Levels of Interleukin-21 in Patients With Untreated Chronic Periodontitis

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Background: A growing body of evidence suggested that interleukin (IL)-21 enhances the effector phase during T-cell responses. The aim of our study is to determine the levels of IL-21 in periodontal sites from patients with chronic periodontitis and controls.

Methods: The population studied consisted of 34 patients (15 with chronic periodontitis and 19 healthy patients). Twenty samples (10 gingival crevicular fluid [GCF] and 10 gingival biopsies) were collected from each group before the patients with periodontitis received periodontal treatment. Total protein concentrations were measured in all samples; the presence of IL-21 was confirmed by immunohistochemistry and Western blot, and IL-21 levels were quantified through an enzyme-linked immunosorbent assay. Statistical analyses were performed using statistical software. Data were expressed as patient means ± SDs or medians (interquartile ranges) by using the x^2, Student t, and Mann-Whitney U tests.

Results: GCF IL-21 was mainly detected in patients with chronic periodontitis (P<0.05). Levels of IL-21 in gingival tissues were significantly higher in patients with chronic periodontitis compared to healthy individuals (P<0.05). The Western blot and immunohistochemical staining confirmed the presence of IL-21 in periodontal tissues and GCF.

Conclusion: IL-21 was highly expressed in patients with chronic periodontitis, especially in gingival biopsies; therefore, IL-21 might play a role in the T-cell response. J Periodontol 2011;82:1483-1489.

KEY WORDS
Cytokines; interleukin-21; pathogenesis; periodontal disease.

Periodontitis is a multifactorial disease that involves bacterial biofilm and the generation of an inflammatory response.¹ Although a chronic bacterial and endotoxin exposure is a prerequisite for gingival inflammation and periodontal tissue destruction to occur, its presence alone is responsible for a relatively small proportion (20%) of the variance in the clinical expression of periodontitis.² The major component of soft- and hard-tissue destruction associated with periodontitis results from the activation of the host’s immune inflammatory response to the bacterial challenge; thereby, the destructive character of the disease is mostly determined by the nature of the inflammatory response³ and involves the active expression of catabolic cytokines and inflammatory mediators, such as interleukin (IL)-1β and -6 and tumor necrosis factor (TNF)-α, through the activation of monocytes/macrophages, lymphocytes, fibroblasts, and other cellular elements.⁴ These cytokines and inflammatory mediators are capable of acting alone or together to stimulate alveolar bone resorption and collagen destruction via tissue-derived matrix metalloproteinases.⁴

IL-21 is the most recently discovered member of the type-1 cytokine family. Structurally, it shows a homologue structure with IL-2, -4, and -15 proteins. IL-21 is mainly produced by activated T cells, but it targets a broad range of lymphoid and myeloid cells of the immune system,

which enables IL-21 to regulate the innate and acquired immunity. Recent studies showed that IL-21 also controlled the generation of T helper 17 cells (Th17) and, in combination with transforming growth factor (TGF)-β, induced IL-17 production from naive CD4+ T cells. These studies also demonstrated that IL-6 and -21 could induce Th17 cells themselves to produce more IL-21. The endogenous production of IL-21 by Th17 cells appeared to be biologically very 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-21 receptor signaling. Therefore, it is possible that Th17 cells induced by TGF-β and either IL-6 or -21 make a qualitatively different contribution to the development of the inflammation depending on the pathogen that is present and according to the tissue that the results affected. The aim of the present study is to determine total amounts and concentrations of IL-21 in gingival crevicular fluid (GCF) and gingival tissue samples in patients with chronic periodontitis and in healthy individuals.

MATERIALS AND METHODS

Patients
Thirty-four patients were consecutively enrolled over a 6-month period (March 2009 to September 2009) from the Diagnosis Center of the Faculty of Dentistry, University of Chile. Of these, 15 patients had a clinical diagnosis of chronic periodontitis, whereas 19 control patients were healthy. Criteria used for patient selection were: ≥14 natural teeth, excluding third molars, and ≥10 posterior teeth. Patients had not received any periodontal treatment before the time of examination, suffered any systemic illness, or received antibiotics or non-steroid anti-inflammatory therapy in the 6-month period before the study. Chronic periodontitis was defined as having ≥5 teeth with periodontal sites with probing depths (PD) ≥5 mm, clinical attachment loss (AL) ≥3 mm, and extensive bone loss determined radiographically. The control healthy group was selected from volunteers among patients with no evidence of periodontal disease determined by the absence of AL or increased PDs. Twenty GCF and tissue samples were collected from all patients. Before sampling, all patients received a supragingival prophylaxis to remove gross calculus to allow the assessment of PDs.

