Original Research

Infliximab attenuates inflammatory osteolysis in a model of periodontitis in Wistar rats

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Abstract

Periodontitis is a chronic inflammatory disease related to tooth loss in adults. Infliximab is a chimeric monoclonal antibody against TNF-α and is prescribed for the treatment of systemic inflammatory diseases. This study aimed to investigate the role of infliximab on experimental periodontal disease (EPD). EPD was induced by passing a 3.0 nylon thread around the upper left second molar in Wistar rats. Animals were either treated with intravenous infliximab (1, 5, 7, and 10 mg/kg) or saline solution 30 min before the periodontitis induction and were followed until they were sacrificed on the 11th day. A subset of rats was euthanized on the third day for analysis of gingival myeloperoxidase (MPO) and the blood MPO granulocyte index. In addition, we analyzed the bone loss index (BLI), the periodontal histopathological score, and the periodontal collagen network using confocal microscopy. We also analyzed metalloproteinase-1/-8, RANK, RANK-L, and osteoprotegerin in maxillary tissue by immunohistochemistry Gingival MPO, IL-1β, TNF-α were measured by ELISA. EPD caused leukocytosis, significant increases in BLI and gingival pro-inflammatory cytokines and cell infiltrates, with worse histopathological scores and periodontal collagen derangement. Infliximab (5 mg/kg) reduced granulocyte blood counts, gingival IL-1β, TNF-α, and MPO levels, diminished MMP-1/-8, RANK, and RANK-L bone immunolabeling with better periodontal histopathological scores and collagen network in comparison with the challenged saline group. We concluded that infliximab had significant anti-inflammatory and bone-protective effects in Wistar rats challenged by periodontitis.

Keywords: Infliximab, TNF-alpha, periodontitis, inflammation, RANK

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Introduction

Periodontitis is a chronic inflammatory disease that affects humans and, despite improvements in oral hygiene, is still considered a leading cause of tooth loss in the adult population worldwide.[1] Periodontitis is characterized by inflammatory-driven gingival edema, marked leukocyte tissue infiltration, release of inflammatory mediators, and destruction of the bones and connective tissues that support the teeth.[2] The gold standard for treating inflammatory periodontal diseases is mechanical surface treatments, which include scaling, root planing, and the removal of the supra and subgingival biofilm.[3] However, in severe cases of periodontitis, conventional treatments are usually unsuccessful.[4,5] If not properly treated, the dental attachment apparatus may be irreversibly damaged, resulting in tooth loss.[6]

There are common factors that characterize inflammatory-induced osteolysis that could negatively affect the periodontal tissues, including overproduction of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) and excessive bone destruction caused by increases in reabsorbing osteoclast recruitment and activation.[7] TNF-α is one of the dominant cytokines active in osteolytic conditions and is produced primarily by activated T cells,

macrophages, and synoviocytes.[7] TNF-α is a key osteoclast-stimulating molecule that promotes receptor activator of nuclear factor kB ligand (RANK-L) production by stromal cells and its secretion by T-lymphocytes. In addition, it also stimulates macrophage colony stimulating factor (M-CSF) production, a key factor for monocyte differentiation.[8,9]

The discovery of the RANK-L/RANK/OPG signaling pathway as the main regulator of osteoclast differentiation and function represented a major breakthrough in bone research.[10] Deletion of RANK or RANK-L genes or over-expression of osteoprotegerin (OPG) (soluble inhibitory receptor for RANK-L) in transgenic mice causes poor osteoclast formation, ultimately leading to osteopetrosis.[11,12]

RANK-L is a homodimeric transmembrane protein expressed on the surface of stromal osteoblasts. RANK-L/RANK signaling regulates osteoclast formation, activation, and survival in normal bone remodeling and in a variety of pathological conditions characterized by increased bone turnover. OPG protects bone from excessive resorption by binding to RANK-L and preventing it from binding to RANK.[13]

Although mechanical and surgical treatments are effective in stopping the progression of periodontal disease, these interventions are ineffective in limiting the tissue damage caused by an over-inflammatory host response.[14] Thus, the manipulation of the host's pro-inflammatory cytokine response may attenuate the periodontal bone loss.

Infliximab is a chimeric, human IgG1 TNF-α monoclonal antibody.[15] The studied fragment is derived from anti-TNF-1 immunoglobulin produced by B-lymphocytes in humans bearing a moiety derived from mouse. The molecule is composed of two light chains and two heavy chain polypeptides linked together by covalent bonds.[16] Anti-TNF-α monoclonal antibodies bind to TNF receptors (TNFRs), producing a reverse signaling through the membrane-anchored ligand, suppressing LPS-induced cytokine production and cell apoptosis, or by downstream binding inhibition of transmembrane TNF (tmTNF) to TNFRs.[17]

In a longitudinal clinical trial, rheumatoid arthritis patients who were treated with infliximab showed lower TNF-α levels in the crevicular fluid compared to patients treated with other bone-trophic compounds, thus suggesting the benefit of infliximab in periodontitis.[18] Therefore, this study aimed to evaluate the role of infliximab in the course of the inflammatory osteolysis induced by experimental periodontitis in Wistar rats, assessing gingival pro-inflammatory cytokines and the bone RANKL-RANK signaling pathway.

Materials and methods

Drug

Infliximab (Remicade® 100 mg, Schering Plough, New Jersey, USA) was obtained from the University Hospital, Federal University of Ceara, kindly provided by Dr F.S. Silva Junior, MD.

Animals

Wistar rats weighing 200–250 g were obtained from the Department of Physiology and Pharmacology vivarium, Federal University of Ceara. Experimental rats were housed in polypropylene boxes with free access to chow diet and water, under controlled temperature, and a 12 h light/dark cycle. All experimental protocols were in compliance with the Brazilian College for Animal Experimentation (COBEA) and the Institutional Animal Care and Use Committee guidelines (protocol #78/2011) from the Department of Physiology and Pharmacology, Federal University of Ceara.

