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# Interleukin-35 inhibits alveolar bone resorption by modulating the Th17/Treg imbalance during periodontitis

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## Abstract

**Aim:** T lymphocytes play a central role during the pathogenesis of periodontitis, and the imbalance between the pathogenic T-helper type 17 (Th17) and protective T-regulatory (Treg) lymphocytes determines the tooth-supporting alveolar bone resorption. Interleukin (IL)-35 is a novel anti-inflammatory cytokine with therapeutic properties in diseases whose pathogenesis is associated with the Th17/Treg imbalance; however, its role during periodontitis has not been established yet. This study aimed to elucidate whether IL-35 inhibits the alveolar bone resorption during periodontitis by modulating the Th17/Treg imbalance.

**Materials and Methods:** Mice with ligature-induced periodontitis were treated with locally or systemically administered IL-35. As controls, periodontitis-affected mice without IL-35 treatment and non-ligated mice were used. Alveolar bone resorption was measured by micro-computed tomography and scanning electron microscopy. The Th17/Treg pattern of the immune response was analysed by qPCR, ELISA, and flow cytometry.

**Results:** IL-35 inhibited alveolar bone resorption in periodontitis mice. Besides, IL-35 induced less detection of Th17 lymphocytes and production of Th17-related cytokines, together with higher detection of Treg lymphocytes and production of Treg-related cytokines in periodontitis-affected tissues.

**Conclusion:** IL-35 is beneficial in the regulation of periodontitis; particularly, IL-35 inhibited alveolar bone resorption and this inhibition was closely associated with modulation of the periodontal Th17/Treg imbalance.

## KEYWORDS

alveolar bone loss, interleukin-35, periodontitis, RANKL, T lymphocytes

## 1 | INTRODUCTION

Periodontitis is a chronic inflammatory disease in which the locally deployed host's immune response against the dysbiotic subgingival microbiome plays a pivotal role in the onset and progression of the disease (Lamont, Koo, & Hajishengallis, 2018). In this context, it has been clearly established that the activity of specific T-lymphocyte subsets plays a key role in the pathogenesis of periodontitis, where T-regulatory (Treg) lymphocytes are protective and T-helper type 17 (Th17) lymphocytes are involved in periodontal inflammation and tissue breakdown (Alvarez et al., 2018; Campbell, Millhouse, Malcolm, & Culshaw, 2016). During periodontitis, the metabolic balance of tooth-supporting alveolar bone relies on the reciprocal relationship between Th17 and Treg lymphocyte activity; in fact, pathogenic alveolar bone resorption is a consequence of the Th17/Treg imbalance in periodontitis-affected tissues (Alvarez et al., 2019; Campbell et al., 2016).

During antigen presentation, Th17 lymphocytes differentiate in the presence of interleukin (IL)-6, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), and IL-23, which are mainly produced by antigen-presenting cells (Campbell et al., 2016; Díaz-Zúñiga et al., 2014), and express the phenotype-specific transcription factor retinoid-related orphan receptor  $\gamma$ t (ROR $\gamma$ t), which is necessary for their activation and function (Díaz-Zúñiga et al., 2015; Sato et al., 2006). Activated Th17 lymphocytes produce pro-inflammatory cytokines, such as IL-6, IL-17A, and IL-23, as well as the pro-bone-resorptive factor termed receptor activator of nuclear factor  $\kappa$ B ligand (RANKL), a signal for osteoclast differentiation and activation (Sato et al., 2006; Vernal et al., 2014). On the other hand, Treg cells differentiate in the presence of IL-2 and TGF- $\beta$ 1 and express the phenotype-specific transcription factor forkhead box P3 (Foxp3), which is necessary for their activation and suppressive function (Alvarez et al., 2018; Cafferata et al., 2019). Activated Treg lymphocytes produce anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ 1, which in turn inhibit the Th17-related response and contribute to the maintenance of immune homeostasis (Alvarez et al., 2018; Cafferata et al., 2019).

