Interferon-γ, Interleukins-6 and -4, and Factor XIII-A as Indirect Markers of the Classical and Alternative Macrophage Activation Pathways in Chronic Periodontitis

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Background: Macrophages account for 5% to 30% of the inflammatory infiltrate in periodontitis and are activated by the classic and alternative pathways. These pathways are identified by indirect markers, among which interferon (IFN)-γ and interleukin-6 (IL)-6 of the classic pathway and IL-4 of the alternative pathway have been studied widely. Recently, factor XIII-A (FXIII-A) was reported to be a good marker of alternative pathway activation. The aim of this study is to determine the macrophage activation pathways involved in chronic periodontitis (CP) by the detection of the indirect markers IFN-γ, IL-6, FXIII-A, and IL-4.

Methods: Biopsies were taken from patients with CP (n = 10) and healthy individuals (n = 10) for analysis of IFN-γ, IL-6, IL-4, and FXIII-A by Western blot (WB), immunohistochemistry (IHC), and enzyme-linked immunosorbent assay (ELISA). The same biopsies of healthy and diseased gingival tissue were used, and the expressions of these markers were compared between healthy individuals and those with CP.

Results: The presence of macrophages was detected by CD68+ immunohistochemistry and their IFN-γ, IL-6, IL-4, and FXIII-A markers by WB, IHC, and ELISA in all samples of healthy and diseased tissue. IL-6, IL-4, and FXIII-A were significantly higher in patients with CP, whereas FXIII-A was higher in healthy individuals.

Conclusion: The presence of IFN-γ, IL-6, IL-4, and FXIII-A in healthy individuals and in patients with CP suggests that macrophages may be activated by both classic and alternative pathways in health and in periodontal disease. *J Periodontol 2014;85:751-760.*

KEY WORDS

Cytokines; factor XIII; immunology; macrophages; macrophage activation; periodontal diseases.

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Periodontitis is an infectious and inflammatory condition that affects the supporting tissues of the teeth[1] and is caused by periodontal pathogens that interact with host tissues and cells, such as macrophages; these cells account for 5% to 30% of the inflammatory infiltrate of periodontal lesions[2] and release a wide range of cytokines, chemokines, and inflammatory mediators, leading to the breakdown of alveolar bone, radicular cementum, and periodontal ligament with the formation of pockets, gingival recession, or both.[1] They play a mainly antigen-presenting role in the innate and acquired immune responses.[3] Macrophages are activated through two distinct pathways[4] to perform a variety of functions, including the production of insulin-like growth factor, prostaglandin, cyclic adenosine monophosphate, and collagenases against bacterial endotoxins and immune complexes.[5]

The classic pathway is promoted by interferon (IFN)-γ, tumor necrosis factor (TNF)-α, or TNF-inducers, such as Toll-like receptor ligands (e.g., lipopolysaccharides [LPS], among others), is associated with T-helper (Th)1,[6] and is responsible for killing intracellular parasites,[7] lysis of tumor cells, and the

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production and secretion of interleukin (IL)-1β, IL-6, TNF-α,[8] reactive oxygen intermediates, and nitric oxide.[9] The alternative pathway, activated by Th2 cytokines, such as IL-4,[6] is involved in immunoregulation, the stimulation of cell-surface receptors, and the secretion of IL-10 and transforming growth factor-β1[7] to promote the resolution of inflammation, wound healing, and other reparative phenomena.[10]

Markers of macrophage activation in periodontitis are needed, and the candidates IFN-γ, IL-6 (classic pathway), and IL-4 (alternative pathway) have been studied widely.[11] Recently, factor XIII-A (FXIII) was reported to be a good marker of alternative pathway activation.[12] This fibrin-stabilizing coagulation factor is present in the circulation in tetrameric form with two A catalytic units and two B inhibitor units,[13] and the A subunit (FXIII-A) is involved in hemostasis, wound healing, and the maintenance of pregnancy.[14,15] The role of FXIII-A as a marker of the alternative pathway has been established because there is a clear increase in FXIII-A messenger RNA and protein in alternatively activated macrophages.[12]

Because of the diversity and relevance of their proinflammatory functions and their participation in reparative processes,[9] it is important to determine how macrophages present in periodontitis are activated. If classic pathway activation is confirmed, its role is most likely a rapid, acute response against bacterial infection,[11] whereas alternative pathway activation would indicate a predominance of reparative processes,[10] which are essential to halt progression of the disease.

The aim of this research is to study IFN-γ, IL-6, FXIII-A, and IL-4 as indirect markers of macrophage activation pathways in chronic periodontitis (CP).

