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ORIGINAL ARTICLE

Boldine inhibits the alveolar bone resorption during ligature-induced periodontitis by modulating the Th17/Treg imbalance

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Abstract

Background: During periodontitis, tooth-supporting alveolar bone is resorbed when there is an increased expression of the pro-osteolytic factor termed receptor activator of nuclear factor κB ligand (RANKL), which is responsible for osteoclast differentiation and activation. In periodontitis-affected tissues, the imbalance between T-helper type-17 (Th17) and T-regulatory (Treg) lymphocyte activity favors this RANKL overexpression. In this context, immunotherapeutic strategies aimed at modulating this Th17/Treg imbalance could eventually arrest the RANKL-mediated alveolar bone loss. Boldine has been reported to protect from pathological bone loss during rheumatoid arthritis and osteoporosis, whose pathogenesis is associated with a Th17/Treg imbalance. However, the effect of boldine on alveolar bone resorption during periodontitis has not been elucidated yet. This study aimed to determine whether boldine inhibits alveolar bone resorption by modulating the Th17/Treg imbalance during periodontitis.

Methods: Mice with ligature-induced periodontitis were orally treated with boldine (10/20/40 mg/kg) for 15 consecutive days. Non-treated periodontitis-affected mice and non-ligated mice were used as controls. Alveolar bone loss was analyzed by micro-computed tomography and scanning electron microscopy. Osteoclasts were quantified by histological identification of tartrate-resistant acid phosphatase-positive cells. Production of RANKL and its competitive antagonist osteoprotegerin (OPG) were analyzed by ELISA, quantitative polymerase chain reaction (qPCR), and immunohistochemistry. The Th17 and Treg responses were analyzed by quantifying the T-cell frequency and number by flow cytometry. Also, the expression of their signature transcription factors and cytokines were quantified by qPCR.

Results: Boldine inhibited the alveolar bone resorption. Consistently, boldine caused a decrease in the osteoclast number and RANKL/OPG ratio in periodontal lesions. Besides, boldine reduced the Th17-lymphocyte detection and response

and increased the Treg-lymphocyte detection and response in periodontitis-affected tissues.

Conclusion: Boldine, administered orally, inhibited the alveolar bone resorption and modulated the Th17/Treg imbalance during experimental periodontitis.

KEYWORDS
boldine, periodontitis, T-lymphocytes, alveolar bone loss, RANKL protein

1 | INTRODUCTION

Plant-derived bioactive compounds play a significant role in modern medicine, mainly due to their beneficial therapeutic properties and minimal side effects, which are frequently a problem in chemically synthesized drugs. Consequently, in recent years, the use of natural products obtained from plants has been strongly suggested for the treatment of oral diseases, including stomatitis, dental caries, and periodontitis.[1,2]

Boldine is the first isoquinoline alkaloid extracted from the bark and leaves of the boldo tree (*Peumus boldus* Molina), one of the most widely known and used medicinal plants in South America.[3,4] Among South American native peoples, traditional applications of boldo as a brew include headache, earache, nasal congestion, rheumatism, and gastrointestinal disorders, and its use has entered some European countries as pharmaceutical boldine-containing concentrates.[3,5] Many pharmacological properties have been attributed to boldine, mainly due to its cytoprotective, hepatoprotective, nephroprotective, neuroprotective, antioxidant, antipyretic, and anti-inflammatory activities.[3] Indeed, accumulating scientific evidence has supported its therapeutic use against hypertension, diabetes mellitus, cancer, cerebrovascular injury, and rheumatoid arthritis.[6–11] Recent studies reported that the oral administration of boldine or its lower homolog norboldine in experimental animal models of rheumatoid arthritis or osteoporosis inhibited pathological bone resorption, and this inhibition was associated with the downregulation of the pro-bone-resorptive factor termed receptor activator of nuclear factor κB ligand (RANKL) and the upregulation of its soluble decoy osteoprotegerin (OPG) in the affected tissues.[6,12] Notably, the decreased bone resorption was associated with modulation of the imbalance between T-helper type-17 (Th17) and T-regulatory (Treg) lymphocyte activity, which directly determines the decreased local RANKL/OPG ratio.[12–16]

As is the case with rheumatoid arthritis and osteoporosis, bone resorption is also the hallmark of periodontitis. Certainly, periodontitis is an inflammatory disease characterized by alveolar bone resorption caused by an increase in the RANKL-mediated osteoclast activity in periodontal lesions.[17] It has been convincingly established that changes in the periodontal RANKL/OPG levels are determined by the pattern of the host's immuno-inflammatory response triggered against the constant subgingival microbial challenge, and the pattern of immuno-inflammatory response deployed in the periodontal tissues is a consequence of reciprocal interactions between different T-lymphocyte subpopulations.[17–21] According to this, alveolar bone resorption is closely associated with a Th17/Treg imbalance, defined by an increase in the activity of RANKL-producing Th17 lymphocytes and a decrease in the activity of Treg lymphocytes in periodontitis-affected tissues.[17–21] Taking together all these pathogenic similarities, we can suggest that natural products used for controlling bone loss in rheumatoid arthritis and osteoporosis, such as boldine, are also useful for periodontitis. We, therefore, hypothesized that boldine inhibits the alveolar bone resorption and modulates the Th17/Treg imbalance during experimental periodontitis.

To determine whether boldine has an effect on the alveolar bone resorption and immune response during periodontitis, in this investigation we assessed whether oral administration of boldine in mice with ligature-induced periodontitis affects the extent of the alveolar bone resorption, detection of osteoclasts and inflammatory cells, production of RANKL and OPG, expression of proinflammatory cytokines, expression of Th17 or Treg-related transcription factors and cytokines, and detection of Th17 and Treg lymphocytes in periodontitis-affected tissues.

2 | MATERIALS AND METHODS

2.1 | Boldine extraction and purification

Boldine was isolated from *P. boldus* bark as previously described.[4] The *P. boldus* bark was collected in early 2019 (summer) in Curacaví township (33°23′ S and 70°59′ W, about 580 m above sea level), Metropolitan Region, Chile. The dried, milled bark was submerged for 4 hours in hexane at 50°C to dewax, repeating the procedure three

times. The plant material was dried and then extracted with 1-M hydrochloric acid (HCl). The filtered solution was made basic (pH 9-10) by adding concentrated (25%) ammonia, and the aqueous solution was extracted 3 times with dichloromethane. The crude alkaloid extract was dried with Na_2SO_4, filtered, and concentrated. Finally, the boldine was purified by column chromatography on silica gel 60 Å (40-63 μm), using an EtOAc:MeOH gradient going from pure EtOAc to pure MeOH, monitoring the fractions by thin layer chromatography (TLC) (EtOAc:MeOH 4:1 and DCM:MeOH 5:3), examining the chromatograms under 254 and 365 nm UV light, and spraying with Dragendorff reagent to detect the presence of alkaloids. Boldine was identified by ^{1}H and ^{13}C-nuclear magnetic resonance (NMR), and its purity was >98% by high performance liquid chromatography-ultraviolet detection (HPLC-UV). The hydrochloride was prepared subsequently by adding the calculated amount of concentrated (37%) HCl to a solution of boldine in acetone, and the water-soluble salt (MW: 363.8 g/mol) was used for animal administration.