After the approval of the institutional board of reviews (Dentistry Faculty, University of Chile), the protocol of the study was explained to all patients, and informed written consent forms were collected. The protocol stated that within 2 weeks of detection of periodontal disease, samples would be taken and 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, excluding third molars. These parameters consisted of PD, clinical AL, dichotomous measurements of supragingival plaque accumulation (plaque index), and bleeding on probing (BOP) to the base of the crevice. Six sites were examined for each tooth: mesio-buccal, mid-buccal, disto-buccal, disto-lingual, mid-lingual, and mesio-lingual. One calibrated examiner (JG) performed all patient evaluations and measurements.

GCF and Tissue Sample Collection
To obtain the GCF, sampled sites were isolated with cotton rolls and dried with a gentle stream of air to prevent saliva contamination. Six sterile strips were used in each site; they were inserted into the gingival crevice and left in place for 30 seconds. The elution was performed with 60 μL NaCl (0.9%) per strip, incubated for 30 minutes at 4°C, and centrifuged at 12,500 rpm at 4°C for 5 minutes. This procedure was repeated twice, and eluates were frozen and kept at −80°C. Samples of gingival tissues were collected from periodontal lesions under local anesthesia according to the protocol described by Ramfjord and Nissle and weighted (52.19 ± 34.89 mg). Incisions were made 1 to 2 mm subgingivally; therefore, specimens were composed of gingival margin, sulcular epithelium, and gingival connective tissue. In healthy patients, gingival tissues for biopsies were obtained during the third-molar extraction, with all of them in the same state of eruption. Protein extracts were obtained by automated homogenization in a lysis buffer (50 mM Tris-HCl [pH 7.5] and 1% Triton x100) with the addition of proteinase inhibitor cocktail (one tablet per 10 mL buffer).

Afterward, aliquots of all gingival tissues and GCF samples were used for the quantification of the total protein concentration by using a kit according to the manufacturer’s indications and for immunobiochemical assays.

Immunohistochemistry
Sections were fixed in 4% paraformaldehyde at 4°C overnight. Tissue sections of 4μm were obtained and deparaffined, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes. After washing, non-specific tissue binding was blocked by incubation with 2.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (pH 7.4) for 30 minutes at room temperature and washed again. Specimens were incubated overnight with...
Interleukin-21 in Patients With Chronic Periodontitis

1:200 diluted primary antibodies against human IL-21** in 2.5% BSA in PBS (pH 7.4). All rinsing steps were performed with PBS (pH 7.4). After washing, goat anti-mouse immunoglobulin (lgG) G secondary antibodies conjugated with peroxidase were used at a 1:400 dilution for 30 minutes.†† A brown color was developed by an exposure for 3,3′-diaminobenzidine tetrahydrochloride (DAB). §§ Sections were counter-stained with Mayer hematoxylin, §§ dehydrated, and permanently mounted. Positive controls were processed with each series. Slides were observed under an optical microscope, III and images were captured and digitized with a camera and software. ##

**Western Blot**
Five micrograms of total protein from extracts of GCF and gingival tissues were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis Tris-glycine gels at 10% under reducing conditions are transferred to a polyvinyl difluoride membrane. For the Western blot, the membrane was blocked with 3% BSA in Tris-buffered saline–Tween (TBS-T) 0.1% for 1 hour at 37°C with constant agitation. The primary antibody against IL-21*** was diluted 1:500 in 3% BSA in TBS-T 0.1% was added and the membrane was incubated overnight at 37°C with constant agitation. The membrane was then washed with TBS-T 0.1%, incubated for 1 hour at 37°C with constant agitation with a secondary stabilized peroxidase-conjugated goat anti-mouse antibody at a 1:10,000 dilution, †††† and washed again with TBS-T 0.1%. The bands were detected with an enhanced chemiluminescence detection kit. ‡‡‡‡ Blots were immediately exposed to radiographic films from 30 seconds to 3 minutes. The films were developed, fixed, dried, and digitized. §§§ Samples of GCF and gingival tissues from all patients were included in this study, and each experiment was repeated three times for each sample.

