Periodontitis-mediated Conversion of CD4+CD25+ Regulatory T Cells into Proinflammatory Interleukin 17-producing T Helper Cells

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Research article

Keywords: Chronic periodontitis, CD4+ T cells, Treg-Th17 imbalance, Treg-Th17 conversion

Posted Date: March 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-361398/v1

Abstract

Background: Among CD4+ T helper (Th) cells, Th17-Treg imbalance is a major pathogenesis of Chronic periodontitis (CP). Treg cells are often considered to play a protective role but they may have plasticity. This study aimed to clarify the regulatory role of Th17-Treg balance in periodontitis and further reveal Treg plasticity.

Methods: An experimental periodontitis model was established by ligation of a silk thread and local injection of Pg-LPS. Inflammatory factors in serum and gingival tissues were measured by ELISA and RT-PCR. The degree of alveolar bone absorption was evaluated by micro-CT and histomorphological analysis. Quantities of Treg and Th17 cell and their related gene expression levels were examined. Furthermore, after magnetic bead-sorting spleen Treg cells, their characteristic genes and Th17 cell-related factors were explored at the mRNA level.

Results: Inflammatory cytokines in serum and gingival tissue increased significantly in experimental periodontitis, which revealed obvious crestal bone loss around maxillary second molars. Consistently, we found increased secretion of RANKL by osteoblasts, thereby promoting the formation of osteoclasts and enhancing their activity in periodontitis. Further analysis showed that the number of Th17 cells and expression of related genes increased more significantly than Treg cells, demonstrating Treg-Th17 imbalance in periodontitis. Flow cytometry showed that the proportions of Treg cells in the blood and spleen of the periodontitis group was lower than those of the control group. Furthermore, Treg cell-related gene Foxp3 was downregulated and their expression of Th17 cell-related genes Rorc and IL-17A were increased.

Conclusions: These results provided evidence that periodontitis may lead to Treg-Th17 conversion, although its mechanisms requires further study.

Background

Chronic periodontitis (CP), an infectious disease occurring in the supporting tissues of teeth, is characterized by gingival inflammation and pathological absorption of alveolar bone. It directly leads not only to tooth loss, but also induces systemic diseases such as cardiovascular diseases (1). The etiology of CP is very complex, involving immunity, heredity, and environmental and other factors, and its pathogenesis is not yet clear. Since the 1980s, there have been two consensuses on the etiology of CP. Specifically, plaque microorganisms in the oral cavity are the initiating factors of CP, and plaque microorganism-induced inflammation and host immune responses play crucial roles in the homeostasis of periodontal tissue (2).

Concerning host immune responses, the cellular immune response mediated by CD4 + T helper cells (CD4 + T cells) has been recognized as a major pathological mechanism of CP (3). Under invasion of periodontal microorganisms, antigen-specific CD4 + T cells in regional lymph nodes activate and differentiate into various cell subsets [Th1, Th2, Th17, regulatory T cells (Treg) cells]. These CD4 + T cell

subsets induced by specific cytokines migrate to periodontal tissues and play inflammatory or anti-inflammatory roles (4, 5). The Th1-Th2 balance has long been a classic model of immune system regulation. The theory that Th1 cells provide protection and Th2 cells destroy in the pathogenesis of periodontal disease has also gained some clinical and experimental evidence (6). However, other studies propose conflicting views, such as expression of Th1 cytokines in periodontal lesions is higher than that of Th2 cytokines (7) and Th1-specific cytokine interferon-γ (IFN- γ) is closely related to the destruction of alveolar bone during periodontal disease (8). Therefore, the traditional Th1/Th2 model is unsufficient to explain the pathogenesis of periodontal disease.

There is another set of CD4 + T cells subgroups, which have opposing functions and play important roles in the maintenance of periodontal tissue homeostasis, namely Th17 cells (proinflammatory effects) and Treg cells (anti-inflammatory effects) (9). Both experimental periodontitis and patients with periodontal disease have decreased Treg cell numbers and/or increased Th17 cell differentiation, referred to as the "Th17-Treg imbalance," which significantly correlate with periodontal tissue destruction of CP (10, 11). The role of Th17 cells and their characteristic cytokine interleukin-17 (IL-17) in the pathogenesis of CP have been studied extensively. For example, periodontal pathogens induce a significant increase of IL-17 (12). The Th17-IL17 axis induces osteoblasts to secrete nuclear factor-kappa B receptor activating factor ligand (RANKL) and promotes osteoclast differentiation, thereby aggravating periodontal bone destruction and absorption (13). In addition, Th17-related cytokines are directly or indirectly involved in Th1-mediated immune responses and promote fibroblasts to secrete inflammatory mediators (14).

Treg cells were found because some CD4 + T cells in normal animals are specialized for immune suppression (15). Characterization of this population indicated that they constitutively express the CD25 molecule and further studies revealed that Treg cells specifically express the transcription factor forkhead box P3 (Foxp3) that is essential for their development and function (15, 16). Much evidence has been provided for the critical roles of Treg cells in establishing immunological self-tolerance and maintaining immune homeostasis (17). Recently, there have been some studies on Treg cells regulating the pathogenesis of CP. For example, Treg cell-related cytokines IL-10 and TGF-β have been reported to slow down the periodontal tissue destruction (18). Treg cells alleviate CP through their own membrane glucocorticoid-induced tumor necrosis factor receptor and negatively regulate the expression of RANKL (18, 19). These studies suggest that Treg cells play protective roles during the pathogenesis of CP, but their exact functions in different stages of CP remain controversial. The number of Treg cells in periodontal tissues increases, especially after the acute stage of inflammation. However, the number of Treg cells decreases significantly in the advanced stage of periodontal inflammation, but increases in the repair stage (18–20). The diversity of these results in different models may be attributed to heterogeneity in the phenotype and functions of Treg cells. Another more plausible explanation for the contradiction is that Treg cells have plasticity (21). Specifically, Treg cells may acquire the characteristics of effector T cells in certain microenvironments and even convert into Th1- or Th17-like Treg cells. The latter two cell types express Th1 or Th17 cell-specific transcription factors and secrete their characteristic cytokines (IFN-γ or IL-17) (21). In particular, conversion of Treg cells into Th17-like Treg cells has gained more evidence under various conditions such as severe lymphocyte depletion, hyperinflammation and the

autoimmune microenvironment (22–24). However, little research has been conducted on the pathogenesis of CP.

In this study, we investigated the relationship between the Treg-Th17 balance and periodontal inflammation, clarified the Treg-Th17 conversion phenomenon, and further revealed its role in the occurrence and development of CP. This study introduces a new theory ‘Treg-Th17 conversion’ in the immunopathology of CP and may guide the development of new CP therapies.

Methods

Animals and tissue preparation

A total number of 20 Male C57BL/6 mice weighing 25 ± 5 g were purchased from Jinan pengyue laboratory animal breeding co.ltd. 20 mice were divided into control group and periodontitis group by the way of simple random sampling. All mice received normal chow diet and were maintained in a temperature-controlled room at 25°C under a 12 h light/dark diurnal cycle. Authors were aware of the group allocation during the conduction of the experiments. However, they were blind during the outcome assessment and the data analysis.