2.2 | Animals

Healthy 7-week-old, ≈20 g C57BL/6 mice were obtained from the Institutional Animal Facility of the Faculty of Dentistry, Universidad de Chile. The mice were maintained under pathogen-free conditions in a controlled environment: temperature 24°C ± 0.5°C, relative humidity 40% to 70%, an air exchange rate of 15-room vol/hour, and 12-hour light/dark cycles, with lights on at 7:00 am. For the duration of the study, the animals were fed with sterile standard food and water *ad libitum*. All experimental procedures were reviewed and approved by the Institutional Ethics Committee for Animal Care and Use of Universidad de Chile (Protocol #061601). The study was conducted in accordance with the NC3Rs ARRIVE guidelines,[22] the recommendations of the American Veterinary Medical Association (AVMA),[23] and the guidelines approved by the Council of the American Psychological Society (1980).[24]

2.3 | Induction of experimental periodontitis and boldine administration

Experimental periodontitis was induced by the ligation method following a protocol previously described.[25] Briefly, mice were anesthetized with an intraperitoneal injection of ketamine and xylazine, and a 5-0 silk ligature was tied around the maxillary second molars by an experienced operator (EAC), without causing damage to the periodontal tissues. Boldine was given by oral gavage at 10, 20, or 40 mg/kg in 100-μL phosphate-buffered saline (PBS), starting from the day of ligature placement, for 15 consecutive days. The doses and frequency of boldine were determined based on previous reports.[8,15,26] Forty animals were randomly allocated to five groups with eight mice in each group: a) non-ligated group, b) non-treated ligated group, c) ligated group receiving low doses of boldine (10 mg/kg), d) ligated group receiving medium doses of boldine (20 mg/kg), and e) ligated group receiving high doses of boldine (40 mg/kg). The non-ligated group (group a) was used as control. The non-treated ligated group (group b), which received PBS vehicle without boldine, was used for comparisons. After 15 days, the animals were euthanized by an intraperitoneal ketamine and xylazine overdose, and samples of maxillae, gingival crevicular fluid, palatal periodontal tissues, and cervical lymph nodes were collected for further analysis.

2.4 | Alveolar bone resorption

The extent of the alveolar bone resorption was quantified in maxillae by microcomputed tomography (micro-CT) and scanning electron microscopy (SEM) as previously described.[27] Maxillae were dissected free of soft tissues, immersed in 2.5% sodium hypochlorite solution for 12 hours, and washed with 70% EtOH followed by 90% and 100% EtOH for 24 hours each. For micro-CT analysis, maxillae were scanned in all three spatial planes using a micro-CT apparatus[*] and the 3D-digitized images were obtained using a reconstruction software.[†] A standardized region of interest (ROI) was acquired using an analysis software,[‡] using the following anatomical parameters: 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. The percentage of bone loss was calculated using the formula: percentage of bone loss = 1 -(remaining bone volume/ROI) × 100, and adjusted to the non-ligated control group. For SEM analysis, maxillae were fixed in 2.5% buffered glutaraldehyde overnight at room temperature, dehydrated, sputter-coated with a 200 nm-thickness layer of gold, and analyzed at an accelerating voltage of 20 kV in a scanning electron microscope.[§] Bone loss analysis was performed at 30× magnification, quantifying the area of bone loss between the mesial surface of the first molar and the distal surface of the third

[*] SkyScan 1278; Bruker, Kontich, Belgium.
[†] Nrecon software; Bruker, Kontich, Belgium.
[‡] Dataviewer software; Bruker, Kontich, Belgium.
[§] Jeol JSMIT300LV; Jeol, Tokyo, Japan.

molar, and between the cemento-enamel junction and the alveolar bone crest, adjusting the area of bone loss of each experimental condition to the area detected in the non-ligated control group. All data were collected by a single observer (EAC) who was masked to experimental specimen conditions.

2.5 | TRAP$^+$ osteoclast detection

Activated osteoclasts were quantified in the periodontal tissues by identifying cells expressing the osteoclast-specific marker tartrate-resistant acid phosphatase (TRAP) as previously described.[28] For this, periodontal tissue biopsies were taken and immediately fixed in 10% formalin pH 7.4 for 24 hours. Then, samples were demineralized in 5% EDTA for 60 days and processed for standard paraffin embedding. Sections of 5 μm were prepared and stained using a TRAP histochemical stain kit[**] according to the manufacturer's protocol. Microphotographic images were obtained using an brightfield optical microscope[††] and analyzed in a masked manner by a single calibrated examiner (CC). TRAP$^+$ multinucleated cells were considered as osteoclasts.

2.6 | RANKL and OPG production

To measure the RANKL and OPG production in the periodontal tissues, gingival crevicular fluid samples were collected using a previously described protocol.[29] Briefly, under intraperitoneal ketamine and xylazine anesthesia, the original ligature used for periodontitis induction was gently removed, and a new 2 cm-length 5-0 silk ligature was tied in the same position. After 10 minutes, the sampling ligature containing the gingival crevicular fluid was collected and immediately processed using a standardized elution procedure. Sampling ligatures were submerged in 60 μL of 0.05% Tween 20 in PBS, containing a cocktail of protease inhibitors,[‡‡] incubated for 30 minutes at 4°C, and centrifuged at 12,000 × g for 5 minutes. The elution protocol was repeated twice. From eluted samples, the RANKL and OPG levels were determined using commercially available ELISA kits[§§] and analyzed in an automatic microplate reader.[***]

2.7 | RANKL and OPG histological expression

The RANKL and OPG detection in the periodontal tissues were also analyzed by immunohistochemistry. Biopsy sections of 5 μm were immunostained by incubating them with anti-RANKL or anti-OPG goat immunoglobulin G polyclonal primary antibodies[†††] overnight at 4°C; followed by a donkey anti-goat immunoglobulin G H&L Alexa Fluor 555-labeled polyclonal secondary antibody,[‡‡‡] diluted in 5% PBS-BSA containing 10 μg/mL DAPI, for 1 hour at 4°C. Confocal images were acquired in a laser scanning microscope[§§§] in a masked manner by a single trained examiner (CC). The series of images obtained from confocal z-stacks were processed and analyzed using image software.[****]

2.8 | Histopathologic analysis

The potential inflammatory changes within periodontal tissues as a consequence of boldine treatment were histologically analyzed by identifying the influx of inflammatory cells as previously described.[30] Biopsy sections of 5 μm were processed for hematoxylin and eosin staining, and microphotographic images were obtained using an brightfield optical microscope.[††††] Lymphocytes, neutrophils, and macrophage-like cells were identified by an oral pathologist (MH) in a single-masked manner. As a complement, the expression levels of the proinflammatory cytokines interleukin (IL)-1β, IL-12p40, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α were determined as detailed in the next section.

