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ORIGINAL ARTICLE

Alveolar bone resorption and Th1/Th17-associated immune response triggered during *Aggregatibacter actinomycetemcomitans*-induced experimental periodontitis are serotype-dependent

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Abstract

Background: *Aggregatibacter actinomycetemcomitans* expresses several virulence factors that may contribute to the pathogenesis of periodontitis. Based on the antigenicity of the O-polysaccharide component of the lipopolysaccharide (LPS), different *A. actinomycetemcomitans* serotypes have been described. Among them, serotype *b* has demonstrated a stronger capacity to trigger Th1 and Th17-associated cytokine, CC-chemokine, and CC-chemokine receptor production on immune cells in vitro. With a murine model of experimental periodontitis, this investigation aimed to analyze the alveolar bone resorption and the pattern of immune response triggered by the different *A. actinomycetemcomitans* serotypes within periodontal lesions.

Methods: For periodontal lesion induction, mice were orally infected with the different *A. actinomycetemcomitans* serotypes or their purified LPS. Alveolar bone resorption was analyzed using microcomputed tomography and scanning electron microscopy. Bacterial infection, receptor activator of nuclear factor-kappa B ligand (RANKL) and Th1 and Th17-associated cytokine, CC-chemokine, and CC-chemokine receptor levels were quantified by quantitative polymerase chain reaction (qPCR). T lymphocytes isolated from periodontal lesions were analyzed by flow cytometry.

Results: In periodontal lesions, serotype *b* of *A. actinomycetemcomitans* induced higher alveolar bone resorption and expression of RANKL compared with the other serotypes. In addition, serotype *b* induced greater levels of Th1- and Th17-related cytokines, CC-chemokines, and CC-chemokine receptors than the others. Similarly, higher numbers of infiltrating Th1 and Th17 lymphocytes were detected in serotype *b*-induced periodontal lesions.

Conclusions: These results demonstrate that periodontal lesions induced with different *A. actinomycetemcomitans* serotypes elicited distinct alveolar bone resorption and immune response. In particular, serotype *b* was more pathogenic than the others and

induced stronger Th1 and Th17 patterns of immune responses during experimental periodontitis.

KEYWORDS

Aggregatibacter actinomycetemcomitans, bone resorption, chemokine receptors, chemokines, cytokines, RANKL, T lymphocytes

1 | INTRODUCTION

Aggregatibacter actinomycetemcomitans is a key pathogen in the etiology of periodontitis;[1–3] however, not all bacteria strains belonging to the *A. actinomycetemcomitans* species have the same immunogenicity. In fact, different serotypes of *A. actinomycetemcomitans* are recognized based on lipopolysaccharide (LPS) antigenicity, corresponding to structurally distinct O-polysaccharide components that function as immuno-dominant antigens.[4]

The *A. actinomycetemcomitans* serotypes differ in their ability to induce response in host immune cells; in particular, the serotype *b* has shown a higher immunogenicity in macrophages, dendritic cells, and T lymphocytes when compared with the other serotypes.[5–10] In fact, the serotype *b* of *A. actinomycetemcomitans*, when exposed to T lymphocytes, induced significantly higher levels of T-helper (Th)1- and Th17-related cytokines, CC-chemokines, and CC-chemokine receptors when compared with the others, thus inducing a pattern of immune response associated with the proinflammatory and destructive events characteristic of periodontitis.[5,7] In addition, T lymphocytes stimulated by serotype *b*-primed dendritic cells, when compared with the same cells stimulated with the other serotypes, elicited higher levels of the pro-bone-resorptive factor termed receptor activator of nuclear factor-kappa B ligand (RANKL), and these increased RANKL levels were associated with greater in vitro osteoclast activation and bone resorption.[11] The *A. actinomycetemcomitans* serotype-disease association during periodontitis in vivo, however, has not been established yet.

With the goal to assess whether the alveolar bone resorption and immune response triggered during *A. actinomycetemcomitans*-induced periodontitis is serotype-dependent, in this investigation we assessed whether the different serotypes of *A. actinomycetemcomitans* induce differential expression of cytokines, CC-chemokines, CC-chemokine receptors, and RANKL within periodontal lesions. We hypothesized that serotype *b*, when compared with the other serotypes, induces higher levels of Th1- and Th17-related cytokines, CC-chemokines, and CC-chemokine receptors, concomitant with higher infiltration of Th1 and Th17 lymphocytes in *A. actinomycetemcomitans*-induced periodontal lesions, and these increased levels associated with the greater levels of RANKL and alveolar bone resorption.

2 | MATERIALS AND METHODS

2.1 | *A. actinomycetemcomitans* strains

The *A. actinomycetemcomitans* strains ATCC 43717 (serotype *a*), ATCC 43718 (serotype *b*), and ATCC 43719 (serotype *c*) were cultured as previously described.[5] Periodontal infections were induced using either whole bacteria or its purified LPS. For periodontal infections with whole bacteria, bacterial growth curves were made using standard procedures,[12] and bacteria were taken at the exponential growth phase to obtain a reliable number of bacteria having their whole antigenic potentiality. For periodontal infections with LPS, purified LPS was obtained from each *A. actinomycetemcomitans* serotype using a modified version of the Tri-reagent protocol as previously described.[9]

2.2 | Animals

Experimental groups were composed of 8-week-old wild-type BALB/c mice obtained from the Institutional Animal Facilities (Faculty of Dentistry, Universidad de Chile). Animals were housed in separate cages and maintained under standard conditions under a 12:12 hours light/dark cycle, lights-on at 07:00 am, at 24°C ± 0.5°C and 40% to 70% of relative humidity, with an air exchange rate of 15-room volumes/hour. Throughout the period of the study, animals had free access to sterile standard solid mice chow and sterile water. The protocol was approved by the Institutional Committee for Animal Care and Use (#061601), and the experiments followed the guidelines approved by the Council of the American Psychological Society (1980) for the use of animal experiments and were conducted in accordance with the American Veterinary Medical Association (AVMA) recommendations.

2.3 | Periodontal infections

The infection protocol compromised the direct microinjection of each of the different *A. actinomycetemcomitans* serotypes or their purified LPS via a 26s-gauge syringe* into the palatal interproximal gingiva between the first, second, and third molars both on the left and right side. The mice were divided

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into eight groups: whole serotype *a*-infected group (n = 15), whole serotype *b*-infected group (n = 15), whole serotype *c*-infected group (n = 15), LPS of serotype *a*-infected group (n = 7), LPS of serotype *b*-infected group (n = 7), LPS of serotype *c*-infected group (n = 7), sham-infected mice (n = 7) which received phosphate buffered saline (PBS) without bacteria or LPS, and non-infected animals (n = 7). Injections containing 1×10^9 colony forming units (CFU)/mL of each of the different *A. actinomycetemcomitans* serotypes or 20 μg of their purified LPS in 2-μL PBS total volume were given three times each week throughout the duration of the experiment.[13,14] The mice were euthanized at 30 days by cervical dislocation under isoflurane anesthesia; the maxilla was dissected free of soft tissues to quantify the extent of the alveolar bone loss; and the whole palatal periodontal tissues between the mesial surface of first molar and the distal surface of the third molar were collected and processed immediately.