All animal experiments comply with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

The establishment of experimental periodontitis was performed according to the method of et al (25). Mice were anesthetized with an intraperitoneal injection of 1 % Pentobarbital (50mg/1000g body weight). Then silk thread ligation was operated in the cervical area of left maxillary second molar with nylon thread (No. 5 – 0). Using microinjectors, 10 μl of 1.0 mg/ml LPS from P. gingivalis (Invivogen, Toulouse, France) was injected into the gingival tissues adjacent to the left upper second molars every other day for 2 weeks. Control mice were injected with the equivalent volume of PBS (pH 7.4) in the similar anatomical regions with no ligatures. After 2 weeks, animals are euthanized by using an overdose of anesthesia (intraperitoneal injection of 1% Pentobarbital, 100mg/1000g body weight). Tissue samples were not collected until the animals were completely unconscious.

For statistical analysis, 10 animals were sacrificed for both control and periodontitis groups. In each experiment, the highest and lowest sample values will be removed, and the rest samples will be reserved for statistical analysis. At 2 weeks after ligation, fresh heparinized peripheral blood samples were collected for flow cytometry and ELISA analysis. Gingival tissues from both experimental and control mice were prepared for RNA extraction. For treg cell sorting, spleens were also taken under sterile conditions from each group. Following that, maxillary bone was extracted and immersed in the 4% paraformaldehyde fixative for 24 h. To evaluate alveolar bone loss, specimens were analyzed using a Micro CT system. After that, all specimens were embedded in paraffin after a series of processes including demineralization with 10% EDTA-2Na solution and dehydration through an ascending ethanol

series. 5 μm thickness serial sections were prepared throughout the specimen for following histological analysis.

Detection of cytokines IL-1β, IL-6, and IL-17 by ELISA

To determine serum cytokine levels, sera were collected from blood drawn of experimental and control mice. IL-1β, IL-6, and IL-17 levels were measured using cytokine-specific enzyme-linked immunosorbent assay (ELISA) kits (ProteinTech Group, Chicago, IL, USA) according to the protocols provided by the manufacturer.

Measurement of cytokines in gingiva by Real-time quantitative PCR

The buccal gingival tissues (ranging from the mesial margin of the first molar to the distal margin of the third molar) from each group were diced into 1-mm fragments and homogenized for RNA extraction. Total RNA was extracted with an RNA extraction reagent (TRIZOL) (Takara, Tokyo, Japan) and then complementary DNA was synthesized in a reverse transcription (RT) reaction (Takara). According to the instructions of the manufacturer, polymerase chain reaction (PCR) analysis of inflammatory cytokines (TNF-α, IL-1β, IL-18, IL-6), Treg cell-related cytokines (TGF-β1, IL-10, Foxp3) and Th17 cell-related factors (Rorc, IL-17A) were performed using Roche Light Cycler 480 system (Roche, Basel, Switzerland). The relative target gene quantification was calculated using the comparative threshold (CT) method following normalization to GAPDH. All experiments were performed in triplicate independently.

Micro CT Analysis

In order to evaluate the severity of bone destruction in experimental periodontitis, all specimens were analyzed using a Micro CT system (Quantum FX, Caliper Life Sciences, USA). Scaning was performed from the mesial margin of the first molar to the distal margin of the third molar, along the long axis of maxillary bone (source voltage 80 kV, source current 100 μA, and image pixel size 9.8). Then, two dimension (2D) images were reconstructed into 3D images for analysis using software package (Quantum FX). Crestal bone loss (mm) was calculated to reflect the degree of alveolar bone resorption.

Histological examination and image analysis

To identify the histological changes of alveolar bone in periodontitis model, H&E staining was performed and then digital images were taken using a light microscope (Olympus BX-53, Tokyo, Japan). Thickness of alveolar bone on buccal side of maxillary second molar (um) were measured with the aid of Image Pro Plus 6.2 software (Media Cybernetics, Silver Spring, MD). For statistical analysis, three tissue slices were collected from each of the three levels (apical area, root center, and cervical part) and the average level of parallel samples were used for each group. Control and periodontitis group were compared using the sections from equal level.

Immunohistochemistry examination and image analysis

For immunolocalization of ALP, RANKL, Foxp3, IL-17, TGF-β and IL-6, 5 μm-thick paraffin sections were dewaxed in xylene and rehydrated in ethanol series. To block endogenous peroxidases, sections were preincubated with 0.3% hydrogen peroxide. Then nonspecific binding was reduced by treating sections with 1% bovine serum albumin in phosphate-buffered saline (BSA-PBS) for 20 min at room temperature. After that, sections were then incubated for 2 h at room temperature with: 1) rabbit antiserum against rat tissue-nonspecific ALPase, generated by Oda et al (Oda et al. 1999) at a dilution of 1:75; 2) anti-RANKL antibody (Abcam, Cambridge, MA, USA) at a dilution of 1:50; 3) anti-Foxp3 antibody (ProteinTech Group, Chicago, IL, USA) at a dilution of 1:50; and 4) anti-IL-17 antibody (Abcam) at a dilution of 1:50 ; 5) anti-TGF-β antibody (Abcam) at a dilution of 1:50; and 6) anti-IL-6 antibody (Proteintech) at a dilution of 1:50 in 1% BSA-PBS. After rinsing with PBS, sections were treated with horseradish peroxidase (HRP)-conjugated Goat Anti-Rabbit IgG H&L (Abcam) at a dilution of 1:100 for 1 h at room temperature. Furthermore, the immunostaining was visualized using diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) as the substrate. The primary antibody was replaced with 1×PBS as a negative control.

After finishing ALP visualizing using DAB, double staining with tartrate-resistant acid phosphatase (TRAP) were performed by employing enzyme histohemistry as previously showed (26)(Li et al., 2013) (26). In brief, sections were submerged in a mixture of 3.0 mg of naphthol AS-BI phosphate (Sigma), 18 mg of red violet LB salt (Sigma), and 100 mM L(+) tartaric acid (0.36 g) diluted in 30 ml of 0.1 M sodium acetate buffer (pH 5.0) for 15 min at 37 ºC. Staining were then assessed by light microscopy (Olympus) after faintly counterstaining with methyl green.

Statisticaly, ten sections were selected for each sample and the mean parameters of parallel samples were calculated. Immunostaining intensity of ALP, RANKL, TGF-β and IL-6 was measured in three randomly selected non-overlapping microscopic by Image-Pro Plus 6.2 software. Also, TRAP-positive osteoclast numbers, Foxp3-positive cell numbers, IL-17-positive cell numbers were counted in similar view of serial sections at an original magnification of ×400.

Isolation of Splenic lymphocytes and Flow Cytometry

Fresh heparinized peripheral blood samples from each group were collected. Spleens were removed and disrupted on 75-mm stainless steel screens. After being isolated using mouse lymphocyte separation medium (Dakewe Biotechnology, Shenzhen, Guangdong, China) centrifugation, single mononuclear cells were suspended in cell culture medium with 10% fetal calf serum. Cell counting was performed in a hemocytometer after assessing cell viability by trypan blue staining.

The cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 and allophycocyanin (APC)-conjugated anti-mouse CD25 antibody (BD Biosciences, San Diego, CA, USA) for 30 min on ice. After being permeabilized with a cytofix/cytoperm solution, the cells were incubated with an phycoerythrin (PE)-conjugated anti-Foxp3 antibody (BD Biosciences) for 1h at 4°C. Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA) was used to detect the labeled cells, and Kaluza Flow Cytometry Analysis Software (Beckman) was used to analyze the data. Lymphocytes were gated according to their forward and side-scatter characteristics. FITC, APC and PE labeled isotype-matched

control mAbs (BD Biosciences) were used to determine the positive and negative expression of each molecule. The results were expressed by the percentage of CD25 + Foxp3 + in gated CD4 + cell. All experiments were explored in triplicate independently.

Isolation of CD4 + CD25 + T cells

Splenic single-lymphocyte suspension was prepared and then CD4 + CD25 + T cells were isolated using the “Regulatory T cell isolation kit” (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. MACS buffer (Bie & Berntsen, Denmark) and the AutoMACS Running Buffer (Miltenyi Biotec) was used for incubation with beads and cell separations on the AutoMACS Cell Separator respectively. The purity of CD4 + CD25 + T cell were above 85%. CD4 + CD25 + T cells were homogenized in TRIZOL for RNA extraction. And, PCR analysis of TGF-β1, IL-10, Foxp3, Rorc, IL-17A were performed as described above.

Statistical Analysis

All statistical analyses were performed using SPSS software and all values are presented as mean ± standard deviation. Differences among groups were assessed by the unpaired t test. $P < 0.05$ was considered statistically significant and $P < 0.001$ represent highly significant.

Results

Successful establishment of an experimental periodontitis model

To explore systemic inflammatory changes in mice after silk ligation and Pg-LPS injection, we first examined the expression levels of proinflammatory cytokines (IL-1β, IL-6, and IL-17) by ELISAs. The serum levels of IL-1β (0.2050 ± 0.0202 in control vs 6.597 ± 0.3735 in periodontitis, $P < 0.001$), IL-6 (6.057 ± 0.3185 in control vs 24.22 ± 0.8333 in periodontitis, $P < 0.001$), and IL-17 (11.03 ± 0.09855 in control vs 13.48 ± 0.2232 in periodontitis, $P < 0.001$) were all increased significantly in the periodontitis group compared to the control group (Fig. 1A-C).

We next determined the gene expression levels of various proinflammatory cytokines in gingival tissue by real-time RT-PCR. The mRNA levels of TNF-α, IL-1β, IL-18, and IL-6, which contribute to alveolar bone loss, were elevated in periodontitis mice (TNF-α: 7.2-fold, $P < 0.001$; IL-1β: 30.5-fold, $P < 0.001$; IL-18: 1.7-fold, $P < 0.001$; IL-6: 65.0-fold, $P < 0.001$) (Fig. 1D-G). Collectively, these results indicated that silk ligation and Pg-LPS injection induced an obvious in vivo inflammatory response in gingival tissue.

Next, micro-CT was used to investigate periodontal bone loss induced by silk ligation and Pg-LPS injection. As shown in Fig. 1H and J, alveolar bone in the control group was intact and the position of the alveolar ridge crest (ABC) was closed to the cemento-enamel junction (CEJ). The 3D images indicted both vertical and horizontal bone resorptions in the periodontitis group and the remaining bone was characterized by an osteoporosis structure (Fig. 1I, K). The distance from the CEJ to the ABC was

measured to quantitate periodontal bone loss, which we refer to as crestal bone loss. Statistical analysis indicated that crestal bone loss was increased markedly after silk ligation and Pg-LPS injection compared with the control group (0.4200 ± 0.01571 in control vs 1.220 ± 0.02082 in periodontitis, $P < 0.001$) (Fig. 1L). Thus, we experimentally achieved the condition of periodontitis by ligature and Pg-LPS injections.

Histological alterations in the maxillary alveolar bone

In control mice, the alveolar bone around maxillary second molars was intact with an abundant bone matrix and the periodontal ligament was arranged regularly (Fig. 2A, C). However, in the periodontitis group, disordered ligature was observed and only a few dotted or needle-shaped bone islands remained around maxillary second molars. Moreover, the periodontal ligament structure presented a deranged distribution (Fig. 2B, D). Statistical analysis indicated significant differences in alveolar bone thickness between periodontitis and control mice (167.1 ± 3.564 in control vs 24.48 ± 1.653 in periodontitis, $P < 0.001$) (Fig. 2E).

Numerous osteoblasts that showed obvious alkaline phosphatase (ALP) activity were found evenly on the surface of alveolar bone as well as the periodontal ligament in control mice (Fig. 2F). In the periodontitis group, osteoblasts were scattered within periodontal fibrous tissue or around residual bone tissue (Fig. 2G). Although ALP activity of osteoblasts in the periodontitis group appeared to have increased slightly, there was no statistical difference between the two groups (0.2884 ± 0.002632 in control vs 0.2865 ± 0.001763 in periodontitis, $P > 0.05$) (Fig. 2J). A small amount of TRAP-positive osteoclasts was observed in the control group, which were distributed on the periosteal side and the periodontal side of the alveolar bone (Fig. 2F). The number of TRAP-positive osteoclasts was increased significantly in the periodontitis group and multiple osteoclasts distributed in clusters were observed (Fig. 2G). Consistent with these observations, statistical analysis indicated that the number of osteoclasts was increased significantly in the periodontitis group (6.500 ± 0.4830 in control vs 21.42 ± 0.4167 in periodontitis, $P < 0.001$) (Fig. 2K).

RANKL expression was detected to further clarify the coupling between osteoblasts and osteoclasts. The control group showed very weak RANKL immunoreactions, whereas RANKL expression was increased significantly in the periodontitis group (Fig. 2H, I). Statistical analysis showed a significant difference in the RANKL immunostaining intensity between the two groups (0.1891 ± 0.001814 in control vs 0.2273 ± 0.004378 in periodontitis, $P < 0.001$) (Fig. 2L)

Th17/Treg proportion becomes disordered in periodontitis mice

The mRNA expression levels of Treg cell-related cytokines (TGF-β1, IL-10, and Foxp3) and Th17 cell-related factors (Rorc and IL-17A) in gingival tissues were measured by real-time RT-PCR. As shown in Fig. 3, increased TGF-β1, Foxp3, Rorc and IL-17A expression in gingival tissues (TGF-β1: 4.0-fold, $P < 0.01$; Foxp3: 3.8-fold, $P < 0.01$; Rorc: 9.9-fold, $P < 0.001$; IL-17A: 17.5-fold, $P < 0.001$) (Fig. 3A, C–E) was observed

in periodontitis mice compared with control mice, although no significant difference was found in IL-10 expression between the two groups (0.8-fold, $P > 0.05$; Fig. 3B). Further analysis showed that expression of Treg cell-related genes (TGF-β1 and Foxp3) was increased by about three times in the periodontitis group, while that of Th17 cell-related genes (Rorc and IL-17A) was increased by nine and seventeen times, respectively. These results indicated that the expression of Th17 cell-related genes was increased more significantly than those of Treg cells during periodontal inflammation.

