Interleukin-21 Expression and Its Association With Proinflammatory Cytokines in Untreated Chronic Periodontitis Patients

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Background: Interleukin-21 (IL-21) controls the differentiation of T-helper Th17 cells and induces the production of IL-17 in this T-cell subtype. The aim of this study is to determine the relative expression of IL-21 in gingival tissues of chronic periodontitis patients and correlate/associate this expression with proinflammatory cytokines and clinical parameters of disease.

Methods: Samples of gingival biopsies were collected from chronic periodontitis patients (n = 10) and controls (n = 8). The mRNA expressions of IL-21, IL-1β, IL-6, IL-17, IL-23, IL-10, and transforming growth factor-β1 (TGF-β1) were quantified using real-time reverse transcription-polymerase chain reaction. IL-21 levels were compared between chronic periodontitis and healthy gingival tissues and correlated with cytokine and clinical parameters of tissue destruction.

Results: A significant overexpression of IL-21, IL-1β, IL-6, IL-17, and IL-23p19 was detected in periodontal disease–affected tissues compared to healthy gingival tissues. IL-10 and TGF-β1 were, however, downregulated in periodontal lesions. IL-21 yielded significant positive correlations with probing depth, clinical attachment level, IL-1β, and IL-6. In addition, IL-21 was negatively correlated with IL-10 and TGF-β1.

Conclusions: IL-21 was overexpressed in chronic periodontitis gingival tissues and correlated with clinical parameters of periodontal destruction and with proinflammatory cytokines. Therefore, IL-21 might play a role in the tissue destruction that characterizes chronic periodontal disease. *J Periodontol 2012;83:948-954.*

KEY WORDS

Adaptive immunity; chronic periodontitis; cytokines; interleukin-21; Th17 cells.

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Chronic periodontitis is an infectious disease that involves a host immune inflammatory response in the periodontal tissues against the microorganisms present in dental plaque.[1] The clinical features of periodontitis include clinical attachment level (CAL), alveolar bone loss, periodontal pockets, and gingival inflammation.[2] Host immune inflammatory reactions are thought to protect the host against the infectious agents, but the persistent release of inflammatory mediators by immune cells results in the destruction of soft and mineralized periodontal tissues.[3]

Interleukin (IL)-21 is one of the most recently discovered members of the type I cytokine family. Structurally, it shows a homolog structure with IL-2, IL-4, and IL-15 proteins. IL-21 is mainly produced by activated T cells, but targets a broad range of lymphoid and myeloid cells of the immune system, which enables IL to regulate the innate and acquired immunity.[4] Recent studies have shown that IL-21 controls the generation of IL-17-producing T-helper (Th17) cells and, in combination with transforming growth factor-β (TGF-β), induces IL-17 production from this T_H subtype.[5-7] These studies also demonstrated that IL-6 and IL-21 could induce Th17 cells to produce higher levels of IL-21. The endogenous

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production of IL-21 by Th17 cells appeared to be biologically significant because the number of IL-17-producing cells stimulated by TGF-β and IL-6 was reduced in the absence of IL-21/IL-21R signaling. Thus, Th17 cells induced by TGF-β and either IL-6 or IL-21 make a distinct contribution to the development of the inflammation, which depends not only on the infective pathogens but also on the affected tissues.[4-7]

IL-21 participates in the immunity against tumor cells and chronic viral infections; nevertheless, excessive production of IL-21 has been associated with the development of immune inflammatory diseases in various organs.[4,8,9] IL-21 is enhanced in skin biopsies of patients with systemic lupus erythematosus, psoriasis, and atopic dermatitis.[9,10] In addition, IL-21 expression correlates with the presence of Th17 cells in synovial fluid and peripheral blood in rheumatoid arthritis patients.[8] Although the role of IL-21 in inflammation has been widely studied, the direct effect of this cytokine on periodontal diseases remains unclear. The aim of the present study is to determine the relative expression of IL-21 mRNA in gingival tissues of patients with chronic periodontitis and to correlate these levels with clinical parameters, Th17-related cytokines (IL-1β, IL-6, IL-17, and IL-23), and T-cell regulatory (Treg)-related cytokines IL-10 and TGF-β1.