2.9 | Th17 and Treg-related transcription factor and cytokine expression

To analyze potential changes in the Th17/Treg-pattern of immune response triggered in the periodontal tissues during experimental periodontitis and treatment with boldine, the mRNA expression levels of the Th17 and Treg-associated transcription factors and their signature cytokines were quantified using quantitative polymerase chain reaction (qPCR). Also, the mRNA expression levels of RANKL, OPG, and proinflammatory cytokines were quantified. From each whole palatal periodontal tissue sample, total cytoplasmic RNA was obtained

[**] Sigma-Aldrich, St. Louis, MO.
[††] AxioStarPlus, Carl Zeiss, Oberkochen, Germany.
[‡‡] cOmplete Mini EDTA-free; Roche Diagnostics, Mannheim, Germany.
[§§] Quantikine; R&D Systems, Minneapolis, MN.
[***] Synergy HT; Bio-Tek Instrument, Winooski, VT.
[†††] R&D Systems, Minneapolis, MN.
[‡‡‡] Abcam, Cambridge, England.
[§§§] Leica TCS SP8; Leica Microsystems, Wetzlar, Germany.
[****] Leica LAS X software; Leica Microsystems, Wetzlar, Germany.
[††††] AxioStarPlus, Carl Zeiss, Oberkochen, Germany.

using a molecular purification reagent[‡‡‡‡] and the first-strand cDNA was synthesized using a reverse transcription kit,[§§§§] following the manufacturer's instructions. Then, 10 ng of cDNA were amplified using the appropriate primers (see Table S1 in online *Journal of Periodontology*) and a qPCR reagent[*****] using a real-time qPCR apparatus.[†††††] The mRNA expression levels of the transcription factors retinoic acid receptor-related orphan nuclear receptor-γt (RORγt) and forkhead box P3 (Foxp3), the cytokines IL-1β, IL-6, IL-10, IL-12 (p40 subunit), IL-17A, IL-23 (p19 subunit), IL-35 (Epstein-Barr virus induced gene 3 [EBI3] subunit), IFN-γ, transforming growth factor (TGF)-β1, and TNF-α and the bone-related factors RANKL and OPG were determined. Amplification reactions were conducted as follows: 95°C for 3 minutes, followed by 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds. For the detection of unspecific product formation and false-positive amplification, a melting curve was carried out as follows: 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. As an endogenous control, 18S rRNA expression levels were measured.

2.10 | Th17 and Treg lymphocyte detection

For further analysis of the Th17/Treg-pattern of immune response triggered during experimental periodontitis and its treatment with boldine, the presence of Th17 and Treg lymphocytes in the cervical lymph nodes that drain the periodontal lesions were analyzed using flow cytometry as previously described.[27] The cervical lymph nodes were removed and disrupted on a 70 μm cell-strainer in PBS, and single cells were counted using an automated cell counter.[‡‡‡‡‡] Previous to the determination of cell viability using a live/dead kit,[§§§§§] cells were extracellularly stained using the following fluorochrome-coupled antibodies[******]: anti-CD4 brilliant violet 605 (BV605; clone GK.1.5), anti-CD8a brilliant ultraviolet 396 (BUV396; clone 53-6.7), and anti-CD45 brilliant ultraviolet 737 (BUV737; clone 30-F11). After that, cells were treated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 μg/mL ionomycin, and 5 μg/mL brefeldin-A at 37°C for 4 hours and fixed/permeabilized using commercial fixation and permeabilization/wash buffers,[†††††††] following the manufacturer's instructions. Subsequently, cells were intracellularly stained using the following fluorochrome-coupled antibodies[‡‡‡‡‡‡]: anti-RORγt phycoerythrin CF594 (PE CF594; clone Q31-378), anti-Foxp3 Alexa Fluor 488 (AF488; clone 150D), anti-IL-10 allophycocyanin-Cy7 (APC-Cy7; clone JES5-16E3), and anti-IL-17A phycoerythrin-Cy7 (PE-Cy7; clone TC11-18H10.1). A flow cytometer[§§§§§§] was used to detect the labeled cells using the following gating strategy: FSC/SSC, FSC/FSC, live/dead/CD45, and CD4/CD8. Isotype-matched control antibodies were used to determine negative cell populations. The experiments were performed separately for each animal.

2.11 | Statistical analysis

The data were expressed as the mean ± SD for eight mice per group unless otherwise stated. Alveolar bone loss was calculated as percentage (micro-CT) and μm^2 (SEM). The ELISA data were calculated as pg/mL using a logistic equation of 4 parameters. The qPCR data were calculated using the $2^{-\Delta\Delta Ct}$ method using a qPCR software[*******] and expressed as fold-change of relative quantities. The flow cytometry data were analyzed using a specific software.[††††††††] Data were statistically analyzed,[‡‡‡‡‡‡‡] the normality of data distribution was established using the Kolmogorov-Smirnov test, and statistical differences were determined using the ANOVA and Bonferroni's tests. *P* values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Alveolar bone loss

Experimental periodontitis was successfully induced by ligature, and mice developed substantial alveolar bone resorption around maxillary molars 15 days after ligation (Fig. 1A). Boldine dose-dependently inhibited alveolar bone resorption in these periodontitis-affected mice (Figs. 1B and 1C). When 10 mg/kg of boldine were administered orally, significantly less alveolar bone resorption was induced compared with non-treated mice. When 20 mg/kg of boldine were used, significantly less alveolar bone

[‡‡‡‡] TRIzol Plus; Invitrogen, Barcelona, Spain.
[§§§§] SuperScript III; Invitrogen, Grand Island, NY.
[*****] KAPA SYBR Fast qPCR; KAPA Biosystems, Woburn, MA.
[†††††] StepOnePlus; Applied Biosystems, Singapore.
[‡‡‡‡‡] Luna II, Logos Biosystems, Annandale, VA.
[§§§§§] Zombie UV Fixable Viability Kit; BioLegendÿ San Diego, CA.
[******] BD Bioscience, San Jose, CA.
[†††††††] R&D Systems, Minneapolis, MN.
[‡‡‡‡‡‡] BioLegend, San Diego, CA.
[§§§§§§] LSR Fortessa X-20; BD Immunocytometry Systems, Franklin Lakes, NJ.
[*******] StepOne 2.2.2 software; Applied Biosystems, Singapore.
[††††††††] WinMDi 2.9 software; The Scripps Research Institute, La Jolla, CA.
[‡‡‡‡‡‡‡] SPSS 22.0 software; IBM Corp., Armonk, NY.

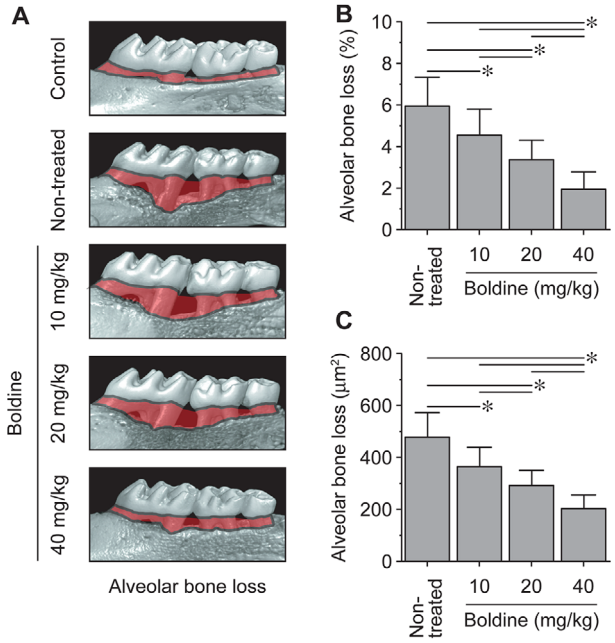

FIGURE 1 Alveolar bone loss. **A**) Representative micro-computed tomography (micro-CT) images of maxilla specimens from mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. **B**) Alveolar bone loss quantified by micro-CT in maxilla specimens of mice with ligature-induced periodontitis treated with boldine or left untreated. Data are represented as percentage of bone loss adjusted to the non-ligated control group and shown as mean ± SD (n = 8). **C**) Area of bone loss quantified by scanning electron microscopy (SEM) in maxilla specimens of mice with ligature-induced periodontitis treated with boldine or left untreated. Data are represented as μm^2 adjusted to the non-ligated control group and shown as mean ± SD (n = 8). $^*P < 0.05$. Non-treated group compared with boldine-treated groups (10, 20, or 40 mg/kg of boldine orally)

resorption was induced compared with both non-treated mice and mice treated with 10 mg/kg of boldine. When 40 mg/kg of boldine were used, significantly less alveolar bone resorption was induced compared with non-treated mice and mice treated with 10 or 20 mg/kg of boldine.