IL-35 is a novel immunoregulatory cytokine with anti-inflammatory capacities, which is mainly produced by Treg lymphocytes and formed from a heterodimer of Epstein-Barr virus-induced gene 3 (EBI3) and IL-12p35 subunits (Olson, Sullivan, & Burlingham, 2013). IL-35 suppresses the Th17-cell differentiation and function by down-regulating the production of Th17-associated cytokines (Niedbala et al., 2007; Okada et al., 2017). Moreover, IL-35 favours the differentiation of naïve T cells into IL-35-producing Treg lymphocytes, a recently described cell population termed iTr35 that mediates immunosuppression mainly via IL-35 (Collin & Bigley, 2018; Collison et al., 2010). Accordingly, IL-35 has been proposed as protective in several diseases whose pathogenesis is closely associated with the Th17/Treg imbalance, such as experimental colitis, asthma, inflammatory bowel disease, and rheumatoid arthritis (Gao, Su, Lv, & Zhang, 2017; Li, Wang, Liu, Zuo, & Lu, 2014; Niedbala et al., 2007; Wirtz, Billmeier, McHedlidze, Blumberg, & Neurath, 2011). Indeed, it

### Clinical Relevance

*Scientific rationale for the study:* Periodontitis is characterized by alveolar bone resorption as a consequence of the Th17/Treg imbalance, with an increase in the Th17-response and decrease in the Treg response in periodontitis-affected tissues. According to this, strategies aimed at modulating this Th17/Treg imbalance could eventually arrest alveolar bone loss.

*Principal findings:* IL-35 inhibited the alveolar bone resorption by modulating the periodontal Th17/Treg imbalance. In particular, IL-35 elicited an expansion of Treg-cell activity and suppression of RANKL-producing Th17-cell activity.

*Practical implications:* Administration of IL-35 shows promising potential as a coadjuvant of conventional therapy for controlling alveolar bone resorption during periodontitis.

has been recently demonstrated that IL-35 prevents bone loss in an experimental model of rheumatoid arthritis (Li et al., 2016; Niedbala et al., 2007).

Even though IL-35 has been detected in periodontitis patients (Jin, Liu, & Lin, 2017; Jing et al., 2019; Kalburgi, Muley, Shivaprasad, & Koregol, 2013; Köseoğlu, Sağlam, Pekbağrıyanik, Savran, & Sütçü, 2015; Mitani et al., 2015; Raj et al., 2018) and a possible role in maintaining the homeostasis of the local immune microenvironment in periodontal tissues has been mildly suggested (Jin et al., 2017), the role of IL-35 on alveolar bone metabolism and periodontal immune response during periodontitis has not been elucidated yet. Therefore, this study aimed to evaluate the effects of systemically or locally administered IL-35 on the alveolar bone resorption and periodontal Th17/Treg imbalance during experimental periodontitis.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Healthy 8-week-old C57BL/6 mice were purchased from the Institutional Animal Facility of the Faculty of Dentistry, Universidad de Chile. Throughout the period of the study, animals had free access to sterile standard food and water and were housed under controlled conditions (12 hr light/dark cycles with lights on at 07:00 am, controlled temperature at  $24 \pm 0.5^\circ\text{C}$ , relative humidity of 40 to 70%, and air exchange rate of 15-room vol/hr). The study was approved by the Institutional Animal Care and Use Committee (Protocol #061601) and conducted in accordance with the ARRIVE guidelines. All the experiments were carried out following the recommendations of the American Veterinary Medical Association (AVMA), and the guidelines approved by the Council of the American Psychological Society (1980) for animal experiments.

## 2.2 | Induction and treatment of experimental periodontitis

Periodontitis was induced using 5–0 silk ligatures, which were tied around the maxillary second molars without causing damage to the periodontal tissues (Abe & Hajishengallis, 2013). Treatment of experimental periodontitis consisted of intragingival injections of 1, 10, or 100 ng of IL-35 (Prospec) in 2  $\mu$ l phosphate-buffered saline (PBS) daily for 15 days, placed into the palatal interproximal gingiva between the first, second, and third molars. For comparison, ligated mice were systematically treated by intraperitoneal injections of 2  $\mu$ g of IL-35 in 100  $\mu$ l PBS daily for 15 days. Selected doses and frequency of IL-35 inoculation were based on previous reports (Li et al., 2016; Niedbala et al., 2007). As controls, sham-treated ligated mice, which received injections of PBS vehicle without IL-35, and non-ligated mice were used. Animals were randomly allocated in six groups with eight mice in each group: (a) control group non-ligated, (b) periodontitis group without receiving IL-35 treatment, (c) periodontitis group receiving 2  $\mu$ g of IL-35 intraperitoneal, (d) periodontitis group receiving 1 ng of IL-35 intragingival, (e) periodontitis group receiving 10 ng of IL-35 intragingival, and (f) periodontitis group receiving 100 ng of IL-35 intragingival. After 15 days of ligature, animals were euthanized by a single overdose of ketamine/xylazine anaesthesia, and samples of maxillae, palatal periodontal tissues, and cervical lymph nodes were collected for further analysis. Sampled palatal periodontal tissues include the posterior palate and exclude the anterior palate, in order to avoid contamination with immune cells from the nasal-associated lymphoid tissues (NALT), as previously described (Bittner-Eddy, Fischer, Tu, Allman, & Costalonga, 2017).