We next detected the expression and distribution of Foxp3 and IL-17 in alveolar bone by immunohistochemical staining. There were few Foxp3-positive cells in the control group (Fig. 3F), whereas the number of Foxp3-positive cells was increased in periodontitis mice (Fig. 3G). Serial sections revealed the presence of IL-17-positive cells in fibrous tissue around the alveolar bone in the control group (Fig. 3H), which was more strongly expressed around the inflamed alveolar bone in periodontitis mice (Fig. 3I). Statistical analysis revealed significantly greater numbers of Foxp3-positive (8.917 ± 0.4362 in control vs in 15.33 ± 0.3575 periodontitis, $P < 0.001$) (Fig. 3J) and IL-17-positive cells (211.8 ± 6.983 in control vs 319.8 ± 4.377 in periodontitis, $P < 0.001$) (Fig. 3K) in periodontitis mice compared with control mice. Further comparisons revealed that the amplification of IL-17-positive cells was more significant than that of Foxp3-positive cells, indicating a disordered Th17/Treg ratio in experimental periodontitis mice.

To explain this Th17/Treg disorder, we further examined the expression of TGF-β and IL-6 that play critical roles during Th17/Treg cell generation. As shown in Fig. 3, the control group exhibited obvious staining of TGF-β and IL-6 that were mainly expressed in fibroblasts and also in osteoblasts (Fig. 3L, N). Periodontitis mice showed more extensive immunoreactivity against TGF-β and IL-6 than control mice, and the amplification of IL-6 was more significant (Fig. 3M, O). Statistical analysis of the integrated optical density revealed significantly higher expression of TGF-β (822.5 ± 15.74 in control vs 863.4 ± 7.984 in periodontitis, $P < 0.05$) and IL-6 (779.8 ± 12.76 in control vs 1016 ± 14.83 in periodontitis, $P < 0.001$) in the periodontitis group than in the control group (Fig. 3P, Q).

Periodontal inflammation may induce Treg-Th17 conversion

To further evaluate the role of Treg cells in periodontitis, the percentages of CD4 + CD25 + Foxp3 + T cells among CD4 + T cells were examined by flow cytometry. As shown in Fig. 4, both flow cytometry and statistical analysis showed that the percentages of CD4 + CD25 + Foxp3 + T cells among CD4 + T cells in the peripheral blood (11.51 ± 0.2194 in control vs 8.820 ± 0.1482 in periodontitis, $P < 0.001$, Fig. 4A, B, E) and spleens (9.105 ± 0.08984 in control vs 6.680 ± 0.1417 in periodontitis, $P < 0.001$, Fig. 4C, D, F) of periodontitis mice were decreased markedly compared with those of control mice.

To understand the possible mechanisms of immunoregulation during periodontitis mediated by Treg cells, CD4 + CD25 + T cells were isolated and then analyzed for the expression patterns of Treg cell markers and Th17-related cytokines. As shown in Fig. 4, although there was no significant difference in mRNA expression levels of TGF-β1 and IL-10 (TGF-β1: 0.7-fold, $P > 0.05$; IL-10: 1.0-fold, $P > 0.05$) (Fig. 4G and H), Foxp3 mRNA expression levels in CD4 + CD25 + T cells were decreased significantly in

periodontitis mice as compared with control mice (Foxp3: 0.6-fold, $P < 0.05$) (Fig. 4I). Furthermore, periodontitis mice had markedly upregulated levels of Rorc and IL-17A mRNA expression comparing with control mice (Rorc: 2.1-fold, $P < 0.05$; IL-17A: 9.7-fold, $P < 0.001$) (Fig. 4J and K). These results indicated that periodontal inflammation may induce Treg-Th17 conversion is manifested by impaired immunosuppressive functions of Treg cells and activation of Th17 inflammatory pathways.

Discussion

Upon invasion of periodontal pathogens, neutrophils and monocytes/macrophages in peripheral blood and gingival crevicular fluid are activated and then release various inflammatory mediators such as TNF-α, IL-1, and IL-6 (27, 28). Consistently, previous studies have shown that the levels of inflammatory factors in serum of patients with periodontitis are significantly higher than those of healthy groups, and the expression of inflammatory factors is closely related to the occurrence and development of CP (29). In the present study, inflammatory factors were used to evaluate the degree of periodontal infection and the systemic inflammatory response after establishment of an experimental periodontitis model. ELISAs of serum samples and quantitative analysis of gene expression in gingival samples showed that TNF-a, IL-1β, IL-6, and IL-18 levels were increased significantly in experimental periodontitis mice, which confirmed local and systemic inflammatory responses induced by silk ligation combined with Pg-LPS injection.

Physiologically, alveolar bone undergoes an orderly and coupled bone resorption and bone formation process, i.e., bone remodeling. CP is essentially the destruction of bone remodeling homeostasis caused by inflammation and immune responses, which ultimately causes alveolar bone resorption (30). In the current study, micro-CT analysis revealed that increased crestal bone loss was involved in periodontitis mice. Histomorphological examination also showed that only a few dotted or needle-shaped bone islands remained around maxillary second molars in periodontitis mice. Next, we found increased secretion of RANKL by osteoblasts, thereby promoting the formation of osteoclasts and enhancing their activity in periodontitis mice. Therefore, we experimentally achieved the condition of periodontitis characterized by greater bone resorption by ligature and Pg-LPS injections.

Accumulating data are indicating that the immune system plays an important role in the process of CP. In particular, the role of the CD4+ T cell-mediated cellular immune response has been studied extensively (3). Th17 and Treg cells are CD4+ T cell subsets with opposite regulatory functions. Th17 cells are characterized by secreting proinflammatory factor IL-17. Their proliferation and differentiation require the involvement of the transcription factor retinoid-related orphan receptor (RORγt). Treg cells secrete anti-inflammatory factors TGF-β and IL-10, and their differentiation and maturation depend on the specific transcription factor Foxp3 (31). Many studies have provided evidence showing that a Th17-Treg imbalance is a major factor in the development of CP (9). Wang et al. found that oral administration of drugs enhances activation of Treg cells, inhibits activation of Th17 cells, and significantly inhibits absorption of alveolar bone (32, 33). In this study, we found that the Treg cell number and their expression of related genes (Foxp3 and TGF-β1) as well as the Th17 cell number and their expression of related genes (Rorc and IL-17A) in periodontal tissue were all increased in the periodontitis group. Further

analysis showed that the number of Th17 cells and expression of related genes were increased more significantly than in Treg cells. These results further validated the phenomenon of the Treg-Th17 imbalance in periodontal inflammation. Regarding the inconsistency with previous studies reporting a decrease of the Treg cell number and increase of Th17 cell differentiation during periodontitis, a possible explanation is the variation of the Th17/Treg balance in different stages of periodontal disease (10, 11).