2.4 | Detection of *A. actinomycetemcomitans* within the periodontal lesions

To demonstrate the periodontal infection, the serotype-specific DNA detection levels of the different *A. actinomycetemcomitans* strains from mice periodontal lesions were determined by conventional polymerase chain reaction (PCR) and the bacterial loads were quantified by quantitative polymerase chain reaction (qPCR). Total DNA extraction was performed using a DNA extraction kit* according to the manufacturer´s protocol. After extraction, DNA was suspended in sterile DNase-free milli-Q water and quantified using a spectrophotometer.† To determine the DNA expression of the different *A. actinomycetemcomitans* serotypes, DNA samples were amplified using serotype-specific primers (see supplementary Table 1 in online *Journal of Periodontology*) and a conventional PCR kit‡ following the manufacturer's recommendations. The reaction was run in a 2% agarose gel stained with DNA staining§ and photographed using a transilluminator.¶ To quantify the DNA expression of the different *A. actinomycetemcomitans* serotypes, DNA samples were amplified using a qPCR reagent# in a real-time qPCR equipment.‖ Standard curves were developed for each *A. actinomycetemcomitans* serotype using as a template known concentrations of genomic DNA. Serial 10-fold dilutions in DNase-free milli-Q water were made to prepare standard DNA ranging from 1×10^2 to 1×10^9 CFU. Amplification reactions were conducted as follows: 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. After amplification, a melting curve analysis of 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds was performed to determine the specificity of the PCR products. As reference, 16S rDNA expression levels were determined.

2.5 | Alveolar bone resorption

To analyze the extent of the alveolar bone loss, the maxilla was split into two halves from the midline between the central incisors. One half was taken for microcomputed tomography (μCT) analysis, while the other half was used for scanning electron microscopy analysis. For μCT analysis, hemisected maxillae were placed in standardized cylindrical sample holders and imaged in μCT equipment** using the following parameters: 59 kV, 588 μA, 0.5° rotation, and 360° of angular range. The specimens were scanned in all three spatial planes and three-dimensional digitized images were generated using a modified cone-beam algorithm[15] and reconstruction software.†† Image data were analyzed using a protocol described previously.[16] Briefly, each image was reoriented such that the cemento-enamel junction (CEJ) from the first and the second molar was parallel to the horizontal axis, and both the CEJ and the root apex appear in the same slice. A standardized region of interest (ROI) was then obtained using analyzer software‡‡ with the following anatomical limits: the furcation roof and root apex of both first and second molar, the medial root surface of the first molar, and the distal root surface of the second molar. For scanning electron microscopy analysis, hemisected maxillae were mechanically defleshed, exposed to 3% hydrogen peroxide, washed with milli-Q water, and fixed in 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer§§ overnight at room temperature. Specimens were then washed three times with PBS, dehydrated through immersion in different concentrations of ethanol (50%, 70%, 95%, and 100%), sputter-coated with gold layer to a thickness of 200 nm, and examined in a scanning electron microscope¶¶ at an accelerating voltage of 20 kV. Bone loss analysis was performed at magnification ×30, quantifying the area of bone loss between the mesial surface of first molar and the distal surface of the third molar, and between the cemento-enamel junction and the alveolar bone crest, as described previously.[17] All data were

* FavorPrep Tissue Genomic DNA Extraction Mini Kit; Favorgen Biotech, Taiwan.

† Synergy HT; Bio-Tek Instrument, Winooski, VT

‡ GoTaq Green Master Mix; Promega, Madison, WI

§ GelRed; Biotium Inc., Hayward, CA

¶ Gel Logic 2200Pro; Carestream Health, Rochester, NY

KAPA SYBR Fast qPCR; KAPA Biosystems, Woburn, MA

‖ StepOnePlus; Applied Biosystems, Singapore

** SkyScan 1278; Bruker, Kontich, Belgium

†† Nrecon software; Bruker

‡‡ Dataviewer software; Bruker

§§ Sigma-Aldrich Chemie, Buchs, Switzerland

¶¶ Jeol JSMIT300LV; Jeol, Tokyo, Japan

collected by a single observer who was masked to conditions of the maxillae samples.

2.6 | Expression of cytokines, chemokines, chemokine receptors, TLRs, and RANKL

The mRNA expression levels for the cytokines interleukin (IL)-1β, IL-6, IL-12, IL-17, IL-23, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, for the CC-chemokines CCL2, CCL5, and CCL20, for the CC-chemokine receptors CCR2, CCR5, CCR6, CCR7, for the surface toll-like receptor (TLR)2 and TLR4, as well as for the pro-bone-resorptive factor RANKL, were quantified by qPCR. Each individual whole palatal periodontal tissue sample was minced to ≈1 mm^3 fragments and manually homogenized in an RNase-free vial using 1 mL of ice-cold molecular purification reagent* according to the manufacturer's instructions. After incubation for 10 minutes at 4°C, 200 μL of chloroform were added and incubated for 10 minutes at 4°C under vigorous uniform vortex. After centrifugation at 12,000 *g* for 20 minutes, the aqueous phase was transferred to a fresh RNase-free vial and the total cytoplasmic RNA was precipitated in 500 μL of isopropyl alcohol and 20 μg/μL glycogen† for 30 minutes. After centrifugation at 12,000 *g* for 20 minutes at 4°C, the total RNA precipitate was washed once with 1 mL 75% ethanol and one-touch vortex. Finally, the purified RNA was resuspended in 10 μL of sterile RNase-free milli-Q water and quantified in a spectrophotometer.‡ The first-strand cDNA was synthesized using 1 μg of total RNA with a reverse transcription kit§ following the manufacturer's instructions. Ten nanograms of cDNA were amplified using the appropriate primers (see supplementary Table 1 in online *Journal of Periodontology*) and a qPCR reagent¶ in real-time qPCR equipment.# Amplification reactions were conducted as follows: 95°C for 3 minutes, followed by 40 cycles of 95°C for 3 seconds, 60°C for 30 seconds, and finally a melting curve of 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds, for detection of non-specific product formation and false-positive amplification. As an endogenous control, 18S rRNA expression levels were determined.

