

# High Expression Levels of Receptor Activator of Nuclear Factor-Kappa B Ligand Associated With Human Chronic Periodontitis Are Mainly Secreted by CD4<sup>+</sup> T Lymphocytes

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**Background:** Chronic periodontitis is an infectious disease characterized by alveolar bone destruction and teeth loss. Receptor activator of nuclear factor-kappa B ligand (RANKL) is an osteoclastogenic cytokine, a central regulatory factor in the osteoclast's lifespan, and a participant in physiological and pathological bone resorption. Gingival T cells synthesize RANKL, contributing to molecular local imbalance that entails the alveolar bone resorption seen in periodontitis. Our study was aimed at associating the levels of RANKL with the CD4<sup>+</sup> T-cell activity present in gingival tissues of chronic periodontitis patients.

**Methods:** Gingival biopsies were obtained from 33 chronic periodontitis patients and 20 healthy controls. Specimens were either formalin fixed and paraffin embedded for real-time reverse transcription-polymerase chain reaction (RT-PCR) and histologic analysis or tissue digestion processed for cell culture and flow-cytometry analysis. RANKL mRNA and protein levels were determined by quantitative RT-PCR and enzyme-linked immunosorbent assay (ELISA) in gingival-cell culture supernatants. Gingival leukocytes were quantified by flow cytometry. RANKL and CD4 immunoreactivity were analyzed by flow cytometry and confocal microscopy.

**Results:** RANKL mRNA levels were higher in patients with periodontitis than in healthy subjects, and spontaneous and lipopolysaccharide (LPS)- and phytohemagglutinin (PHA)-stimulated RANKL synthesis were higher also in patients than controls. CD4<sup>+</sup> T lymphocytes were the predominant infiltrate cell subset present in gingival tissues of periodontitis patients. Furthermore, an association between RANKL and CD4<sup>+</sup> T cells was determined by double-staining flow cytometry and confocal microscopy.

**Conclusion:** Taken together, these data demonstrate that gingival CD4<sup>+</sup> T cells are the main cells responsible for higher levels of RANKL observed in human chronic periodontitis patients. *J Periodontol* 2006;77:1772-1780.

## KEY WORDS

CD4; chronic periodontitis; lymphocytes; RANKL; receptor activator of nuclear factor-kappa B ligand; T cells.

Three novel members of the tumor necrosis factor (TNF) ligand and TNF receptor (TNFR) superfamily, osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B (RANK) and RANK ligand (RANKL), have recently been identified as participants of the key system of physiological and pathological regulation of bone.<sup>1-3</sup> RANKL, also known as osteoprotegerin ligand (OPGL), osteoclasts differentiation factor (ODF), TNF-related activation-induced cytokine (TRANCE), and TNF superfamily member 11 (TNFSF11), exerts its biologic effects directly through binding to its receptor RANK, inducing the differentiation of osteoclast precursors and activation of mature osteoclast.<sup>4,5</sup> The biologic activity of RANKL is neutralized by binding to its decoy OPG.<sup>5</sup>

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Chronic periodontitis is a multifactorial infection elicited by a complex of bacterial species that interact with host tissues and cells causing the release of a broad array of inflammatory cytokines, chemokines, and mediators, some of which lead to the destruction of the periodontal structures, including tooth-supporting tissues, alveolar bone, radicular cement, and periodontal ligament.<sup>6,7</sup> The disease is probably triggered by the increase of specific periodontopathogenic bacteria in the complex microbial biofilms that colonize the sulcular regions between the tooth surface and the gingival margin. The imbalance of the biofilm ecology can induce important changes of the specific adherence interactions and architectural changes in the sulcus, including attachment loss and pocket formation.<sup>8</sup> The destruction of the osseous support of the dentition is the hallmark of chronic periodontitis, which is a principal cause of teeth loss.<sup>9</sup>

Previous *in vivo* studies have demonstrated the association of RANKL to periodontitis.<sup>10-13</sup> In rheumatoid arthritis studies, it has been proposed that CD4<sup>+</sup> T are the primary cells responsible for the synthesis of RANKL;<sup>14</sup> however, its correlation with periodontitis in humans has not yet been clarified. Therefore, the aim of our study was to associate the RANKL levels in gingival tissue samples from adult patients affected with chronic periodontitis to gingival infiltrate CD4<sup>+</sup> T-cell activity.

## MATERIALS AND METHODS

### Subjects

Fifty-three subjects aged 35 to 65 years were consecutively enrolled during a 3-month period (March to May 2004) from the Center of Diagnostic and Treatment Dra. Eloisa Diaz, Northern Metropolitan Health Services, Santiago, Chile. Of these, 33 subjects had a clinical diagnosis of chronic periodontitis, whereas 20 control subjects were healthy. Criteria used for patient selection were: 1) a minimum of 14 natural teeth, excluding third molars, and with at least 10 posterior teeth; 2) no periodontal treatment received prior to the time of examination; 3) no systemic illness; and 4) no antibiotics or non-steroidal anti-inflammatory therapy in the 6-month period prior to the study. Chronic periodontitis patients were defined as individuals having at least five teeth with periodontal sites with probing depth (PD)  $\geq 5$  mm, clinical attachment level (CAL) loss  $\geq 3$  mm, and extensive radiographically determined bone loss. The control group was selected from volunteers with no evidence of periodontal disease determined by the absence of CAL or increased PD. The protocol was clearly explained to all participants, and an approved Institutional Review Board informed consent was signed prior to enrollment. When periodontal disease was detected, patients were provided with periodontal therapy.