Induction of experimental periodontal disease

The animals were anesthetized with 10% chloral hydrate (400 mg kg) intraperitoneally (ip). The experimental periodontal disease (EPD) was induced by surgical insertion of a nylon suture (3.0) around the left upper second molar. The ligature around the tooth enables plaque accumulation and induces inflammation and subsequent alveolar bone resorption.[19] Prior to the thread passage, we used a guide through the medial and distal interproximal spaces to facilitate suture placement to ensure that the surgical knot remained facing the labial surface of the animal's mouth. The rats were euthanized on the 3rd or 11th day post-challenge, based on previous studies that demonstrated significant destruction of the alveolar process and cementum and increased neutrophil activity.[19,20]

Experimental groups

Initially, a pilot experiment was conducted to identify the most effective dose of infliximab in preventing alveolar bone loss induced by EPD. The pilot infliximab doses were based on data from a murine model of intestinal mucositis in which infliximab (5 mg/kg) was found to be anti-inflammatory.[21] In our pilot study, rats were divided into unchallenged controls and EPD-challenged groups (at least 6 animals each) receiving either intravenous infliximab (via penile or tail vein) 30 min before surgery at doses of 1, 5, 7, or 10 mg/kg (IFX 1, IFX 5, IFX 7, and IFX 10, respectively) or saline. On day 11 following EPD, we analyzed the total maxillary bone loss area to assess EPD-induced bone resorption. Infliximab at a dose of 5 mg/kg was found to be the most effective dose at protecting against alveolar bone loss, and therefore this dose was chosen for the molecular biology experiments.

Experiments were conducted thereafter with the following groups: untreated and unchallenged rats (controls), untreated and EPD-challenged rats receiving saline intravenously 30 min prior to surgery (saline), and EPD-challenged rats receiving infliximab (5 mg/kg) intravenously (via penile or tail vein) 30 min prior to surgery (IFX 5).

Measurement of alveolar bone loss

Experimental animals were sacrificed after 11 days of periodontitis induction, and the maxillae were excised and fixed in 10% neutral formalin. Both maxillary halves were then defleshed and stained with methylene blue (1%) to

differentiate bone from teeth. The maxillae were fixed in a piece of wax and photographed under low magnification (2.5×) using a Nikon camera. Photographs were digitized and processed with ImageJ software (ImageJ, 1.32, National Institute of Health, Bethesda, USA) for the measurement of alveolar bone loss using a modification of the area method.[22] The alveolar bone loss was measured by the exposed molar root surface area (mm^2) on the buccal aspect of the maxillary left side by subtracting the total area of the left maxillae tooth and bone resorption from the right maxilla tooth area (unligated control).

Periodontal histology and collagen autofluorescence

Decalcified maxillary bone was paraffin-embedded and stained with HE and examined either in a light microscope (Olympus, CX31) for histology analyses and image capture of the periodontal tissue or a laser scanning confocal microscope (FV-100, Olympus) to examine the periodontal collagen autofluorescence. The periodontal region between the two first molars was chosen for the analyses. To evaluate autofluorescence, we used a 488 nm wave-length laser and emission channel for FITC-green fluorescence. Images were electronically generated by a computer connected to the confocal microscope; the images were exported as a TIFF file.

Periodontal histopathology scores

Six micrometer thick sections of hematoxylin-eosin-stained demineralized maxillae corresponding to the area between the first and second right molars were evaluated under light microscopy (low and high magnification). To evaluate IFX's effects on the periodontal tissues, we utilized histopathological scores (range: 0–3), as described previously,[23] to assess the severity of the periodontal damage. A score of 0 indicates the absence of or only discrete inflammatory cell infiltration (restricted to the region of the marginal gingiva), preserved alveolar process, and cementum; a score of 1 indicates moderate inflammatory cell infiltration all over the gingival insert, some minor alveolar bone loss, and intact cementum; a score of 2 indicates accentuated inflammatory cell infiltration (present in both the gingival and periodontal ligament), accentuated alveolar process resorption, and partial cementum breakdown; and a score of 3 was defined as having accentuated cell infiltrates, complete alveolar bone resorption, and severe cementum breakdown.

Blood cell counts

Under 10% chloral hydrate anesthesia, blood was drawn via intracardiac puncture from the experimental rats 11 days after the EPD induction and placed in collection tubes impregnated with ethylenediaminetetraacetic acid (EDTA). Analyses were processed through an automated ADVIA-2120 Hematology System (Siemens Healthcare Diagnostics Inc., Tarrytown, New York, USA). For differential leukocyte counts, blood smears were prepared and stained with Wright-Giemsa and analyzed with the aid of a high-resolution light microscope by an experienced technician.

Gingival MPO assay

Myeloperoxidase (MPO) tissue activity is used as a marker of inflammatory cell infiltrates (especially neutrophils) in the inflamed tissue.[23] After sacrifice on either the 3rd or 11th day after the induction of EPD, a portion of the buccal gingiva was collected, weighed, and frozen in a −80°C freezer until assayed. Thawed gingival tissue was immersed in 0.5% HTAB (hexadecyltrimethylammonium bromide) solution at a ratio of 50 mg of tissue/mL of buffer, homogenized, and centrifuged (1500 *G*/15 min at 4°C). The supernatant was transferred to an Eppendorf tube and further centrifuged (10 min). After plating of the supernatant (7 μL onto a 96-plate well), 200 μL of the reading solution (5 mg of O-dianisidine, 15 μL of 1% H_2O_2, 3 mL of phosphate buffer, and 27 mL of H_2O) was added and read at 450 nm ($t_0 = 0$ min and t1 = 1 min). The change in absorbance was obtained and expressed as MPO/mg tissue (MPO activity).

Granulocyte MPO index by flow cytometry

The MPO granulocyte index is a marker of blood granulocyte activity in the presence of systemic inflammation.[24] The granulocyte MPO index was assayed using blood harvested by intracardiac puncture on the third post-operative day with the aid of the IntraStain flow cytometry kit (FACS Calibur, Becton Dickinson, Franklin, New Jersey, USA). Anti-MPO monoclonal antibody (IQProducts BV) was used to react with the intracellular MPO. Initially, each sample was incubated for 15 min in 100 μL of reagent A for fixation. After the addition of 2 mL PBS and centrifugation for 5 min, reagent B was added for permeabilization. Afterwards, an appropriate volume of anti-myeloperoxidase antibody conjugated to FITC was added for incubation in tubes protected from light and at RT for 15 min. After that, washing was performed with 2 mL of PBS added to each tube, followed by 5-min centrifugation at 1500 rpm. Finally, 500 μL of PBS were added to complete the sample volume and immediately analyzed in the flow cytometer. At least 10,000 cells were analyzed.

The samples were analyzed by the CellQuest program (Becton Dickinson). By this method, granulocytes are separated by internal complexity and size characteristics and then analyzed for MPO. The results were reported as an index, which was calculated as the percentage of positive granulocytes and mean MPO reactivity.

