ORIGINAL ARTICLE

Journal of
PERIODONTAL RESEARCH WILEY

Higher expression of Th1/Th2-related cytokines in the intestine of Wistar rats with ligature-induced periodontitis

João Martins de Mello-Neto[1,2] | Gayathiri Elangovan[1] | Edilson Ervolino[3] |
Newell Walter Johnson[1] | Anders Gustafsson[4] | Carlos Marcelo da Silva Figueredo[1,4]

[1]School of Medicine and Dentistry, Griffith University, Southport, Queensland, Australia

[2]College of Medicine and Dentistry, James Cook University, Cairns, Queensland, Australia

[3]Department of Basic Sciences, School of Dentistry, São Paulo State University-UNESP, Araçatuba, Brazil

[4]Division of Oral Diseases, Department of Dental Medicine, Karolinska Institutet, Stockholm, Sweden

Correspondence

Carlos Marcelo da Silva Figueredo, School of Medicine and Dentistry, Griffith University, Gold Coast campus Parklands Drive, Southport, Qld 4222, Australia.
Email: c.dasilvafiguered@griffith.edu.au

Funding information

Australian Dental Research Foundation (ADRF); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Abstract

Objective: This study aimed to investigate the impact of ligature-induced periodontitis (LIP) on histopathological and immunological outcomes in the colon of Wistar rats.

Background: It has been repeatedly shown that inflammatory bowel disease (IBD) patients are at higher risk of developing periodontitis and presenting worse oral health than non-IBD patients. However, whether the chronic inflammatory process around teeth contributes to the pathophysiology of IBD needs to be further explored.

Materials and Methods: Thirteen Wistar rats were allocated into LIP ($n=7$) and controls ($n=6$). Half of the colon was processed for histopathological analyses and immunohistochemical (CD45); the other half was homogenized for immunological analyses. Periodontal destruction was confirmed by measuring the distance from the cementum-enamel junction to the mandible's apical position of the mesial interproximal bone. The immunological analyses were performed with the Bio-Plex Th1/Th2 assay.

Results: There was a significantly higher interproximal bone loss in LIP compared to controls. The LIP group showed a moderate infiltrate of inflammatory cells, predominantly mononucleated cells in the intestinal tissues. There was significantly higher expression of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, and TNF-α in the intestinal tissues of LIP group compared to controls.

Conclusion: Ligature-induced periodontitis was associated with an overexpression of Th1/Th2-related cytokines in the colon of Wistar rats.

KEYWORDS

cytokines, inflammatory bowel diseases, intestine, periodontitis, rats

1 | INTRODUCTION

Periodontal disease is a multifactorial disorder that affects the teeth-supporting tissues. It occurs as an aberrant immunological response against the constant polymicrobial biofilm challenge, leading to the destruction of tooth-supporting tissues, attachment loss, and in severe cases, tooth loss.[1] The periodontal inflammation expresses critical proinflammatory cytokines, such as IL-1, IL-6, TNF-α, and IFN-γ that, together with hematogenous translocation of periodontal microorganisms, could disseminate in the peripheral circulation and contribute to the pathogenesis of important systemic diseases such as diabetes, cardiovascular diseases, and inflammatory bowel disease (IBD).[1,2]

Inflammatory bowel disease, such as Crohn's disease and ulcerative colitis, are chronic inflammatory diseases of the gastrointestinal

tract characterized by intestinal inflammation and epithelial injury.[3] A higher level of proinflammatory cytokines has also been implicated in the disease pathogenesis.[4] The coexistence of IBD and periodontal disease seems to be associated with a more intense inflammatory response,[5,6] and animal models have been used to demonstrate how one disease can affect the other.[7,8] Qiao et al.[8] reported that IL-10 knockout mice express an accelerated alveolar bone loss, more correlated with intestinal inflammation than local periodontal inflammation. The authors also showed that macrophage polarization was associated with alveolar bone loss, suggesting that IBD's abnormal alveolar bone metabolism was related to intestinal immune-mediated pathological changes. Kitamoto et al.[7] used a ligature-induced periodontitis (LIP) model in mice to demonstrate that oral pathobiont-reactive Th17 cells can be found in the guts. Once activated by translocated oral pathobionts, they can cause the development of colitis. Interestingly, the same cells are not activated by gut-resident microbes.

Our group has used an animal model to control the initiation and progression of experimental periodontitis and dextran sulfate sodium (DSS)-induced colitis in the same animal aiming to investigate the interplay between both diseases. In addition, this model enables us to control variables that cannot be controlled in human studies. We have recently reported that DSS-induced colitis was associated with an overexpression of Th1/Th2-related cytokines in the gingival tissue of Wistar rats. We have shown that the induction of DSS-induced colitis in rats led to a significantly increased expression of IL-1α, IL-1β, IL-2, IL-6, IL-12, IL-13, GM-CSF, INF-γ, and TNF-α in the gingival tissue when compared to healthy controls.[9] We believe that DSS-induced colitis triggered a basal cytokine response leading to overexpression in the periodontal tissues, which might have a clinical impact on the establishment and severity of IBD. Herein, we hypothesized that LIP could similarly impact gut pathogenesis and cause overexpression of Th1/Th2-related cytokines in the colon. Therefore, we aimed to investigate the impact of LIP on the expression of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, and TNF-α in the colon of Wistar rats.