### Clinical Measurement

All subjects received a supragingival prophylaxis to remove gross calculus and to allow probing access during clinical examination. CAL, PD, and dichotomous measurement of supragingival bacterial plaque (SBP) accumulation and bleeding on probing (BOP) were taken at six sites per tooth, in all teeth, by a single calibrated investigator. Measurements were made at mesio-buccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual periodontal sites using an automated probe.<sup>||</sup>

### Gingival Tissue Biopsies

In periodontitis patients, gingival biopsies were obtained from periodontally affected sites (PD  $\geq 5$  mm and CAL  $\geq 3$  mm) under troncular anesthesia and according to surgical therapy requirements. In healthy controls, gingival biopsies were taken during the surgical removal of wisdom teeth. Specimens were either fixed in 10% neutral buffered formalin for 24 hours at 4°C and paraffin embedded for real-time reverse transcription-polymerase chain reaction (RT-PCR) and histologic analysis or placed in a vial containing 5 ml cold sterilized transport media (Roswell Park Memorial Institute [RPMI] 1640 supplemented with 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin, and 200 mM L-glutamine)<sup>¶</sup> for cell culture and flow cytometry analysis.

### Total RNA Extraction

Total RNA extraction was performed on formalin-fixed and paraffin-embedded tissues using an RNA isolation kit according to the manufacturer's instructions.<sup>#</sup> Isolated RNA was resuspended in 30  $\mu$ l RNase-free diethyl pyrocarbonate<sup>¶</sup> (water DEPC) 0.1% for 15 minutes at 60°C. Total RNA samples were quantified using a spectrophotometer and stored at -80°C at a final concentration of 250 ng/ $\mu$ l.

### RT-PCR

First-strand cDNA was synthesized using 500 ng total RNA with a reverse transcription kit according to the manufacturer's instructions.<sup>\*\*</sup> A 30- $\mu$ l volume reaction, containing 3  $\mu$ l 10 $\times$  RT buffer, 6.6  $\mu$ l MgCl<sub>2</sub> 25 mM, 6  $\mu$ l deoxynucleotide triphosphate [dNTP] mixture, 1.5  $\mu$ l Oligo d(T), 0.6  $\mu$ l RNase inhibitor, 0.75  $\mu$ l reverse transcriptase, 9.55  $\mu$ l RNase-free water DEPC 0.1%, and 2  $\mu$ l total RNA, was retrotranscribed in a thermal cycler<sup>††</sup> under the following conditions: 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C. To examine the mRNA expression of RANKL, 83.3 ng cDNA, in duplicate 1 $\times$  and 0.1 $\times$  dilutions,

|| Florida Probe, Gainesville, FL.

¶ Sigma Chemical, St. Louis, MO.

# Optimun FFPE RNA Isolation Kit, Ambion, Austin, TX.

\*\* TaqMan Reverse Transcription Reagents, Roche Molecular Systems, Belleville, NJ.

†† Primus 96 plus Thermal Cycler, MWG Biotech, Ebersberg, Germany.

were amplified by real-time quantitative PCR using a specific RANKL gene expression set,<sup>††</sup> containing a forward and a reverse primer at non-limiting concentrations and a 6-carboxyfluorescein (6-FAM) dye-labeled probe, specifically designed to detect and quantify cDNA sequences from the multiexon RANKL gene, without amplifying genomic DNA. A 25- $\mu$ l volume reaction that contained 12.5  $\mu$ l of a universal PCR master mix,<sup>§§</sup> 1.25  $\mu$ l of a 20 $\times$  RANKL gene expression set, 6.25  $\mu$ l RNase-free water DEPC 0.1%, and 5  $\mu$ l cDNA, were analyzed under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, in a quantitative thermal cycler.<sup>|||</sup> As an endogenous control assay, 33.3 ng cDNA were amplified to determine the GAPDH dehydrogenase mRNA expression, using a specific GAPDH gene expression set,<sup>¶¶</sup> in the same dilutions and conditions as RANKL.

### Isolation of Gingival Cells

The isolation of cells, flow cytometry, and gingival cell cultures were performed as previously described.<sup>15</sup> Briefly, tissue samples were incubated in a tissue digestion medium RPMI 1640 supplemented with 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin, and 200 mM L-glutamine, plus 200 U/ml of type IV collagenase,<sup>##</sup> ~10 ml tissue digestion medium per 50 mg gingival sample, at 37°C for 90 minutes. Cells obtained were washed twice with phosphate buffered saline (PBS) and resuspended in complete RPMI 1640 medium with penicillin, streptomycin, L-glutamine, and 10% fetal bovine serum.<sup>\*\*\*</sup> Cell counting was performed on a Neubauer chamber using phase contrast microscopy,<sup>†††</sup> and cell viability was calculated by Trypan blue dye exclusion.