## 2.3 | Alveolar bone loss

Maxillae were dissected free of soft tissues to quantify the extent of the alveolar bone resorption by micro-computed tomography (micro-CT) and scanning electron microscopy (SEM) as previously described (Monasterio et al., 2018). For micro-CT analysis, maxillae were scanned in all three spatial planes using a micro-CT equipment (SkyScan 1278, Bruker) and 3D-digitized images were obtained using a reconstruction software (Nrecon software, Bruker, Kontich, Belgium). A standardized region of interest (ROI) was acquired using an analysis software (DataViewer software, Bruker) 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. The percentage of bone loss was calculated using the formula: percentage of bone loss =  $1 - (\text{remaining bone volume}/\text{ROI}) \times 100$ , and adjusted to the non-ligated control group. For SEM analysis, maxillae were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma-Aldrich Chemie, Buchs, Switzerland) overnight at room temperature, dehydrated, sputter-coated with gold layer to a thickness of 200 nm and examined using a scanning electron microscope (Jeol JSMIT300LV; Jeol, Tokyo, Japan) at an accelerating voltage of 20 kV. Bone loss analysis

was performed at magnification 30x, quantifying the area of bone loss between mesial surface of the first molar and the distal surface of the third molar, and between the cement–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 who was masked to conditions of the maxillae specimens.

## 2.4 | Histological analysis

For histopathology analysis, tartrate-resistant acid phosphatase (TRAP) staining and immunohistochemistry (IHC) for RANKL and osteoprotegerin (OPG) detection were performed. The specimens were fixed in 10% formalin pH 7.4 for 24 hr and then demineralized in 5% EDTA (Sigma-Aldrich) for 60 days. The specimens were then processed for paraffin embedding, and serial 5  $\mu$ m sections were prepared using standard histological protocols. The slides were stained using a TRAP histochemical stain kit (Sigma-Aldrich) according to the manufacturer's instructions and photographed with an optical microscope (AxioStarPlus, Carl Zeiss) in a masked manner by a single calibrated examiner. TRAP<sup>+</sup> multinucleated cells were considered as osteoclasts. For IHC, RANKL and OPG immunostaining were performed by incubating the samples with an anti-RANKL or anti-OPG goat IgG polyclonal primary antibodies (R&D Systems) overnight at 4°C, followed by a donkey anti-goat IgG H&L Alexa Fluor 555-labelled polyclonal secondary antibody (Abcam, Cambridge, England) for 1 hr at 4°C. Previous DAPI staining for 1 min at room temperature, images were acquired in a Leica TCS SP8 laser scanning microscope (Leica Microsystems) in a blinded manner by a single calibrated examiner. The series of images obtained from confocal z-stacks were processed and analysed using the Leica LAS X software (Leica Microsystems).

## 2.5 | RANKL and OPG production

For each animal, gingival crevicular fluid samples (GCF) were collected as previously described (Matsuda et al., 2016). The secreted levels of RANKL and OPG were measured by ELISA (Quantikine, R&D Systems Inc.) using an automatic microplate reader (Synergy HT, Bio-Tek Instrument Inc.).