MATERIALS AND METHODS

Patients

Participants were recruited at the School of Dentistry, University of Chile, Santiago, Chile, and at the periodontics unit at the Dr. Eloisa Diaz Diagnosis and Treatment Centre of the Northern Hospital Complex of the North Metropolitan Health Service, Santiago, Chile. In total, 23 patients with CP (nine males and 14 females, mean age: 47.30 ± 4.47 years) and 23 healthy individuals (11 males and 12 females, mean age: 46.38 ± 3.20 years) met all inclusion criteria. The study protocol was fully explained, and the participants signed an informed consent. The protocol and design of this study was approved by the Ethics Committee of the School of Dentistry, University of Chile, and by the National Fund for Scientific and Technological Development. Inclusion criteria for patients with CP included the following: 1) patients aged ≥35 years; 2) ≥14 natural teeth present (≥10 posteriors after excluding third molars); 3) individuals diagnosed with moderate to advanced CP, having ≥6 teeth with probing depth (PD) ≥5 mm and clinical attachment level (CAL) ≥3 mm with bone loss in ≥30% of the periodontal sites[1]; 4) those without previous periodontal treatment; and 5) no systemic diseases, such as diabetes, arthritis, ulcerative colitis, blood dyscrasias, Crohn's disease, human immunodeficiency virus infection, cancer, or coronary artery disease.[16] The control group criteria were the same except individuals did not have periodontal disease. Exclusion criteria included the following: 1) smoking; 2) pregnancy; and 3) patients who underwent treatment with antibiotics and/or anti-inflammatory drugs within 6 months before the initial examination. Of the total patients enrolled, 10 biopsies of healthy tissue and 10 of participants with periodontitis were obtained to perform Western blot (WB), immunohistochemistry (IHC), and enzyme-linked immunosorbent assay (ELISA).

Clinical Examination

Supragingival scaling was performed in all patients, and a trained technician (JG) recorded clinical parameters such as PD, CAL, O'Leary's plaque index (PI), and bleeding on probing (BOP) at six sites (mesio-buccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual) for each tooth, excluding third molars, using a periodontal probe.‖

Sample Collection

Biopsies were obtained from sites of posterior teeth with periodontitis, PD ≥5 mm and CAL ≥3 mm, and in healthy individuals during crown-lengthening surgery by prosthetic prescription, with no gingival inflammation or healthy gums associated with third molars in which extraction was indicated. An incision was made with a no. 15 blade, 1 to 2 mm from the gingival margin, directed toward the alveolar crest, following the major axis of the tooth, so that the biopsy contained epithelium and connective tissue (CT), according to the 1974 protocol of Ramfjord and Nissle.[17] Biopsies were then immersed in 5 mL sterile culture medium¶ supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin, and 200 mM L-glutamine and then transported on ice to the Periodontal Biology Laboratory (PBL), School of Dentistry, University of Chile, where they were washed three times in 0.9% NaCl, weighed on an analytical balance, and kept dry at –80°C for additional processing. The same biopsies of healthy and diseased gingival tissue were used for WB, IHC, and ELISA.

Extraction and Measurement of Total Protein

For each milligram of sample used in WB and ELISA, 10 mL lysis buffer was added containing 50 mM Tris-hydrochloride (HCl) (pH 7.5), 1% non-ionic surfactant,# and 1 tablet of protease inhibitor cocktail.**

‖ Hu-Friedy, Chicago, IL.
¶ Roswell Park Memorial Institute 1640, Sigma-Aldrich, St. Louis, MO.
# Triton X-100, Sigma-Aldrich, St. Louis, MO.
** Mini EDTA-Free Proteinase Inhibitor Cocktail, Roche, Indianapolis, IN.

Samples were homogenized with a high-performance dispersing machine[††] three times for 10 seconds each time, rested on ice, and then centrifuged for 5 minutes at 4,500 rpm. The total protein concentration of the supernatant was measured using a kit[‡‡] following the instructions of the manufacturer. Then, the absorbance was measured with a spectrophotometer[§§] at 562 nm. To determine the protein concentration of each unknown sample, a standard curve was constructed using linear regression of the calibration curve, calculating protein concentration by interpolating the curve, and multiplying by the dilution factor of the sample.

WB

There were 10 samples of healthy tissue and 10 of diseased gingival tissue. WB was performed for all markers, using actin as a loading control. First, 30 μL sample was lyophilized at –50°C and then resuspended in loading buffer, agitated, boiled for 5 minutes, and allowed to cool.

Electrophoresis in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Samples were resolved on 15% polyacrylamide Tris-glycine gels and transferred to polyvinyl difluoride membrane.[‖‖] After blocking in 3% bovine serum albumin (BSA),[¶¶] the membranes were incubated with primary antibodies, including the following: 1) monoclonal antihuman IL-6[##] at a dilution of 1:250; 2) antihuman FXIII-A[***] at 1:10,000; 3) antihuman IL-4[†††] at 1:1,000; 4) antihuman IFN-γ[‡‡‡] at 1:2,000; and 5) β-actin[§§§] at 1:5,000, overnight. The secondary antibody stabilized goat antimouse immunoglobulin G (heavy and light chain), peroxidase conjugate[‖‖‖] was added for actin and IL-6 at a 1:10,000 dilution, and goat antirabbit horseradish peroxidase-conjugated antibody[¶¶¶] was added at 1:10,000 for IL-4, FXIII-A, and IFN-γ at 37°C for 1 hour. The blots were developed by chemiluminescence[###] for 5 minutes and exposed on photographic plates,[****] followed by developing and fixing with a film processing system.[††††] Finally, the plates were digitized under standard conditions using specific software.[‡‡‡‡]

Densitometry

Films were scanned,[§§§§] and then bands were quantified using the software[‖‖‖‖] to establish a numerical relationship between each marker and actin expressed in pixels per actin.