### Flow Cytometry Analysis

To quantify the immune cells that infiltrate the gingival tissues, 200,000 total cells in 50  $\mu$ l PBS were incubated separately with 10  $\mu$ l phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (CD4<sup>+</sup> T cells), anti-CD8 (CD8<sup>+</sup> T cells), anti-CD14 (monocytes), anti-CD19 (B cells), anti-CD16, anti-CD56 (natural killer cells), anti-CD62L (neutrophils), and anti-CD84 (dendritic cells) monoclonal antibodies (mAb) for 30 minutes at 4°C in the dark.<sup>†††</sup> To determine the gingival immune cell responsible for the RANKL expression, double-staining flow-cytometry analysis was performed with a mouse anti-human RANKL mAb,<sup>§§§</sup> 20  $\mu$ g/ml at 4°C overnight, and incubated with FITC-conjugated rabbit anti-mouse immunoglobulin G (IgG)<sup>||||</sup> (1:64) for 1 hour at room temperature. Cells were washed once in PBS and resuspended in 300  $\mu$ l PBS to be analyzed by flow cytometry.<sup>¶¶¶</sup> Double-staining<sup>####</sup> CD45/CD14 was used to determine the leukocyte cellular population, and triple-staining<sup>\*\*\*\*\*</sup> CD4/CD8/CD3 was used to

determine the CD4/CD8 ratio. Cells were gated according to their forward- and side-scatter characteristics and their specific CD marker. FITC- and PE-conjugated isotype-matched control mAb were used to determine the positive and negative populations. Gates of each specific cell population were evaluated for RANKL expression.

### Cell Culture and Determination of RANKL Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Tissue-derived cells were cultured at 10<sup>6</sup> cells/ml in 200  $\mu$ l complete culture medium in a 96-well culture plate and stimulated with 5  $\mu$ g/ml of phytohemagglutinin<sup>††††</sup> (PHA) for T-lymphocyte activation and 0.1  $\mu$ g/ml LPS of *Escherichia coli*,<sup>††††</sup> for 24 hours at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Aliquots of supernatant cellular cultures were assayed by ELISA to determine the RANKL levels according to the manufacturer's recommendations.<sup>§§§§</sup>

### Histologic Analysis

Patient and control gingival samples were analyzed to identify CD4<sup>+</sup> and RANKL<sup>+</sup> immunoreactions by immunofluorescent microscopy.

Tissue sections of 5- $\mu$ m thickness were cut and mounted on poly-L-lysine<sup>|||||</sup> glass slides. After rinsing in PBS, sections were digested with 0.2% pepsin at 37°C for 30 minutes for antigen unmasking<sup>¶¶¶¶</sup> and were preincubated with 3% PBS/bovine serum albumin (BSA)<sup>#####</sup> for 30 minutes at room temperature to avoid unspecific background staining. Thereafter, sections were incubated with a primary mAb of mouse origin raised against human RANKL<sup>\*\*\*\*\*</sup> in 20  $\mu$ g/ml working solution in 3% PBS/BSA and a primary polyclonal antibody (pAb) of rabbit origin raised against human CD4<sup>†††††</sup> at 4°C overnight in a humidified chamber. Afterwards, the sections were incubated with either a goat anti-mouse<sup>†††††</sup> or a goat

†† Assay-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA.

§§ TaqMan Universal PCR Master Mix, Applied Biosystems.

||| ABI PRISM 7700 Sequence Detector System, Applied Biosystems.

¶¶ TaqMan Pre-Developed Assay Reagent Human GAPDH, Applied Biosystems.

## Gibco Invitrogen, Grand Island, NY.

\*\*\* Gibco Invitrogen.

††† Axiovert 100, Zeiss, Göttingen, Germany.

†††† Becton Dickinson Immunocytometry Systems, San Jose, CA.

§§§ R&D Systems, Minneapolis, MN.

|||| R&D Systems.

¶¶¶ Becton Dickinson Immunocytometry Systems.

#### LeucoGate, Becton Dickinson Immunocytometry Systems.

\*\*\*\*\* TriTest, Becton Dickinson Immunocytometry Systems.

††††† Roche Molecular Biochemicals, Mannheim, Germany.

†††††† Sigma Chemical.

§§§§§ Alpco Diagnostics, Windham, NH.

||||| Sigma Chemical.

¶¶¶¶ Difco Laboratories, Detroit, MI.

##### Sigma Chemical.

\*\*\*\*\* R&D Systems.

†††††† Ready to use, NeoMarkers, Lab Vision, Warm Springs, CA.