Gingival IL-1β expression by immunoblotting

Western blots were performed on samples from the surrounding maxilla gingival tissue corresponding to the periodontal lesion area in the operative rats. The contralateral gingiva was used as a negative control. Gingival tissue was removed, immediately frozen in liquid nitrogen and stored in a −80°C freezer until use. Briefly, tissues were homogenized in 0.2 mL of lysis buffer containing protease inhibitors (50 mM Tris–HCl [pH 8.5]; 50 mM NaCl; 0.1 mM EDTA; 1% Tween 20; 1 mM [each] dithiothreitol, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride). Total protein (50 μg protein/well) was resolved on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred

to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Amersham, UK). The membranes were blocked with 5% skimmed milk/Tris-buffered saline with 0.1% Tween 20 for 14 h at 4°C, followed by an incubation period of 1 h at room temperature with the primary antibodies (rabbit polyclonal anti-IL-1β, 1:500 or anti-β-Actin, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). The blots were washed, followed by incubation with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit immunoglobulin G, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The membranes were washed, incubated with ECL (Amersham Pharmacia Biotech), and exposed to Kodak MIN-R film (Kodak, New York, USA). Data were expressed as the relative density of IL-1 β/β-actin bands.

Gingival IL-1β and TNF-α ELISA assays

TNF-α and IL-1β concentrations were determined in the buccal gingiva surrounding the upper left molars 11 days after periodontitis induction in rats that received 5 mg/kg infliximab or intraperitoneal saline. The gingiva were removed and stored at −80°C. The material was homogenized and the supernatant was used to determine the cytokine levels by enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates were coated overnight at 4°C with an antibody against murine TNF-α and IL-1β (10 μg/mL). After blocking the plates, the samples and standard at various dilutions were added in duplicate and incubated at 4°C for 24 h. The plates were washed three times with buffer and a second biotinylated polyclonal antibody against TNF-α and IL-1β diluted 1:1000 was added (100 μL/well). After further incubation at room temperature for 1 h, the plates were washed and 100 μL of avidin-horseradish peroxidase (HRP) diluted to 1:5000 was added. The color reagent o-phenylenediamine (OPD; 100 μL) was added 15 min later and the plates were incubated in the dark at 37°C for 15–20 min. The enzyme reaction was stopped with H_2SO_4 and absorbance was measured at 490 nm. TNF-α and IL-1β concentrations were expressed as picograms/mL.

Immunohistochemistry for MMP-1/-8, RANK, RANK-L, and OPG

Immunohistochemistry was performed using the streptavidin–biotin–peroxidase method in formalin-fixed, paraffin-embedded tissue sections (4 μm thick) mounted onto poly l-lysine-coated microscope slides. Analyses were made from the maxillary bone surrounding the upper left molars removed from experimental rats and demineralized in a 10% ethylenediamine tetra-acetic acid (EDTA) solution. All the sections were deparaffinized and rehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (v/v) hydrogen peroxide and washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4°C) with primary rabbit anti-rat MMP, RANK, RANK-L, and OPG antibodies diluted 1:200 in PBS plus bovine serum albumin (PBS-BSA). The slides were then incubated with biotinylated goat anti-rabbit diluted 1:400 in PBS-BSA. After washing, the slides were incubated with avidin–biotin–horseradish peroxidase conjugate (Strep ABC complex by Vectastain® ABC Reagent and peroxidase substrate solution) for 30 min according to the Vectastain protocol. MMP, RANK, RANK-L, and OPG were visualized with the chromogen 3,3′-diaminobenzidine. Negative control sections were processed simultaneously as described above but with the first antibody being replaced by PBS-BSA 5%. None of the negative controls showed immunoreactivity. Slides were counterstained with Harry's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene, and cover slipped.

Statistical analysis

Data are presented as the mean ± SEM. Analysis of variance (ANOVA) followed by Bonferroni's test was used to compare means between multiple groups and the unpaired Student's t-test was used for comparisons between two groups. In the histopathological analyses, median scores were analyzed using Kruskal–Wallis test. A $P < 0.05$ value was set to indicate significant differences.

Results

Measurement of alveolar bone loss

Infliximab (5 mg/kg) given intravenously 1 h before the induction of experimental periodontitis was able to significantly reduce ($P = 0.0002$) the total alveolar bone loss area compared to the saline-challenged group. Other doses tested (1, 7, and 10 mg/kg) failed to protect against bone loss when compared with the saline group ($P > 0.05$) (Figure 1).

Periodontal histology and collagen autofluorescence

EPD caused marked inflammatory cell infiltrates and intense destruction of alveolar crest bone and cementum. Infliximab-treated rats showed improved inflammatory infiltrates in the periodontal tissue and greater integrity of the alveolar process and cementum when compared to the saline group. In addition, HE-stained maxilla slides from experimental rats were examined under confocal microscope to evaluate collagen autofluorescence. The periodontal region between the two first molar was chosen for analysis. Untreated EPD-challenged rats showed marked decreases in the alveolar crest and collagen fiber network of the periodontal ligament (detected by reductions in autofluorescence) with intense collagen disarrangement compared to the non-operated group. EPD-challenged rats treated with infliximab (5 mg/kg) showed a more preserved alveolar process and collagen network architecture (Figure 2).

Histopathology scores

Untreated EPD-challenged rats (saline group) exhibited accentuated inflammatory cell infiltration, breakdown of alveolar bone, collagen fiber derangement within the periodontal ligament, and resorption of cementum, receiving a median score of 3 (ranging from 2