2 | MATERIALS AND METHODS

2.1 | Animals

This research follows ARRIVE (Animal Research: Reporting of in vivo Experiments).[10] The present study used thirteen 3-month-old male rats (Wistar—*Rattus norvegicus*) supplied by the Animal Resources Centre (Harrison Road, Forrestfield, Western Australia), with body weights ranging from 405 to 485 g. They were housed in two per cage, acclimatized for 2 weeks before the experiment started under a 12-h light/dark cycle, and received food and water ad libitum. The animals were randomly divided into a LIP group (LIP, $n = 7$) and a control group (C, $n = 6$). For all experimental procedures involving pain or discomfort, animals were anesthetized using inhalation of 4% atmospheric isoflurane (Mediquip Pty Ltd) with an oxygen flow rate

of 400 mL/min at the induction chamber, subsequently reduced to 1%–2% isoflurane with an oxygen flow rate of 200 mL/min to the animal's nose for the duration of the procedure. The research protocol followed institutional guidelines for treating laboratory animals and was approved by the Griffith University Animals Ethics Committee (Ref No: DOH/02/20/AEC).

2.2 | Ligature-induced periodontitis

To induce periodontitis, a non-absorbable sterile surgical silk ligature 5-0 (PERMA-HAND Suture; Ethicon) was placed around the right first mandibular molar and stabilized in a sub-gingival position with a surgical knot. The ligature was retained in situ for 2 weeks to induce LIP.

2.3 | Euthanasia and sample collection

Animals were deeply anesthetized and euthanized using the cervical dislocation technique 14 days after ligature placement. To isolate the colon and cecum, we separated them from the small intestine at the ileocecal junction and from the anus at the distal end of the rectum. The colon was then measured with a metal ruler. After that, the colon was separated from the cecum at the ileocecal junction and quickly flushed with a 5–10 mL syringe with a feeding needle using cold PBS to remove feces and blood. The cecum was discarded, and the colons were cut into two equal pieces. The proximal colon was processed for the immunological analyses and the rectal region for histopathological analyses (Figure A1). Hemimandibles were also collected to confirm LIP presence.

2.4 | Cytokine analysis

Bio-Plex Pro™ Rat Cytokine Th1/Th2 Assay #171K1002M (Analytes: GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, and TNF-α) were used to evaluate the immunological changes in the presence of LIP. The entire colon tissue was first measured with a plastic ruler, excised in two equal sections, and weighed on an analytical balance (Ohaus), then transferred to a microtube containing two ultrapure 3.0 mm zirconia beads, 300 μL of phosphate-buffered saline (PBS; Sigma-Aldrich), and 50 μL of protease inhibitor (Sigma-Aldrich). Tissue homogenization was performed using a cell disruptor (TissueLyser II; QIAGEN) at 30 Hz for 4 min. Homogenate was then centrifuged at 11 200 *g* for 10 min. The supernatant was stored at −80°C until multiplex analysis.

2.5 | Histological processing

The colons were fixed using 4% paraformaldehyde (Chemical® Sigma) at room temp for 72 h and submitted to conventional histological processing for paraffin embedding. For histopathological

analysis, sections 4 μm thickness were stained with hematoxylin–eosin (H&E). Aiming to confirm the presence of LIP, the hemimandibles were demineralized in 10% ethylenediamine tetraacetic acid (EDTA) (Chemical® Sigma) in PBS for 60 days. They were also submitted to conventional histological processing for paraffin embedding.

2.6 | Histopathological analyses

H&E slides were scanned using a VS200 Research Slide Scanner (Olympus®) and analyzed using QuPath 0.3.0 and Image J software. A calibrated and blinded to the groups certified histologist (EE) performed the histopathological analyses, evaluating the following: the presence of the inflammatory infiltrate; extension of the inflammatory infiltrate; a cellular pattern of epithelial and connective tissue; and the pattern of structuring of the epithelial and connective tissue in the intestinal mucosa.