††††††† ML Texas Red, Molecular Probes, Eugene, OR.

anti-rabbit<sup>§§§§§</sup> secondary mAb, in 5 µg/ml working solution, for 1 hour at 37°C in a humidified chamber. After being cover slipped in fluorescence mounting media, slides were observed by fluorescence microscopy.<sup>|||||||</sup>

The colocalization of RANKL<sup>+</sup> and CD4<sup>+</sup> immunoreaction was detected using a defined protocol.<sup>|||||||</sup> Briefly, sections were incubated with the anti-CD4 primary pAb detected with secondary mAb<sup>#####</sup> and, thereafter, incubated with the anti-RANKL primary mAb detected with secondary mAb,<sup>\*\*\*\*\*</sup> as previously described. Finally, the media slides (cover slipped in fluorescence mounting media) were observed by confocal microscopy.<sup>+++++</sup>

To prove the specificity of immunoreactions, negative controls were carried out by omitting the primary Ab and both the primary and secondary Ab and using PBS/BSA instead. A rat maxillae tooth sample under orthodontic distal force, which induces distal bone resorption, was used as the positive control of RANKL immunoreaction.

#### Data Analysis

Clinical and experimental data were statistically analyzed using software<sup>+++++</sup> and expressed as subject means ± SD. The unpaired Student *t* test was used to analyze differences in clinical and RT-PCR data between patient and control groups. In the flow cytometry analysis, the differences were evaluated using the analysis of variance (ANOVA) test. The significance (*P* < 0.05) of differences was assessed using the Tukey test.

The RT-PCR data were analyzed with software.<sup>§§§§§</sup> The data were plotted as the ΔRn fluorescence signal versus the cycle number, using the equation  $\Delta Rn = Rn^+ - Rn^-$ , where  $Rn^+$  is the fluorescence signal of the product at any given time and  $Rn^-$  is the fluorescence signal of the baseline emission during cycles 3 to 15. An arbitrary threshold was set by the replicate method. The Ct value is defined as the cycle number at which the ΔRn crosses this threshold. The relation between RANKL and GAPDH in each individual was defined as ΔCt, where  $\Delta Ct = Ct_{RANKL} - Ct_{GAPDH}$ . The amplification efficiency between GAPDH and RANKL was evaluated analyzing the ΔCt variation with template dilutions having a 1,000-fold range. A plot of ΔCt versus log cDNA dilution was made, and data were fitted using least-square linear regression analysis. The change in RANKL relative to the GAPDH endogenous control was determined by the  $2^{-\Delta\Delta Ct}$  method.<sup>16</sup>

The flow cytometry data were analyzed using software.<sup>|||||||</sup> CD data were represented as histograms and CD4/CD8/CD3<sup>|||||||</sup> as a contour plot. The fraction of RANKL-positive cells was expressed as a percentage.

## RESULTS

Eight males and 25 females were included in the periodontitis patient group (age: 46.8 ± 7.33 years) and five males and 15 females were studied as healthy controls (age: 43.0 ± 5.50 years). Table 1 summarizes the clinical characteristics of subjects included in this study. No statistically significant differences in age and gender were observed between groups. Sites with SBP and BOP were significantly higher in periodontitis patients than in the control group (74.0 versus 42.1 and 59.8 versus 3.2, respectively). Furthermore, PD and CAL were also significantly higher in patients than controls (3.2 ± 0.32 versus 2.1 ± 0.33 and 3.4 ± 0.37 versus 1.3 ± 0.49, respectively).

Mean variations and standard deviations of Ct and ΔCt of RANKL and GAPDH of patients and healthy controls are shown in Table 2. GAPDH Ct was similar between patients and controls (21.678 ± 1.62 versus 21.505 ± 0.86), but the RANKL Ct and ΔCt were significantly lower in patients compared to the control group (28.791 ± 3.27 versus 36.514 ± 2.67 and 7.113 ± 3.00 versus 15.009 ± 2.80, respectively). The amplification efficiency of GAPDH and RANKL was similar with a slope of 0.0752 (N = 3); thus, amplification efficiencies of both amplicons are similar, and the fold change in RANKL gene expression may be represented by the  $2^{-\Delta\Delta Ct}$  method. Using the  $2^{-\Delta\Delta Ct}$  method, it was determined that the RANKL expression in periodontitis patients incremented 238.3-fold (range: 29.8 to 1,903.4) over control subjects (Table 2).

The characterization of gingival cells obtained by the tissue digestion of periodontitis and healthy gingival samples is shown in Table 3. Figure 1 shows a flow cytometry CD4/CD8/CD3<sup>#####</sup> contour plot of a representative sample of a periodontitis patient and a control individual. Higher levels of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes are associated with periodontitis samples (28.1 ± 7.09 versus 4.4 ± 1.46 and 15.4 ± 3.80 versus 3.2 ± 0.95, respectively), whereas the presence of granulocytes is associated with healthy samples (26.9 ± 7.28 versus 44.8 ± 7.29). No statistical differences in the CD4/CD8 ratio between groups were found. In the periodontitis samples, 19.4 ± 2.81 of the total isolated cells expressed RANKL, from which 17.1 ± 2.54 of the RANKL expression was

§§§§§ ML Alexa Fluor 488, Molecular Probes.

||||||| Exioskop 20, Carl Zeiss, Germany.

||||||| Jackson Immuno Research Laboratories, West Probe, PA.

##### ML Alexa Fluor 488, Molecular Probes.

\*\*\*\*\* ML Texas Red, Molecular Probes.

+++++ Leica Microsystems, Heidelberg, Germany.