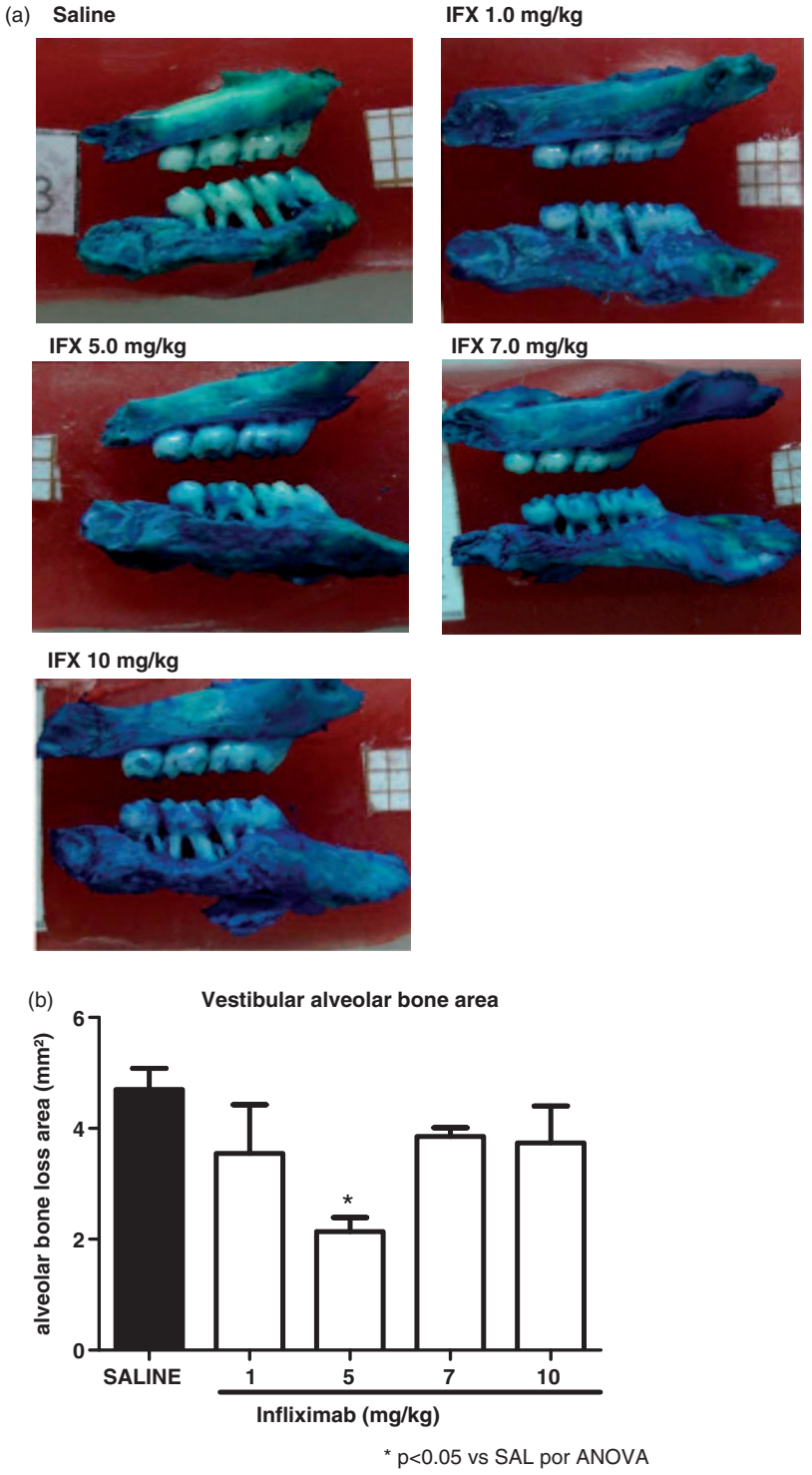

Figure 1 (a) Macroscopic aspect of the maxillae from study rats either subjected or not to experimental periodontal disease (EPD), stained by 10% methylene blue on the 11th day post-challenge. (b) Morphometric analyses of the alveolar bone total area after proper calibration. Data are shown as the mean ± SEM, by ANOVA followed by Bonferroni's test. At least $N = 6$ per group were used for analyses. *$P < 0.05$ versus saline by ANOVA. (A color version of this figure is available in the online journal.)

to 3) (Table 1). The periodontium of rats treated with 5 mg/kg of infliximab showed preservation of the alveolar process and cementum, reduction of the inflammatory cell infiltration, and partial preservation of collagen fibers of the PDL, receiving a median score of 1 (range 0–2). These values were significantly different ($P < 0.05$) when compared with the control and EPD-challenged saline group.

Blood cell counts

A dose of 5 mg/kg infliximab (single preoperative dose) improved hematological parameters on the 11th day

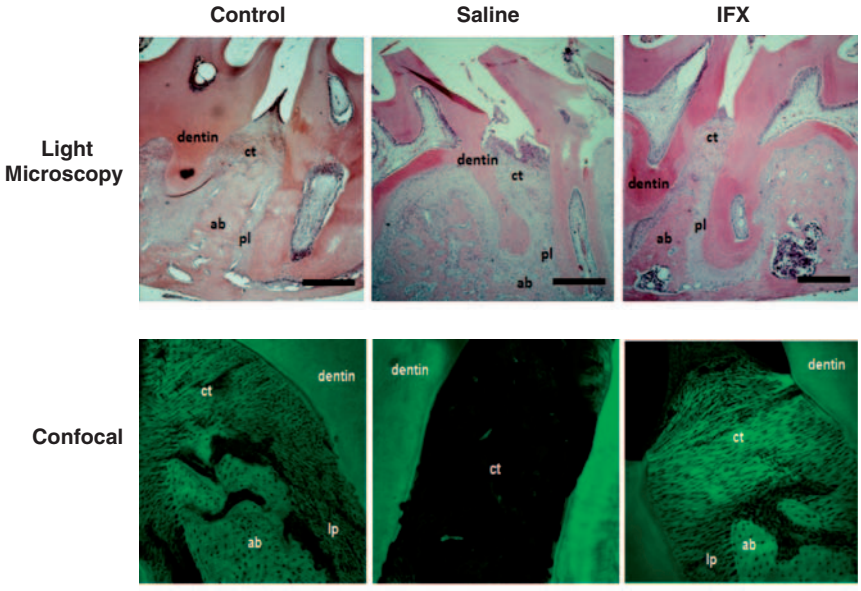

Figure 2 Representative histology of the periodontal tissues from study rats on the 11th post-EPD day either subjected or not to experimental periodontal disease (EPD) (H&E, X100). Images were obtained by light microscopy (upper panel) and scanning confocal microscopy (lower panel). Confocal microscopy allowed visualization of the collagen autofluorescence. Scale bar: 100 μm; Control: unchallenged rats; Saline: EPD-challenged rats receiving 0.9% saline solution; IFX: EPD-challenged rats receiving infliximab (5 mg/kg). (A color version of this figure is available in the online journal.)
AO: alveolar bone; PL: periodontal ligament; CT: connective tissue

Table 1 Effect of Infliximab on histopathological analysis of rat maxillae challenged with EPD (11 days post-surgery)

Groups	Scores (median values and range)
Control	0 (0–1)
Saline	3.0 (2–3)#
Infliximab (5 mg/kg)	1.5 (1–2)#*

Note: Data are reported as median scores and score variation for four rats per group (by Kruskal–Wallis test).
#$P < 0.05$ compared to the control group.
*$P < 0.05$ compared to the saline group.

post-challenge in experimental rats subjected to periodontitis in relation to the total number of red blood cells, total hemoglobin, and the hematocrit when compared to the untreated challenged group ($P < 0.05$). The percentage of blood granulocytes (%), which was found to be elevated with experimental periodontitis, was reduced in the infliximab-treated group when compared to the untreated EPD-challenged group ($P < 0.05$). In addition, there was a marked lymphopenia following the periodontitis challenge, an effect that was partially recovered with infliximab treatment, see Table 1.