Histological sections stained with H&E were used to analyze the number of inflammatory cells, blood vessels, and fibroblasts in the colon. Briefly, the intestine was divided into four equal quadrants. In each quadrant, a rectangle measuring 320 by 280 μm was positioned equidistant from each other on the bowel wall. The major axis of this rectangle was parallel to the muscularis of the intestinal mucosa. Each of these rectangles contained a small part of the lumen, the epithelium, the lamina propria, and the muscularis of the intestinal mucosa. The area occupied by the connective tissue of the lamina propria was delimited using Image J software (LOCI, University of Wisconsin). In this area, inflammatory cells, fibroblasts, and blood vessels were quantified. A magnification of 400× was used to acquire each image. The number of inflammatory cells, blood vessels, and fibroblasts occupying the area was expressed as the mean ± standard deviation of cells or blood vessels per $0.01 \, mm^2$ (Figure A2).

2.7 | Immunolabeling for CD45 in the colon

The immunohistochemical processing followed the protocol described previously by Ervolino et al.[11] In the histological sections of the colon submitted to the CD45 detection, four images of the intestinal wall were obtained, as previously described. Six slides from each group were allocated for the immunohistochemistry. The immunolabeling pattern for CD45 was categorized according to the following scores: SCORE 0—the absence of immunoreactive cells; SCORE 1—from 1 to 25 immunoreactive cells/$0.01 \, mm^2$; SCORE 2—from 26 to 50 immunoreactive cells/$0.01 \, mm^2$; and SCORE 3—more than 50 immunoreactive cells/$0.01 \, mm^2$. The scores for CD45 immunolabeling were expressed as median and interquartile range.

2.8 | Histometric analysis

Alveolar bone loss was measured using QuPath 0.3.0 and Image J software (LOCI®; University of Wisconsin) (Figure A3). The alveolar
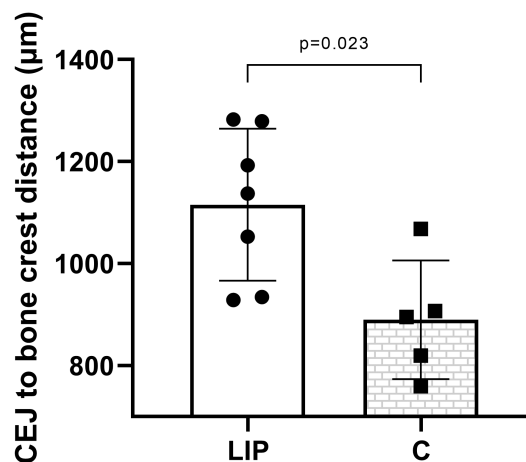

FIGURE 1 Graphic with a mean and standard deviation of the linear distance from CEJ to alveolar bone crest (μm). Ligature-induced periodontitis and control. μm, micrometers; CEJ, cementum enamel junction.

bone loss was determined by measuring the distance between the bone crest and cementum-enamel junction (CEJ) on the mesial aspect of the lower right first molar ($n = 12$).

2.9 | Statistical analysis

Data were analyzed with SPSS 21.0 (IBM Corporation). The normality of data was checked with the Kolmogorov–Smirnov test. The continuous variables are presented as mean and standard deviation (SD) or median and interquartile range. Student's *t*-test was used when the data sets followed a normal distribution and had unknown variances. For non-parametric data, Mann–Whitney test was used for immunological analyses to compare continuous variables between groups. Statistical significance was set at $p \leq .05$.

3 | RESULTS

3.1 | Clinical and histometric findings

There were no significant differences in the final weight between the LIP group (mean: 499.4 g SD ± 28.8 g) and the control group (mean: 506.6 g SD ± 42.2 g). There was also no significant difference in the clinical intestinal length between the LIP group (mean: 17.9 cm, SD ± 1.85 cm) and the Control group (mean: 19.2 cm, SD ± 1.5 cm).

Regarding the impact of the ligatures in the periodontium, the LIP group presented a higher distance between the mesial alveolar bone crest and the cementoenamel junction (mean: 1115.3 μm; SD ± 148.9 μm) compared to the control group (mean: 889.6 μm; SD ± 116.3 μm) confirming the bone loss in LIP group ($p = .023$) (Figure 1).

—WILEY |

3.2 | Histopathology and immunohistochemistry

In the LIP group, the intestinal mucosa presented with isolated and sparsely distributed foci of discontinuity of the epithelial tissue. The lamina propria showed a moderate infiltrate of inflammatory cells in these regions and their surroundings, predominantly mononucleated. The lamina propria showed fibroblasts, a delicate network of excellent collagen fibers and numerous blood vessels in these regions. The structure of the intestinal glands was shown to be little affected, even in the vicinity of sites with discontinuity of the epithelium (Figure 2A).

In the control group, the intestinal mucosa was constituted by a simple columnar epithelium composed of absorptive cells, where several goblet cells were incorporated into its structure. The intestinal glands were intact from their surface to their base (Figure 2B). The lamina propria was composed of loose connective tissue with many fibroblasts, rare inflammatory cells, a moderate amount of fine collagen fibers, and numerous blood vessels. The LIP group presented a significantly higher amount of inflammatory cells, blood vessels, and a significantly lower number of fibroblasts per $0.01\,mm^2$ in the intestine lamina propria than the control (Figure 3).