+++++ Stata 7.0, Statistics/Data Analysis, Stata, College Station, TX.

§§§§§ ABI PRISM 1.7, Applied Biosystems.

||||||| WinMDi 2.8, The Scripps Research Institute, La Jolla, CA.

||||||| TriTest, Becton Dickinson Immunocytometry Systems.

##### TriTest, Becton Dickinson Immunocytometry Systems.

associated with CD4<sup>+</sup> T cells,  $1.7 \pm 1.05$  with dendritic cells, and  $0.6 \pm 0.63$  with monocytes (Table 4).

Under non-stimulatory conditions, higher levels of RANKL were detected in patients versus control samples ( $18.4 \pm 5.79$  versus  $8.0 \pm 1.34$ ). Also, lymphocytes from patients were strongly induced to secrete RANKL by LPS and PHA compared to control-derived lymphocytes ( $33.8 \pm 7.30$  versus  $21.0 \pm 5.60$  and  $30.1 \pm 7.77$  versus  $14.5 \pm 3.57$ , respectively) (Fig. 2). Furthermore, it was observed that LPS and PHA significantly stimulate the RANKL synthesis in periodontitis ( $33.8 \pm 7.30$  and  $30.1 \pm 7.77$ , respectively, versus  $18.4 \pm 5.79$ ) and control subjects ( $21.0 \pm 5.60$  and  $14.5 \pm 3.57$ , respectively, versus  $8.0 \pm 1.34$ ).

Cytoplasmic and on-surface granular RANKL immunoreactivity by fluorescent microscopy of various intensities were detected in a gingival section of a rep-

resentative periodontitis sample (Fig. 3A). Lower RANKL immunoreaction was observed in a representative healthy sample (Fig. 3B). Figure 3C shows RANKL<sup>+</sup> expression and its CD4<sup>+</sup> colocalization by confocal microscopy in a gingival specimen of a periodontitis patient (yellow arrowhead). All immunoreactive cells showed granular surface staining with moderate to strong intensities where most of the staining is scattered on the inflammatory focus of the periodontal infection.

## DISCUSSION

This work analyzed RANKL levels in gingival tissues from periodontitis patients and healthy control subjects, and associated these RANKL levels with the gingival CD4<sup>+</sup> T-cell activity. Our data demonstrate that higher RANKL levels observed in patients are associated with gingival CD4<sup>+</sup> T cells during periodontal infection.

Periodontitis, one of the most prevalent human pathologies, is an inflammatory disease elicited by a specific bacterial infection that involves an immune host response where a broad array of inflammatory cytokines lead to the destruction of the tooth-supporting alveolar bone.<sup>6,7</sup> Although several cytokines, such as interleukin (IL)-1 $\beta$ , TNF- $\alpha$ , prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), interferon (IFN)- $\gamma$ , and IL-6, have been proposed as key regulators of pathologic bone resorption, the precise underlying mechanisms and key cytokines of in vivo alveolar bone destruction in human periodontitis remain unknown.

The RANKL/RANK/OPG cytokine complex has been associated with different physiological activities and diverse metabolic and inflammatory bone diseases. RANKL has been associated with lymph-node formation,<sup>17</sup> mammalian gland formation,<sup>18</sup> bone modeling and remodeling,<sup>19</sup> rheumatoid arthritis,<sup>14,18</sup> osteoporosis,<sup>5</sup> Paget's bone disease,<sup>5</sup> bone tumors,<sup>20</sup> and facial osteolytic lesions.<sup>21</sup>

**Table 1.**

### Clinical Characteristics of Periodontitis Patients and Healthy Control Subjects

| Clinical Characteristic               | Patients (N = 33) | Controls (N = 20) |
|---------------------------------------|-------------------|-------------------|
| Age (years [mean $\pm$ SD])           | 46.8 $\pm$ 7.33   | 43.0 $\pm$ 5.50   |
| Gender (% females [mean])             | 75.8              | 75.0              |
| PD* (mm [mean $\pm$ SD])              | 3.2 $\pm$ 0.32    | 2.1 $\pm$ 0.33    |
| CAL <sup>†</sup> (mm [mean $\pm$ SD]) | 3.4 $\pm$ 0.37    | 1.3 $\pm$ 0.49    |
| SBP <sup>‡</sup> (% sites [mean])     | 74.0              | 42.1              |
| BOP <sup>§</sup> (% sites [mean])     | 59.8              | 3.2               |

\* PD in periodontitis versus control groups:  $P < 0.0001$ .

<sup>†</sup> CAL in periodontitis versus control groups:  $P < 0.0001$ .

<sup>‡</sup> SBP in periodontitis versus control groups:  $P < 0.0001$ .

<sup>§</sup> BOP in periodontitis versus control groups:  $P < 0.0001$ .