Gingival MPO assay

To analyze the MPO (an enzyme found in azurophilic granules of neutrophils and other inflammatory cells) activity in the early stages of the inflammatory response, gingival samples were collected on the 3rd and 11th post-operative days. A significant increase in gingival MPO levels was found following the experimental periodontitis, both in the 3rd and 11th post-EPD days. Infliximab (5mg/kg) significantly reduced gingival MPO levels on the third day ($P = 0.03$) (Figure 3a). However, we found no significant difference between the infliximab-treated group and the unchallenged group on the 11th day post-challenge (Figure 3b).

MPO granulocyte index

Flow cytometry MPO analyses were performed on EDTA-treated blood samples from experimental rats on the third post-operative day (acute phase of periodontitis). A significant decrease in the MPO granulocyte index was found in the untreated EPD-challenged group compared with the unchallenged group. EPD-challenged rats treated with infliximab (5 mg/kg) did not show reductions when compared with the untreated group and reached the index level of the unchallenged control group (Figure 3c).

Gingival IL-1β and TNF-α assays

We found significantly higher gingival IL-1β levels after EPD in untreated challenged rats when compared to the unchallenged saline group detected by ELISA (Figure 4a). Gingival TNF-α levels were also found to be increased in the EPD-challenged group ($P < 0.05$) (Figure 4b). Infliximab treatment was able to significantly reduce gingival IL-1β and TNF-α levels when compared with the saline-challenged group (Figure 4). Likewise, gingival IL-1β expression was found to be augmented in EPD-challenged rats as seen by immunoblotting, an effect that was significantly reduced by infliximab (Figure 5).

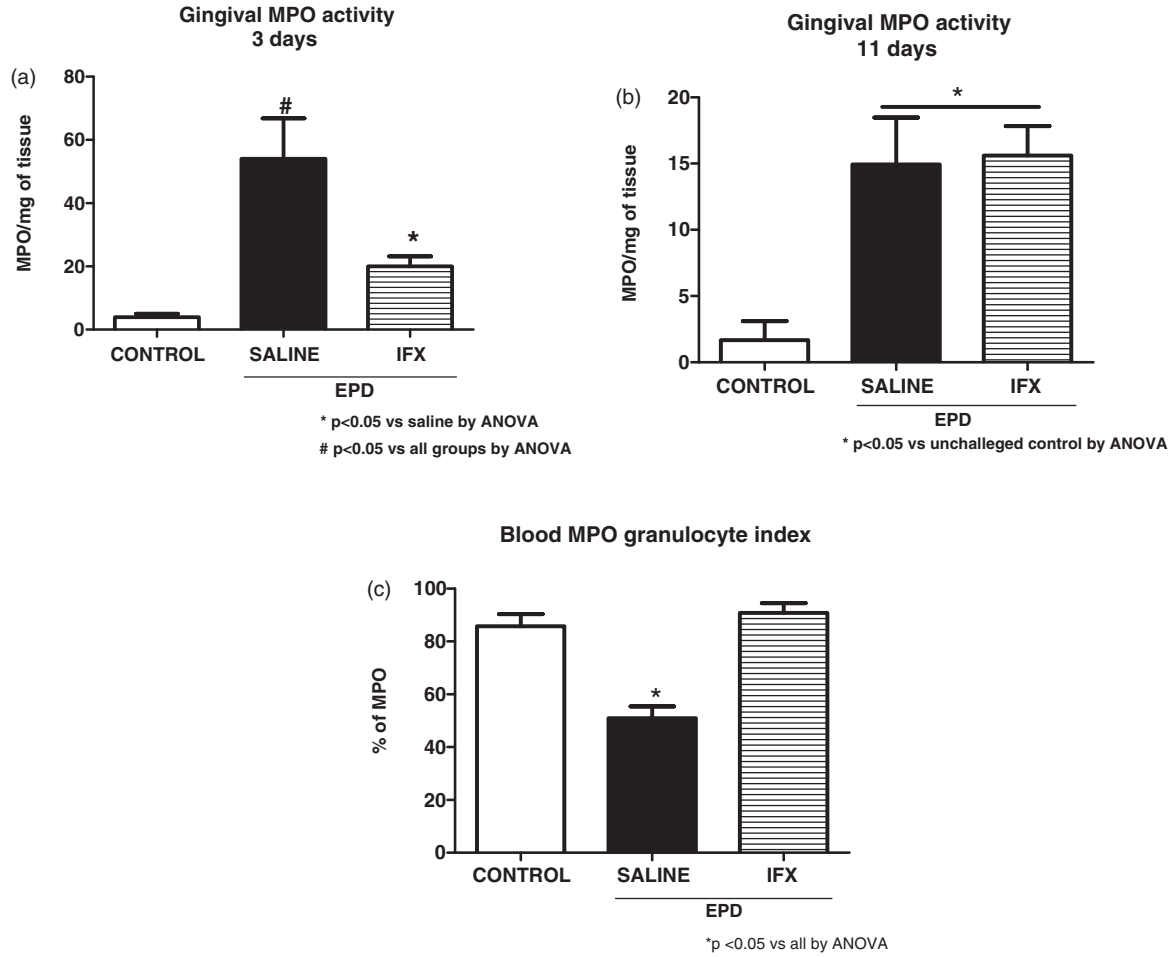

Figure 3 Gingival myeloperoxidase (MPO) activity from study rats either subjected or not to experimental periodontal disease (EPD) and assayed on the 3rd (a) and 11th (b) post-EPD days. (c) Blood granulocyte myeloperoxidase (MPO) index detected by flow cytometry on the third day after EPD induction. Data are shown as the mean ± SEM. At least six samples per group were used for the analyses. *$P < 0.05$ versus saline; #$P < 0.05$ vs all groups by ANOVA. Control: unchallenged rats; Saline: EPD-challenged rats receiving 0.9% saline solution; IFX: EPD-challenged rats receiving infliximab (5 mg/kg)