IHC

Biopsies were fixed in 4% buffered formalin, dehydrated, and embedded in paraffin for storage. Then, 6-μm sections were cut, deparaffinized, and rehydrated using a standard protocol.

The tissue processing began with peroxidase blocking and antigen retrieval with 0.01 M citrate buffer (pH 6). Later, non-specific sites were blocked with 50 μL 2.5% phosphate-buffered saline (PBS)/BSA for 30 minutes at room temperature. PBS/BSA at 2.5% was removed from the positive controls, and 50 μL of the appropriate monoclonal antibody was added as follows: 1) antihuman IL-6 primary antibody[¶¶¶¶] at a dilution at 1:50; 2) antihuman FXIII-A[####] at 1:100; 3) antihuman IL-4[*****] at 1:100; 4) antihuman INF-γ[†††††] at 1:25, incubated in a humid chamber at 4°C overnight, with the exception of the CD68 monoclonal antibody,[‡‡‡‡‡] which was incubated for 1 hour at 1:50 according to the protocol. Later, biotinylated secondary antibody[§§§§§] was incubated at a concentration of 1:400 for 30 minutes and was developed with a diaminobenzidine kit[‖‖‖‖‖] as chromogen, with hematoxylin for nuclear counterstaining. Negative controls were used in each procedure, and human tonsil biopsies were used as positive controls. Finally, the samples were dehydrated and mounted on hydrophobic media.[¶¶¶¶¶] These slides were then analyzed with an optical microscope,[#####] and photographs were taken of selected areas with a camera.[******] The images were analyzed using software.[††††††]

Quantification

Labeled cells were quantified using the methodology described and validated by Atkinson et al.[18] and Gamonal et al.[19] Quantification was performed for markers of IFN-γ, IL-6, IL-4, and FXIII-A gingival tissue samples per field counting the total number of

††	Ultra-Turrax, Sigma-Aldrich.
‡‡	Micro BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL.
§§	Unicam UV/VIS Spectrometer UV2-100, Unicam, Santa Cruz, CA.
‖‖	Thermo Fisher Scientific.
¶¶	Rockland, Gilbertsville, PA.
##	R&D Systems, Minneapolis, MN.
***	Epitomics, Burlingame, CA.
†††	Epitomics.
‡‡‡	Epitomics.
§§§	Abcam, Cambridge, MA.
‖‖‖	Pierce Biotechnology, Rockford, IL.
¶¶¶	Pierce Biotechnology.
###	SuperSignal West Femto, Pierce Biotechnology.
****	Blue x-ray, catalog no. 34090, Pierce/Thermo Fisher Scientific.
††††	Periomat Intra, Durr Dental, Bietigheim-Bissingen, Germany.
‡‡‡‡	Control Center 3, MultiFunction Center Applications, Brother, Bridgewater, NJ .
§§§§	DCP-130 C, Brother.
‖‖‖‖	UN-SCAN-IT gel automated digitizing system v4.1 for Windows, Silk Scientific, Orem, UT.
¶¶¶¶	Monoclonal antihuman IL-6, catalog no. MAB2061, R&D Systems.
####	Catalog no. 2559-1, Epitomics.
*****	Catalog no. 2177-1, Epitomics.
†††††	Catalog no. 1833-1, Epitomics.
‡‡‡‡‡	Mouse anti-CD68, KP1 clone, catalog no. 08-0125, Invitrogen, Carlsbad, CA.
§§§§§	Vectastain Elite ABC Kit PK Universal 6200, Vector Laboratories, Burlingame, CA.
‖‖‖‖‖	Peroxidase Substrate Kit DAB SK4100, Zymed Laboratories, San Francisco, CA.
¶¶¶¶¶	Flotex, Lerner Laboratories, Pittsburgh, PA.
#####	Axiostar Plus model, serial no. 3109005987 1159-151, Carl Zeiss, Oberkochen, Germany.
******	Power Shot A640, Canon, Melville, NY.
††††††	Vision Axiovision Rel software, Carl Zeiss.

Table 1.