**Table 2.**

### Ct and $\Delta$ Ct of RANKL and GAPDH and Fold Change of RANKL Using the $2^{-\Delta\Delta$ Ct Method in Periodontitis Patients and Healthy Control Subjects

|   | Patients (N = 33)                      | Controls (N = 20)                      |
|---|--|--|
| Ct <sub>GAPDH</sub> (mean $\pm$ SD and data range)    | 21.678 $\pm$ 1.62<br>19.026 to 24.072  | 21.505 $\pm$ 0.86<br>20.845 to 23.117  |
| Ct <sub>RANKL</sub> (mean $\pm$ SD and data range)    | 28.791 $\pm$ 3.27*<br>24.189 to 35.644 | 36.514 $\pm$ 2.67*<br>36.426 to 40.000 |
| $\Delta$ Ct (mean $\pm$ SD)                           | 7.113 $\pm$ 3.00 <sup>†</sup>          | 15.009 $\pm$ 2.80 <sup>†</sup>         |
| $2^{-\Delta\Delta$ Ct (fold change [estimated error]) | 238.3 (29.8 to 1,903.4)                | 1.0 (0.1 to 7.0)                       |

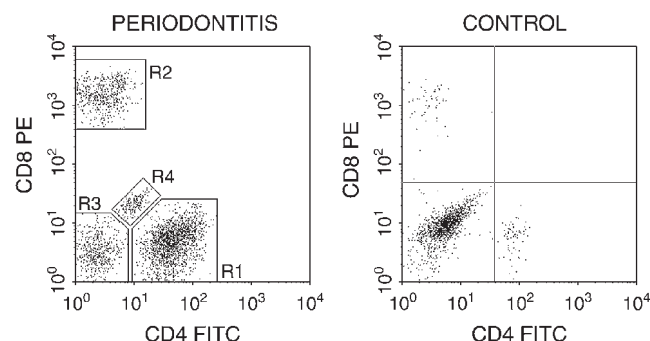
\* Ct<sub>RANKL</sub> in periodontitis versus control groups:  $P < 0.0001$ .

<sup>†</sup>  $\Delta$ Ct in periodontitis versus control groups:  $P < 0.0001$ .

**Table 3.**  
**Gingival Immune Cells of Periodontitis Patients and Healthy Control Groups Obtained by Tissue Digestion and Analyzed by Flow Cytometry (% [mean ± SD])**

| Gingival Immune Cell           | Cell Marker | Patients (N = 33) | Controls (N = 20) |
|--------------------------------|-------------|-------------------|-------------------|
| Lymphocytes T CD4 <sup>+</sup> | CD4         | 28.1 ± 7.09*      | 4.4 ± 1.46*       |
| Lymphocytes T CD8 <sup>+</sup> | CD8         | 15.4 ± 3.80†      | 3.2 ± 0.95†       |
| CD4/CD8 ratio                  | CD4/CD8     | 1.9 ± 0.50        | 1.6 ± 0.61        |
| Lymphocytes B                  | CD19        | 6.0 ± 2.00        | 4.5 ± 1.38        |
| NK cytotoxic cells             | CD16        | 3.3 ± 0.49        | 2.0 ± 1.20        |
| NK secretory cells             | CD56        | 4.8 ± 1.02        | 4.6 ± 2.35        |
| Monocytes                      | CD14        | 6.6 ± 3.86        | 9.1 ± 1.84        |
| Dendritic cells                | CD83        | 2.7 ± 0.55        | 3.5 ± 1.10        |
| Neutrophils                    | CD62L       | 26.9 ± 7.28‡      | 44.8 ± 7.29‡      |

\* CD4<sup>+</sup> T cells in periodontitis versus control groups: *P* < 0.0001.  
 † CD8<sup>+</sup> T cells in periodontitis versus control groups: *P* < 0.0001.  
 ‡ Neutrophils in periodontitis versus control groups: *P* < 0.0001.



**Figure 1.**  
Immunotyping by flow cytometry of gingival cells obtained by tissue digestion of gingival samples. CD4/CD8/CD3 contour plot of a representative sample of a periodontitis patient and a healthy control subject. Periodontitis: R1 = CD4<sup>+</sup> T cells (52.4%); R2 = CD8<sup>+</sup> T cells (19.3%). Healthy: CD4<sup>+</sup> T cells (4.1%); CD8<sup>+</sup> T cells (3.2%). PE = phycoerythrin; FITC = fluorescein isothiocyanate.

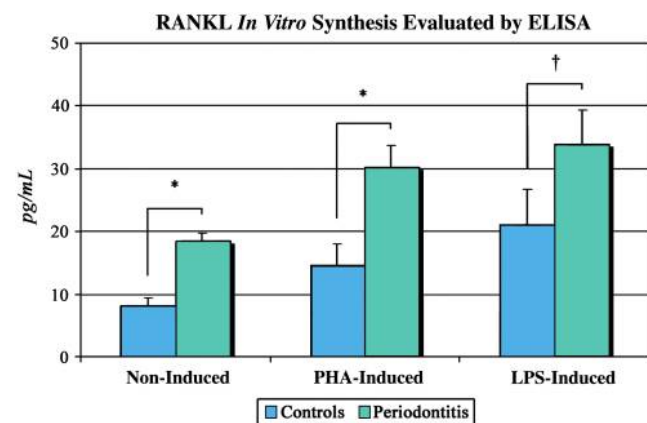
In periodontal tissues, the RANKL/OPGL/OPG cytokine complex has been associated with physiological events that regulate the alveolar bone homeostasis,<sup>22</sup> odontogenesis,<sup>23</sup> alveolar bone resorption to form the eruption pathway during tooth eruption,<sup>24</sup> physiological deciduous root resorption,<sup>25</sup> alveolar bone destruction in periapical granuloma,<sup>26</sup> and periodontitis.<sup>10-13,27,28</sup>