## 2.6 | Th17 and Treg-related transcription factor and cytokine expression

The mRNA expression levels of the Th17-related transcription factor (ROR $\gamma$ t) and cytokines (IL-6, IL-17A, and IL-23 -p19 chain-) and the Treg-related transcription factor (Foxp3) and cytokines (IL-10, IL-35 -EBI3 chain-, and TGF- $\beta$ 1) were quantified by qPCR. As a complement, the RANKL and OPG mRNA levels were also analysed. First, total cytoplasmic RNA was purified from each palatal

periodontal tissue sample using an ice-cold molecular purification reagent (TRIzol Plus; Invitrogen) as previously described (Monasterio et al., 2018). Then, the first-strand cDNA was synthesized using a reverse transcription kit (SuperScript III; Invitrogen), following the manufacturer's instructions. Finally, 10 ng of cDNA was amplified using the appropriate primers (Table S1) and a qPCR reagent (KAPA SYBR Fast qPCR; KAPA Biosystems) in real-time qPCR equipment (StepOnePlus; Applied Biosystems). Amplification reactions were conducted as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s, and finally a melting curve of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s, for detection of unspecific product formation and false-positive amplification. As an endogenous control, 18S rRNA expression levels were determined.

## 2.7 | Th17 and Treg-cell detection

The presence of Th17 and Treg lymphocytes within the periodontal lesions and cervical lymph nodes that drain these periodontal lesions was analysed by flow cytometry, as previously described (Monasterio, Budini, et al., 2019; Monasterio, Fernandez, et al., 2019). Cells were isolated from the palatal periodontal tissue samples using procedures previously described (Pandiyan, Bhaskaran, Zhang, & Weinberg, 2014; Vernal et al., 2005). From cervical lymph nodes, cells were mechanically isolated using a 70 µm cell strainer in PBS. Previous cell counting using an automated cell counter (Luna II, Logos Biosystems) and determination of live cells using a live/dead kit (Zombie Aqua fixable viability kit, BioLegend), cells were extracellularly stained using the following monoclonal antibodies: anti-CD4 brilliant violet 605 (BV605)-labelled, anti-CD8 brilliant violet 711 (BV711)-labelled, and anti-CD45 brilliant violet allophycocyanin-Cy7 (APC-Cy7) (BioLegend). Cells were then treated with 5 µg/ml brefeldin-A (Invitrogen) at 37°C for 6 hr, fixed for 10 min at 4°C using a cold-flow cytometry fixation buffer (R&D Systems), and permeabilized using a flow cytometry permeabilization/wash buffer (R&D Systems). Subsequently, cells were intracellularly stained using the following monoclonal antibodies: anti-RORγt phycoerythrin CF594 (PE CF594)-labelled, anti-Foxp3 Alexa Fluor 488-labelled, anti-IL-10 brilliant violet 421 (BV 421)-labelled, anti-IL-17A allophycocyanin-Cy5.5 (APC-Cy5.5)-labelled, and anti-RANKL phycoerythrin (PE)-labelled (BioLegend). Cell analysis was performed in a flow cytometer (LSR Fortessa X-20, Becton Dickinson Immunocytometry Systems) using a sequential gating strategy according to the FS/SS parameters, live/dead staining, and CD45, CD4, and CD8 markers. Isotype-matched control antibodies were used to determine negative cell populations. For each animal, the experiments were performed separately.

## 2.8 | Statistical analysis

Alveolar bone loss was calculated as percentage (micro-CT) and µm<sup>2</sup> (SEM). The ELISA data were calculated as pg/ml using a logistic

equation of 4 parameters. The qPCR data were calculated using the 2<sup>-ΔΔCt</sup> method with the StepOne v.2.2.2 software (Applied Biosystems) and expressed as fold change of relative quantities. The flow cytometry data were analysed using the WinMDi v.2.9 software (The Scripps Research Institute). Data were statistically analysed using the SPSS v.22.0 software (IBM Corp.). The normality of data distribution was established using the Kolmogorov–Smirnov test, and statistical differences were determined using the ANOVA and Bonferroni's tests. The level of significance was set at *p* < .05.