In the gingiva, the LIP group presented with an inflammatory infiltrate predominantly composed of mononucleated cells in the supra-alveolar connective tissue. The LIP group also identified partial disintegration of connective tissue attachment and alveolar bone loss (Figure 4A). In the control group, periodontal tissues had a regular pattern of cellularity and structure (Figure 4B).
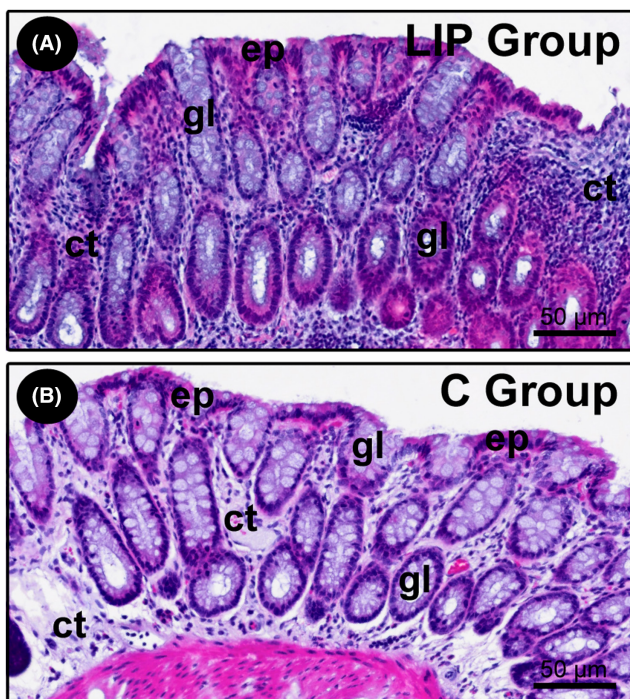

FIGURE 2 Photomicrographs of the colon in ligature-induced periodontitis group (A) and control (C) group (B) at 14 days. Scale bars: 50 μm. Staining: Hematoxylin and eosin (H&E). μm, micrometers; ct, connective tissue; ep, epithelium; gl, glands.

Regarding the immunolabeling pattern for CD45 in the colon, the LIP group (median: 2; IR: 2–3) showed greater immunolabeling when compared to the C group (median: 2; IR: 1–2) ($p = .014$). The immunolabeling pattern for CD45 in LIP and Control groups is depicted in Figure 5.

3.3 | Cytokine's expression in the colon

Animals from the LIP group presented significantly higher levels of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, and TNF-α in the intestinal tissues compared to controls. Figure 6 depicts the immunological differences between the groups (pg/mg).

4 | DISCUSSION

Our study has demonstrated that LIP was associated with significant overexpression of both Th1- and Th2-related cytokines in the colon of Wistar rats. The T helper dichotomy (Th1/Th2) has been extensively studied in inflammatory bowel disease, where CD4+ Th cells have been distinguished into two major types, designated type 1 (Th1) and type 2 (Th2), based on the different cytokines they produce. Based on the immunological status of the host or the type and phase of the inflammatory process, these cytokines may play a protective and/or proinflammatory role.[12] Although Crohn's disease seems to be driven by a Th1/Th17 response related to IL-12 and IL-23 cytokines, ulcerative colitis has been associated with a Th2-like response driven by IL-13 and IL-5 cytokines.[13] Still, according to the authors, cells from the lamina propria produce increased amounts of IL-5 without an increased IFN-γ response. Herein, we observed an overexpression of IL-5 and IFN-γ in the intestines of animals exposed to LIP, suggesting that the periodontal inflammation was associated with a broader cytokine expression in the intestine, inducing a polarised ulcerative colitis-related Th2 response.