Using RT-PCR analysis of gingival tissues, we demonstrated that the Ct and ΔCt of RANKL in periodon-

**Table 4.**  
**RANKL Expression in Gingival Immune Cells of Periodontitis Patients Analyzed by Double-Staining Flow Cytometry (mean ± SD)**

| Gingival Immune Cell           | Cell Marker                          | Percentage    |
|--------------------------------|--------------------------------------|---------------|
| Total RANKL <sup>+</sup> cells | RANKL <sup>+</sup>                   | 19.4 ± 2.81   |
| Lymphocytes T CD4 <sup>+</sup> | CD4 <sup>+</sup> RANKL <sup>+</sup>  | 17.1 ± 2.54*† |
| Monocytes                      | CD14 <sup>+</sup> RANKL <sup>+</sup> | 0.6 ± 0.63*   |
| Dendritic cells                | CD83 <sup>+</sup> RANKL <sup>+</sup> | 1.7 ± 1.05†   |

\* RANKL<sup>+</sup> in CD4<sup>+</sup> T cells versus monocytes: *P* < 0.00001.  
 † RANKL<sup>+</sup> in CD4<sup>+</sup> T cells versus dendritic cells: *P* < 0.00001.

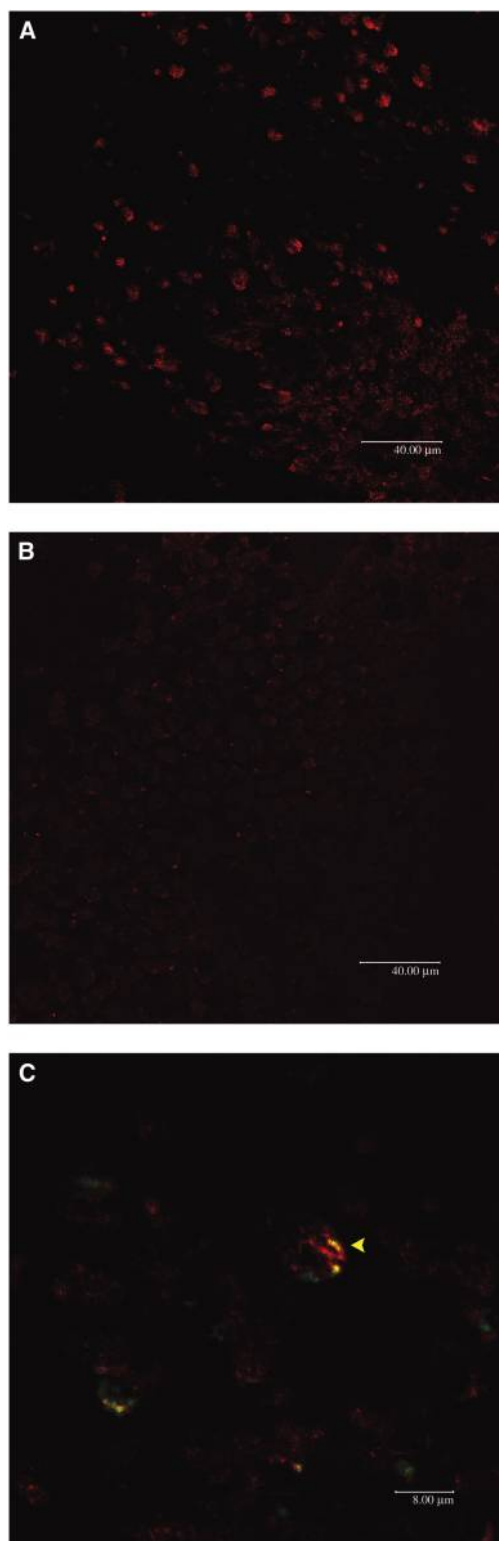


**Figure 2.**  
Non-induced, LPS-, and PHA-induced RANKL in vitro synthesis evaluated by ELISA in supernatant of gingival cell cultures in periodontitis patients and healthy subjects. \**P* = 0.002; †*P* = 0.02.

titis subjects were lower than in healthy subjects. Because Ct values decrease linearly with increasing input-target quantity,<sup>29</sup> RANKL mRNA levels were higher in the periodontitis group.

In agreement with Liu et al.,<sup>11</sup> our periodontitis subjects expressed RANKL mRNA. Our data show that all periodontitis subjects present a lower Ct of RANKL mRNA than the mean Ct of the control group (36.514 ± 2.67), except in one case (35.644) (Table 2).