2.7 | Detection of Th1 and Th17 lymphocytes within periodontal lesions

The presence of Th1 and Th17 lymphocytes infiltrating the infected palatal periodontal tissues was demonstrated by flow cytometry using double staining of intracellular expression of transcription factor T-box expressed in T cells (T-bet)/IFN-γ and transcription factor orphan nuclear receptor (ROR)γt/IL-17 as described previously.[7] Cells were isolated from the palatal periodontal tissue samples using procedures described previously.[18,19] Briefly, samples were collected, weighed, minced into ≈1-mm^3 pieces, and incubated in 1-mL tissue digestion medium supplemented with 200 U/mL of type IV collagenase,‖ in a water bath under mild agitation at 37°C for 90 minutes. Cells obtained were washed twice with PBS, counted, and fixed for 10 minutes at 4°C using a cold-flow cytometry fixation buffer** to ensure the stability of antigens and to retain the target protein in the original cellular location. Previous cell permeabilization using flow cytometry permeabilization/wash buffer,** cells were stained using the following monoclonal antibodies: T-bet peridinin chlorophyll protein complex (PerCP)-Cy5.5-labeled, RORγt fluorescein isothiocyanate (FITC)-labeled, IFN-γ allophycocyanin (APC)-labeled, and IL-17 phycoerythrin (PE)-labeled,†† and then analyzed by flow cytometry.‡‡ Cells were gated according to their forward and side scatter characteristics and the CD4 marker. Isotype-matched control antibodies were used to determine the negative cell populations.

2.8 | Statistical analysis

To quantify the number of bacteria detected within mice periodontal lesions, standard curves were obtained by plotting cycle-threshold values versus log CFU. Data were expressed as bacterial load normalized by the tissue weight and shown as mean ± SD. Alveolar bone loss data were presented as percentage (μCT) and μm^2 (scanning electron microscopy), and shown as mean ± SD. The percentage of alveolar bone loss was calculated using the formula %bone loss = 1-(remaining bone volume/ROI) ×100. The cytokine, CC-chemokine, CC-chemokine receptor, TLR, and RANKL data were analyzed using a qPCR software§§ and presented as mean ± SD of fold-change of relative quantities, using the $2^{-\Delta\Delta Ct}$ method. The flow cytometry data were analyzed using the WinMDi 2.9 software¶¶ and expressed as mean ± SD percentage of double-positive cells over the total. Data were statistically analyzed,## the normality of data distribution was

* TRIzol Plus; Invitrogen, Barcelona, Spain

† Roche Diagnostics, Mannheim, Germany

‡ Synergy HT; Bio-Tek Instrument, Winooski, VT

§ SuperScript III; Invitrogen, Grand Island, NY

¶ KAPA SYBR Fast qPCR; KAPA Biosystems, Woburn, MA

StepOnePlus; Applied Biosystems, Singapore

‖ Gibco Invitrogen, Grand Island, NY

** R&D Systems, Minneapolis, MN

†† BioLegend, San Diego, CA

‡‡ BD FACSCanto; Becton Dickinson Immunocytometry Systems, San Jose, CA

§§ StepOne software 2.2.2; Applied Biosystems, Singapore.

¶¶ The Scripps Research Institute, La Jolla, CA

SPSS 22.0 software; IBM, Armonk, NY

determined using the Kolmogorov-Smirnov test and differences among groups and within each group regarding the *A. actinomycetemcomitans* load, alveolar bone resorption, and cytokine, CCL, CCR, TLR, and RANKL mRNA expression were analyzed using the ANOVA and Tukey post-hoc tests. Differences regarding the transcription factors and cytokine double-expression levels analyzed by flow cytometry were determined using the χ^2 test. Correlation coefficients were obtained using the Pearson test. A value of $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Detection of *A. actinomycetemcomitans* within the periodontal lesions

Each of the periodontal infections was serotype-specific, as confirmed by the bacteria DNA amplifications obtained using serotype-specific primers (Figure 1A). In addition, there were no differences in the periodontal bacterial load between all experimental conditions 30 days after *A. actinomycetemcomitans* oral inoculation (Figure 1B).

3.2 | Alveolar bone resorption

When the strain ATCC 43718 belonging to the serotype *b* of *A. actinomycetemcomitans* was used for periodontal infection, significantly higher alveolar bone resorption was observed by μCT ($P < 0.001$) when compared with periodontal infections induced with the strains ATCC 43717 or ATCC 43719 belonging to the serotypes *a* or *c*, respectively (Figures 1C and 1D). Similarly, in serotype *b*-induced periodontal infections, an increase in the area of bone loss between the mesial surface of first molar and the distal surface of the third molar and between the cemento-enamel junction and the alveolar bone crest was detected using scanning electron microscopy, as compared with the others ($P = 0.010$ and $P = 0.029$) (Figures 1E and 1F).

3.3 | Expression of cytokines

Figure 2 shows the expression of IL-1β, IL-6, IL-12, IL-17, IL-23, IFN-γ, and TNF-α in periodontal lesions induced with 1×10^9 CFU/mL of each of the *A. actinomycetemcomitans* serotypes or 20 μg of their purified LPS. When the serotype *b* of *A. actinomycetemcomitans* was used for periodontal infection, significantly higher expression levels of IL-1β ($P < 0.001$), IL-6 ($P < 0.001$), IL-12 ($P < 0.001$), IL-17 ($P < 0.001$), IL-23 ($P < 0.001$), IFN-γ ($P < 0.001$), and TNF-α ($P < 0.001$) were detected when compared with periodontal infections induced with the other serotypes