There is a competitive effect in the process of initial CD4 + T cells producing Th17 or Treg cells, which depends on the local concentrations of TGF-β and IL-6 (34). A high concentration of TGF-β inhibits RORγt functions and promotes Treg cell formation by increasing Foxp3 expression. However, under the action of a low concentration of TGF-β, IL-6 promotes expression of RORγt that silences the Foxp3 gene and promotes differentiation of Th17 cells. We further found that both immunoexpression of TGF-β and IL-6 was increased in the periodontitis group, although the expression of IL-6 was amplified more significantly. Therefore, we believe that a certain concentration of TGF-β promotes the formation of Treg cells, whereas a higher concentration of IL-6 may promote more distinct differentiation of Th17 cells. This explains, to a certain extent, why the Th17 cell number and related gene expression levels were increased more significantly than those of Treg cells.

Treg cells have plasticity that enables them to convert into Th17 cells during tumorigenesis, inflammatory conditions, and autoimmune diseases (35, 36). There are currently two theories explaining Treg cell plasticity (23). The first theory is the reprogramming model in which Foxp3+ Treg cells are recoded and convert into Foxp3+ Treg cells secreting IL-17. Another theory is the heterogeneous model suggesting that Foxp3+ Treg cells, which are unstable and easily lose Foxp3 expression, convert into Foxp3- Treg cells and then into Th17 cells. In our study, flow cytometry showed that the proportion of Treg cells in the blood and spleen of periodontitis mice was lower than that in control mice. Furthermore, Treg cell-related gene Foxp3 was downregulated and their expression of Th17 cell-related genes (Rorc and IL-17) was increased. These results indicated that CD4+CD25+ T cells isolated from the spleen heterogeneously expressed Foxp3 and Rorc simultaneously. The periodontitis microenvironment decreases the expression of Foxp3 and upregulates Rorc expression, leading to Treg-Th17 conversion (Fig. 5). Previous studies have shown that various cytokines (e.g., IL-1β, IL-6, and IL-23) promote the conversion of Treg cells into Th17 cells (36-38). Singh et al. reported that defective expression of CD18 also induces Treg cells producing IL-17 (39). However, this issue of Treg cell plasticity remains controversial because unequivocal evidence for lineage reprogramming and the relevant mechanisms need to be studied further.

List Of Abbreviations

Chronic periodontitis (CP)

CD4+ T helper cells (CD4+ T cells)

Regulatory T cells (Treg) cells

Interferon-γ (IFN- γ)

Interleukin-17 (IL-17)

Nuclear factor-kappa B receptor activating factor ligand (RANKL)

Forkhead box P3 (Foxp3)

Retinoid-related orphan receptor (RORγt)

Declarations

Ethics approval and consent to participate:

All studies involving animals were carried out adherence to the ARRIVE guidelines (PLoS Bio 8(6), e1000412,2010). Also, anaesthesia and euthanasia of animals complied with the American Veterinary Medical Association (AVMA) Guidelines (2020). All experimental protocols were approved by Ethics Committee of Shandong University (No.GD201815).

Consent to publish:

Not applicable

Availability of data and materials

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

Competing interests:

Hongrui Liu declare that she has no conflict of interest.

Dongfang Li declare that he has no conflict of interest.

Minqi Li declare that he has no conflict of interest.

Fundings:

This research was supported by the National Natural Science Foundation of China (No. 81800982), The Construction Engineering Special Fund of “Taishan Scholars” of Shandong Province (No.ts20190975), The Fundamental Research Funds of Shandong University (No. 2019GN042), Young Scholars Program of Shandong University, The Special fund for postdoctoral innovation project of Shandong Province (No. 201702006). The funders had no role in study design, data collection and analysis, decision to publish or

preparation of the manuscript. We thank Mitchell Arico from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Authors' Contributions:

All authors have made substantial contributions to conception and design of the study. HL and DL have been involved in data collection and data analysis. HL and ML have been involved in data interpretation, drafting the manuscript and revising it critically and have given final approval of the version to be published.

Acknowledgements:

Not applicable

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Figures

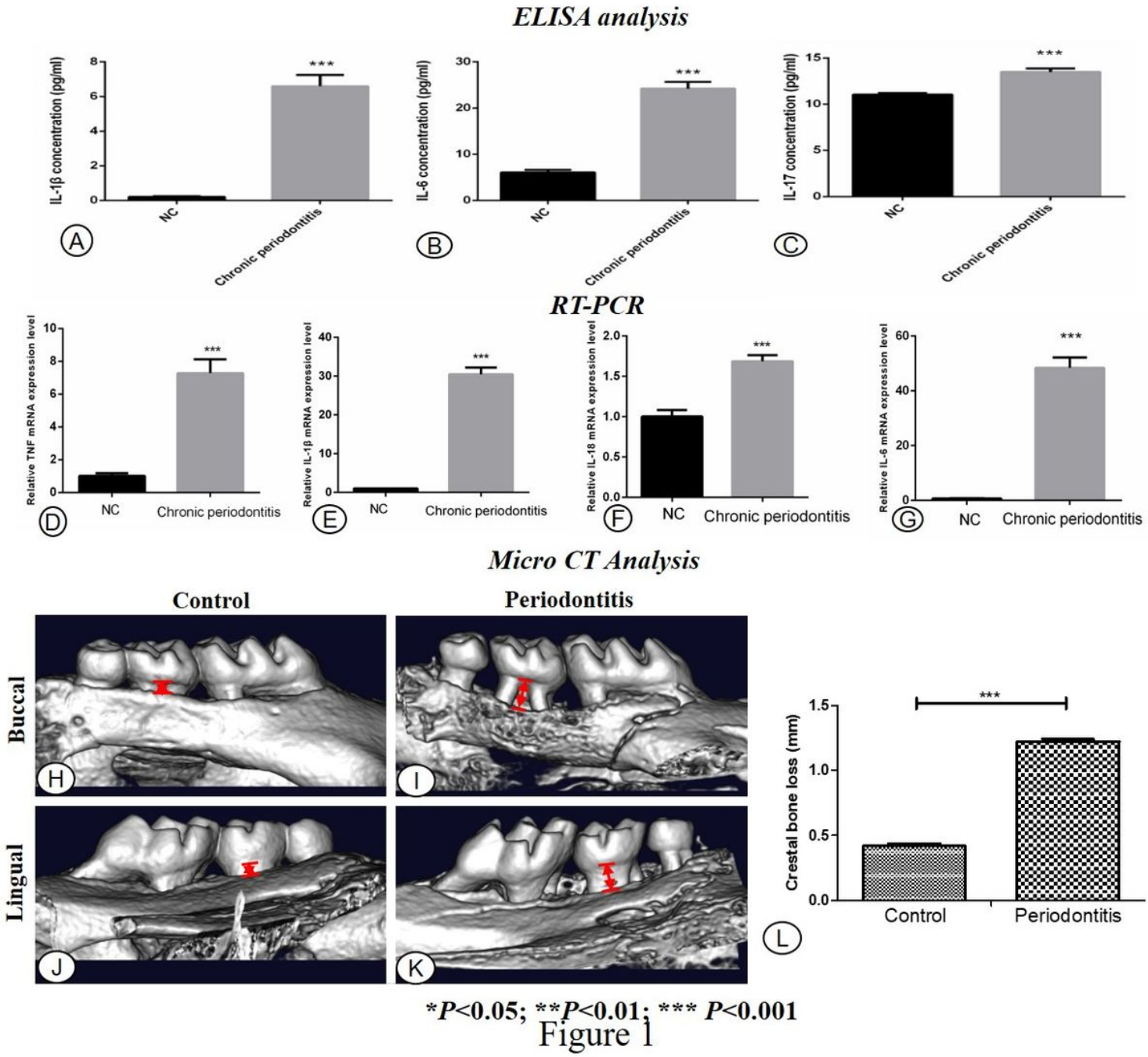


Figure 1

Figure 1

Successful establishment of an experimental periodontitis model. The serum expression levels of pro-inflammatory cytokines IL-1β (A), IL-6 (B), and IL-17 (C) by ELISA and statistical analysis. Relative mRNA expression level of TNF-α (D), IL-1β (E), IL-18 (F) and IL-6 (G) in gingiva and statistical analysis. 3D images of buccal/lingual maxillary bone in control group (H, J) and periodontitis group (I, K). Statistical analysis for crestal bone loss (L). The serum levels of IL-1β, IL-6, and IL-17 all significantly increased in periodontitis group compared to the control group (A-C, n=10, P<0.001). The mRNA levels of TNF-α, IL-1β, IL-18 and IL-6 were elevated in periodontitis mice (D-G, n=10, P<0.001). Alveolar bone in control group was intact, and the position of the ABC was closed to CEJ (H, J). Both vertical and horizontal bone resorption were involved in periodontitis groups and the remaining bone is characterized by osteoporosis structure (I, K). Statistical analysis indicated that the crestal bone loss increased markedly after silk ligation and Pg-LPS injection compared to the control group (L, n=10, P<0.001). Error bars indicate ± SD.

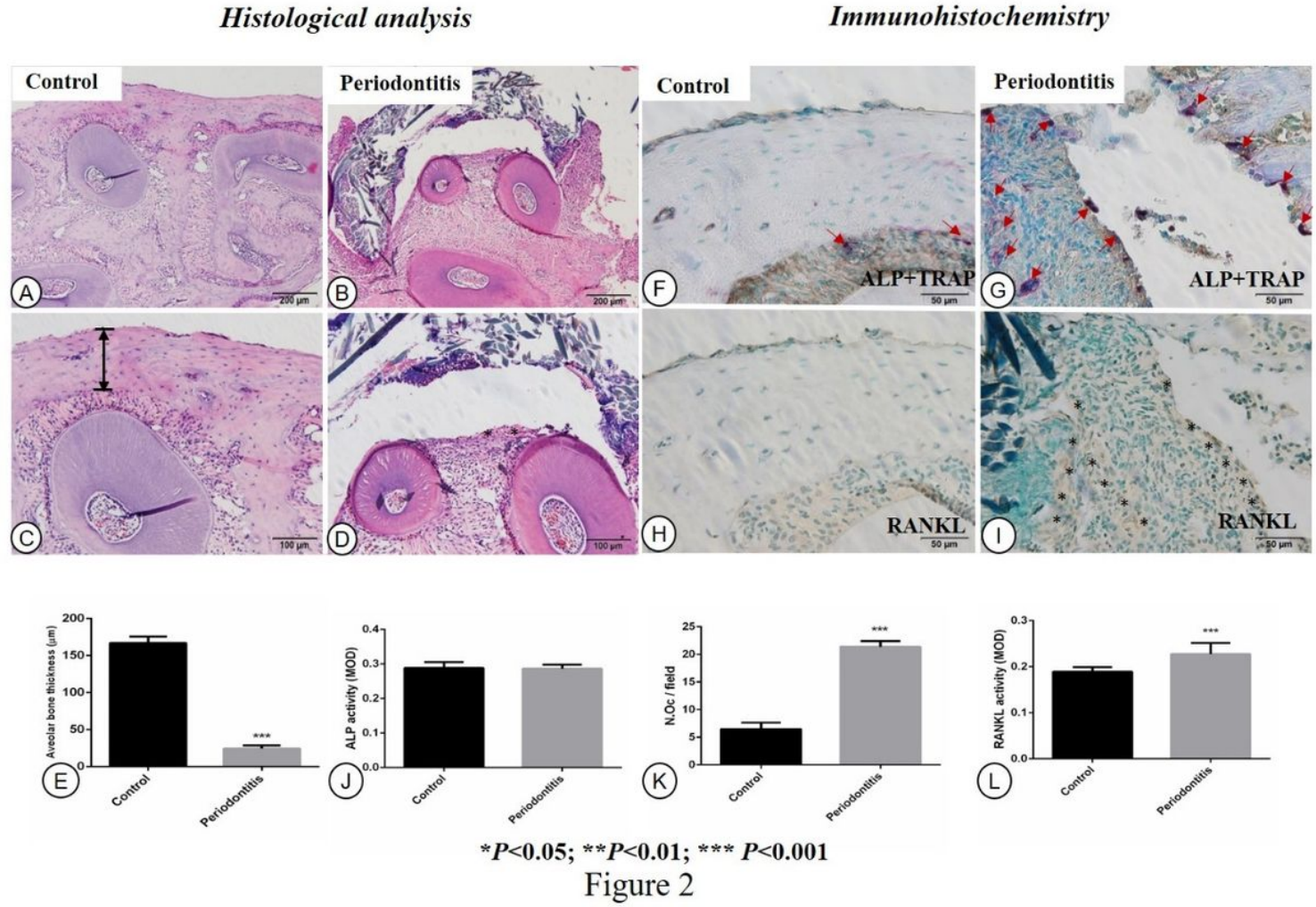


Figure 2

Figure 2

Histological alterations in the maxillary alveolar bone. Low/high magnification images of the alveolar bone in control group (A, C) and periodontitis group (B, D). Statistical analysis for alveolar bone thickness (E). Double staining for ALP (brown) and TRAP (red) in alveolar bone of control group (F) and

periodontitis group (G). Immunostaining of RANKL in control group (H) and periodontitis group (I). Statistical analyses for activity of ALP (J), TRAP positive osteoclast number (K) and activity of RANKL (L). In control mice, the alveolar bone was intact with abundant bone matrix, whereas only a few dotted or needle-shaped bone islands remained in periodontitis (A-D). Statistical analysis indicated significant decreased alveolar bone thickness (E, n=10, P<0.001). Although ALP activity of osteoblasts in periodontitis group seemed to have increased slightly but there was no statistical difference between the two groups (F, G, J, n=10, P>0.05). The number of TRAP-positive osteoclasts increased significantly in periodontitis group, and it was detectable that multiple osteoclasts distributed in clusters (F, G, K, n=10, P<0.001). Control group showed very weak RANKL immunoreaction, while RANKL immunostaining intensity significantly increased in the periodontitis group (H, I, L, n=10, P<0.001). Error bars indicate ± SD.