Quantification of CD68, IFN-γ, IL-6, IL-4, and FXIII-A Markers Present in Gingival Tissue Samples From Healthy Individuals and Patients With CP by IHC

Marker	Healthy Tissue (n = 10)	CP Tissue (n = 10)	*P* Values
CD68	4.5 (4.5)	17.5 (73.5)	0.015*
IFN-γ	4.3 ± 1.28	7.9 ± 5.9	0.056
IL-6	15 ± 7.9	62.3 ± 21.4	<0.0001*
IL-4	9 (5.5)	105 (21.5)	0.001*
FXIII-A	1 (1)	15.5 (3.5)	0.001*

Data are expressed as median (interquartile range) or mean ± SD.
* Statistically significant difference: $P < 0.05$ (unpaired *t* test and Mann-Whitney *U* test).

inflammatory cells positive for immunohistochemical staining, total count of stained cells for markers of CD68, IFN-γ, IL-6, IL-4, and FXIII-A, and as percentages of the total number of inflammatory cells (Table 1).

To perform a double-masked measurement, a trained member (LH) of the PBL randomly assigned each sample a number. Then, the researcher in charge (MN) scored the sections based on eight ×40 fields per sample under the microscope. Each field was divided into four quadrants for total quantitation of labeled inflammatory cells in the CT, and the results were verified by a trained reviewer (ND). Both researchers were masked to the antibody that had been incubated in the samples and to the clinical condition of donor patients.

ELISA

To perform the assay, 10 samples of healthy gingival tissue and 10 of diseased gingival tissue were used. A specific kit was used for each marker as follows: IFN-γ,‡‡‡‡‡‡ IL-6,§§§§§§§ FXIII-A,‖‖‖‖‖‖‖ and IL-4.¶¶¶¶¶¶ Samples and reagents, including wash buffer, enzyme immunoassay diluent, standards, biotinylated antibody, and streptavidin–peroxidase, were prepared in advance according to the manufacturer's protocol and were kept at room temperature. Later, 50 μL standard, sample, and biotinylated antibody were added to each well, covered with plastic strips, and incubated at room temperature for 2 hours. Then, the 20 samples were washed three to five times with wash buffer, incubated with streptavidin–peroxidase for 30 or 60 minutes, and again washed three to five times with wash buffer; chromogen substrate was added and incubated for 10 to 30 minutes according to the manufacturer's instructions. Finally, a solution was added to stop the reaction. The results were quantified by an ELISA reader###### at 450 nm and expressed in picograms per milliliter per sample.

Statistical Analyses

Statistical analyses were performed with statistical software.*******††††††† Age, PD, and CAL were compared using unpaired *t* tests, and sex, BOP, and PI were compared using the χ^2 test. The normality of the data distributions were tested using the Shapiro-Wilk test based on the results expressed as mean ± SD and analyzed by unpaired *t* tests. In case of non-normal distributions, the Mann-Whitney *U* test was used, and results were expressed as medians with interquartile ranges. The results were considered statistically significant when *P* values were <0.05.

RESULTS

Demographic and clinical features of all participants and samples tested are summarized in Table 2, which shows no significant differences by age or sex. However, all clinical parameters were significantly increased in periodontitis.

The presence of macrophages was detected through anti-CD68 IHC (Figs. 1A through 1D). IFN-γ, IL-6, IL-4, and FXIII-A were detected by WB (Fig. 2), IHC (Figs. 1E through 1T), and ELISA (Fig. 3) in all samples from healthy individuals and patients with CP. In WB by densitometric analysis (Fig. 3), no statistically significant differences were found in the observed bands for each marker in healthy individuals and patients with CP. IL-6, IL-4, and FXIII-A levels were significantly different between healthy and diseased gingival tissue samples by IHC presented as the total count of CD68, IFN-γ, IL-6, IL-4,

‡‡‡‡‡‡ Human IFN-γ ELISA Kit, catalog no. EHIFNG, Thermo Fisher Scientific.
§§§§§§§ Human IL-6 ELISA Kit, catalog no. EH2IL6, Thermo Fisher Scientific.
‖‖‖‖‖‖‖ Factor XIII (Human) ELISA Kit, catalog no. A0474, Abnova, Taipei City, Taiwan.
¶¶¶¶¶¶ Human IL-4 ELISA Kit, catalog no. EH2IL4, Thermo Fisher Scientific.
Expert 96, ASYS Hitech, Salzburg, Austria.
******* GraphPad Prism v.4.03, GraphPad Software, San Diego, CA.
††††††† Stata v.11.1, StataCorp, College Station, TX.