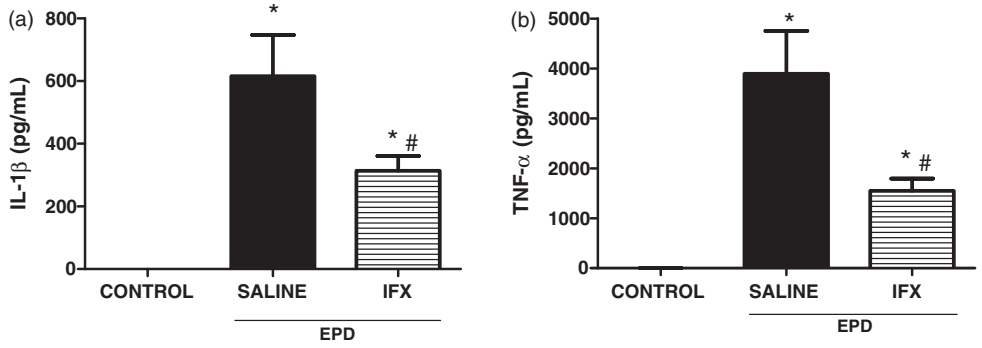

Figure 4 Gingival IL-1β (a) and TNF-α (b) levels from study rats either subjected or not to experimental periodontal disease (EPD) and assayed on the third post-challenge day. Data are shown as the mean ± SEM. Five samples per group were used for the analyses. *$P < 0.05$ versus normal; #$P < 0.05$ versus all groups by ANOVA. Control: unchallenged rats; Saline: EPD-challenged rats receiving 0.9% saline solution; IFX: EPD-challenged rats receiving infliximab (5 mg/kg)

Effect of infliximab on RANK, RANK-L, and OPG immunostaining

There was a marked RANK and RANK-L immunolabeling in the periodontal tissue from the untreated EPD-challenged group, especially within infiltrated leukocytes (mostly polymorphonuclear cells, PMN) and in the osteoclasts near the alveolar process compared to the unchallenged group (Figure 6) Infliximab treatment (5 mg/kg) was able to reduce RANK immunolabeling. In addition, a conspicuous reduction in PMN infiltrates was found in the infliximab treated group as opposed to the saline challenged group (Figure 6). Interestingly, epithelial

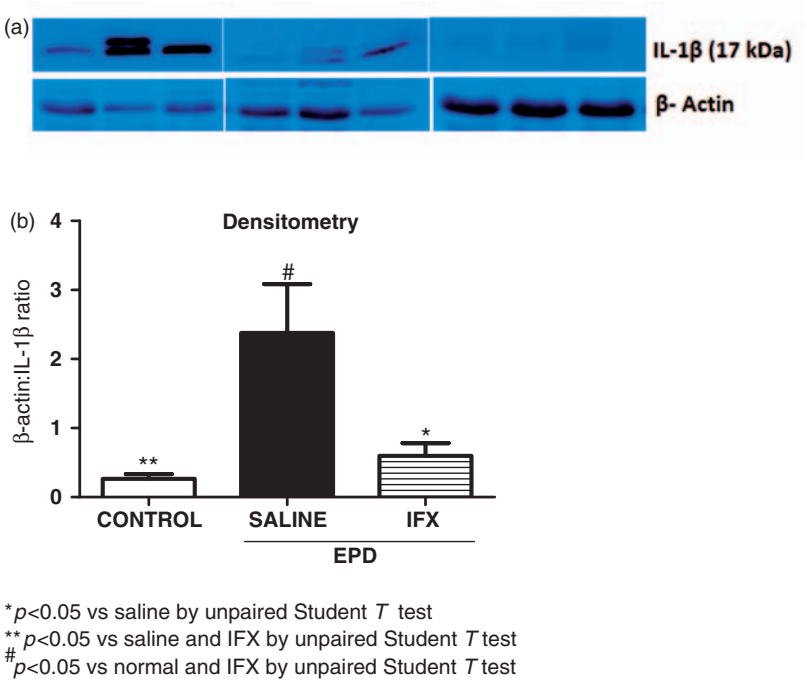

Figure 5 (a) Representative gingival immunoblot of the experimental groups for interleukin 1-β (IL-1β). (b) The band densitometry after normalizing for the housekeeping protein β-actin. Statistical analyses were performed using Student's *t*-test. The results are presented as the mean ± SEM. *$P < 0.05$, significant increase versus EPD-challenged group receiving saline; **$P < 0.05$, significant increase versus EPD-challenged group receiving saline and infliximab; #$P < 0.05$ significant increase versus all groups. Control: unchallenged rats; Saline: EPD-challenged rats receiving 0.9% saline solution; IFX: EPD-challenged rats receiving infliximab (5 mg/kg). (A color version of this figure is available in the online journal.)

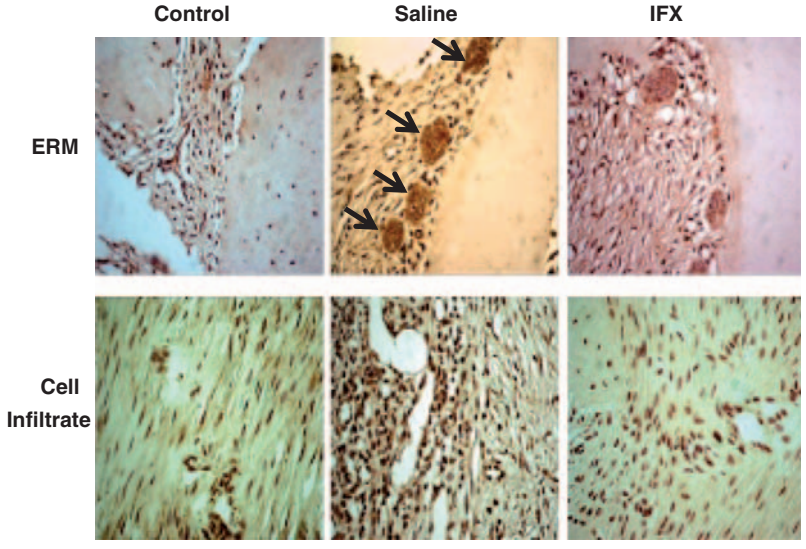

Figure 6 Representative immunohistochemistry of the periodontal tissues for RANK-L (upper panel) and RANK (lower panel) from study rats on the 11th day post-challenge either subjected or not to experimental periodontal disease (EPD) (H&E, X100). The upper panel shows increased RANK-L immunolabeling of epithelial rest of Malassez (ERM) cells near the root cementum (arrows), an effect that was reduced by infliximab treatment. The lower panel shows increased RANK immunolabeling of the cell infiltrate after EPD-challenge, an effect that was reduced by infliximab treatment (IFX). Control: unchallenged rats; Saline: EPD-challenged rats receiving 0.9% saline solution; IFX: EPD-challenged rats receiving infliximab (5 mg/kg). (A color version of this figure is available in the online journal.)

rests of Malassez, often seen in the tooth root of the challenged rats, were found with increased RANK-L immunostaining near the maxillary second molar root among the untreated challenged rats, an increase which was not seen in the infliximab-treated group (Figure 6). OPG immunolabeling was found in the periodontal cells, especially in osteoclasts from animals that did not undergo EPD (control group) in relation to the marked immunostaining found in the saline challenged group. Infliximab treatment (5 mg/kg) increased OPG immunostaining compared with both the saline group and the control group.