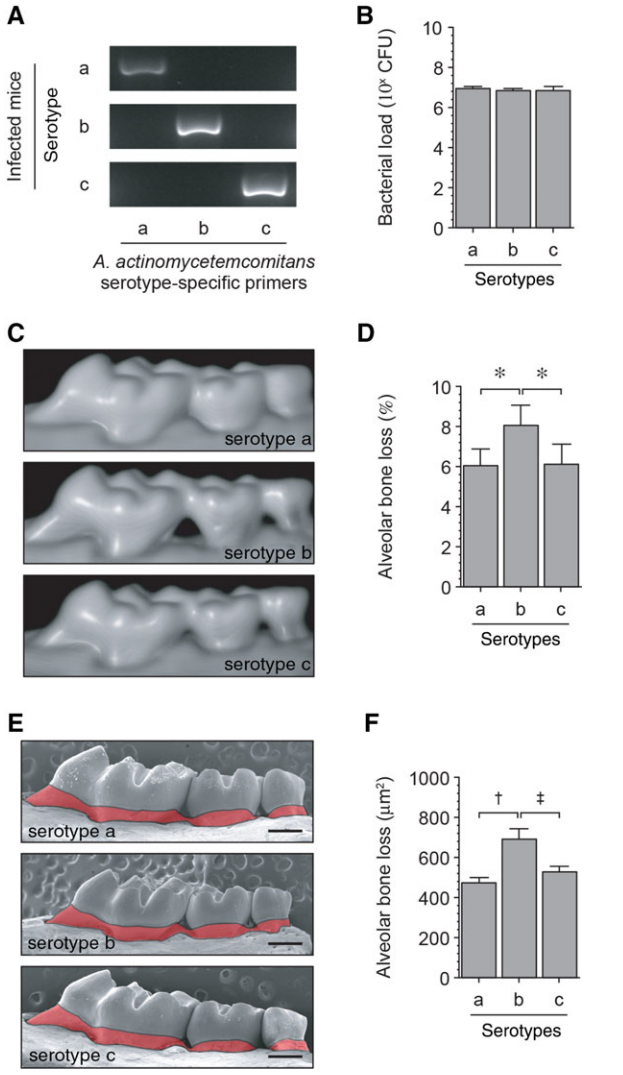

FIGURE 1 Periodontal infection and alveolar bone resorption. **A)** Serotype-specific detection of the different *A. actinomycetemcomitans* strains from mice periodontal lesions 30 days after oral inoculation. **B)** Quantification of the different *A. actinomycetemcomitans* serotypes from mice periodontal lesions 30 days after oral inoculation. Bacterial loads from periodontal lesions normalized to tissue weight are represented as 1×10^x CFU and shown as mean ± SD from three independent experiments. **C)** Representative microcomputed tomography (μCT) three-dimensional images of maxillae specimens of mice infected with the different *A. actinomycetemcomitans* serotypes. **D)** Alveolar bone loss quantified by μCT in palatal specimens of mice infected orally with 1×10^9 CFU/mL of the different *A. actinomycetemcomitans* serotypes. Data are represented as percentage and shown as mean ± SD from 10 independent experiments. **E)** Representative scanning electron microscopy photomicrographs of maxillae specimens of mice infected with the different *A. actinomycetemcomitans* serotypes, showing area of alveolar bone loss (Scale bar = 500 μm). **F)** Area of bone loss quantified by scanning electron microscopy in palatal specimens of mice infected orally with 1×10^9 CFU/mL of the different *A. actinomycetemcomitans* serotypes. Data are represented as μm^2 and shown as mean ± SD from 10 independent experiments. $^*P < 0.001$, $^\dagger P < 0.01$, and $^\ddagger P < 0.05$ *A. actinomycetemcomitans* serotype *b* compared with serotypes *a* and *c*

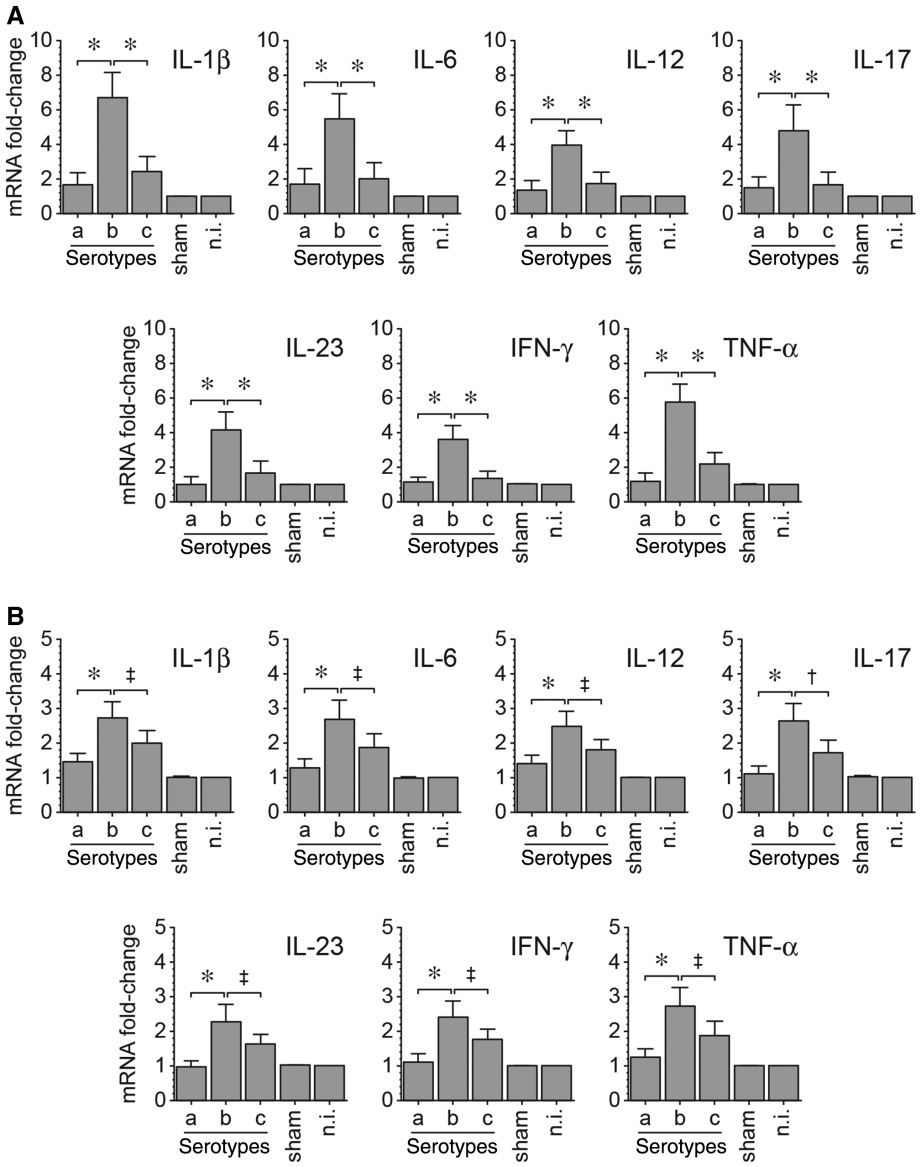

FIGURE 2 Cytokine expression. **A)** Cytokine mRNA expression in mice infected orally with 1×10^9 CFU/mL of the different *A. actinomycetemcomitans* serotypes. **B)** Cytokine mRNA expression in mice infected orally with 20 μg of LPS purified from the different *A. actinomycetemcomitans* serotypes. For relative expression, the cytokine mRNA expression in non-infected (n.i.) mice was considered as 1, as a reference for fold-change in expression. Sham-infected (sham) mice were used as controls. Data are represented as mRNA fold-change and shown as mean ± SD from seven independent experiments. Each experiment was performed in duplicate. $^{*}P < 0.001$, $^{\dagger}P < 0.01$, and $^{\ddagger}P < 0.05$ *A. actinomycetemcomitans* serotype *b* compared with serotypes *a* and *c*

(Figure 2A). The described changes in cytokine mRNA levels were consistent when purified LPS were used for periodontal lesion inductions. In fact, significantly higher expression levels of IL-1β ($P < 0.001$ and $P = 0.020$), IL-6 ($P < 0.001$ and $P = 0.023$), IL-12 ($P < 0.001$ and $P = 0.017$), IL-17 ($P < 0.001$ and $P = 0.004$), IL-23 ($P < 0.001$ and $P = 0.026$), IFN-γ ($P < 0.001$ and $P = 0.027$), and TNF-α ($P < 0.001$ and $P = 0.015$) were detected in periodontal lesions induced with LPS purified from the serotype *b* of *A. actinomycetemcomitans*, compared with those induced with LPS purified from the serotypes *a* or *c*, respectively (Figure 2B).