**Total Amounts and Concentrations of IL-21**
Levels of IL-21 in GCF and gingival tissues were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. Standards and samples were incubated in the 96-well polystyrene microplate precoated with anti-IL-21 antibody. The plate was read at 450 nm. **** Concentrations of IL-21 in GCF and in tissue extracts from gingival tissues were determined by interpolation from the curve. The sensitivity of the ELISA kit used was 16 pg/mL. Results were expressed as IL-21 levels standardized in milligrams of weighted gingival tissue (picograms per milligram of tissue) and by the total protein concentration (picograms per milligram of total protein).

**Statistical Analyses**
Data analyses were performed using software. ††††† The Shapiro-Wilk test was applied to analyze the distribution of data. Results were expressed in means ± SDs and medians (interquartile ranges). Measurements were analyzed with the unpaired t or Mann-Whitney U test, depending if the data distribution was normal or not, and the x² test (Table 1) for frequencies. A statistical significance was considered when P was <0.05.

**RESULTS**
Clinical characteristics of patients with chronic periodontitis and healthy volunteers included in this study are grouped in Table 1. Six males and nine females were studied (age range: 35 to 62 years; mean age: 46.20 ± 14.86 years) in the periodontitis group, and six males and 13 females were studied (age range: 37 to 53 years; mean age: 44.47 ± 7.59 years) in the control group. No statistically significant differences in age or sex existed between both groups. Compared with the control group, significantly higher percentages of sites with plaque and BOP and higher PDs and CALs were observed in the chronic periodontitis group (P<0.05).

The immunohistochemical stain revealed that IL-21 was expressed in gingival tissues from healthy patients and patients with chronic periodontitis (Fig. 1). IL-21 immunoreactivities in GCF and tissue samples of patients with periodontitis and healthy patients were seen at 15.5 kDa (Fig. 2). IL-21 bands were observed in all samples from patients with disease and healthy patients.

Mean variations of IL-21 levels in gingival tissues and GCF from periodontitis and control groups are shown in Tables 2 and 3 and Figure 3. GCF IL-21 was mainly detected in patients with chronic periodontitis (P<0.05). In gingival tissue samples, levels of IL-21 were significantly higher in patients with chronic periodontitis than in healthy patients (P<0.05).

**DISCUSSION**
Our results showed, for the first time to our knowledge, the expression of IL-21 in patients with chronic periodontitis. IL-21 was more frequently detected in the GCF from adult patients with chronic periodontitis than in healthy patients, whereas concentrations of

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**Notes:**
- **BioLegend,** San Diego, CA.
- Kit Legend, Burlington, VT.
- ImmPact DAB, Vector Laboratories.
- Phosphate-buffered saline (PBS), pH 7.4.
- Tris-glycine gels at 10% under reducing conditions.
- Goat anti-mouse immunoglobulin (lgG) G secondary antibodies conjugated with peroxidase.
- 1:10,000 dilution.
- 3,3′-diaminobenzidine tetrahydrochloride (DAB).
- Mayer hematoxylin.
- Tris-buffered saline–Tween (TBS-T) 0.1%.
- Goat anti-mouse antibody against IL-21.
- All rinsing steps were performed with PBS (pH 7.4).
- After washing, goat anti-mouse immunoglobulin (lgG) G secondary antibodies conjugated with peroxidase were used.
- 1 hour at 37°C with constant agitation.
- 1:500 dilution.
- 3% BSA in Tris-buffered saline–Tween (TBS-T) 0.1%.
- Incubated overnight at 37°C with constant agitation.
- Goat anti-mouse antibody at a 1:10,000 dilution.
- Radiographic films.
- Developed, fixed, dried, and digitized.
- Samples of GCF and gingival tissues from all patients were included in this study, and each experiment was repeated three times for each sample.