3.2 | TRAP$^+$ osteoclast detection

Boldine also reduced the detection of TRAP$^+$ osteoclasts. TRAP$^+$ osteoclasts were observed in all experimental conditions (Fig. 2A). Dose-dependently, periodontitis-affected mice treated with boldine showed significantly less TRAP$^+$ osteoclasts compared with periodontitis mice without treatment, in which numerous osteoclasts were observed (Fig. 2B). In turn, TRAP$^+$ osteoclast detection in non-ligated healthy mice was scarce.

3.3 | RANKL and OPG production

Based on the finding that boldine confers protection against alveolar bone resorption during experimental periodontitis and that osteoclast differentiation and activation depends on the local RANKL/OPG levels, the production of RANKL and OPG in the periodontitis-affected tissues was analyzed (Fig. 3). In a dose-dependent manner, the expression (mRNA levels) and secretion (protein levels) of RANKL were significantly lower in periodontitis mice treated with boldine than non-treated mice (Figs. 3A and 3B). Conversely, an increase in the expressed and secreted levels of OPG was detected in boldine-treated periodontitis mice as compared with non-treated mice (Figs. 3A and 3B). These changes resulted in a diminished RANKL/OPG ratio in the periodontal lesions when periodontitis mice were treated with boldine (Fig. 3C). In agreement with this, boldine reduced the RANKL-immunopositive staining and increased the OPG-immunopositive staining in the periodontitis-affected tissues (Fig. 3D).

3.4 | Inflammatory cell detection and proinflammatory cytokine expression

Boldine also induced changes in the inflammatory cell infiltration of the mice periodontium (Fig. 4A). Ligature caused accentuated leukocyte infiltration in periodontal tissues. When boldine was administered, less lymphocytes, neutrophils, and macrophage-like cells were identified. Besides, periodontitis-affected mice treated with boldine showed dose-dependently less mRNA expression of the proinflammatory cytokines IL-1β, IL-12, IFN-γ, and TNF-α as compared with non-treated mice (Fig. 4B).

3.5 | Th17 and Treg-related transcription factor and cytokine expression

Alveolar bone resorption and RANKL overexpression during periodontitis are closely associated with the local Th17/Treg imbalance. Thus, to assess whether the inhibitory effect of boldine on the alveolar bone loss and RANKL-mediated osteoclast activation was associated with the modulation of the Th17/Treg imbalance, the expression of the Th17 or Treg-related transcription factors and cytokines was analyzed in the periodontal lesions (Fig. 5). In a dose-dependent manner, the mRNA expression of the transcription factor RORγt, associated with the differentiation and function of Th17 lymphocytes, was significantly lower in periodontitis mice treated with boldine than in non-treated mice (Fig. 5A). Conversely, a

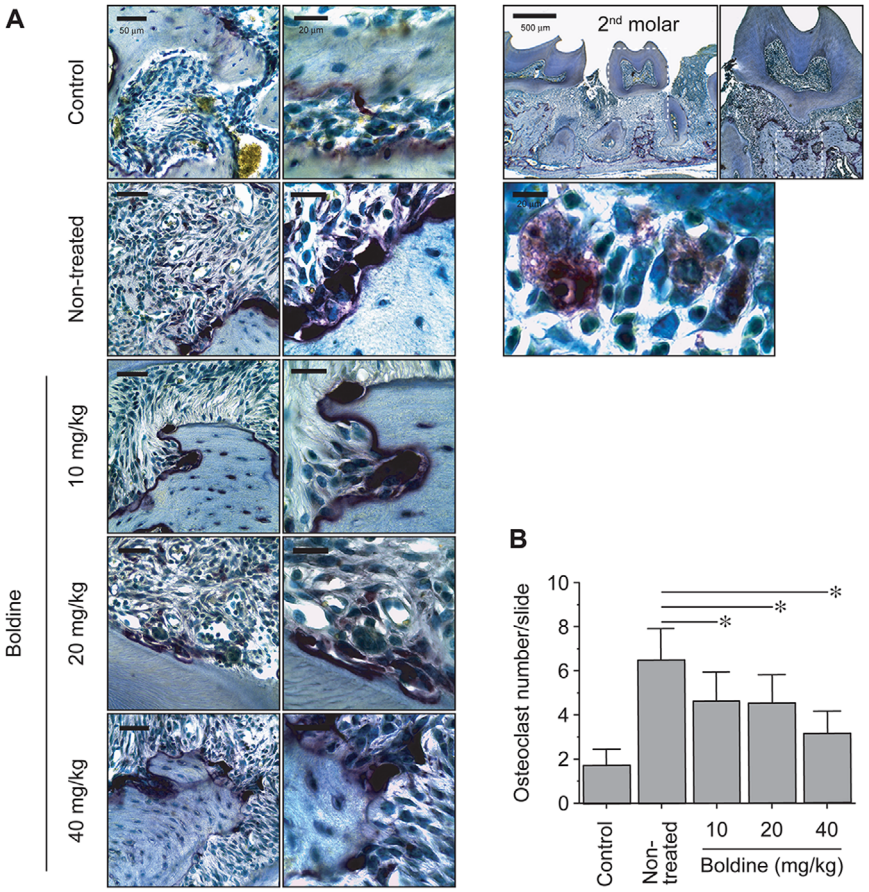

FIGURE 2 Osteoclast detection. **A**) Representative tartrate-resistant acid phosphatase (TRAP) staining of periodontal tissues of mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. Scale bar: 50 μm (40× objective) and 20 μm (100× objective). **B**) Quantification of TRAP$^+$ osteoclasts in periodontal tissues of mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. Data are represented as osteoclast number/slide and shown as mean ± SD (n = 6). $^*P < 0.05$. Non-treated group compared with boldine-treated groups (10, 20, or 40 mg/kg of boldine orally)

dose-dependent increase in the mRNA expression of the transcription factor Foxp3, associated with the differentiation and function of Treg lymphocytes, was detected in boldine-treated periodontitis mice compared with non-treated mice (Fig. 5B). Besides, periodontitis-affected mice treated with boldine showed significantly less mRNA expression of the Th17-related cytokines (IL-6, IL-17A, and IL-23) as compared with non-treated mice (Fig. 5C). Conversely, in a dose-dependent manner, the mRNA levels of the Treg-related cytokines (IL-10, IL-35, and TGF-β1) were significantly higher in periodontitis mice treated with boldine than in non-treated mice (Fig. 5D).