3.4 | Expression of chemokines and chemokine receptors

Figure 3 shows the expression of CCR2, CCR5, CCR6, CCR7, CCL2, CCL5, and CCL20 in periodontal lesions induced by oral inoculation of 1×10^9 CFU/mL of each of the *A. actinomycetemcomitans* serotypes or 20 μg of their purified LPS. When the serotype *b* of *A. actinomycetemcomitans* was used for periodontal infection, significantly higher expression levels of CCR2 ($P = 0.001$ and $P = 0.002$), CCR5 ($P < 0.001$), CCR6 ($P = 0.001$), CCR7 ($P < 0.001$), CCL2 ($P < 0.001$), CCL5 ($P < 0.001$), and CCL20 ($P < 0.001$) were detected

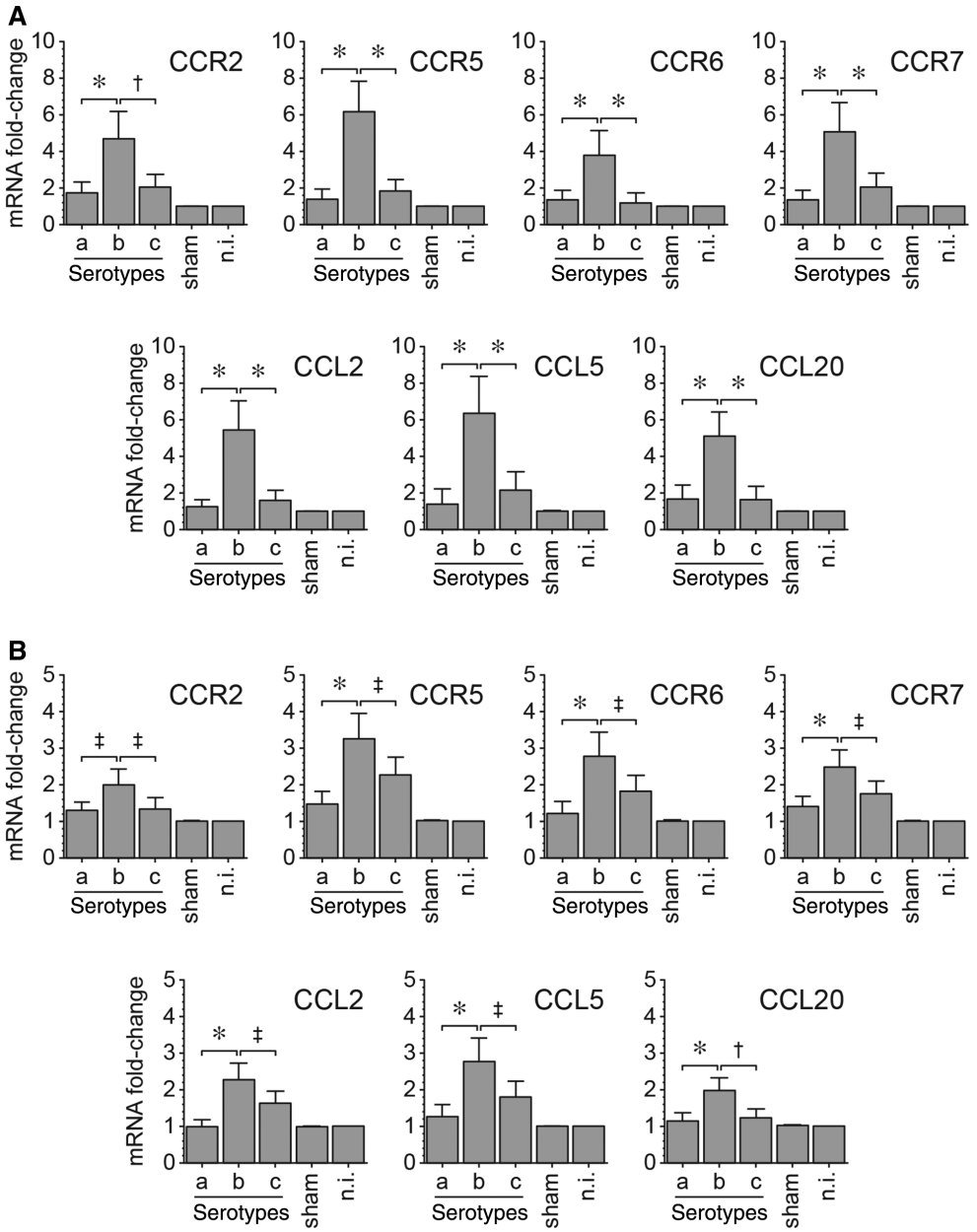

FIGURE 3 CC-chemokine and CC-chemokine receptor expression. **A)** CC-chemokine and CC-chemokine receptor mRNA expression in mice infected orally with 1×10^9 CFU/mL of the different *A. actinomycetemcomitans* serotypes. **B)** CC-chemokine and CC-chemokine receptor mRNA expression in mice infected orally with 20 μg of LPS purified from the different *A. actinomycetemcomitans* serotypes. For relative expression, the CCR and CCL mRNA expression in non-infected (n.i.) mice was considered as 1, as a reference for fold-change in expression. Sham-infected (sham) mice were used as controls. Data are represented as mRNA fold-change and shown as mean ± SD from seven independent experiments. Each experiment was performed in duplicate. $^{*}P < 0.001$, $^{\dagger}P < 0.01$, and $^{\ddagger}P < 0.05$ *A. actinomycetemcomitans* serotype *b* compared with serotypes *a* and *c*

when compared with periodontal infections induced with the other serotypes (Figure 3A). Similarly, significantly higher expression levels of CCR2 ($P = 0.011$ and $P = 0.015$), CCR5 ($P < 0.001$ and $P = 0.028$), CCR6 ($P < 0.001$ and $P = 0.023$), CCR7 ($P = 0.001$ and $P = 0.022$), CCL2 ($P < 0.001$ and $P = 0.028$), CCL5 ($P < 0.001$ and $P = 0.018$), and CCL20 ($P = 0.001$ and $P = 0.002$) were detected in periodontal lesions induced with LPS purified from the serotype *b* of *A. actinomycetemcomitans*, compared with those induced with LPS purified from the serotypes *a* or *c*, respectively (Figure 3B).