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**References:**
- BioLegend, San Diego, CA.
- Thermo Scientific.
- Super Signal West Femto, Pierce, Rockford, IL.
- CL-X Posure Film, Thermo Scientific.
- Perimetra Intra Developer and Fixer, Durr Dental, Bietigheim-Bissingen, Germany.
- Camera Power Shot A640, Canon, Lake Success, NY.
- AxioVision 4.8, Zeiss.
- BioLegend.
IL-21 in gingival tissues from periodontal sites were found in significantly higher levels than in gingival tissues of healthy individuals. Chronic periodontitis is an inflammatory process that affects the teeth’s attachment structures and is a significant cause of tooth loss in adults. Periodontitis is also one of the most prevalent forms of bone pathologies 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 patients with diabetes. An epidemiologic survey in Chile assessed the periodontal status of the adult population and showed that the number of sites with severe AL (>6 mm) was 38% in younger adults and 69% in senior adults (P < 0.05). Periodontitis is considered to be an infection in which putative periodontopathogens trigger a chronic inflammatory and immune response against periodontal structures. Moreover, an imbalanced host response to periodontopathogens was described as an important determinant in the disease’s outcome. The ultimate determinant of the disease progression and clinical outcome is the host’s immune response, which involves generation of cytokines, activation of osteoclasts, and recruitment of inflammatory cells. There is evidence that the major tissue destruction in periodontal lesions results from the recruitment of host cells via the activation of monocytes/macrophages, lymphocytes, fibroblasts, and other cell types. Considerable efforts were made to study the T-cell type associated with periodontitis. These studies demonstrated that the multiple roles of T cells in promoting and developing periodontal lesions are complex and not fully elucidated.

Table 1. Clinical Characteristics of Patient Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Periodontitis (n = 15)</th>
<th>Healthy (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years; mean ± SD)</td>
<td>46.20 ± 14.86</td>
<td>44.47 ± 7.59</td>
</tr>
<tr>
<td>Females (%)</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>PD (mm; mean ± SD)*</td>
<td>3.30 ± 1.02</td>
<td>1.20 ± 0.45</td>
</tr>
<tr>
<td>Attachment level (mm; mean ± SD)†</td>
<td>4.10 ± 1.80</td>
<td>1.85 ± 0.90</td>
</tr>
<tr>
<td>Sites with plaque (%)*</td>
<td>80.20</td>
<td>25.50</td>
</tr>
<tr>
<td>Sites with BOP (%)†</td>
<td>51.60</td>
<td>2.20</td>
</tr>
</tbody>
</table>

* P = 0.001 for periodontitis versus control groups (unpaired t and X² tests).
† P = 0.0001 for periodontitis versus control groups (unpaired t and X² tests).

Recently, CD4+ CD25+ regulatory T cells (Treg) were described as one distinct subset from T cells. The development of autoimmune pathologies in healthy individuals is limited by this specialized subset of T cells, named Treg cells. Treg cell differentiation and functions are driven by the transcription factor Forkhead box P3, and they have an anti-inflammatory role, the purpose of which is to keep the tolerance to self-components by contact-dependent suppression or releasing anti-inflammatory cytokines, such as IL-10 and TGF-β. TGF-β induces the differentiation of Treg cells, whereas TFG-β in combination with IL-6 results in the differentiation of Th17 cells. These Treg cells may counteract the generation of proinflammatory responses to prevent immune-mediated diseases. However, under pathologic conditions...

![Figure 1](image-url) Presence of IL-21 detected by immunohistochemistry. A brown color was developed by exposure for DAB, and sections were counterstained with Mayer hematoxylin. A) Human tonsil as a positive control. B) Gingival tissue of a healthy patient. C) Gingival tissue of a patient with periodontitis.
interleukin-21 detection has been demonstrated in patients with chronic periodontitis. This suggests a potential role for IL-21 in the regulation of immune responses, particularly in the context of periodontal disease. Further research is needed to fully understand the role of IL-21 in the pathogenesis of periodontal disease and the potential for therapeutic interventions targeting this cytokine.

Table 3. Sample Data and IL-21 Detection in GCF From Patient Groups

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Periodontitis</th>
<th>Healthy</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein concentration</td>
<td>1.09 ± 0.51</td>
<td>0.18 ± 0.17</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-21 (frequency of detection)</td>
<td>8/10</td>
<td>2/10</td>
<td><strong>0.007</strong></td>
</tr>
</tbody>
</table>

Bold values = P < 0.05.