We also observed a significantly higher intestinal expression of the proinflammatory cytokines IL-1β and TNF-α associated with LIP. IL-1β is a critical initiating factor in the cascade of events resulting in gut inflammation. It has recently been associated with promoting colitis by activating inflammasome-mediated secretion from intestinal mononuclear phagocytes.[7] Conversely, TNF-α is chronically elevated in patients with IBD, and suppressing TNF-α function is one of the most effective therapies for treating refractory IBD.[14] Moreover, in Chron's disease patients, TNF-α blockage down-regulates CD40 expression by intestinal microvascular cells in vivo and in vitro and circulating sCD40L.[15] TNF-α and IL-1β are key proinflammatory molecules that play critical roles in the development of colorectal cancer.[16] Thus, we could speculate that periodontal disease might be essential in initiating and progressing inflammatory bowel disease by affecting the expression of critical proinflammatory cytokines in the gut. Mechanistic studies are needed to confirm such an impact, as our present study could only evaluate the association.
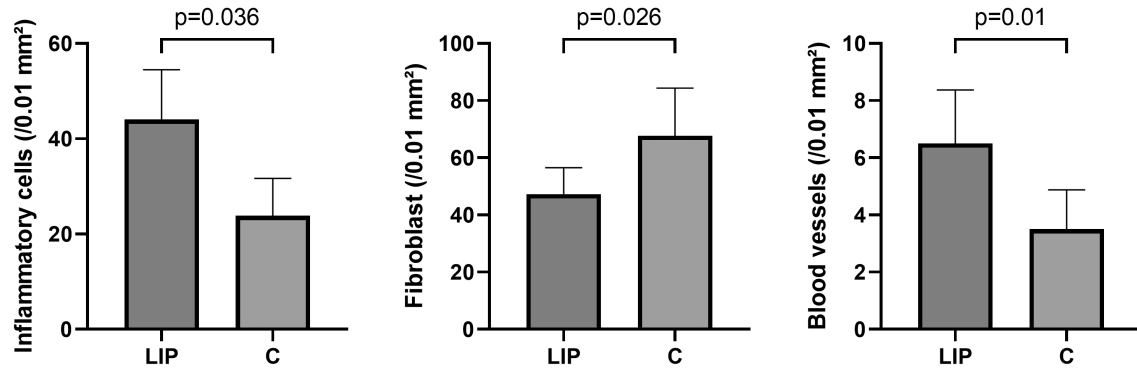
FIGURE 3 Graphics with the mean and standard deviation of the number of inflammatory cells, fibroblasts, and blood vessels per 0.01 mm² in the intestinal lamina propria (Student's T-test).
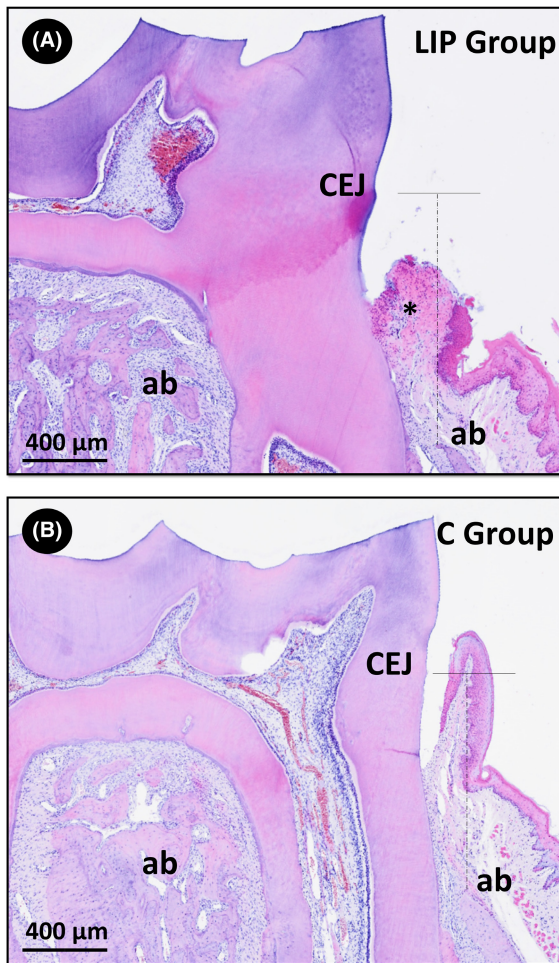

FIGURE 4 Photomicrographs of the right mandibular first molar showing the course of the inflammatory response and bone level in ligature-induced periodontitis (A) and control group (B), at 14 days. Ab, alveolar bone; CEJ, cementum enamel junction; *inflammatory infiltrate. Scale bars: 400 μm. Staining: hematoxylin and eosin (H&E).

FIGURE 5 Photomicrographs showing the immunolabeling pattern for CD45 in the intestine of ligature-induced periodontitis (A) and control group (B) at 14 days. Scale bars: 20 μm. μm, micrometers; ct, connective tissue; gl, glands.