3.6 | Th17 and Treg lymphocyte detection

The changes in the Th17/Treg-pattern of the immune response induced by boldine were ratified when the frequency and absolute number of Th17 and Treg lymphocytes were analyzed within the cervical lymph nodes that drain the periodontal tissues (Fig. 6). In a dose-dependent manner, boldine induced a significant reduction in the frequency and number of CD45$^+$CD4$^+$CD8$^-$RORγt$^+$IL-17A$^+$ Th17 lymphocytes in periodontitis-affected mice compared with non-treated mice (Figs. 6A and 6C). Interestingly, boldine also induced a significant increment in the frequency and number of CD45$^+$CD4$^+$CD8$^-$Foxp3$^+$IL-10$^+$ Treg lymphocytes in periodontitis-affected mice compared with non-treated mice (Figs. 6B and 6D).

4 | DISCUSSION

Nowadays, there is a growing demand for safe and effective traditional medicines obtained from natural sources and medicinal plants to treat human diseases. As a result, many studies have assayed different botanical products as coadjuvant therapies aimed to control oral diseases.[1,2,30–32] Alveolar bone resorption, however, remains an important clinical problem, and none of the approaches proposed to date have proven to be very effective.[1,33] In this context, boldine is a plausible alternative, since recent in vivo studies have reported its beneficial effects on pathological osteoclast activity and bone loss.[6,12] In the present study, we have demonstrated for the first time that boldine ameliorates periodontitis by inhibiting the alveolar bone resorption, and this inhibition is associated with the modulation of the Th17/Treg imbalance that determines the RANKL-mediated osteoclast activity in the periodontal lesions.

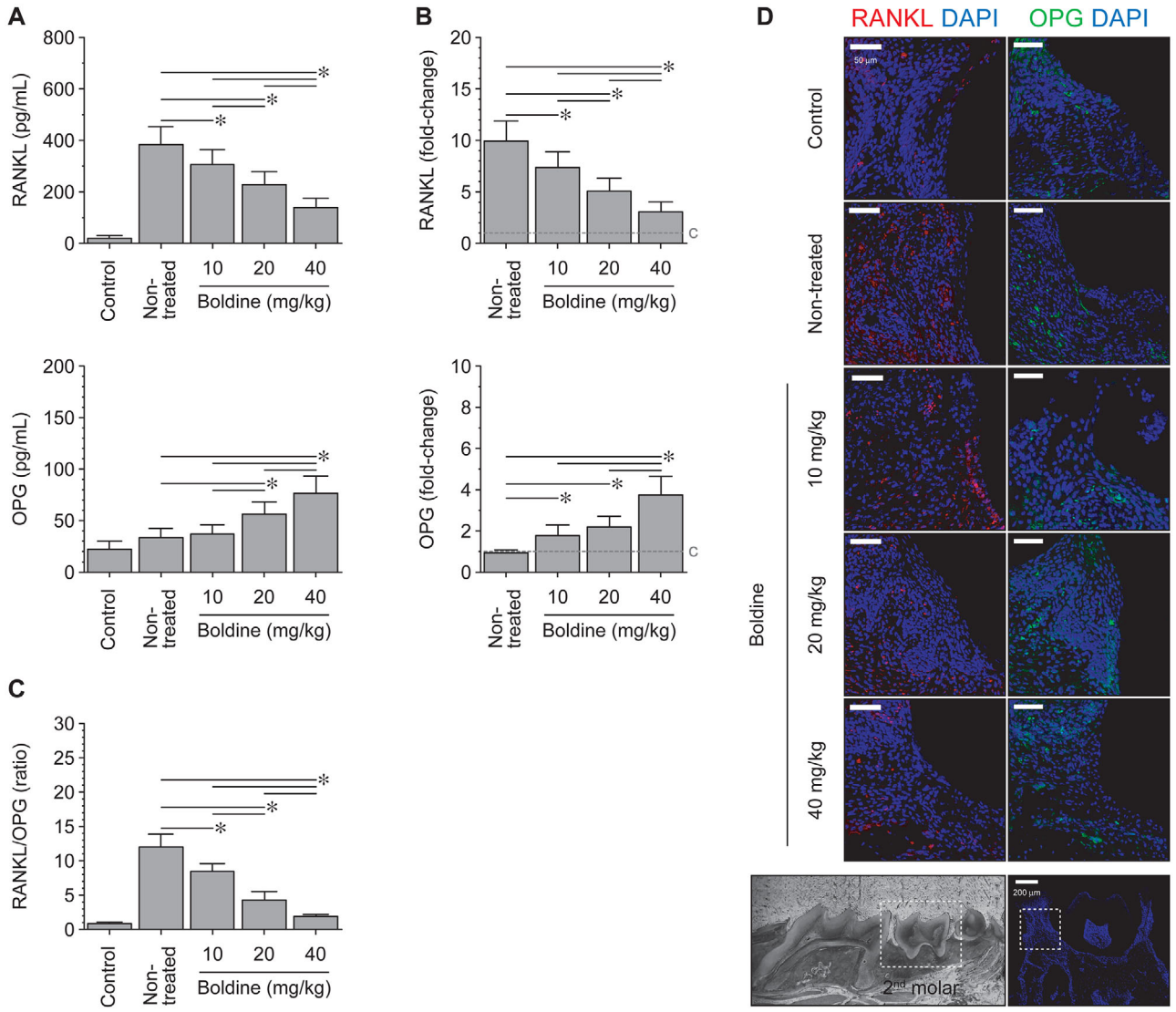

FIGURE 3 RANKL and OPG production. **A**) RANKL and OPG secretion in the gingival crevicular fluid (GCF) samples of mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. Data are represented as pg/mL and shown as mean ± SD (n = 8). Each experiment was performed in duplicate. **B**) RANKL and OPG mRNA expression in the periodontal lesions of mice with ligature-induced periodontitis treated with boldine or left untreated. As references for fold-change in expression, the RANKL and OPG mRNA expressions in control (c) non-ligated mice were considered as 1. Data are represented as mRNA fold-change and shown as mean ± SD (n = 8). Each experiment was performed in duplicate. **C**) Ratios between the secreted levels of RANKL and OPG in GCF samples of mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. Data are represented as RANKL/OPG ratio and shown as mean ± SD (n = 8). **D**) RANKL (in red) and OPG (in green) immunoreactivity by fluorescence microscopy in representative periodontal lesions of mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. DAPI staining (in blue) was used to visualize the cellular nuclei. Scale bar: 50 μm (40× objective). $^{*}P < 0.05$. Non-treated group compared with boldine-treated groups (10, 20, or 40 mg/kg of boldine orally)

Mounting evidence has shown that boldine has a beneficial effect in distinct human diseases.[6–11] In the context of osteoimmunological diseases, it is noteworthy that boldine has also been demonstrated to protect against bone loss.[6,12] By using an animal model of collagen-induced rheumatoid arthritis, oral administration of boldine inhibited osteoclast differentiation and the consequent sub-chondral articular bone loss.[6] In particular, boldine induced an increase in the expression levels of OPG and a decrease in the expression levels of RANKL in the joint lesions.[6] Similarly, using an animal model of ovariectomy-induced osteoporosis, boldine prevented pathological bone loss and conducted this protective effect through the inhibition of RANKL-induced osteoclastogenesis.[12] Consistent with