Figure 2. Presence of IL-21 detected by a Western blot. Every lane contained 5 μg total protein from extracts of GCF (A) or gingival tissues (B). A purified primary antibody against human IL-21 was used at a 1:500 dilution, and secondary stabilized peroxidase conjugated goat anti-mouse antibody was used at a 1:10,000 dilution. C = sample from a healthy patient. P = sample from a patient with periodontitis.

Table 2. Sample Data and IL-21 Levels in Gingival Tissue in Patient Groups

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Periodontitis (n = 10)</th>
<th>Healthy (n = 10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue weight (mg; mean ± SD)</td>
<td>38.61 ± 35.25</td>
<td>65.77 ± 30.28</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Total protein concentration</td>
<td>2.30 ± 1.01</td>
<td>1.92 ± 0.60</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-21 (pg/mg tissue; median [interquartile range])</td>
<td>3.35 (2.34)</td>
<td>0.98 (0.64)</td>
<td><strong>0.006</strong></td>
</tr>
<tr>
<td>IL-21 (pg/mg total protein; mean ± SD)</td>
<td>44.00 ± 14.80</td>
<td>34.33 ± 12.65</td>
<td><strong>0.07</strong></td>
</tr>
</tbody>
</table>

Bold values = P < 0.05.

conditions (e.g., during bacterial infections) the development of T<sub>reg</sub> cells may be blocked or limited and, thus, facilitate the expansion of effector T cells, which orchestrate the immune response against tissue-infiltrating pathogens. The functional antagonism of IL-21 on T<sub>reg</sub> cells could amplify the effector or pathogenic responses by counteracting T<sub>reg</sub> cell activity. Volpe et al.<sup>31</sup> showed that TGF-β, IL-23, and proinflammatory cytokines were essential in driving and regulating key aspects of human Th17 differentiation: the IL-17 production and acquisition of Th17-specific features and Th17-derived cytokines. Manel et al.<sup>32</sup> showed that TGF-β, IL-1β and IL-6, IL-21, or IL-23 in serum-free conditions were necessary and sufficient to induce IL-17 expression in naive human CD4+ T cells from cord blood. In this context, we considered it reasonable to assume that a patient’s susceptibility to periodontitis could be determined, at least in part, by the balance between the response against periodontopathogens and its regulatory mechanism mediated by the balance between Th17 and T<sub>reg</sub> cells during the immune response. Recently, a single study<sup>33</sup> demonstrated that a Th17/T<sub>reg</sub> functional imbalance was evidenced in patients with acute coronary syndrome (including unstable angina and acute myocardial infarction), suggesting a potential role for the Th17/T<sub>reg</sub> balance in plaque destabilization and the onset of acute coronary syndrome. The functional antagonism of IL-21 on T<sub>reg</sub> cells could amplify the effector or pathogenic responses by counteracting the T<sub>reg</sub> cell activity.<sup>33</sup>

Finally, it is still not clear whether the presence of IL-21 in some organs, such as the gingival tissue in normal conditions, evidences the role of this cytokine in keeping an immunologic homeostasis. In this context, IL-21 plays a decisive role in regulating the functional activities of other immune cells such as B cells, CD8+ T cells and, natural killer cells, and there is evidence that IL-21 signaling may attenuate the course of IgE-mediated diseases.<sup>34</sup> IL-21 was suggested to primarily maintain the expression of specific naive cell-surface markers, such as CD45RA, CD27, CD62L, and CCR7 on human CD4+ T lymphocytes. Thus, a potential role of IL-21 in healthy gingival tissues might be the preservation of a pool of CD4+ T lymphocytes in a naive phenotype. The expression of CCR7 by these cells induces the cell migration by means of CCL21 chemotaxis, keeping the homeostatic role of T cells and their homing to secondary lymphoid organs.<sup>35</sup>

**CONCLUSION**

These data demonstrate that IL-21 levels are significantly higher in patients with chronic periodontitis...
than in healthy individuals, which suggests a role of IL-21 in the pathogenesis of chronic periodontitis.

ACKNOWLEDGMENTS

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