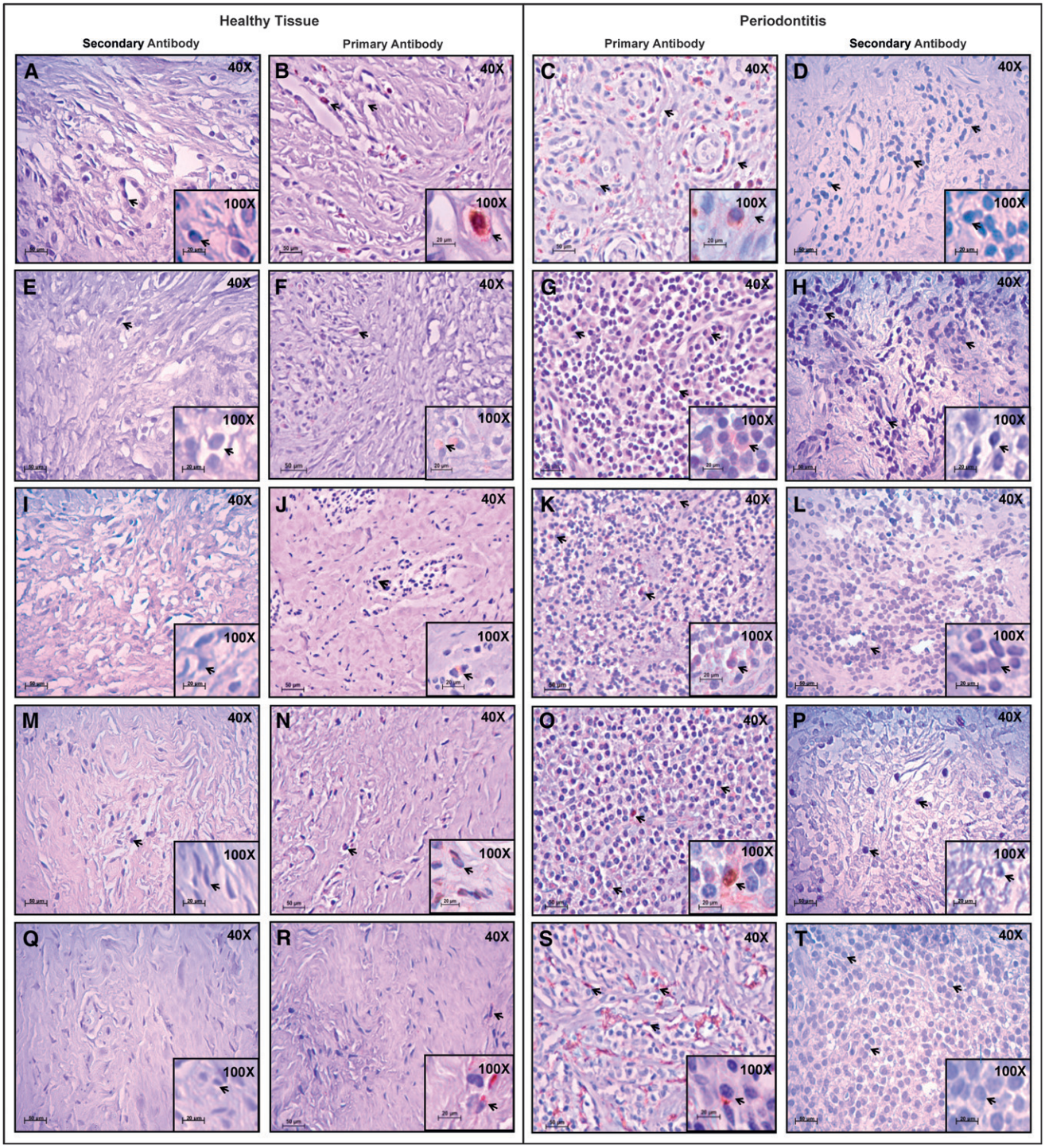

Figure 1.

*Immunohistochemistry CD-68 macrophage marker **(A, through D)**, activation of the classical IFN-γ **(E through H)** and IL-6 **(I through L)** and alternative IL-4 **(M through P)** and FXIII-A **(Q through T)** pathways. Periodontal tissue biopsies labeled with antibodies to CD-68, IFN-γ, IL-6, IL-4, and FXIII-A. The presence of the markers (black arrows), stained with at 40× and 100× magnification in healthy tissue samples (n = 10) and CP patients (n = 10), and the presence of cells (blue) whose morphology is compatible with lymphocytes can be observed.*