3.5 | Expression of TLR2 and TLR4

An increment in the expression levels for TLR2 and TLR4 mRNAs was detected in periodontal lesions induced by oral inoculation of 1×10^9 CFU/mL of each of the *A. actinomycetemcomitans* serotypes (Figure 4). When the serotype

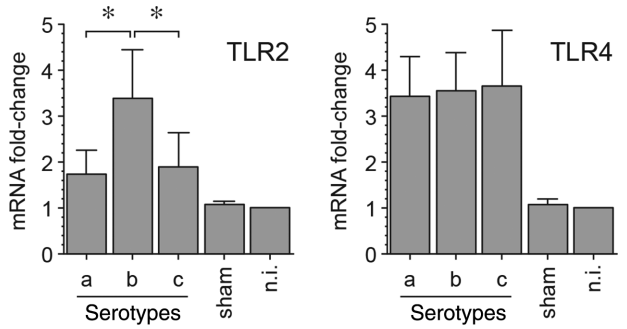

FIGURE 4 TLR2 and TLR4 expression. TLR2 and TLR4 mRNA expression in mice infected orally with 1×10^9 CFU/mL of the different *A. actinomycetemcomitans* serotypes. For relative expression, the TLR2 and TLR4 mRNA expression in non-infected (n.i.) mice were considered as 1, as a reference for fold-change in expression. Sham-infected (sham) mice were used as controls. Data are represented as mRNA fold-change and shown as mean ± SD from seven independent experiments. Each experiment was performed in duplicate. $^{*}P < 0.001$ *A. actinomycetemcomitans* serotype *b* compared with serotypes *a* and *c*

b was used for periodontal infection, significantly higher expression levels of TLR2 ($P < 0.001$) were detected when compared with infections induced with the serotypes *a* or *c*. The levels of TLR4, however, were no different when periodontal lesions were induced with *A. actinomycetemcomitans* serotypes *a*, *b*, or *c*.

3.6 | Detection of Th1 and Th17 lymphocytes within periodontal lesions

Figure 5 shows the flow cytometry analysis of the double staining of the intracellular expression of T-bet/IFN-γ and RORγt/IL-17 in CD4$^+$ T lymphocytes isolated from the palatal periodontal tissues infected with the different *A. actinomycetemcomitans* serotypes, confirming that the Th1 and Th17 phenotypes are associated with their specific function. When the serotype *b* of *A. actinomycetemcomitans* was used for periodontal infection, significantly higher detection of T-bet$^+$IFN-γ^+ Th1 lymphocytes (57.23% versus 44.56% and 47.66%, $P < 0.05$) was detected when compared with periodontal infections induced with the serotypes *a* or *c*, respectively. Similarly, significantly higher detection of RORγt$^+$IL-17$^+$ Th17 lymphocytes (18.82% versus 9.57% and 10.71%, $P < 0.01$) was detected when the serotype *b* of *A. actinomycetemcomitans* was used for periodontal infection, as compared with the serotypes *a* or *c*, respectively.

3.7 | Expression of RANKL

An increase in the RANKL mRNA expression levels was detected in periodontal lesions induced with the serotype *b* of *A. actinomycetemcomitans* or its purified LPS ($P < 0.001$) when compared with lesions induced by serotypes *a* or *c*

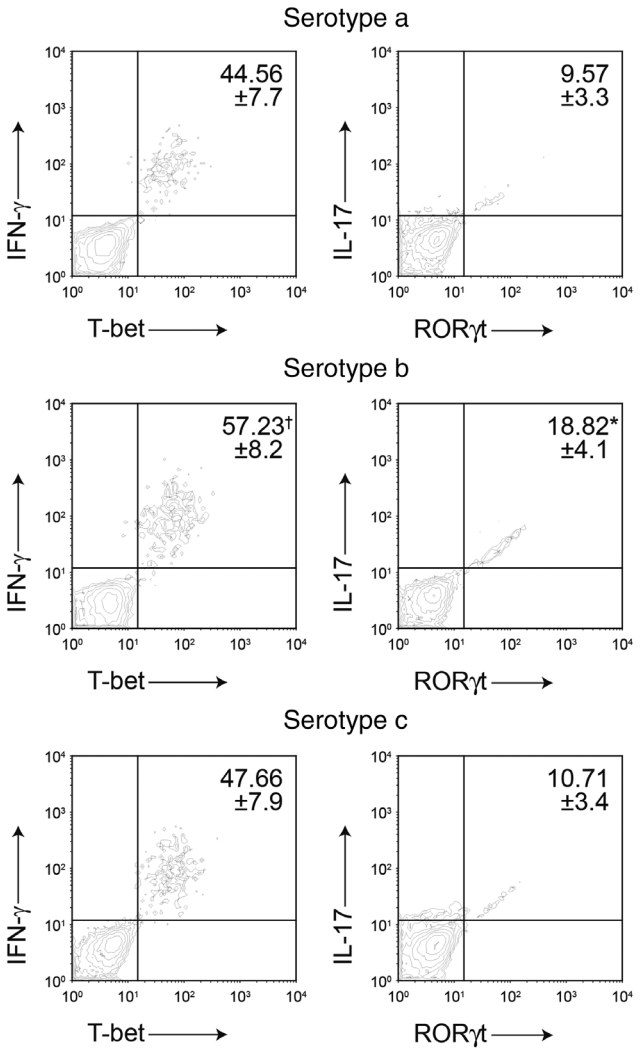

FIGURE 5 Th1 and Th17 lymphocyte detection. Flow cytometry analysis of the double staining of intracellular expression of T-bet/IFN-γ and RORγt/IL-17 demonstrating the presence of IFN-γ producing Th1 cells and IL-17 producing Th17 cells within periodontal lesions induced with 1×10^9 CFU/mL of the different *A. actinomycetemcomitans* serotypes. The flow cytometry data from each experiment are represented as the percentage of double positive cells over the total and shown as mean ± SD from five independent experiments. Each experiment was performed in duplicate. $^{*}P < 0.01$ and $^{\dagger}P < 0.05$ *A. actinomycetemcomitans* serotype *b* compared with serotypes *a* and *c*

(Figure 6). Correlation analyses performed between the RANKL and cytokine expression upon whole bacteria or LPS inductions, respectively, yielded significant positive correlation between RANKL and IL-1β ($r = 0.968$ and $r = 0.990$), IL-6 ($r = 0.969$ and $r = 0.990$), IL-12 ($r = 0.970$ and $r = 0.983$), IL-17 ($r = 0.975$ and $r = 0.995$), IL-23 ($r = 0.935$ and $r = 0.983$), IFN-γ ($r = 0.942$ and $r = 0.997$), and TNF-α ($r = 0.964$ and $r = 0.975$), suggesting that the increased RANKL expression was associated with the Th1- and Th17-pattern of immune response elicited within the periodontal lesions.