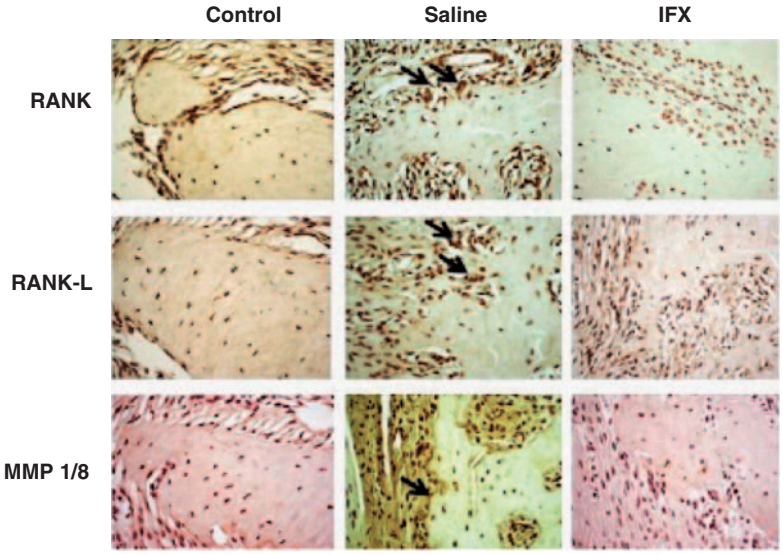

Figure 7 Representative immunohistochemistry of the periodontal tissues for RANK (upper panel), RANK-L (middle panel), and MMP 1/8 (lower panel) from study rats on the 11th day post-challenge either subjected or not to experimental periodontal disease (EPD) (H&E, ×100). The upper panel shows increased RANK immunolabeling of osteoclasts on the surface of eroded bone (arrows) after the EPD-challenge, an effect that was reduced by infliximab treatment. The middle panel shows increased RANK-L immunolabeling of osteoblasts on the surface of remodeling bone (arrows) after the EPD-challenge, effect that was reduced by infliximab treatment. The lower panel shows increased MMP 1/8 immunolabeling of the osteoid in the remodeling bone (arrows) after the EPD-challenge, an effect that was reduced by infliximab treatment. (A color version of this figure is available in the online journal.)
Control: unchallenged rats; Saline: EPD-challenged rats receiving 0.9% saline solution; IFX = EPD-challenged rats receiving infliximab (5 mg/kg).

Effect of infliximab on collagenase (MMP-1/-8) immunostaining

To evaluate the degree of bone reabsorption, we assessed collagenase immunolabeling on the 11th post-EPD day. We found increased collagenase immunostaining in the alveolar bone crest region, especially in areas of osteoclast-induced bone erosion from rats subjected to periodontitis and receiving saline. Infliximab treatment markedly reduced collagenase immunolabeling in the alveolar bone (Figure 7).

Discussion

In this study, we sought to investigate the effect of infliximab on the inflammatory osteolysis in the maxillary bone induced by experimental periodontitis and to examine the mechanisms by which infliximab could prevent bone reabsorption with a focus on the RANK-RANK-L-OPG signaling pathway.

We have demonstrated that infliximab at a dose of 5 mg/kg significantly prevented the inflammatory changes and alveolar bone loss typically seen in rats subjected to EPD. The lowest infliximab dose tested (1 mg/kg) did not prevent the inflammatory response and bone resorption seen in experimental periodontitis, despite the use of the same experimental protocol. We speculate that this dose of infliximab was not enough to elicit a significant TNF-α inhibitory effect in the bone tissue required to reduce TNF-mediated bone absorption. Additionally, the highest doses tested, 7 and 10 mg/kg, under the same conditions did not inhibit the alveolar bone loss or the local inflammatory changes. These apparently contradictory results raise the possibility that basal levels of TNF-α may be necessary for physiological homeostasis of the periodontal tissue.

Recently, Huang and colleagues have shown that low concentrations of TNF-α up-regulates Runx2, Osx, OC, and ALP expression in a murine mesenchymal stem cell line, while cell cultures treated with TNF-α at higher concentrations cause downregulation of these bone markers.[25] We speculate that high doses of infliximab may have ablated TNF-α bone levels and the TNF-α driven-osteogenic response leading to impaired bone formation. However, chronic low dose TNF-α may suppress early bone accrual by inhibiting osteoblasts.[26] More studies are warranted to dissect these contradictory findings.

Alveolar bone and extensive cementum collapse, collagen network disorganization, and increased polymorphonuclear cell infiltrates have been found in the periodontal tissue of rats subjected to experimental periodontitis. All of these features were significantly reduced in the periodontal tissue from infliximab-treated rats (5 mg/kg). Our data are in agreement with previous protective findings seen with thalidomide and pentoxifylline therapy (e.g. reduced bone loss and inflammatory cell influx) in experimental periodontitis. These compounds are known to act as inhibitors of TNF-α (among other pro-inflammatory cytokines) by reducing transcription of the TNF-α gene.[27] In addition, chlorpromazine, a well-known modulator of TNF-α signaling, has been found to diminish RANK-L-induced osteoclastogenesis in bone marrow cells[28] and has been found to reduce alveolar bone loss in experimental periodontitis.[29]

In our model of experimental periodontitis, infliximab (5 mg/kg) was able to improve hemoglobin blood levels compared with saline-challenged groups. A similar result

was found in a clinical trial with infliximab in Crohn's disease, in which the blood infusion of this drug allowed a gradual increase in hematocrit and hemoglobin in Crohn's patients.[30]

Neutrophils are the first line of the host's defense against tissue injury. These phagocytic cells are able to produce and release inflammatory mediators that further amplify and sustain the inflammatory response by activating and recruiting more polymorphonuclear cells; however, tissue damage is often seen due to over-inflammatory responses and increased release of free radicals and proteolytic enzymes.[31]

Increased blood neutrophil counts, which are most frequently seen in acute bacterial infections,[32] and decreases in the MPO granulocyte index, which often indicate an increase in cell degranulation,[33] were observed with the periodontitis challenge but not in the unchallenged and the infliximab-treated groups. These findings suggest a systemic effect of the experimental periodontitis utilized in our studies, which is likely associated with oral-to-blood bacterial translocation and inflammation-mediated LPS release. Those differences were not seen with infliximab treatment, suggesting a more controlled inflammatory process and reduced bacterial translocation.