Our quantitative data demonstrated higher levels of RANKL in periodontitis patients compared to healthy subjects. Similar results were established in semi-quantitative studies of RANKL and OPG protein expression in periodontal tissues by immunohistochemistry and RT-PCR.<sup>10,11</sup> We studied RANKL mRNA levels in periodontitis tissues by RT-PCR because it is the method of choice to quantify the mRNA expression of cytokines expressed at low levels.<sup>29</sup>



### Figure 3.

**A)** RANKL immunoreactivity by fluorescent microscopy in a gingival section of a representative periodontitis sample. **B)** RANKL immunoreactivity in a gingival section of a representative healthy sample. **C)** RANKL<sup>+</sup> expression and its CD4<sup>+</sup> colocalization by confocal microscopy in a gingival specimen of a periodontitis patient (yellow arrowhead).

Furthermore, the change in expression of the RANKL target gene normalized to GAPDH permits the relative quantification of RANKL in periodontal tissues.<sup>16,29</sup> The GAPDH internal standard was selected because of its low fluctuating synthesis in all nucleated cell types.<sup>30</sup> Our results indicate that there was no significant difference in the level of GAPDH gene expression between groups.

Using the  $2^{-\Delta\Delta C_t}$  method,<sup>16</sup> we observed a 238.3-fold (range: 29.8 to 1,903.4) increase in RANKL expression in the periodontitis group compared to the control group, indicating that the RANKL gene is highly overexpressed in periodontal tissues associated with bone destruction.

In gingival cell cultures, spontaneous and LPS- and PHA-stimulated RANKL levels were higher in periodontitis patients. LPS, widely used in *in vitro* cytokine synthesis, was selected as positive control of RANKL synthesis.<sup>31</sup> PHA is a vegetal lectin that links the T-cell receptor (TCR) and CD3 in T lymphocytes, inducing a polyclonal functional response, and is widely used in *in vitro* activation of T cells.<sup>32</sup> It was established that LPS and PHA significantly stimulated the RANKL synthesis in periodontitis and control cultures.

Total gingival cells obtained by enzymatic digestion of gingival samples of periodontitis patients and healthy controls have been used. By flow cytometry, it was determined that total CD4<sup>+</sup> lymphocytes were the predominant subset of cells observed in chronic periodontitis. In agreement with previous reports,<sup>33-35</sup> our study established that lymphocytes are the predominant cells observed in the inflammatory focus developed in infected periodontal tissues. By immunohistochemistry, we confirmed that CD4<sup>+</sup> T cells were the main subset of lymphocytes in patient samples (data not shown).

Additionally, an association of RANKL-positive immunoreaction with periodontitis gingival samples was shown. The anti-RANKL immunoreaction was observed mainly deeper in the connective tissue in the inflammatory focus characterized by a predominance of CD4<sup>+</sup> T cells. Similar data were found by Crotti et al.,<sup>10</sup> who observed a strong RANKL staining in infiltrated leukocytes in the granulation tissue from periodontitis lesions. Confocal microscopy established that RANKL colocalized with the CD4<sup>+</sup> T cells in periodontitis gingival samples. Using an animal chimerical model of periodontitis, Teng et al.<sup>27</sup> demonstrated that bacteria stimulated RANKL production by seeded human CD4<sup>+</sup> T cells. Therefore, whereas flow cytometry analysis demonstrated that CD4<sup>+</sup> T was the main cell isolated from gingival samples, the confocal observation links this CD4<sup>+</sup> expression to RANKL synthesis. To confirm that the RANKL expression was associated with CD4<sup>+</sup> T cell activity in gingival periodontitis tissues, double-staining flow

cytometry analysis was performed. Our data demonstrate that CD4<sup>+</sup> lymphocytes were the major cell type synthesizing RANKL in periodontitis gingival tissues. In a lower level, the RANKL on-surface expression was also detected in macrophages and dendritic cells. It has been established that RANKL is an important regulator of T-lymphocyte and dendritic cell interactions during the antigen presentation.<sup>18</sup> Taking these data together, we proposed that the bone loss adjacent to the inflamed area in periodontitis might be regulated by CD4<sup>+</sup> activity through the RANKL expression. To our knowledge, this is the first study demonstrating the expression of RANKL by CD4<sup>+</sup> T cells in human periodontal diseased tissues.

It has long been known that the immune and skeletal systems have a variety of regulatory molecules, such as cytokines, in common; thus, the physiology and pathology of one system may affect the other.<sup>3</sup> The abnormal activation of the immune system during the periodontal infection leads to alveolar bone destruction. Activated T cells have a positive effect on bone destruction in an indirect manner, stimulating macrophages to secrete proinflammatory cytokines, which strongly induce RANKL in osteoblast-lineage cells in periodontitis.<sup>28</sup> Our work demonstrates that CD4<sup>+</sup> T cells express RANKL themselves. T-cell infiltration is a hallmark of gingival tissues during periodontitis, and CD4<sup>+</sup> T cells are the major RANKL-expressing cells, indicating that they are largely responsible for the molecular imbalance on aberrant activation of osteoclasts in periodontitis.

Therefore, understanding the osteoimmunobiology should provide greater insight into the immunopathogenesis of chronic periodontitis and many other chronic human diseases. This understanding may facilitate the development of immunotherapeutics for these diseases.

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