The impact of our data is still unknown, and mechanistic host–parasite interactions need to be accessed in further studies to evaluate the impact of periodontitis on IBD. However, our results showed overexpression of the cytokines GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, and TNF-α that have a critical biological role in the start and progression of IBD, as mentioned in previous paragraphs. It is difficult to define the possible clinical translatability of our findings. Still, an inflammatory process around the teeth might trigger the expression of proinflammatory cytokines and inflammatory cells in the colon, which could help exacerbate gut inflammation in susceptible hosts. The impact of intestinal inflammation in the periodontal tissues has been shown in an animal study where animals with DSS-induced colitis presented a higher expression of Th1- and Th2-related cytokines in the periodontium.[9]
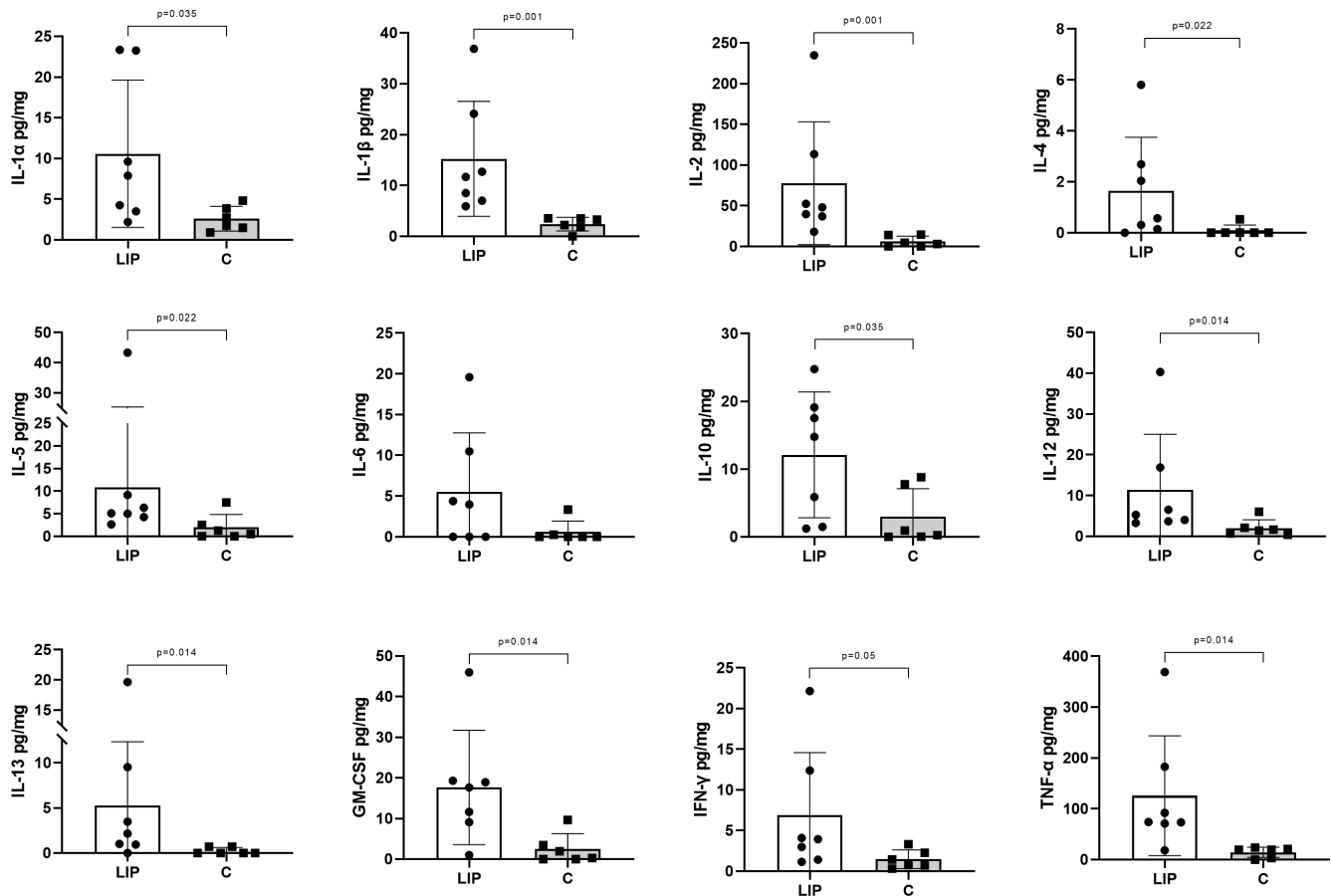
FIGURE 6 Levels of GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, and TNF-α in gingival tissue of in ligature-induced periodontitis (LIP) and control (C) (Mann–Whitney test).

We also found a significantly higher expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the intestine after LIP induction. GM-CSF promotes the migration and activation of myeloid cells to inflammation sites and calibrates macrophage defense and wound healing during intestinal infection and inflammation. Thus, a higher expression of GM-CSF may be associated with inappropriate macrophage activation and impact IBD.[17,18]

There are some limitations in the present study. The lack of microbiological analyses affects the evaluation of the host–parasite interactions, and a similar model using such interactions would help to better understand the interplay between both diseases. Thus, we could not relate the immunological response to a bacterial load. In addition, although there is no doubt that animal models are essential for the investigation of complex interactions of cells and immunological response, no animal model can fully mirror a human chronic disease such as periodontitis. In addition, the reduced number of animals and time points (14 days) used in the present study also unbale us to draw further conclusions. Thus, caution is needed in interpreting our results.

In conclusion, LIP causes histopathological changes and over-expression of both Th1- and Th2-related cytokines in the colon of Wistar rats.

DATA AVAILABILITY STATEMENT

The authors elect not to share data (research data are not shared). The data that support the findings of this study are available on request from the corresponding author.