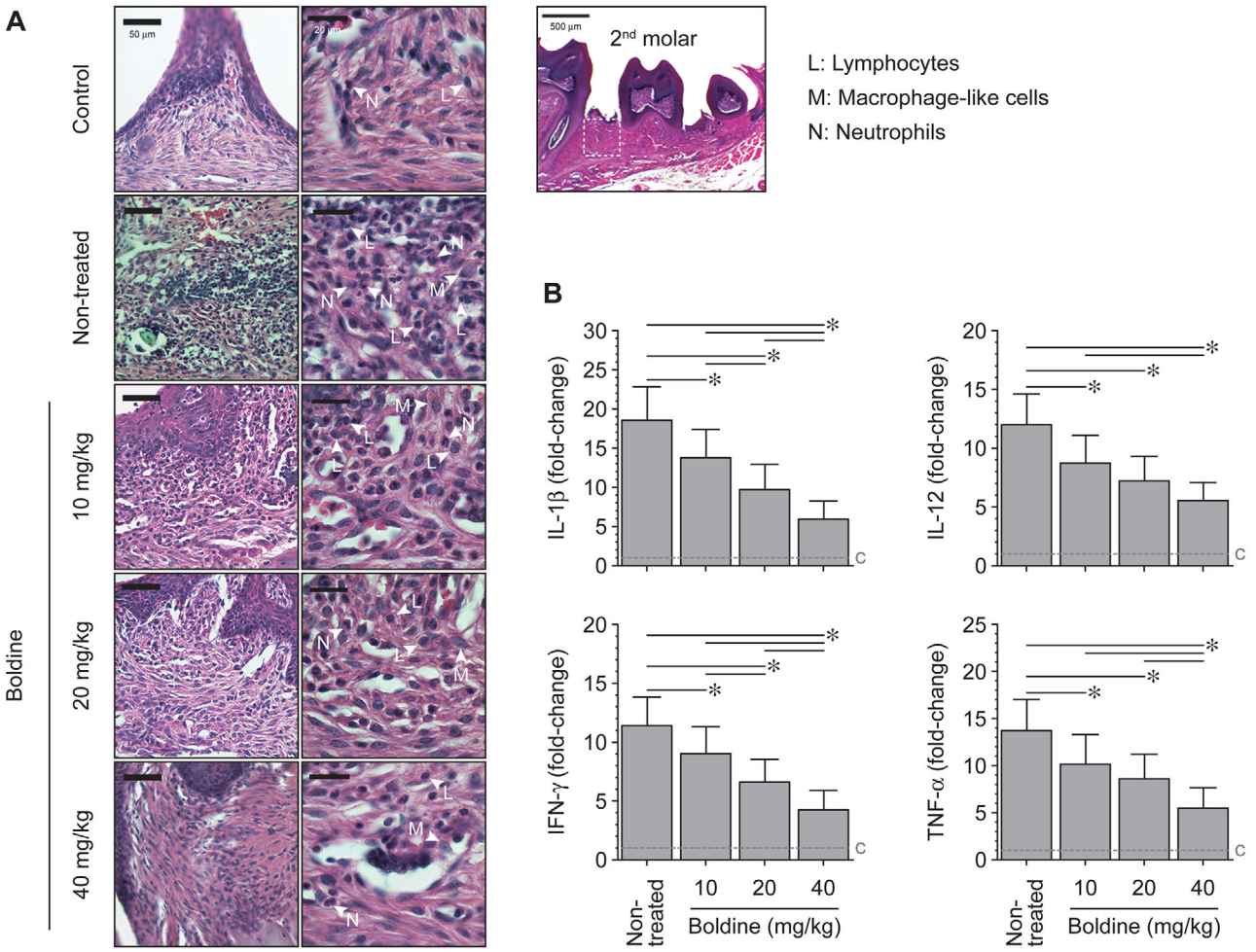

FIGURE 4 Periodontal inflammation. **A**) Representative hematoxylin and eosin (H&E) staining of periodontal tissues of mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. Inflammatory cells were identified as lymphocytes (L), neutrophils (N), and macrophage-like cells (M). Scale bar: 50 μm (40× objective) and 20 μm (100× objective). **B**) Expression levels of the proinflammatory cytokines IL-1β, IL-12, IFN-γ, and TNF-α mRNAs in the periodontal lesions of mice with ligature-induced periodontitis treated with boldine or left untreated. As references for fold-change in expression, the cytokine mRNA expressions in control (c) non-ligated mice were considered as 1. Data are represented as mRNA fold-change and shown as mean ± SD (n = 8). Each experiment was performed in triplicate. $^{*}P < 0.05$. Non-treated group compared with boldine-treated groups (10, 20, or 40 mg/kg of boldine orally)

these findings, in the present study, ligature-induced periodontitis was inhibited after oral administration of boldine, and this inhibition was associated with changes in the production of RANKL and OPG in the periodontal tissues, which resulted in attenuation of the RANKL/OPG signal pathway that determines osteoclast-mediated periodontal bone resorption.

Boldine has been shown to be effective in controlling inflammation. In an experimental animal model of colitis, boldine reduced the occurrence of macroscopic lesions, edema, and neutrophil infiltration.[34] Similarly, boldine reduced carrageenan-induced paw edema in rats and guinea pigs and xylene-induced ear edema in mice, and these anti-inflammatory properties were associated with decreased infiltration of proinflammatory cells, downregulated expression of proinflammatory cytokines, and inhibition of the phosphorylation levels of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3).[35,36] The anti-inflammatory capacity of boldine in the periodontitis-affected tissues was demonstrated in the present study since the administration of boldine diminished the cell inflammatory infiltration and downregulated the expression of IL-1β, IL-12, IFN-γ, and TNF-α. The molecular mechanisms related to this anti-inflammatory effect of boldine in periodontal tissues could be a consequence of JAK/STAT and nuclear factor (NF)-κB signaling pathway involvements, which are critical in the regulation of the inflammatory response.[37] Nevertheless, this reasonable hypothesis needs to be further investigated.

