages during chronic periodontitis. After repeated stimulation of THP-1 cells by Pg lps, IL-1β expression was reduced. Pena et al. (37) revealed that after pre-stimulation of inflammation, human monocyte macrophages were polarized to the M2 phenotype to avoid inflammatory damage and repair tissue. The results of this experiment demonstrated that pg stimulation for 10 days decreased the proportion of pro-inflammatory M1 macrophages in the spleen-activated macrophages and downregulated the inflammatory factor IL-1βmRNA, suggesting long-term periodontal pathogen infection may cause the peripheral immune system to produce endotoxin immune tolerance, and whether this response is systemic and universal still needs further investigation.

In the progress of periodontitis, the immune response to bacteria and bacterial products is the main form of periodontal destruction, and it is also the key factor of bone destruction in periodontitis (10,11,6). Stimulated by local factors, bacteria and their products can induce a large number of immune cells, such as T cells, B cells, and macrophages, to gather in periodontal connective tissue (38,30) This process is called immune cell infiltration, and the immune cells focused on periodontal tissue release a large number of inflammatory factors, so as to kill or devour invading bacteria. The process is an immune response, which will lead to a host inflammatory reaction in periodontal tissue and lead to alveolar bone destruction (27,9,18). Alveolar bone destruction includes two aspects: osteogenesis weakening and osteoclast strengthening, which shows the imbalance of osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation by forming the mature bone mineral matrix, and excessive inflammatory factors including IL-1, IL-6 and TNF-α can inhibit their proliferation, differentiation and mineralization, eventually leading to bone destruction and loss (39,12). At the same time, RANKL secreted by osteoblasts is the key factor of osteoclast formation and plays a key role in bone destruction (40,7). As the precursor cells of osteoclasts, the inflammatory factors secreted by macrophages can stimulate bone destruction bone cell maturation affects bone resorption (19,13). Inflammatory also suppressed osteoblasts proliferate, differentiate and mineralize, destroying and degrading bone matrix by secreting matrix metalloproteinases, thus providing more attachment sites for osteoclasts and further accelerating bone absorption (12,10). IHC staining of the alveolar bone showed that the RANKL expression in the LFP group was higher than LFC group, demonstrating that periodontitis resulted in increased osteoclast expression, as also demonstrated by the increased trap expression in the periodontitis group in TRAP staining. The role of macrophages in inducing alveolar bone resorption is related to the imbalance between M1 and M2 macrophages. It is considered that M1 and M2 are closely related to the injury of periodontal supporting tissues in the local periodontal microenvironment, and the secreted inflammatory factors and chemokines are involved in the progressive injury and destruction of periodontal tissues (23,18).

Periodontitis is a chronic infectious disease (8). After early stimulation by bacterial endotoxin, the immune cell or organ may negatively regulate the inflammation response in periodontitis to protect our body from the inflammatory storm (13,5). It has been shown that macrophages regulate adaptive immunity in the spleen (21).

The results of this experiment demonstrated that pg stimulation for 10 days increased the proportion of M2 macrophages in the spleen-activated macrophages and downregulated the inflammatory factor IL-1β mRNA in the spleen suggesting periodontal pathogen infection may cause the peripheral immune system to produce immune tolerance

The immune system, in order to avoid self-inflammatory damage, may develop an immunologic tolerance (adaptive) response or immunosuppression in the face of specific antigenic substances stimulation (exogenous or self) by T, B cells after low pre-stimulation of specific antigens, which manifests as immune non-response or diminished response. Adaptive immune response reduces the release of various inflammatory cytokines to protect the relevant tissues from immune damage (5,35,41). As an immune regulatory organ, the spleen will make an endotoxin immune tolerance when the periodontitis inflammatory factors continue to rise to avoid the further spread of local inflammation (20).

In this experiment, we found that periodontitis induced alveolar bone resorption by increasing the expression of RANKL, which causes the M2-type macrophages to increase in the spleen as an immunologic tolerance response. But the molecular mechanism of this process needs to be further studied. And considering the macrophages are recruited from the circulation, M1 and M2 type inflammatory cytokines in serum could be detected to explore the consistency of the splenic macrophage phenotype with the systemic response.

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Conflicts of interest
The authors declare that they have no competing interests.

Data availability statement
The data used to support the findings of this study are included in the article.

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