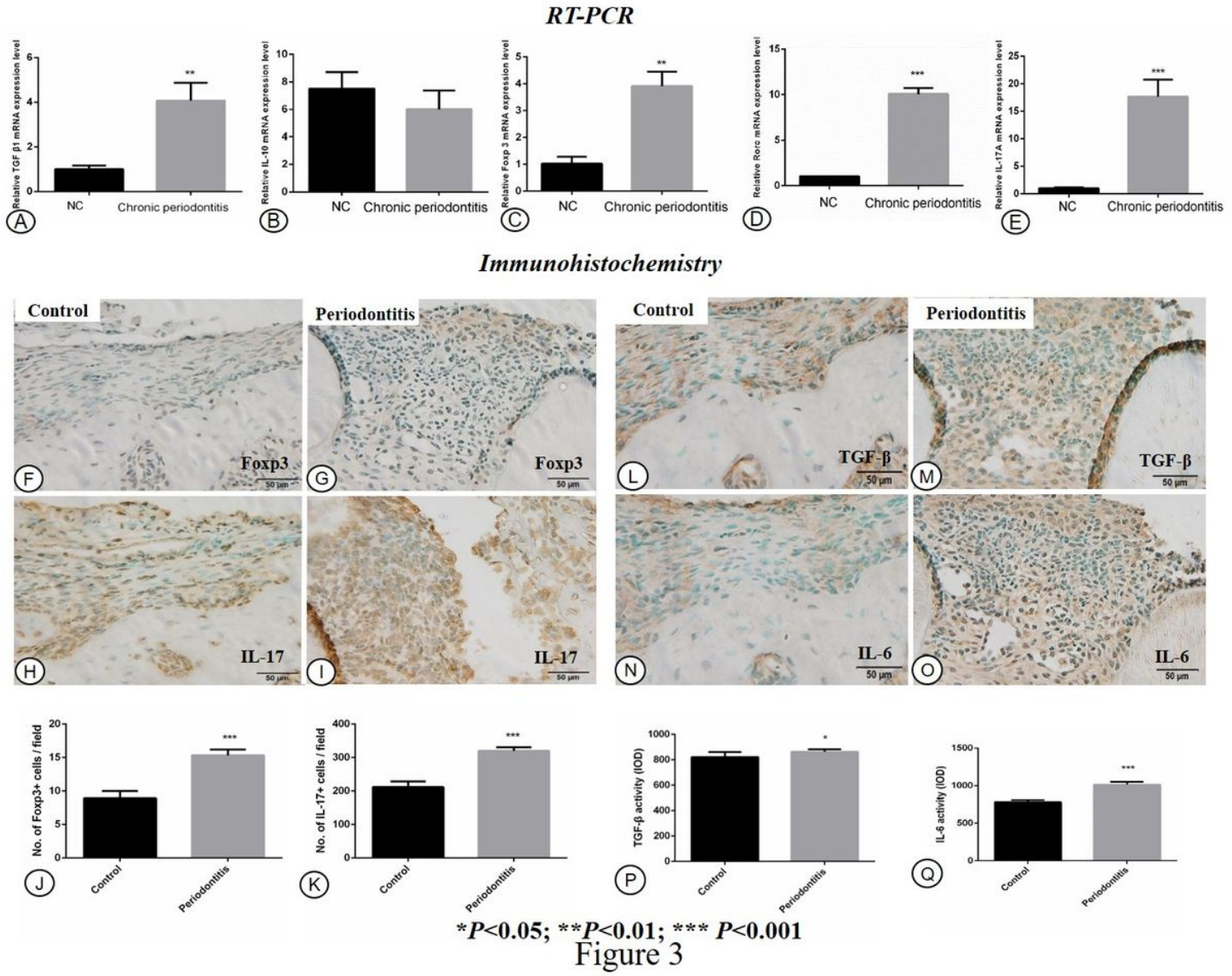
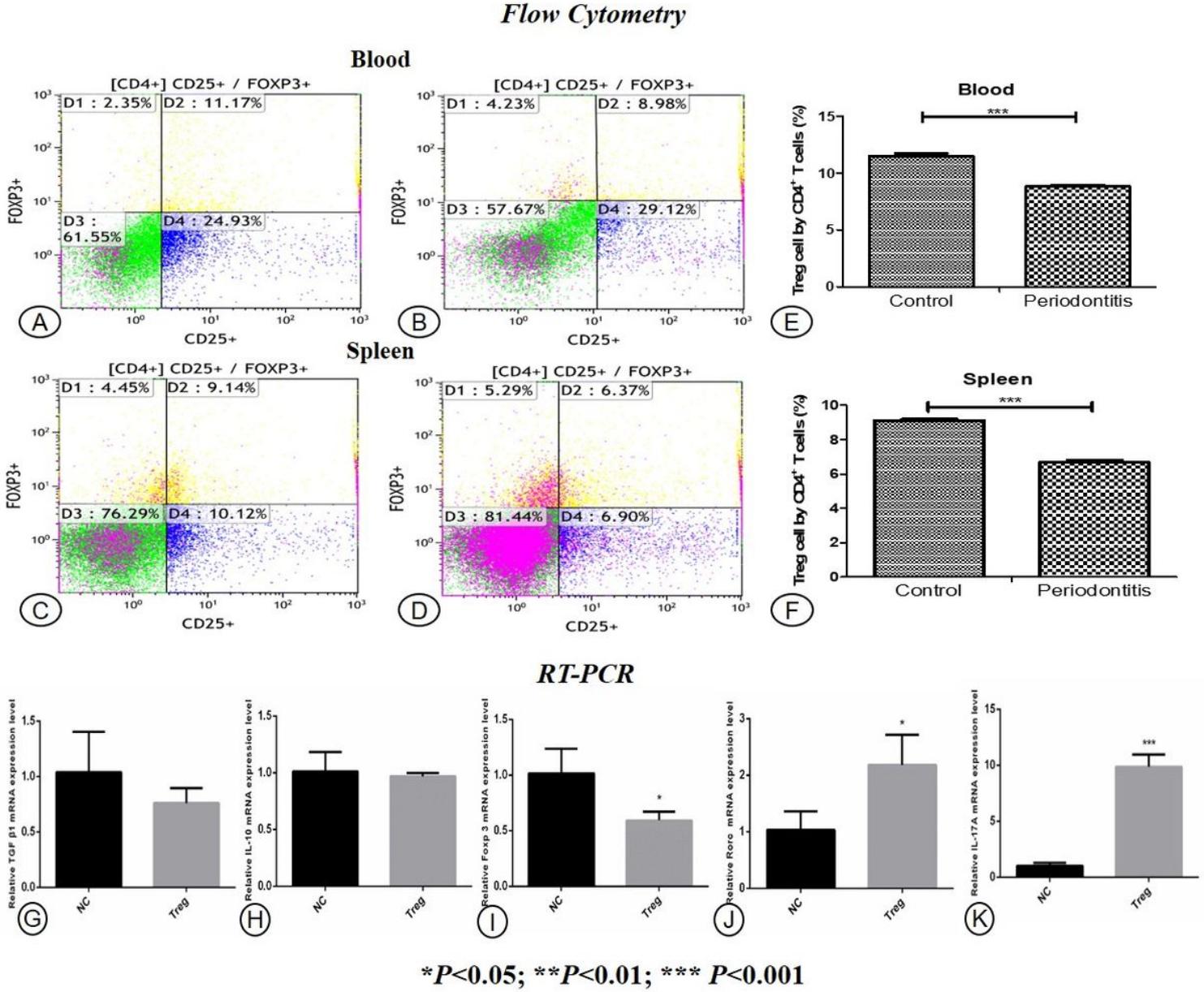