MATERIALS AND METHODS

Patients

Forty-seven participants (16 males and 31 females, aged 35 to 60 years) were consecutively enrolled during a 4-month period (April to July 2009) at the postgraduate Clinic of Periodontology, Dental School, Complutense University of Madrid, Madrid, Spain. Among them, 10 participants were diagnosed as having chronic periodontitis, whereas eight healthy individuals were included as controls. The inclusion criteria were: 1) having ≥14 teeth, excluding third molars, and 2) having ≥10 posterior teeth. Participants had not received any periodontal treatment before the time of examination, had not sustained any systemic illness, and had not received antibiotics or non-steroidal anti-inflammatory therapy in the 3-month period before the study. Chronic periodontitis was defined as having ≥5 teeth with periodontal sites with probing depth (PD) ≥5 mm, CAL ≥3 mm, and extensive bone loss determined radiographically. The healthy control group was selected from volunteers with no evidence of periodontal disease determined by the absence of CAL or increased PD. Tissue samples were collected from both groups. Before sampling, all periodontitis and healthy individuals received supragingival prophylaxis and signed a written informed consent approved by the Institutional Review Board of the Complutense University of Madrid. The protocol stated that within 2 weeks of detection of the periodontal disease, samples should be taken and then patients would receive periodontal treatment. Periodontal therapy consisted of tooth scaling, root planing, and oral hygiene instructions.

Clinical Measurements

Clinical parameters were measured and evaluated in all teeth at six sites for each tooth (mesio-buccal, mid-buccal, disto-buccal, disto-lingual, mid-lingual and mesio-lingual); these parameters consisted of PD,[11] CAL,[12] dichotomous measurements of supragingival plaque accumulation (plaque index),[13] and bleeding on probing (BOP) to the base of the crevice.[13] Clinical parameters were measured in third molars, but they were excluded to diagnose chronic periodontitis. One calibrated examiner (RV) performed the evaluations and measurements in the patients.

Tissue Sample Collection

In patients with chronic periodontitis, samples of gingival tissue were collected from periodontal lesions under local anesthesia according to the protocol described by Ramfjord and Nissle (1974).[14] Incisions were made 1 to 2 mm subgingivally; specimens were conformed by gingival margin, sulcular epithelium, and gingival connective tissue. One sample of gingival tissue per chronic periodontitis patient was taken, and the criteria of selection included the following: 1) PD ≥5 mm, 2) CAL ≥3 mm, 3) BOP, 4) presence of plaque (before supragingival prophylaxis), and 5) bone loss determined radiographically. In healthy individuals, gingival tissues for biopsies were obtained during third molar extraction surgery. All were in the same stage of eruption with no evidence of tissue inflammation or destruction, and were evaluated with the clinical parameters described previously. Samples were immediately submerged in 500 mL ribonucleic acid (RNA)-stabilizing reagent‖ and transported at room temperature to be processed for RNA extraction.

Isolation of Cytoplasmic Total RNA

Tissue samples were homogenized in 2 mL of 4 M guanidinium thiocyanate in 0.05 M sodium citrate (pH 7.0), supplemented with 1% sarcosyl and 100 mM β-mercaptoethanol.¶ Homogenized samples were transferred to a ribonuclease (RNase)-free tube and 200 mL of 2 M sodium acetate (pH 4.5), 2 mL water-saturated phenol, and 400 μL chloroform/isoamylalcohol# (24:1) were added. After incubation for 15 minutes at 4°C and centrifugation at 3,600 rpm for 30 minutes at 4°C, the aqueous phase was transferred to a fresh RNase-free tube, mixed with 1 volume of

‖ RNASafer Stabilizer Reagent, Omega Bio-Tek, Norcross, GA.
¶ Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland.
# Fluka, Sigma-Aldrich Chemie.