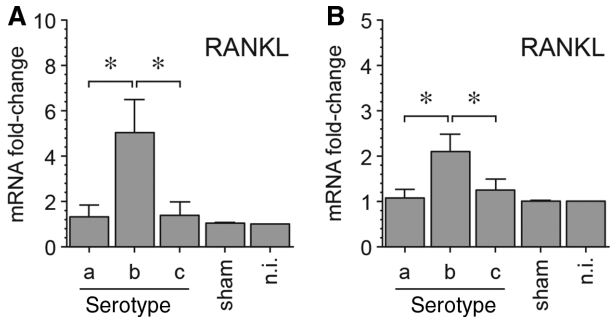

FIGURE 6 RANKL expression. **A)** RANKL mRNA expression in mice infected orally with 1×10^9 CFU/mL of the different *A. actinomycetemcomitans* serotypes. **B)** RANKL mRNA expression in mice infected orally with 20 μg of LPS purified from the different *A. actinomycetemcomitans* serotypes. For relative expression, the RANKL mRNA expression in non-infected (n.i.) mice was considered as 1, as a reference for fold-change in expression. Sham-infected (sham) mice were used as controls. Data are represented as mRNA fold-change and shown as mean ± SD from seven independent experiments. Each experiment was performed in duplicate. $^*P < 0.001$ *A. actinomycetemcomitans* serotype *b* compared with serotypes *a* and *c*

4 | DISCUSSION

A. actinomycetemcomitans play a pivotal role in the pathogenesis of chronic, aggressive, and syndromic forms of periodontitis.[1–3,20,21] In fact, *A. actinomycetemcomitans* and its purified LPS have been shown both in vitro and in vivo to promote inflammation and osteoclast differentiation and activation, resulting in alveolar bone resorption.[14,22–31] These *A. actinomycetemcomitans*-induced pathological events are mediated by activation of a Th1- and Th17-dominated immune response at the local site of the periodontal lesion.[28,31–34] Indeed, LPS purified from the strain ATCC 43718 belonging to the serotype *b* of *A. actinomycetemcomitans* induced an increment on inflammatory cells infiltrating periodontal tissues with higher expression levels of IL-1β, IL-6, and TNF-α compared with non-infected controls, and these cytokine increments were associated with a greater number of osteoclasts and alveolar bone resorption.[31]

Proinflammatory cytokines, chemokines, and chemokine receptors have been largely related to the progression of periodontitis, which involves recruitment of immune cells and production of pro-bone-resorptive factors, such as RANKL.[25,35] Thus, IL-1β, IL-6, IL-17, IL-23, IFN-γ, and TNF-α have been shown to be key mediators that favor the production of RANKL and, finally, osteoclastogenesis and bone loss.[25,35]

Increased production of IL-1β, IFN-γ, and TNF-α has been associated with both greater expression of CCL5 and CCR5, and differentiation of Th1 lymphocytes.[36] Indeed, CCR5 favors the migration of distinct leukocyte subpopulations towards infected periodontal tissues,[29,30] which include pro-osteoclastogenic RANKL-positive Th1 lymphocytes.[23] Accordingly, Th1 lymphocytes are regarded as key in the production of RANKL within periodontal lesions and infiltrating CCR5-positive Th lymphocytes are also positive for RANKL staining.[37] Similarly, increased production of IL-6, IL-17, and IL-23 has been associated with both greater expression of CCL2, CCR6, and CCR7, and subsequent differentiation, activation, and migration of Th17 lymphocytes.[38,39] In fact, CCL19 and CCL21, chemokines that bind to CCR7, have been implied in the chemotaxis of IL-17-producing cells towards infected tissues, which in turn favours the activity of Th1 and Th17 lymphocytes and the production of RANKL.[24,35] The higher pathogenic potential of serotype *b* of *A. actinomycetemcomitans* compared with the others could be associated with local overexpression of Th1- and Th17-related cytokines, chemokines, and chemokine receptors, as demonstrated in the present study, which are involved in the overexpression of RANKL, differentiation and activation of osteoclasts, and resorption of alveolar bone.[23,24,29,32,34,35,40,41]

Alveolar bone resorption is positively regulated by the overexpression of RANKL, which in turn is regulated by TLR-dependent signaling.[42,43] In fact, TLR2 and TLR4 were associated with RANKL-dependent alveolar bone resorption in a mouse model of periodontitis.[44] In this study, *A. actinomycetemcomitans* induced overexpression of TLR2 and TLR4 in infected periodontal tissues; however, the expression levels of TLR2 were higher when infections were induced with serotype *b* in comparison with the others. Thus, the higher immunostimulatory potential of serotype *b* demonstrated in this study could be explained by TLR2-dependent signaling. Indeed, the variable expression of cytokines and chemokine receptors detected in dendritic cells stimulated with different serotypes of *A. actinomycetemcomitans* was established as TLR2 dependent.[8] In general terms, TLR4 responds against the lipid A of LPS; while TLR2 responds against the O-polysaccharide of LPS.[45] The different serotypes of *A. actinomycetemcomitans* have been defined based on the distinct antigenicity of the O-polysaccharide component of their LPS;[4] therefore, the higher capacity to trigger Th1- and Th17-type immune response and alveolar bone resorption of serotype *b* may be explained by the TLR2-mediated higher immunogenicity of its LPS. In fact, our data demonstrated that the LPS purified from the serotype *b* of *A. actinomycetemcomitans* is a more potent immunogen and induces higher levels of RANKL compared with the others.

The number of Th1 ($CD4^+$T-$bet^+IFN\text{-}\gamma^+$) and Th17 ($CD4^+ROR\gamma t^+IL\text{-}17^+$) lymphocytes obtained from infected periodontal tissues was found to be increased when the serotype *b* of *A. actinomycetemcomitans* was used for periodontal infection. CCL2, CCL5, and CCL20, and their respective receptors CCR2, CCR5, CCR6, and CCR7, are involved

in the selective chemoattraction of Th1 and Th17 lymphocytes towards infected tissues.[5] On the other hand, IL-6 and IL-12 are directly involved in the lineage commitment of Th1 and Th17 lymphocytes from naïve T cells during antigen presentation, whereas IL-23 is required to stabilize and strengthen the Th17 phenotype and its pathogenic potential.[46] In this investigation, expression levels of CCL2, CCL5, CCL20, IL-6, IL-12, and IL-23 were significantly higher in serotype *b*-induced periodontal lesions compared with the others. Thus, it could be hypothesized that the increased detection of Th1 and Th17 lymphocytes in serotype *b*-infected periodontal lesions could be a consequence not only of the increased recruitment of differentiated Th1 and Th17 lymphocytes from regional lymph nodes that drain the periodontium towards the infected tissues, but also of the differentiation of these T-cell phenotypes locally in the infected periodontal tissues, in a site-specific manner. Indeed, formation of periodontal lymphoid clusters in which dendritic cells present bacterial antigens locally to naïve or memory T lymphocytes has been previously reported.[47,48]