REFERENCES

1. Pan W, Wang Q, Chen Q. The cytokine network involved in the host immune response to periodontitis. *Int J Oral Sci.* 2019;11(3):30.
2. Hajishengallis G. Interconnection of periodontal disease and comorbidities: evidence, mechanisms, and implications. *Periodontology 2000.* 2022;89(1):9-18.
3. Martini E, Krug SM, Siegmund B, Neurath MF, Becker C. Mend your fences: the epithelial barrier and its relationship with mucosal

immunity in inflammatory bowel disease. *Cell Mol Gastroenterol Hepatol.* 2017;4(1):33-46.

4. Friedrich M, Pohin M, Powrie F. Cytokine networks in the pathophysiology of inflammatory bowel disease. *Immunity.* 2019;50(4):992-1006.

5. de Mello-Neto JM, Nunes JGR, Tadakamadla SK, da Silva Figueredo CM. Immunological traits of patients with coexistent inflammatory bowel disease and periodontal disease: a systematic review. *Int J Environ Res Public Health.* 2021;18(17):8958.

6. Menegat JS, Lira-Junior R, Siqueira MA, et al. Cytokine expression in gingival and intestinal tissues of patients with periodontitis and inflammatory bowel disease: an exploratory study. *Arch Oral Biol.* 2016;66:141-146.

7. Kitamoto S, Nagao-Kitamoto H, Jiao Y, et al. The intermucosal connection between the mouth and gut in commensal pathobiont-driven colitis. *Cell.* 2020;182(2):447-462.e414.

8. Qiao D, Chen R, Li L, Zhu F, Zhang Y, Yan F. Accelerated alveolar bone loss in a mouse model of inflammatory bowel disease and its relationship with intestinal inflammation. *J Periodontol.* 2022;93(10):1566-1577.

9. de Mello-Neto JM, Elangovan G, Ervolino E, Johnson NW, Gustafsson A, da Figueredo CM. Colitis induced by dextran sulphate sodium causes histopathological and immunological changes in the periodontal tissues of Wistar rats. *J Periodontal Res.* 2022;57(6):1267-1276.

10. Kilkenny C, Browne WJ, Cuthi I, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *Vet Clin Pathol.* 2012;41(1):27-31.

11. Ervolino E, Statkievicz C, Toro LF, et al. Antimicrobial photodynamic therapy improves the alveolar repair process and prevents the occurrence of osteonecrosis of the jaws after tooth extraction in senile rats treated with zoledronate. *Bone.* 2019;120:101-113.

12. Bamias G, Corridoni D, Pizarro TT, Cominelli F. New insights into the dichotomous role of innate cytokines in gut homeostasis and inflammation. *Cytokine.* 2012;59(3):451-459.

13. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology.* 2011;140(6):1756-1767.

14. Jones-Hall YL, Nakatsu CH. The intersection of TNF, IBD and the microbiome. *Gut Microbes.* 2016;7(1):58-62.

15. Danese S, Sans M, Scaldaferri F, et al. TNF-alpha blockade downregulates the CD40/CD40L pathway in the mucosal microcirculation: a novel anti-inflammatory mechanism of infliximab in CrCrohn'sisease. *J Immunol.* 2006;176(4):2617-2624.

16. Muthusami S, Ramachandran IK, Babu KN, et al. Role of inflammation in the development of colorectal cancer. *Endocr Metab Immune Disord Drug Targets.* 2021;21(1):77-90.

17. Lotfi N, Thome R, Rezaei N, et al. Roles of GM-CSF in the pathogenesis of autoimmune diseases: an update. *Front Immunol.* 2019;10:1265.

18. Castro-Dopico T, Fleming A, Dennison TW, et al. GM-CSF calibrates macrophage defense and wound healing programs during intestinal infection and inflammation. *Cell Rep.* 2020;32(1):107857.
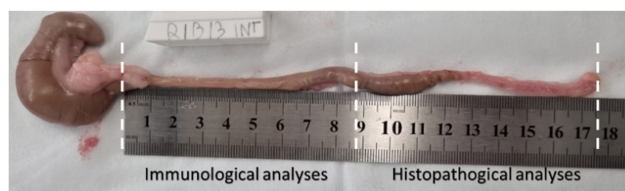
APPENDIX 1