Figure 3

Figure 3

Th17/Treg proportion becomes disordered in periodontitis mice. Relative mRNA expression level of TGF-β1(A), IL-10 (B), Foxp3 (C), Rorc (D) and IL-17A (E) in gingiva and statistical analysis. Immunostaining of

Foxp3/IL-17 in control group (F, H) and periodontitis group (G, I). Statistical analysis for Foxp3 positive cell number (J) and IL-17 positive cell number (K). Immunostaining of TGF-β/IL-6 in control group (L, N) and periodontitis group (M, O). Statistical analysis for TGF-β activity (P) and IL-6 activity (Q). Increased TGF-β1, Foxp3, Rorc and IL-17A expression in gingival tissues (A, C, D, E, n=10) were observed, although no significant difference was found in IL-10 expression between two groups (B, n=10, P>0.05). Serial sections and statistical analysis revealed significantly greater numbers of Foxp3 positive (F, G, J, n=10, P< 0.001) and IL-17 positive cells (H, I, K, n=10, P< 0.001) in periodontitis than control group. Further comparisons revealed that the amplification of IL-17-positive cells was more significant than that of Foxp3-positive cells. Periodontitis mice showed more extensive immune reactivity against TGF-β and IL-6 than control mice, and the amplification of IL-6 was more significant (L-O). Statistical analysis of IOD revealed that significantly higher expression of TGF-β (P, n=10, P< 0.05) and IL-6 (Q, n=10, P< 0.001) in periodontitis group than that of control group. Error bars indicate ± SD.



*P<0.05; **P<0.01; *** P<0.001

Figure 4

Figure 4

Periodontal inflammation may induce Treg-Th17 conversion. Flow cytometry of CD4+CD25+Foxp3+ T cells among CD4+T cells in the peripheral blood and spleens in control group (A, C) and periodontitis group (B, D). Statistical analysis of CD4+CD25+Foxp3+ T cells by CD4+T cells in blood (E) and spleen (F). Relative mRNA expression level of TGF-β1(G), IL-10 (H), Foxp3 (I), Rorc (J) and IL-17A(K) in CD4+CD25+ T cells and statistical analysis. Both flow cytometry and statistical analysis showed that the percentages of CD4+CD25+Foxp3+ T cells among CD4+T cells in the peripheral blood (A, B, E, n=10, P< 0.0001) and spleens (C, D, F, n=10, P<0.0001) were markedly decreased. There was no significant difference in mRNA expression levels of TGF-β1 and IL-10 (G, H, n=10, P>0.05). Foxp3 mRNA expression levels were significantly decreased in periodontitis mice as compared to control mice (I, n=10, P<0.05). Furthermore, periodontitis mice present markedly up-regulated levels of Rorc and IL-17A mRNA expression when comparing to those levels of control mice (J, K, n=10) (Rorc, P<0.05; IL-17A, P<0.001). Error bars indicate ± SD.

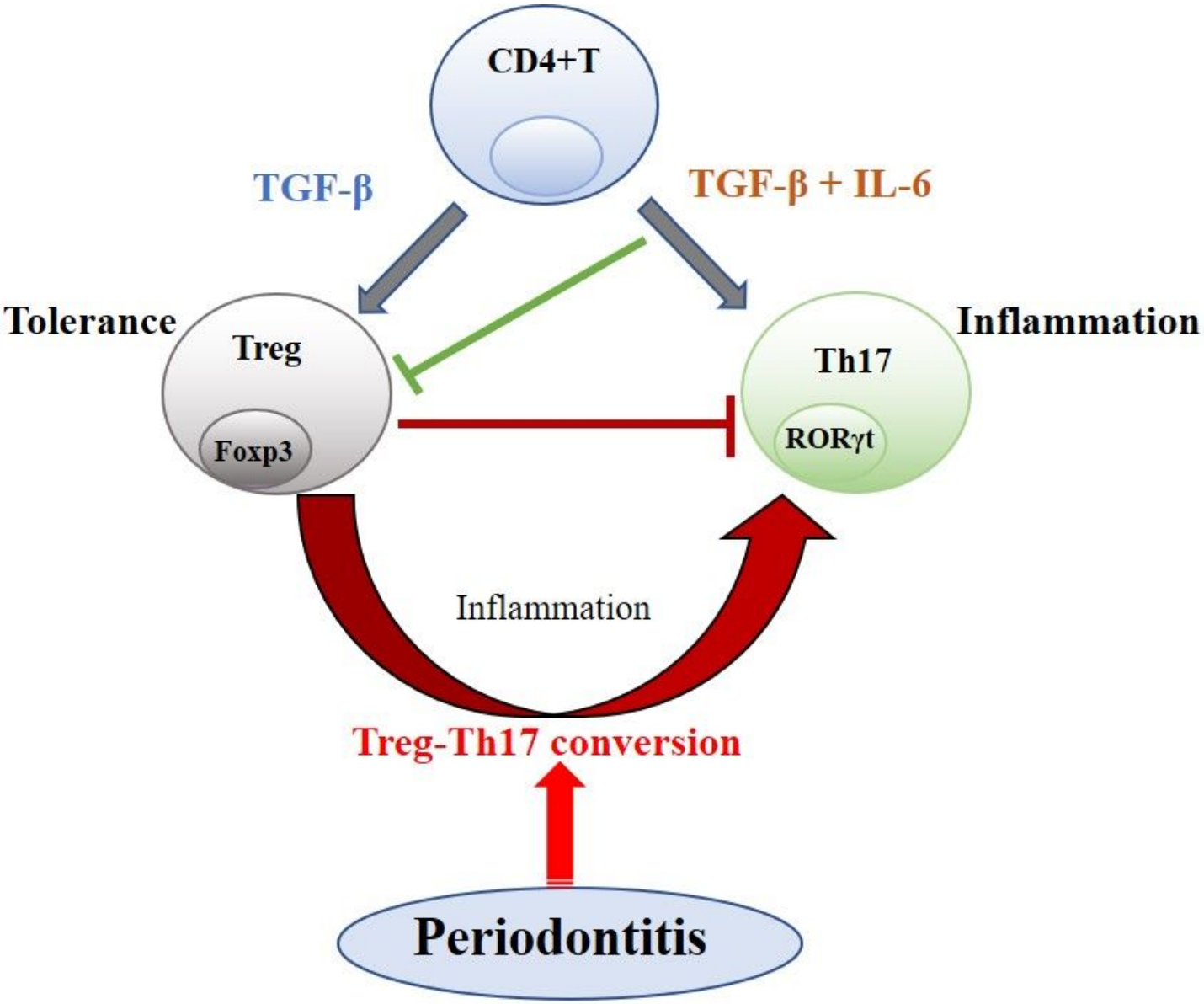


Figure 5

Figure 5

A hypothetical scheme illustrating periodontitis mediated conversion of CD4+CD25+ T cells into proinflammatory interleukin 17-producing Th17 cells. Periodontitis microenvironment can decrease the expression of Foxp3 in CD4+CD25+T cells and up-regulate Rorc, leading to Treg-Th17 conversion.