isopropyl alcohol,** and precipitated overnight at –20°C. Then, samples were centrifuged at 8,000 rpm for 30 minutes at 4°C, lysed in 400 μL ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% sodium dodecyl sulfate, 10 mM EDTA (pH 8.0),†† and 25 μg/mL proteinase K‡‡ and incubated for 30 minutes at 37°C. Subsequently, RNA was extracted with 400 μL chloropan (phenol/chloroform/isoamylalcohol at 25:24:1) and centrifuged for 5 minutes at 14,000 rpm at room temperature.§§ The RNA present in the aqueous solution was precipitated overnight at –20°C with 0.3 M Na-acetate, 2.5 volumes of 100% ethanol, and 1 μL glycogen 20 μg/μL;‖‖ recovered by centrifugation performed for 30 minutes at 14,000 rpm at 4°C; and resuspended in 20 μL RNase-free H_2O. RNA quality was determined in a microfluidic-based platform.¶¶ Cytoplasmic RNA was quantified using a spectrophotometer## and stored at –80°C at a final concentration of 1 μg/μL.

First-Strand Complementary Deoxyribonucleic Acid Synthesis

Reverse transcription (RT) was performed using a complementary deoxyribonucleic acid (cDNA) synthesis kit*** following the recommendations of the manufacturer. A 20 μL reaction containing 4 μL 5× RT buffer, 0.5 μL RNase inhibitor (20 U), 2 μL deoxynucleotide mix (1 mM each), 2 μL random hexamer primer (60 μM), 0.5 μL RT (10 U), 10 μL RNA-grade H_2O, and 1 μL RNA sample (1 μg) were retro-transcribed for 10 minutes at 25°C and 1 hour at 50°C.††† The RT activity was subsequently inactivated by incubating the sample at 85°C for 5 minutes.

Real-Time Quantitative Polymerase Chain Reaction Analysis

The mRNA expression levels of the cytokines IL-1β, IL-6, IL-10, IL-17, IL-21, IL-23p19, and TGF-β1 were quantified using real-time quantitative polymerase chain reaction (qPCR). As endogenous control, the mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined. The cDNA product was used as a template (50 ng) for RT-qPCR analysis using a mix for real-time PCR applications,‡‡‡ specific primers, and a real-time PCR detection system apparatus.§§§ Specific human primers were designed using software‖‖‖ (Table 1).

Statistical Analyses

Clinical and experimental data were analyzed using statistical software.¶¶¶ For each patient, the mean results from each analyzed clinical parameter were generated and expressed as participant mean ± SD (Table 2). The Shapiro-Wilk test was used to analyze the clinical and experimental data distribution. Comparisons in age, PD, and CAL results were made using the two-tailed unpaired Student *t* test, whereas for periodontal sites with supragingival bacterial plaque, BOP, and sex data, the χ^2 test was used. Real-time qPCR data were expressed as C_t (cycle threshold), ΔC_t (C_t mRNA blank – C_t GAPDH ribosomal RNA), and relative quantification (RQ). RQ was obtained using the $2^{-\Delta\Delta Ct}$ method, adjusting the blank mRNA expression to GAPDH mRNA expression and considering the adjusted expression in healthy tissues as a reference (RQ = 1).[15] When the RQ levels of IL-10 and TGF-β1 were calculated, the adjusted expression in periodontal lesions was considered as a reference.

The differences among the mRNA expressions of the analyzed cytokines were determined analyzing the ΔC_t data and using the Mann-Whitney *U* test. Correlation coefficients were obtained using the Spearman

Table 1.

Forward and Reverse Primers Used for Cytokine Amplifications by Real-Time qPCR

Cytokine	Primers
IL-17	5′ CCGCCACTTGGGCTGCATC 3′ AGCCGGAAGGAGTTGGGGCA
IL-21	5′ AGCTCCCAAGGTCAAGATCGCCA 3′ AGTTGGGCCTTCTGAAAGCAGGA
IL-23p19	5′ GGCAGCGTCTCCTTCTCCGC 3′ GGCCTTGCTGGGCCATGGAG
IL-1β	5′ ACGCTCCGGGACTCACAGCA 3′ TGAGGCCCAAGGCCACAGGT
IL-6	5′ AGCGCCTTCGGTCCAGTTGC 3′ GTGGCTGTCTGTGTGGGGCG
TGF-β1	5′ GGCTTTCGCCTTAGCGCCCA 3′ GTTGGTGTCCAGGGCTCGGC
IL-10	5′ CCTGGAGGAGGTGATGCCCCA 3′ CCTGCTCCACGGCCTTGCTC
GAPDH	5′ GAAGGTGAAGGTCGGAGTC 3′ GAAGATGGTGATGGGATTTC