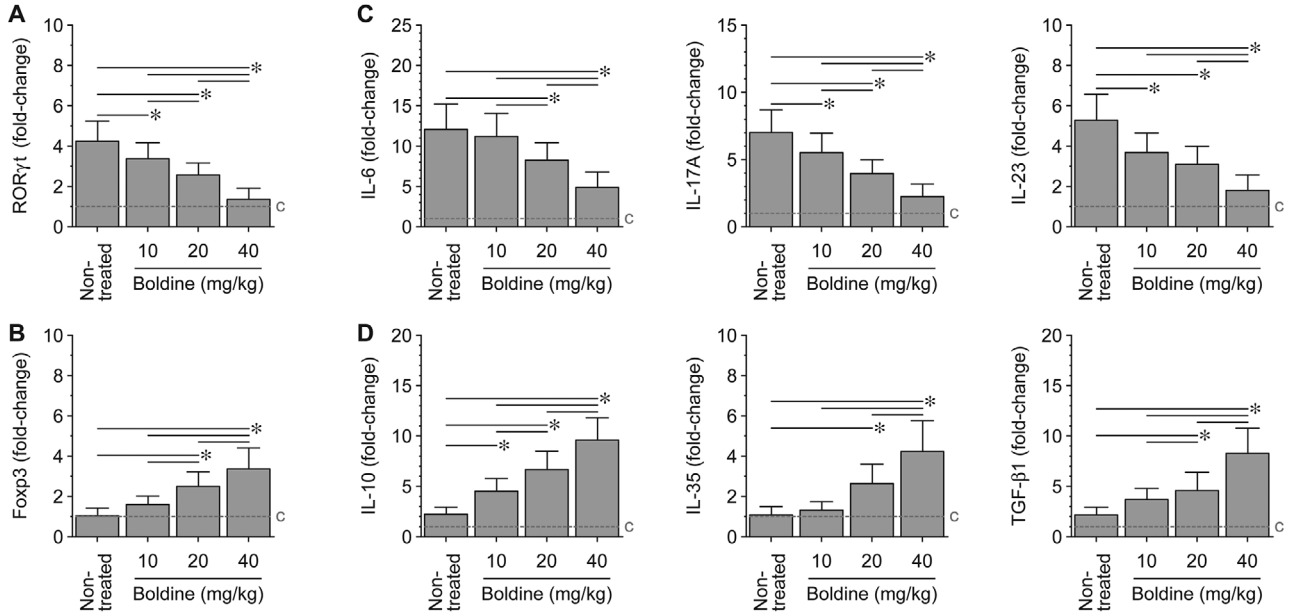

FIGURE 5 Th17 and Treg-related transcription factor and cytokine expression. **A** and **B**) Expression levels of the transcription factors RORγt (Th17) and Foxp3 (Treg) mRNAs in the periodontal lesions of mice with ligature-induced periodontitis treated with boldine or left untreated. **C** and **D**) Expression levels of the cytokines IL-6, IL-17A, and IL-23 (Th17) and IL-10, IL-35, and TGF-β1 (Treg) mRNAs in the periodontal lesions of mice with ligature-induced periodontitis treated with boldine or left untreated. As references for fold-change in expression, the transcription factor and cytokine mRNA expressions in control (c) non-ligated mice were considered as 1. Data are represented as mRNA fold-change and shown as mean ± SD (n = 8). Each experiment was performed in triplicate. $^{*}P < 0.05$. Non-treated group compared with boldine-treated groups (10, 20, or 40 mg/kg of boldine orally)

Boldine has proven to be capable of inhibiting the NF-κB signaling pathway, which is crucial for the induction of osteoclast function.[36,38] Indeed, osteoclast formation is a complex process regulated by multiple signaling cascades, RANKL-dependent NF-κB activation being the central player in the regulation of osteoclast differentiation, activation, and survival.[39,40] The use of anti-RANKL antibodies significantly inhibits T-cell-mediated alveolar bone loss through RANKL blockade in periodontitis-affected tissues, suggesting that T-cell-mediated periodontal bone resorption is RANKL-dependent.[41] Among the T-cell subsets, Th17 lymphocytes are the key responsible for the increment of RANKL in the periodontal lesions by producing IL-17A, which stimulates adjacent osteoblasts and stromal cells to overexpress RANKL.[18,42] Additionally, Th17 lymphocytes can induce the differentiation and activation of osteoclasts directly through the production of RANKL.[18,42] Conversely, Treg lymphocytes produce anti-inflammatory cytokines that suppress the Th17 activity, inhibit the RANKL production, maintain the periodontal immune homeostasis, and control the immune-mediated tissue injury.[43,44] Thereby, a Th17/Treg imbalance is closely associated with the pathogenesis of periodontitis, and the enhanced Th17 response and weakened Treg response might be the cause of upregulated levels of RANKL detected in the periodontitis-affected tissues.[17,18] In this study, we have shown that boldine modulates the Th17/Treg imbalance characteristic of periodontitis by promoting the expansion of the Treg-response and suppression of the Th17-response in a dose-dependent manner. In turn, this Th17/Treg modulation provided a paracrine environment of cytokines in the periodontal tissues that affected the RANKL/OPG ratio, thus targeting the osteoclastogenesis and consequently inhibiting the alveolar bone resorption. Interestingly, the articular modulation of the Th17/Treg imbalance in rheumatoid arthritis-affected mice treated with norboldine has been related to a regulation of the Th17/Treg balance in the gut-associated lymphoid tissues.[14] Indeed, oral administration of this natural product led to an increase in the number of $\alpha4\beta7^{+}$ Treg lymphocytes in the joint lesions, a specific marker of their origin in the gut.[14] To gain insight into how boldine is able to modulate the periodontal Th17/Treg imbalance, more studies are essential. In the present investigation, it was not analyzed how boldine induced the increased Treg response and Treg-cell detection in periodontitis-affected tissues. An indirect effect of boldine in the gut-derived Treg response is plausible, for instance, by modulating the intestinal microbiota and promoting short-chain fatty acid-producing microorganisms, as it was demonstrated for