Furthermore, increases in gingival MPO on the third day after the induction of EPD indicate significant leukocyte traffic to the inflamed tissue; that effect was also partially abrogated by infliximab treatment, indicating less inflammatory-driven chemotaxis. Our findings differ from those by Pers *et al.*,[34] who examined the periodontal tissue in rheumatoid arthritis patients treated with infliximab, showing decreased periodontal attachment but without changes in the overall inflammatory status.

TNF-α is a key cytokine modulating the inflammatory-induced osteolysis response in models of rheumatoid arthritis and periodontal diseases.[35] In our studies, we found that infliximab-treated rats had improved maxillary bone area along with markedly reduced gingival TNF-α levels compared to the challenged untreated group, although those levels were higher than the unchallenged group. TNF-induced osteoclastogenesis is required for the inflammatory osteolytic process, which is further enhanced through IL-1β interactions.[36]

As seen in studies with experimental inflammatory osteolysis,[10,37] we found increased RANK immunolabeling in active resorbing osteoclasts residing upon the eroded maxillary bone in the challenged untreated group compared to unchallenged controls. We also found conspicuous RANK immunostaining in periodontal neutrophilic infiltrates. Infliximab treatment was able to reduce TNF-dependent neutrophil infiltrate and RANK immunostaining. Our data are in line with findings from patients suffering from rheumatoid arthritis. Synovial fluid neutrophils from patients with osteoarthritis show increased RANK and OPG expression, which suggests that these cells are important in bone remodeling in inflamed sites.[38] Infliximab treatment was capable of reducing the expression of RANK in osteoclasts and neutrophils, key players of the inflammatory osteolysis seen in experimental periodontitis. Likewise, RANK-L immunostaining was found to be higher in osteoblasts covering the alveolar crest in the challenged untreated group, and this was partially reversed by infliximab treatment. RANK-L is key for osteoclast activation, leading to the striated border-bone active resorbing cell phenotype.[39]

Interestingly, epithelial rests of Malassez (ERMs) were consistently found in the challenged groups with a more intense RANK-L labeling than the controls, in which ERMs were barely seen. The presence of ERMs in the tooth root at the level of the cementum and alveolar bone insertion among challenged groups has been previously described.[40] Our histological findings suggest that the greater the inflammatory process and increased osteolytic resorption of the tooth root, the greater the activation of the ERM cells. It is noteworthy that infliximab treatment reduced RANK-L staining in ERMs. The precise mechanism of this reduction is unclear and seems not to involve OPG, because OPG immunostaining was not different among the challenged groups. Although we cannot rule out other signaling pathways, it seems to be more an indirect and unspecific effect due to reduced inflammation.

The importance of ERMs lies not only in maintaining the normal function of periodontal cellular elements, but also in their contribution to periodontal regeneration.[40] ERMs may have an important role in tissue repair, especially regarding the cement tissue, through the secretion of matrix proteins expressed early during the development of the dental organ.[41] In addition, challenged rats treated with infliximab showed marked OPG labeling in the alveolar bone region, especially in fibroblasts, which implies that these cells might be active in OPG secretion to counteract the osteoclast-driven osteolytic activity. Our data are in agreement with those of other studies showing that periodontal ligament cells, gingival fibroblasts, and epithelial cells are capable of OPG secretion.[42,43] OPG soluble receptors could act through a decoy antagonism to inhibit RANK-L-driven osteoclast activation, that is, OPG can prevent RANK-L from binding to RANK, ultimately leading to a reduction in osteoclast function.[44]

To assess the degree of bone resorption, we conducted 1/-8 metalloproteinase (MMP-1/-8) immunohistochemistry. MMP-1, found in fibroblasts, and MMP-8, found in neutrophils, are part of a family of proteins called metalloprotein collagenases.[45] In our study, the EPD-challenged and untreated rats showed intense MMP-1/-8 immunostaining with more inflamed gingival tissue. Increased collagenase immunostaining was found in the osteoid from eroded alveolar bone. Previous studies have documented increased MMP-8 levels in saliva, periodontal pockets, and plasma from patients with periodontitis compared to non-affected controls. Periodontal-based therapy was able to reduce these levels, suggesting that MMP-8 would be a good marker for periodontitis in patients with active disease.[46]

Infliximab-treated rats that were challenged with EPD showed marked reductions in collagenase immunostaining, suggesting a TNF-α-mediated collagenase regulation, but this may also be an indirect effect based on the infliximab anti-inflammatory role in decreasing neutrophil infiltration and osteoclastic activity in the periodontal tissue.

In summary, this study shows that infliximab prevents alveolar bone resorption and has anti-inflammatory effects in experimental periodontitis, suggesting its use as an adjuvant therapy in periodontal disease treatment, especially in patients with rheumatoid arthritis, because recent studies have shown that these patients have a higher prevalence of periodontitis.[47,48] Infliximab has been successfully used in rheumatoid arthritis therapy; however, it must be borne in mind that adverse effects have been reported with its prolonged use, such as increased risk for opportunistic infections which may be in some cases life-threatening.[49,50] The benefits and potential side effects of prolonged use must be balanced against the untreated outcome.

Conclusions

In this study, we demonstrate that infliximab treatment (5 mg/kg) had anti-inflammatory effects in challenged rats, resulting in better alveolar bone preservation and improved periodontal tissue in experimental periodontitis, which is an attractive model to evaluate inflammatory bone loss. Our findings suggest beneficial effects of anti-TNF-α agents in inflammatory conditions associated with bone resorption, in which TNF-α plays a pivotal role. Clinical trials are needed to establish a safe therapeutic protocol in humans.

Author contributions: All authors participated in the design and interpretation of the studies, the data analysis, and the review of the manuscript; DCG, RCE, and RRS conducted all *in vivo* animal handling and experiments. RBO, GACB, and KSA conducted the immunohistochemistry, ELISA, and Western blot experiments. HBML conducted the flow cytometry studies. FSSJ and MJSS contributed with the statistical analyses and participated in the preparation of the manuscript. RBO, DCG, and RCL coordinated the study design and manuscript preparation.

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