** Merck, Whitehouse Station, NJ.
†† Fluka, Sigma-Aldrich Chemie.
‡‡ Roche Applied Science, Indianapolis, IN.
§§ Fluka, Sigma-Aldrich Chemie.
‖‖ Roche Applied Science.
¶¶ Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA.
Nanodrop ND-1000, Thermo Fisher Scientific, Wilmington, DE.
*** Roche Applied Science.
††† Primus 96 plus, MWG Biotech, Ebersberg, Germany.
‡‡‡ iQ SYBER Green Supermix, Bio-Rad, Hercules, CA.
§§§ iCycler, Bio-Rad.
‖‖‖ Primer-BLAST, National Center for Biotechnology Information, Bethesda, MD.
¶¶¶ GraphPad Software, La Jolla, CA.

rank correlation test. A statistical significance was considered when $P < 0.05$.

RESULTS

Table 2 summarizes the demographic and clinical characteristics of all individuals evaluated in this study. No statistically significant differences in age or sex were observed between the groups. Significantly higher percentages of sites with plaque and BOP, as well as higher PD and CAL, were observed in patients with chronic periodontitis compared to healthy controls.

The expression of mRNA for IL-1β, IL-6, IL-10, IL-17, IL-21, IL-23p19, and TGF-β1 was detected in all tissue samples of both diseased and healthy gingival tissues. The RQ expressed as fold change for each studied cytokine is shown in Figure 1. A significant overexpression of IL-21 was detected in chronic periodontitis–affected tissues compared to healthy ones (120-fold). Similarly, IL-1β (5.4-fold), IL-6 (2-fold), IL-17 (2.2-fold), and IL-23p19 (3-fold) were also significantly overexpressed in chronic periodontitis tissues. Conversely, IL-10 and TGF-β1 were downregulated in periodontal lesions. When the RQ was recalculated considering the adjusted IL-10 and TGF-β1 expression in the chronic periodontitis tissues as RQ = 1, the expression of IL-10 and TGF- β1 in the healthy gingival tissues was 3.2-fold and 2.1-fold higher, respectively, than the levels detected in the chronic periodontitis tissues.

The analyses of correlation of IL-21 yielded significant positive correlations between IL-21 and PD ($P = 0.002$), IL-21 and CAL ($P = 0.01$), IL-21 and IL-1β ($P = 0.005$), and IL-21 and IL-6 ($P = 0.03$) (Table 3). In addition, IL-21 was negatively correlated with IL-10 ($P = 0.02$) and TGF-β1 ($P = 0.02$).

Table 2.

Demographic and Clinical Characteristics of Periodontitis Patients and Control Individuals

Characteristics	Chronic Periodontitis (n = 10)	Healthy (n = 8)
Age (years)	43.76 ± 6.83	40.0 ± 3.2
Females (%)	75.80	75
PD (mm)	5.79 ± 0.69*	2.15 ± 0.63*
CAL (mm)	6.99 ± 1.73*	0.77 ± 0.31*
BP (%)	72.07*	22.50*
BOP (%)	73.92*	0.06*

Clinical data are expressed as mean ± SD or relative frequencies (%).
* $P < 0.05$.
BP = periodontal sites with supragingival bacterial plaque.

DISCUSSION

The present study shows a significant mRNA overexpression of IL-21 and Th17-related cytokines in chronic periodontitis–affected tissues compared to healthy gingival tissues. In addition, Treg-related cytokines were downregulated in chronic periodontitis tissues. The analysis of correlations showed that IL-21 mRNA was positively correlated with clinical parameters of tissue destruction and proinflammatory cytokine mRNAs, such as IL-1β and IL-6. Conversely, IL-21 mRNA was negatively correlated with IL-10 and TGF-β1 mRNAs, cytokines related with the Treg profile.