Our results are in general agreement with previous studies demonstrating differences in the pathogenic potential among different strains belonging to a bacteria species.[49–52] In fact, a pathogenic increase of the encapsulated *Porphyromonas gingivalis* strains 381 and ATCC 53977, when compared with the non-encapsulated strain ATCC 33277, has been demonstrated when these pathogens were inoculated in a mouse model of experimental periodontitis.[53] In this context, different strains of *P. gingivalis* impact host immune and non-immune cell function differently. Proliferation of human gingival epithelial cells after infection with *P. gingivalis* strain W50 was higher than cell proliferation detected upon infection with the strain ATCC 33277.[54] The invasive capacity of *P. gingivalis* strains KUMC-P5 and KUMC-P7 into human gingival fibroblasts was higher than that of strains W50 and W83, belonging to the serotype K1.[55] A heterogenic immunostimulatory potential on dendritic cells and T lymphocytes has been described with the different capsular serotypes of *P. gingivalis*. Indeed, *P. gingivalis* strains W83 and HG184, belonging to the serotypes K1 or K2, respectively, induce a more robust cytokine expression than the other serotypes.[56,57] Moreover, when the serotypes K1 or K2 were used for T lymphocyte stimulation, they induced higher expression of the transcription factors T-bet and RORC2, which are the master-switch genes implied in the Th1 and Th17 differentiation and function in humans.[56] Similarly, the serotypes K1 and K2 of *P. gingivalis* induced higher production of RANKL and activation of osteoclast than the others.[49] Therefore the different serotypes of *P. gingivalis* could elicit variable bone resorption in vivo, just like the different serotypes of *A. actinomycetemcomitans*.

Several studies have investigated the possible association between the different serotypes of *A. actinomycetemcomitans* and periodontal condition in humans. Of the seven *A. actinomycetemcomitans* serotypes, *a-c* are the most prevalent in whites, Asians, Africans, and Latin Americans; and serotype *b* is isolated most often from diseased periodontal sites in patients affected with periodontitis and seldom detected in subjects without periodontitis.[58–64] In fact, the highly leukotoxic JP2 clone strains of *A. actinomycetemcomitans*, which belong to the serotype *b*, have been strongly associated with initiation and progression of aggressive forms of periodontitis and frequently detected in periodontitis patients living in geographically widespread areas, particularly individuals of African descent.[20,58,65–68]

The JP2 clone strains of *A. actinomycetemcomitans* are characterized by a 530-bp deletion in the promoter region of the leukotoxin operon, whereas the non-JP2 clonal types consist of a large number of clones with a great genetic variability and an intact leukotoxin operon.[20] Thereby, the JP2 clone strains of *A. actinomycetemcomitans* have a high leukotoxic activity, producing 10 to 20-fold higher levels of leukotoxin than the non-JP2 strains,[69] such as the *A. actinomycetemcomitans* strains used in the present study. Leukotoxin of *A. actinomycetemcomitans* selectively causes apoptosis in T lymphocytes by binding to the lymphocyte function associated receptor 1 (LFA-1) and disrupting their membrane integrity.[70] Thus, leukotoxin of *A. actinomycetemcomitans* could contribute to an impaired acquired immune response locally in the infected periodontal tissues by affecting the Th1/Th2/Th17 lymphocyte differentiation and function.[20,70] In this study, it was demonstrated that the pathogenic role of the *A. actinomycetemcomitans* in the etiology of periodontitis differ among serotypes and specifically the *b* serotype induced a stronger Th1 and Th17 immune response in infected periodontal tissues. Thus, it could be suggested that the JP2 clone strains of *A. actinomycetemcomitans*, which belong to the serotype *b*, could induce differential leukotoxin-mediated apoptosis in distinct T-helper phenotypes.

Various animal models have been used to obtain a better understanding of the host-bacteria interactions and immunoinflammatory mechanisms that determine the destructive events characteristic of periodontitis.[13,71] In our study, BALB/c mice were used instead of C57BL/6 mice used in previous investigations[25,27,72] because it has been established that C57BL/6 mice are more resistant to experimental periodontitis induction than BALB/c mice.[73] When different strains of *P. gingivalis* were used for induction of experimental periodontitis, increased alveolar bone resorption was detected in BALB/c, AKR/J, and DBA/2J mice, whereas lower or absent alveolar bone loss was observed in C57BL/6, A/J, A/Hej, and SJL/J mice, demonstrating variable genetic susceptibility to induce experimental periodontal infection among different mice strains.[73]

Similarly, to initiate experimental periodontal lesions, different infection protocols have been described, being

ligature, bacterial oral gavage, and localized injection of bacteria or their pathogenic components into the palatal gingival tissues the most widely used.[13,17,25,27,72,74] A limitation of the ligature-induced periodontitis is the mechanical trauma caused during the placement of a ligature in the gingival margin that could favour the periodontal tissue breakdown.[13] In fact, ligature-induced bone loss occurs primarily during the first 7 to 15 days, being considered a model of acute periodontal disease.[13,72] On the other hand, oral gavage models have been frequently used in an attempt to reproduce chronic periodontitis, involving local and systemic host immune responses and alveolar bone loss that takes longer than 45 to 60 days after last inoculation.[72,74]

The localized injection of bacteria or LPS into the palatal gingival tissues has been widely used to analyze the gene expression of proinflammatory cytokines and pro-bone-resorptive factors.[13,14,31,33] This method promotes significant periodontal inflammation, apical migration of the junctional epithelium, osteoclast differentiation and activation, and alveolar bone loss 20 to 30 days after inoculation and increasing with time.[13,14,31,33] Together with the ligature method, the bacterial injection method has been defined as the most representative model of experimental periodontitis[13] and thus this protocol was used in the present study.

5 | CONCLUSIONS

During periodontitis, a Th1 and Th17-dominated response has been reported in destructive periodontal disease and, in presence of *A. actinomycetemcomitans*, the detection of the serotype *b* has been associated with a stronger Th1 and Th17 immuno-destructive response. Our data demonstrate significant differences in the expression of Th1- and Th17-related cytokines, chemokines, and chemokine receptors in periodontal lesions induced by different serotypes of *A. actinomycetemcomitans* and they indicate that the strain ATCC 43718 belonging to the serotype *b* of *A. actinomycetemcomitans* can induce a more robust immune response, which might lead to the described increased alveolar bone resorption.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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