## 3 | RESULTS

### 3.1 | Alveolar bone loss

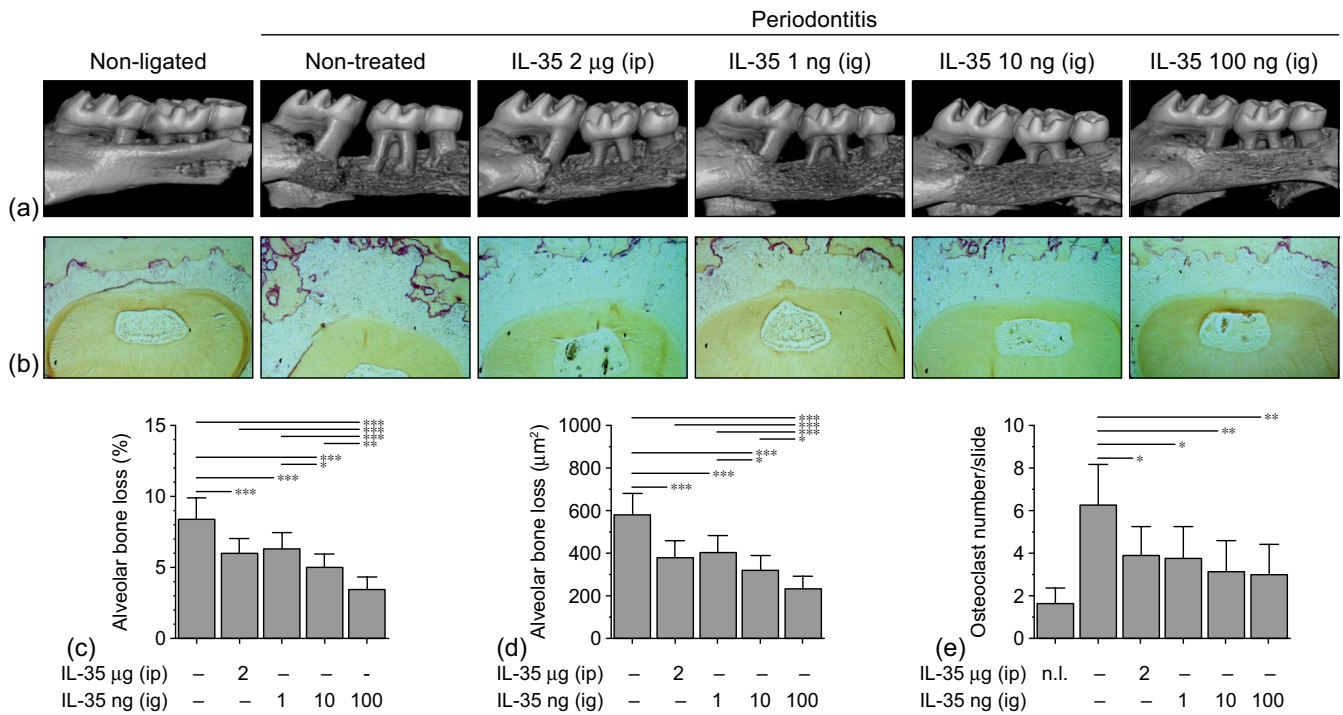
Figure 1 shows the micro-CT, SEM, and histological analysis of alveolar bone resorption in ligature-induced periodontitis mice with or without IL-35 treatment. Experimental periodontitis was successfully achieved, and ligatures caused periodontal destruction and alveolar bone loss around maxillary molars (Figure 1a,b). When IL-35 was locally administrated, periodontitis-affected mice showed a dose-dependently lower alveolar bone resorption compared with periodontitis mice without treatment (Figure 1c,d). Similarly, systemically administrated IL-35 induced lower alveolar bone resorption in treated periodontitis mice compared with non-treated mice, and these diminished levels of bone loss were similar to those detected when ligated mice were locally treated with 1 ng of IL-35 (Figure 1c,d).

### 3.2 | Osteoclast detection

Based on the finding that IL-35 confers protection against alveolar bone resorption during periodontitis, we quantified the number of osteoclasts responsible for this bone loss (Figure 1e). TRAP<sup>+</sup> osteoclasts were observed in all experimental conditions. Periodontitis-affected mice locally or systemically treated with IL-35 showed significantly less TRAP<sup>+</sup> osteoclasts compared with periodontitis mice without treatment, in which numerous osteoclasts were identified. In turn, TRAP<sup>+</sup> osteoclast detection was similar in mice treated with locally or systemically administrated IL-35. TRAP<sup>+</sup> osteoclast detection in non-ligated controls was scarce.