Chronic periodontitis is an inflammatory process, elicited by periodontal pathogens, which affects the attachment structures of the teeth and constitutes a significant cause of tooth loss associated with poor quality of life in adults.[16,17] Periodontitis is also one of the most prevalent forms of bone pathology in humans and is considered an important modifying factor of other chronic systemic diseases that affect the adult population, such as cardiovascular diseases and poor metabolic control in diabetes.[18,19]

The ultimate determinant of periodontitis progression and clinical outcome is the immune response of the host, which involves the generation of cytokines, the activation of osteoclasts, and the recruitment of inflammatory cells.[3] There is enough evidence supporting that the major tissue destruction in periodontal lesions results from the recruitment of host cells and activation of monocytes/macrophages, lymphocytes, fibroblasts, and other cell types.[1] A considerable effort has been made to study the T-cell types associated with periodontitis. These studies have demonstrated that the multiple roles of T cells in promoting and developing periodontal lesions are complex and have not been fully elucidated.[20,21] Recent studies have shown the presence of Th17 and Treg cell types in chronic periodontitis lesions, incorporating new players in the pathogenesis of periodontal disease.[22-25]

The overexpression of IL-21 mRNA in association with the overexpression of the other Th17-related cytokines described in this study leads us to speculate that this cytokine could be involved in the differentiation of Th17 cells from naive $CD4^+$ T cells. The presence of IL-21 in periodontal pathogenesis has been described recently. Previous work in our laboratory showed that IL-21 is present in gingival crevicular fluid and tissue of patients with untreated chronic periodontitis.[26] In line with these findings, a study of the effect of non-surgical periodontal therapy on the levels of Th17/Th1/Th2 cytokines revealed that, after periodontal therapy, IL-21 expression was downregulated when it was compared to basal levels.[27] Altogether,

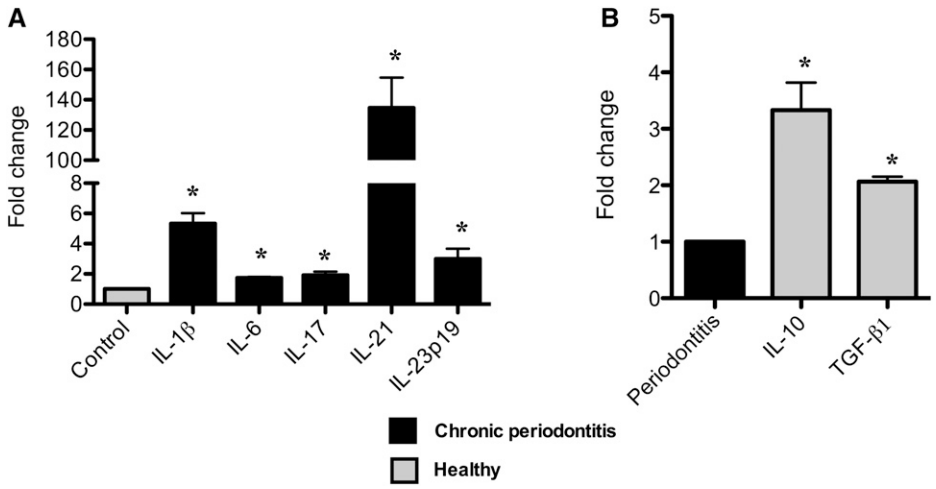

Figure 1.
*IL-21 and Th17/Treg gene expression in chronic periodontitis and healthy gingival tissues. The RQ of each gene expressed as fold change. The RQ in chronic periodontitis tissues was obtained considering the normalized expression in healthy tissues as reference (RQ = 1). IL-21 (120-fold), IL-1β (5.4-fold), IL-6 (2-fold), IL-17 (2.2-fold), and IL-23p19 (3-fold) were significantly overexpressed in chronic periodontitis tissues **(A)**. When the RQ of IL-10 (3.3-fold) and TGF-β1 (2-fold) were calculated, the adjusted expression in chronic periodontitis tissues was considered as reference **(B)**. *P <0.05 (Mann-Whitney U test).*

Table 3.