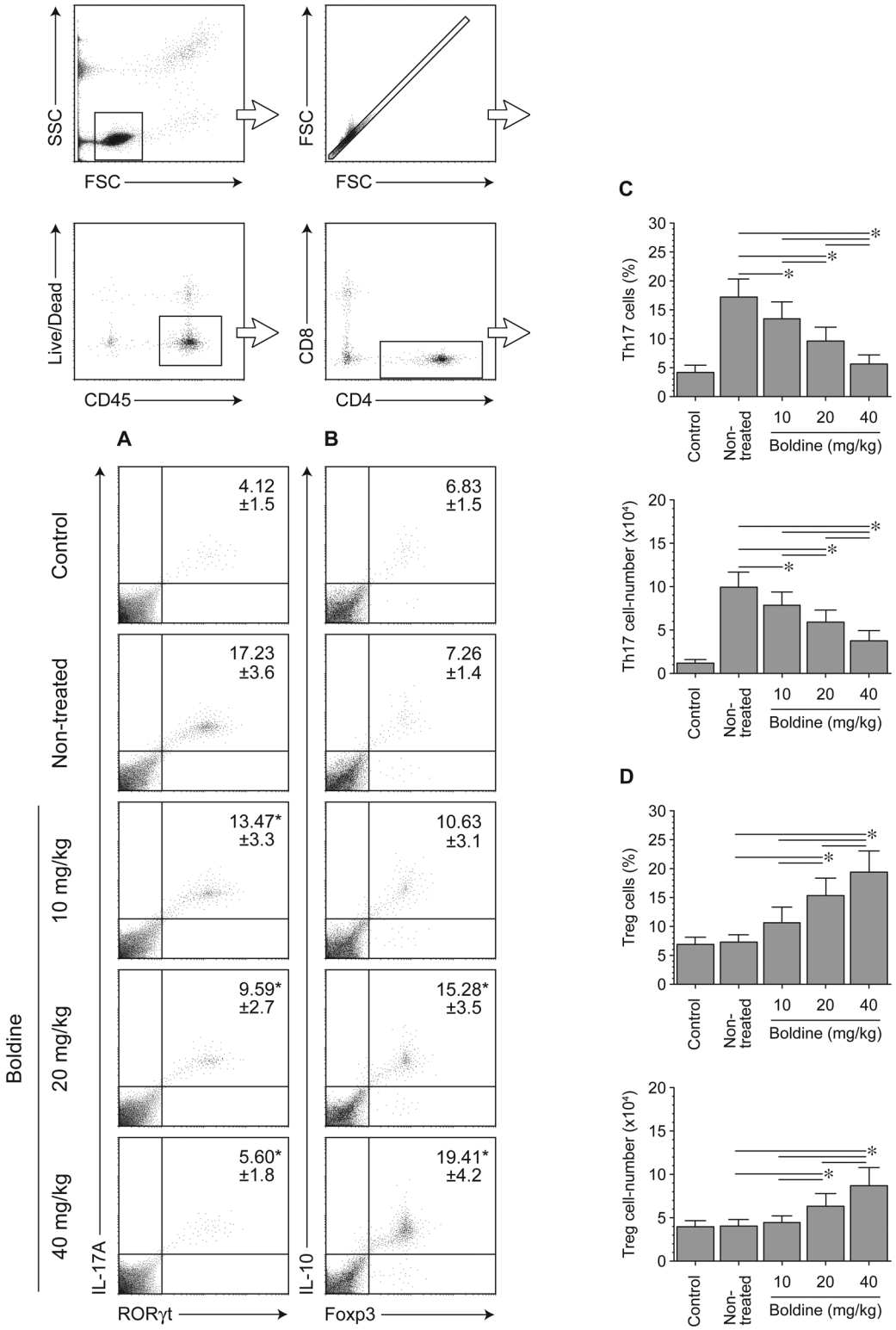

FIGURE 6 Th17 and Treg lymphocyte detection. Flow cytometry analysis demonstrating the presence of **A**) $CD45^+CD4^+CD8^-ROR\gamma t^+IL\text{-}17A^+$ Th17 lymphocytes and **B**) $CD45^+CD4^+CD8^-Foxp3^+IL\text{-}10^+$ Treg lymphocytes within the cervical lymph nodes that drain the periodontal tissues of mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. Frequency and number of **C**) $CD45^+CD4^+CD8^-ROR\gamma t^+IL\text{-}17A^+$ Th17 lymphocytes and **D**) $CD45^+CD4^+CD8^-Foxp3^+IL\text{-}10^+$ Treg lymphocytes within the cervical lymph nodes that drain the periodontal tissues of mice with ligature-induced periodontitis treated with boldine or left untreated. Healthy, non-ligated mice were used as control. Data are represented as Th17 or Treg-cell percentage and absolute number and shown as mean ± SD (n = 8). Each experiment was performed in duplicate. $^*P < 0.05$. Non-treated group compared with boldine-treated groups (10, 20, or 40 mg/kg of boldine orally)

berberine, like boldine an isoquinoline alkaloid, extracted from *Berberis* plants.[31,45] A direct effect of boldine on the intestinal lymph nodes and the migration of $\alpha 4\beta 7^+$ Treg lymphocytes from the gut toward the periodontal lesions is also a reasonable hypothesis that requires investigation.

In general terms, periodontal osteoimmunology implies that immune cells such as T lymphocytes molecularly communicate with both types of effector bone cells: the bone-resorbing osteoclasts and the bone-forming osteoblasts. Thus, together with the catabolic events of inflammatory bone loss, the osteoimmunology integrates anabolic events, particularly the influence of immune cells on alveolar bone regeneration.[17,46] According to our findings, boldine reduces the catabolic role of Th17 lymphocytes during inflammatory bone loss by modulating the periodontal RANKL/OPG production and RANKL-induced osteoclast activity. Even so, boldine might also play an anabolic role in bone metabolism and impact on alveolar bone regeneration by directly or indirectly stimulating osteoblast activity. In the present study, oral administration of boldine caused an increase in the periodontal levels of the Treg-derived anti-inflammatory cytokines, which favors the resolution of chronic inflammation. Thus, an IL-10 and TGF-β1-regulated local and transient physiological inflammation could be reached in the periodontal tissues, where low levels of IL-17A can still be produced by $\gamma\delta$-T cells and the canonical Wnt/β-catenin pathway can be activated in mesenchymal stem cells. These are critical events related to osteoblast differentiation and function, intramembranous ossification, and alveolar bone regeneration.[46,47] In this context, boldine could also have a more direct effect on bone regeneration, similar to that described for berberine, which induces osteoblastogenesis from bone marrow-derived mesenchymal stem cells by activating the Wnt/β-catenin signaling pathway and consequently upregulating Runx2 expression, the essential transcription factor that controls the entire process of osteoblast differentiation and bone-forming activity.[48,49] Considering this, the potential direct effect of boldine on osteoblast differentiation and function is plausible and needs to be investigated.

Non-surgical periodontal therapy is regarded as the gold-standard treatment option for controlling periodontitis, being able to efficiently arrest the inflammatory alveolar bone resorption most of the time. However, in some clinical cases, periodontal healing is not achieved completely. For these cases, the conventional alternatives are surgical periodontal therapy and antibiotic treatment, which may lead to undesired side effects.[50] Currently, the main research efforts are to understand the physiologic and pathologic axes of the microbiome, immune cells, and bone cells to develop novel therapies with a particular focus on the cellular and molecular mechanisms of periodontal disease.[46] In this scenario, boldine has shown promise for its potential to arrest bone loss during osteoporosis, rheumatoid arthritis, and now periodontitis, as demonstrated in the present study. Thus, boldine is an attractive option to be proposed as a natural bioactive coadjuvant in periodontal therapy to achieve successful periodontitis control. Clearly, the clinical potential of boldine for controlling periodontitis requires further investigation.

5 | CONCLUSIONS

The present study demonstrates that the oral administration of boldine ameliorates experimental periodontitis by inhibiting alveolar bone resorption. This inhibited bone loss is associated with the modulation of the Th17/Treg imbalance, which in turn contributes to attenuating the RANKL/OPG signal pathway responsible for osteoclast activity. Thereby, boldine shows promising potential for its application as a coadjuvant for controlling alveolar bone resorption during periodontitis.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design of the study. Emilio A. Cafferata organized the study, performed the animal model of periodontitis, and critically evaluated and supplemented the article. Sebastián Castro-Saavedra and Gonzalo Fuentes-Barros performed the boldine extraction and purification, designed the protocol of boldine administration, and were involved in drafting the article. Emilio A. Cafferata and Samanta Melgar-Rodríguez performed the boldine administration, performed the data analysis, and were involved

in drafting the article. Emilio A. Cafferata and Rolando Vernal performed the micro-CT and SEM experiments and performed the data analysis. Emilio A. Cafferata and Paola Carvajal performed the qPCR experiments and performed the data analysis. Emilio A. Cafferata and Marcela Hernández performed the GCF sampling and ELISA experiments. Bastián I. Cortés and Cristian Cortez performed the histological experiments. Emilio A. Cafferata and Rolando Vernal performed the flow cytometry experiments. Bruce K. Cassels was involved in drafting the article and gave final approval of the version to be published. Rolando Vernal designed and implemented the research protocol, performed the data analysis, and prepared the figures and the article for submission. All the authors read and approved the final version of the article.

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