### 3.3 | RANKL and OPG production

RANKL and OPG determine the osteoclast activity and subsequent bone loss; therefore, we examined the RANKL and OPG production in mice periodontal tissues by qPCR, ELISA, and IHC (Figure 2). In a dose-dependent manner, the mRNA and protein levels of RANKL were significantly lower in periodontitis-affected mice treated with IL-35 than in mice without treatment (Figure 2a,b). Conversely, an increase in the expressed and



**FIGURE 1** Alveolar bone loss. (a) Representative micro-computed tomography (micro-CT) images of maxillae specimens of mice with ligature-induced periodontitis treated with IL-35. (b) Representative tartrate-resistant acid phosphatase (TRAP) staining of periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. (c) Alveolar bone loss quantified by micro-CT in maxillae specimens of mice with ligature-induced periodontitis treated with IL-35. Data are represented as percentage adjusted to the non-ligated control group and shown as mean  $\pm$  SD from 8 independent experiments. (d) Area of bone loss quantified by scanning electron microscopy (SEM) in maxillae specimens of mice with ligature-induced periodontitis treated with IL-35. Data are represented as  $\mu\text{m}^2$  adjusted to the non-ligated control group and shown as mean  $\pm$  SD from 8 independent experiments. (e) Quantification of TRAP<sup>+</sup> osteoclasts in periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. Data are represented as osteoclast number/slide and shown as mean  $\pm$  SD from 8 independent experiments. \* $p < .05$ , \*\* $p < .01$ , and \*\*\* $p < .001$

secreted levels of OPG was detected in IL-35-treated periodontitis mice as compared with mice without treatment (Figure 2a,b). These changes led to diminished RANKL/OPG ratio in periodontal lesions when periodontitis-affected mice were treated with IL-35 (Figure 2c). Similarly, IL-35 reduced the RANKL-immunopositive staining and enhanced the OPG-immunopositive staining in mice periodontal lesions (Figure 2d).