Correlation Coefficients for Cytokine mRNA Expression, PD, and CAL of the Sampled Sites (N = 18)

	IL-1β	IL-6	IL-10	IL-17	IL-21	IL-23p19	TGF-β1
PD	0.73†	0.72†	–0.61‡	0.21	0.71†	0.35	–0.44
CAL	0.8*	0.73†	–0.69†	0.14	0.60‡	0.37	–0.53‡
IL-1β		0.35	–0.37	0.08	0.67†	0.57‡	–0.40
IL-6			–0.28	0.58‡	0.53‡	0.65†	–0.30
IL-10				0.30	–0.59‡	–0.10	0.88*
IL-17					0.10	0.48	0.32
IL-21						0.24	–0.58‡
IL-23p19							0.02

* Correlation significance at the P <0.001 level by Spearman rank correlation test.
† Correlation significance at the P<0.01 level by Spearman rank correlation test.
‡ Correlation significance at the P<0.05 level by Spearman rank correlation test.

these data support an important role of IL-21 in the pathogenesis of periodontal disease.

The overexpression of IL-1β, IL-6, IL-17, and IL-23p19 in chronic periodontitis has been well demonstrated.[5,21,22,24,28,29] These cytokines may enhance periodontal tissue destruction, perpetuating the inflammatory response and the activation of resident cells to produce proteases.[30,31] Furthermore, it has been demonstrated that IL-6 and IL-21 could induce Th17 cells themselves, to produce more IL-21 in a positive feedback loop.[6,29,32]

IL-21 has been reported to be expressed at increased levels in inflammatory bowel disease and rheumatoid arthritis and to prevent the induction and expression of Treg cell activity, thus allowing ongoing inflammation.[8,33] These data are in accordance with the present findings, which showed a downregulation of Treg-related cytokines in chronic periodontitis patients compared to controls, along with a negative correlation between IL-21 and these cytokines.

T-cell phenotype has been proposed as a risk factor for periodontal disease.[34] Studies regarding the role of cytokines in bone tissue have associated Th1 and Th2 cytokines with activation and suppression of bone destruction during periodontitis, respectively; however, additional subtypes of T cells, with cytokine profiles and/or immunosuppressive functions distinct from either Th1 or Th2 cells, termed Th17 and Treg cells, have been associated with periodontal bone destruction.[22,23,25,28,35] Selective T-cell differentiation would depend on the local cytokine milieu during infection, which would determine the predominance of either pathologic (Th1 and/or Th17 cells) or protective (Th2 and/or Treg cells) activities and, thus, the outcome of the periodontal disease.

Previous studies showed an important presence of Treg cells in diseased gingival tissues, and immunohistologic analyses have shown higher numbers of Treg cells in samples from periodontitis-affected patients compared to control patients with gingivitis.[35] Moreover, an overexpression of transcription factor forkhead box P3 (master switch of Treg cells) had been demonstrated in active versus inactive periodontal sites.[24] Conversely, our study shows a downregulation of IL-10 and TGF-β1 (related to Treg cells) in diseased gingival tissues compared to healthy tissues, and a previous study from our group has shown a downregulation of these Treg-related cytokines in active sites compared to inactive sites in patients with periodontitis.[24] The previous and current findings may lead us to speculate that Treg cells do not have a regulatory function but might have a role in the pathogenesis of periodontal lesions by downregulating TGF-β1 and

IL-10 synthesis, which leads to the overexpression of Th17-associated cytokines.

CONCLUSIONS

In summary, our data demonstrate that IL-21 mRNA is overexpressed in untreated chronic periodontitis patients. Additionally, a positive correlation between IL-21 with Th17-related cytokines and a negative correlation with Treg-related cytokines was found in association with clinical parameters of periodontal tissue destruction. Together, these results allow us to attribute a role to IL-21 mRNA in chronic periodontitis pathogenesis.

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