FIGURE A1 Dissected colon being measured and allocated for immunological or histopathological analyses.
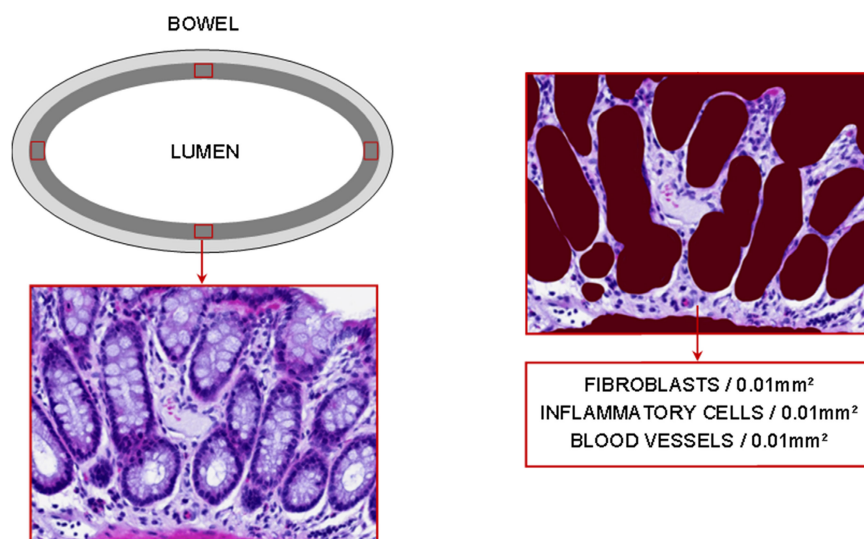

FIGURE A2 Methodology used for quantifying the number of fibroblasts, inflammatory cells, and blood vessels in the intestinal lamina propria.
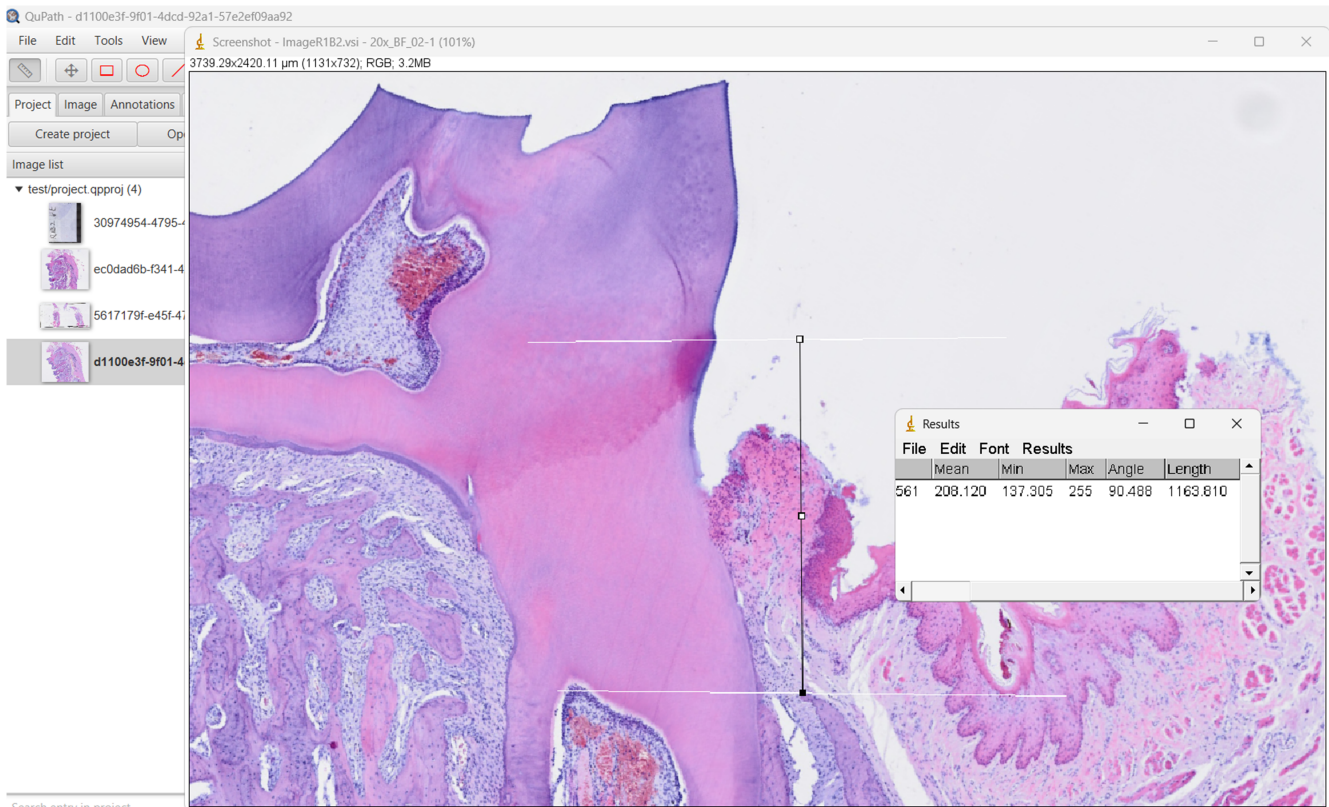
FIGURE A3 Methodology used for measuring the distance between the cementum enamel junction and alveolar bone.