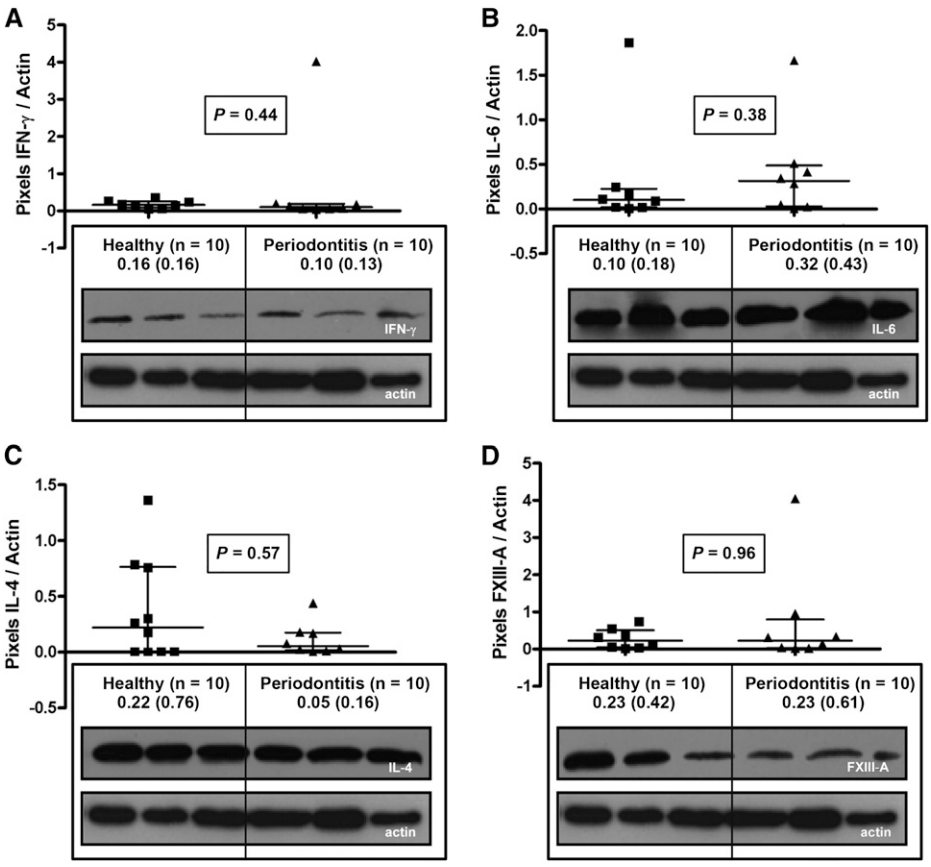

Figure 2.
*Presence of actin and markers of macrophage activation pathways, data sample and densitometric analysis of the bands of IFN-γ **(A)**, IL-6 **(B)**, IL-4 **(C)** and FXIII-A **(D)**, detected by WB in samples of gingival tissue from healthy individuals (n = 3) and patients with CP (n = 3). Data expressed as median (interquartile range = IQR) or mean ± SD. Statistically significant difference (P <0.05) (unpaired t-test and Mann-Whitney U test). Triangles = periodontitis; squares = healthy.*

and FXIII-A marker-positive cells (Table 1) and as percentages of the total number of inflammatory cells (Table 3). The ELISA data also reflect this relationship, with concentrations of IL-6 (7.0215 [2.553] mL versus 99.5745 [126.383] mL, $P = 0.0001$) and IL-4 (1.056 ± 0.4882 mL versus 1.562 ± 0.4713 mL; $P = 0.0299$) that were higher in patients with CP, whereas FXIII-A (94.96 ± 18.38 mL versus 69.68 ± 21.43 mL, $P = 0.011$) is higher in healthy individuals (Fig. 3).

DISCUSSION

Macrophages and their activation pathways are critical in periodontitis because it is considered an immunoinflammatory condition; however, we believe the insufficiency of data describing their role in this disease[20] is enough to justify the present study.

The presence of IFN-γ, IL-6, IL-4, and FXIII-A was detected in all samples of healthy tissue and those with periodontitis by IHC, ELISA, and WB. With IHC, significantly higher levels of IL-6, IL-4, and FXIII-A were observed in patients with CP. With WB, bands of similar size and intensity were observed at their expected molecular weights in healthy individuals and patients with CP. Quantitation by densitometric analysis normalized to actin showed no significant differences. This discrepancy could be attributable to the specific sensitivities of each technique. Moreover, in WB, a standardized amount of protein from each sample lysate is loaded per well, and the samples do not contain only macrophages.

CD68+ macrophages were observed in all gingival tissue samples, with a higher expression in patients with CP. Previous studies indicate that macrophages are present in large numbers in tissues adjacent to the oral epithelium[15,21] and are distributed similarly in patients with gingivitis and in those undergoing periodontal treatment.[21] In contrast, Ralph[22] notes more macrophages in chronically inflamed sites, in agreement with the present results. Therefore, it is possible that the density of macrophages found in periodontal tissues corresponds to the sum of the resident macrophages in gingival tissue plus monocytes that migrate from the lumen of blood capillaries to CT during stimulation of the inflammatory mediators present in periodontitis. Alternatively, Chapple et al.[20] note that the recruitment and activation of macrophages could fail at sites affected by periodontitis as a result of the unique environment of gingival tissues and the unusual pathologic features of an untreated lesion.

When quantifying the expression of indirect markers for the activation pathways, the present results are novel. For IFN-γ, significant differences in the mean concentration in healthy individuals versus patients with CP ($P > 0.05$) were not observed. In contrast, Gorska et al.[23] reported significantly higher concentrations of IFN-γ in the sera and gingival tissues of patients with CP compared with healthy controls. Regarding periods of activity and remission of periodontitis, Garlet et al.,[24] Chapple et al.,[20] and Schlegel Gómez et al.[21] showed that the concentration of IFN-γ in gingival crevicular fluid (GCF) and gingival tissue was higher in active versus inactive

sites. These findings were supported by Ralph[22] and Teng et al.[25] regarding the ability of IFN-γ to induce bone loss. It has been shown that macrophages activated by IFN-γ generate a destructive response associated with Th1 cytokines,[26,27] whose concentration in GCF is 10 times higher than the Th2 cytokines IL-4 and IL-6[28] in severe periodontitis. In addition, increased levels of IFN-γ favor the phagocytic activity of monocytes and neutrophils, limiting infection in periodontal tissues[29] and stimulating monocyte response to LPS, producing proinflammatory molecules, such as prostaglandin E2, IL-1β, and TNF-α, which promote the breakdown of the soft and hard tissues of the periodontium.[27,30]

A higher concentration of IL-6 was found in gingival tissue with periodontitis (P <0.05), in agreement with Rodriguez[31] and Kono et al.,[32] who showed lower concentrations in healthy periodontal tissues versus gingivitis[32] or periodontitis[31] samples. Significantly higher levels of IL-6 were found in GCF in individuals with refractory periodontitis compared with periodontally stable individuals[33,34] and in active versus inactive periodontal sites at initial measurement and at 3 months.[35] It is noteworthy that T cells require previous stimulation by monocytes to secrete IL-6; these are able to synthesize IL-6 without needing other cells,[36] which could explain its high concentration in periodontal tissue. Because of this, IL-6 is considered an indicator of periodontal disease.[37]

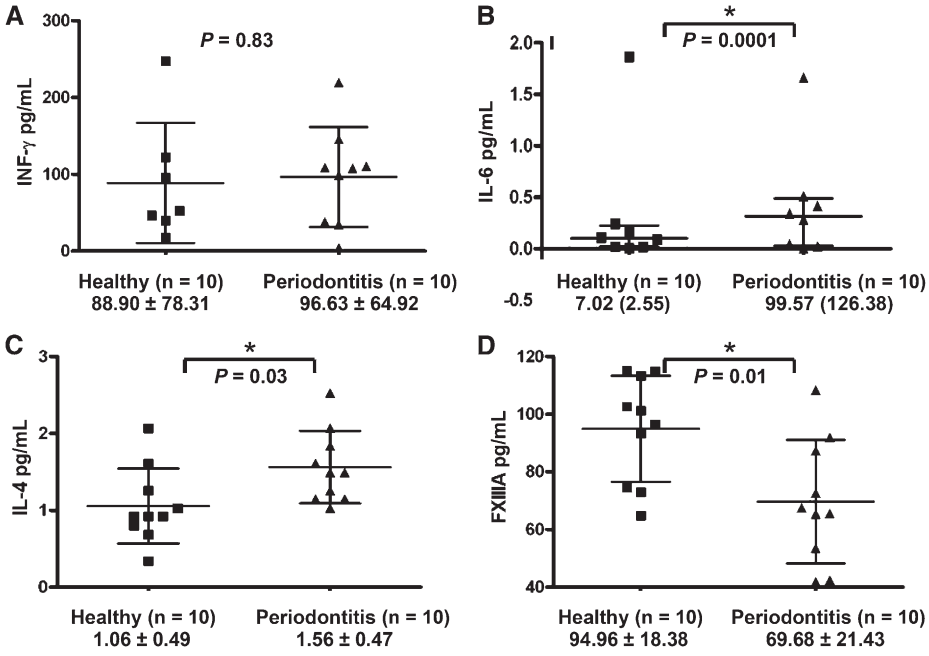

Figure 3.
*ELISA markers of macrophage classical and alternative activation pathways. Total concentration of IFN-γ **(A)** and IL-6 **(B)**, IL-4 **(C)** and FXIII-A **(D)** measured by ELISA in samples of gingival tissue from healthy individuals (n = 10) and patients with CP (n = 10), expressed as pg/mL, as median (interquartile range = IQR) or mean ± SD. ***** Statistically significant difference (P <0.05). Unpaired t-test and Mann-Whitney U test). Triangles = periodontitis; squares = healthy.*

Table 2.

Demographic and Clinical Features of Healthy Individuals and Patients With CP

	Total Recruited		Total Samples Tested	
Features	Healthy (n = 23)	CP (n = 23)	Healthy (n = 10)	CP (n = 10)
Age (mean ± SD)	46.38 ± 3.20	47.30 ± 4.47	45.58 ± 2.20	46.80 ± 4.43
Females (%)	52.17	60.86	50	60
PD (mm, mean ± SD)	1.20 ± 0.45*	3.06 ± 0.65*	1.18 ± 0.2*	3.04 ± 0.55*
CAL (mm, mean ± SD)	0.77 ± 0.89†	4.10 ± 1.80†	0.7 ± 0.69†	4.1 ± 1.70†
Sites with plaque (%)	20.5‡	90.63‡	19.5‡	89.64‡
Sites with BOP (%)	0.02§	47.6§	0.02§	45.8§

* Mean PD in healthy individuals versus patients with CP: P = 0.001.
† Mean CAL in healthy individuals versus patients with CP: P = 00001.
‡ Percentage of sites with plaque in healthy individuals versus patients with CP: P = 00001.
§ Percentage of sites with BOP in healthy individuals versus patients with CP: P = 0.0007 (unpaired t test and χ^2 test).

Table 3.

Relationship Among CD68, IFN-γ, IL-6, IL-4, and FXIII-A Marker-Positive Cells and Total Count Inflammatory Cells in Healthy Gingival Tissue and CP Tissue Detected by IHC

Marker	Healthy Tissue (n = 10)	CP Tissue (n = 10)	*P* Values
CD68 (% cell)	29.19	80.29	<0.0001*
IFN-γ (% cell)	6.24	8.56	0.1207
IL-6 (% cell)	24.05	40.72	<0.0001*
IL-4 (% cell)	22.17	67.58	<0.0001*
FXIII-A (% cell)	5.59	24.34	<0.0001*

Data are expressed as percentage of the total number of inflammatory cells.
* Statistically significant difference: $P < 0.05$ (X^2 test.)

For IL-4, higher concentrations in diseased gingival tissue ($P < 0.05$) were also found. However, Pradeep et al.[38] note that, in GCF, the concentration of IL-4 measured by ELISA decreases from periodontal health toward periodontitis, suggesting the involvement of IL-4 in the periods of remission of periodontal disease related to effector functions expressed by the macrophage in response to alternative activation. A later study using ELISA also showed a higher expression of IL-4 in healthy patients when compared with patients with CP.[39]

This activity would promote anti-inflammatory functions, healing, and tissue repair, which are features compatible with periodontal health. Moreover, IL-4 is a potent inhibitor of macrophage functions and induces monocyte apoptosis.[40,41] In periodontal lesions, IL-4 inhibits mac-rophages and CD14, a key receptor for LPS, which acts as an inducer of classic activation. Because of this, Holla et al.[42] proposed that the absence of monocyte inhibition by IL-4 leads to tissue breakdown in periodontitis. The present data coincide with previous studies that prove a strong association between gingival inflammation, severity of periodontitis, and IL-4.[23] These results could be explained by the complexity of the periodontal disease, which includes two types of immune response, in which the immune response mediated by cells promoted by Th1 cytokines (IFN-γ, IL-6) would be present in the first stage of periodontitis.[43] Conversely, some studies note that the prevailing immune response in progressive periodontal lesions is the humoral, promoted by the IL-4.[24] So, the expression of IL-4 may be associated rather to the type of lesion (stable or progressive) present in the patient with periodontitis than to a state of health or disease. Concerning the latter, a study showed that there are no significant differences in the expression of IL-4 in gingival tissue samples of health versus periodontitis.[44]

Regarding FXIII-A, a higher concentration in samples of healthy individuals compared to patients with CP ($P < 0.05$) was observed, indicating that, because of their plasticity,[45] macrophages would respond to signals generated by this specific microenvironment through the alternative activation pathway, contributing to tissue integrity and homeostasis. Results cannot be compared because, to our knowledge, there are no previous reports.

Exacerbations of periodontitis are related to the Th1 response as a result of the classic activation pathway, and remissions associated with the Th2 response triggered by the alternative pathway are significant. It is also possible that, in the same patient, some sites may be active, with progressive breakdown, whereas other may be stable over time. Therefore, macrophages present in periodontitis may be activated by both pathways. For these reasons, the activation pathways have become a topic of great importance because of their decisive influence.[45] Although different macrophage groups are currently classified according to their activation states, their functional properties and molecular repertoires are somewhat unclear. These uncertainties suggest future interesting investigations.

In summary, the classic and alternative pathways are present in health and disease; therefore, macrophages could be activated by both pathways in healthy individuals and in patients with CP.

The authors suggest additional studies using the markers studied here and especially FXIII-A involving the monitoring of patients diagnosed with and treated for periodontitis, for changes that might relate to the macrophage activation pathways. It would also be interesting to know whether post-treatment levels of these markers are similar to those found in periodontally healthy patients.

CONCLUSIONS

Macrophages and their markers IFN-γ, IL-6, IL-4, and FXIII-A were present in all tissue samples, with a higher density of IL-6, IL-4, and FXIII-A expression in individuals with CP. Additionally, the concentrations of IL-6 and IL-4 were significantly higher in patients with CP, whereas FXIII-A was higher in healthy individuals. Overall, because IFN-γ, IL-6, IL-4, and FXIII-A are present in both healthy individuals and patients with CP, macrophages could be activated by both pathways in health and in periodontal disease. The results of the present study need to be interpreted with caution because of its cross-sectional nature and a relatively small sample size.

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