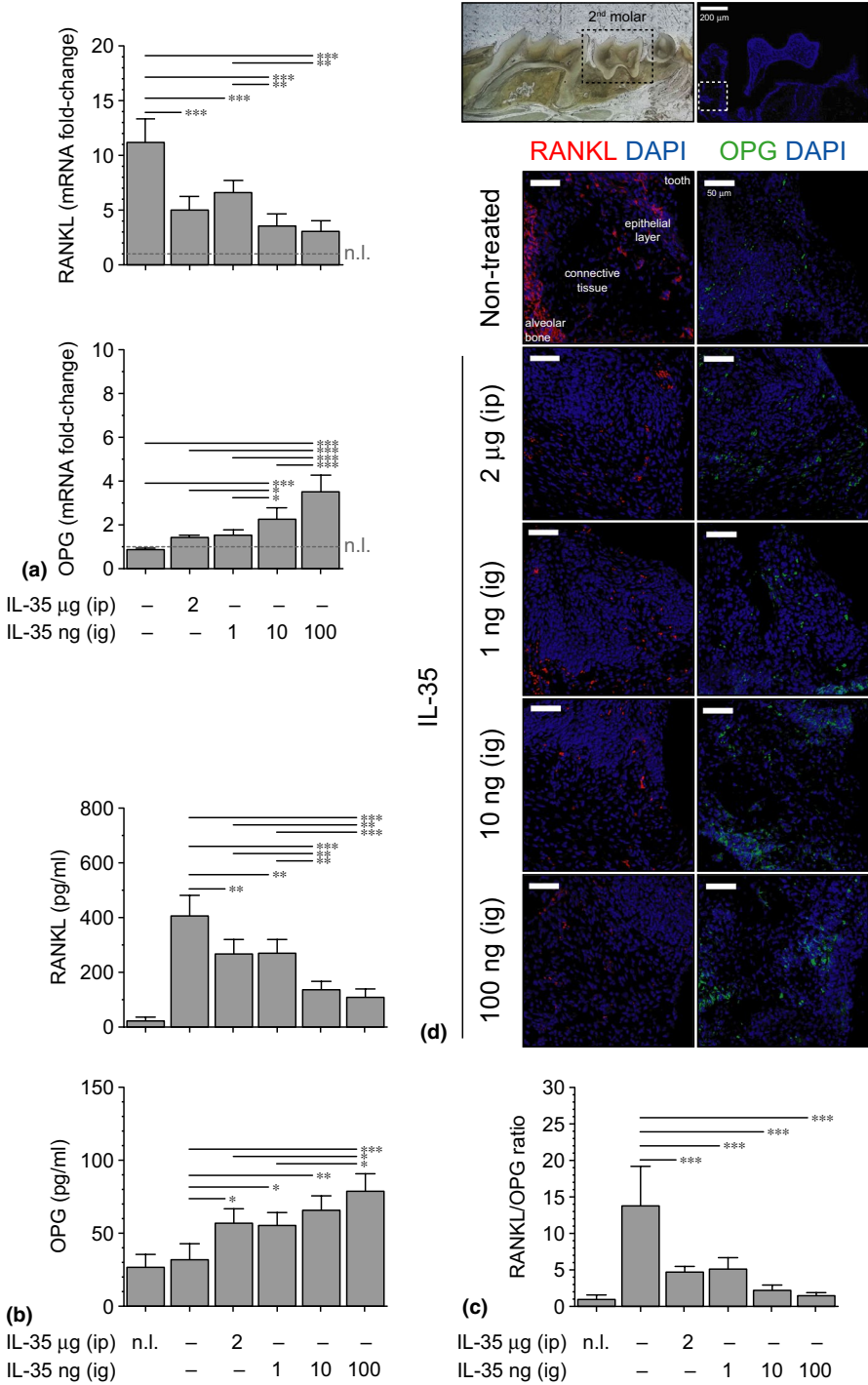
### 3.4 | Th17 and Treg-cell detection

To assess whether the inhibitory effect of IL-35 on the alveolar bone loss was associated with modulation of the Th17/Treg-cell detection during periodontitis, we quantified the frequency and number of IL-17A-expressing Th17 lymphocytes and IL-10-expressing Tregs by flow cytometry in live/single/CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> T lymphocytes obtained from the cervical lymph nodes (Figure 3a) and periodontal lesions (Figure 4a). IL-35 dose-dependently led to a significant reduction in the frequency and number of CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>ROR $\gamma$ t<sup>+</sup>IL-17A<sup>+</sup> Th17 lymphocytes in periodontitis-affected mice compared with non-treated mice (Figures 3b and 4b). Interestingly, a significant increment in the

frequency and number of CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>Foxp3<sup>+</sup>IL-10<sup>+</sup> Treg lymphocytes was detected in IL-35-treated periodontitis mice at all doses as compared with non-treated mice (Figures 3c and 4c).

### 3.5 | Th17 and Treg-related transcription factors and cytokines

For further analysis of the effect of IL-35 on the modulation of the Th17/Treg imbalance during periodontitis, we quantified the expression levels of the Th17 and Treg-related transcription factors and cytokines by qPCR in mice periodontal tissues (Figure 5). At all doses, IL-35 induced significantly higher expression of the IL-35 chain EB13 in periodontitis mice compared with non-treated mice. Similarly, 10 and 100 ng of IL-35 intragingival induced significantly higher expression of the Treg-related transcription factor Foxp3 and cytokines IL-10 and TGF- $\beta$ 1 in periodontitis mice as compared with mice without treatment. Conversely, a reduction in the expression of the Th17-related transcription factor ROR $\gamma$ t and cytokines IL-6, IL-17A (10 and 100 ng of IL-35 intragingival), and IL-23 (all doses) was detected in periodontitis mice treated with IL-35 as compared with non-treated mice.



**FIGURE 2** RANKL and OPG production. (a) RANKL and OPG mRNA expression in periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. For relative expression, the RANKL and OPG mRNA expression in non-ligated control (n.l.) mice were considered as 1, as a reference for fold change in expression. Data are represented as mRNA fold change and shown as mean  $\pm$  SD from 8 independent experiments. (b) RANKL and OPG secretion in the gingival crevicular fluid (GCF) of mice with ligature-induced periodontitis treated with IL-35. Data are represented as pg/ml and shown as mean  $\pm$  SD from 8 independent experiments. (c) Ratio between the RANKL and OPG secreted levels in the GCF of mice with ligature-induced periodontitis treated with IL-35. Data are represented as RANKL/OPG ratio and shown as mean  $\pm$  SD from 8 independent experiments. (d) RANKL (in red) and OPG (in green) immunoreactivity by fluorescence microscopy in representative periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. DAPI staining (in blue) was used to visualize the cellular nucleus. Bar: 50  $\mu\text{m}$ . \* $p < .05$ , \*\* $p < .01$ , and \*\*\* $p < .001$

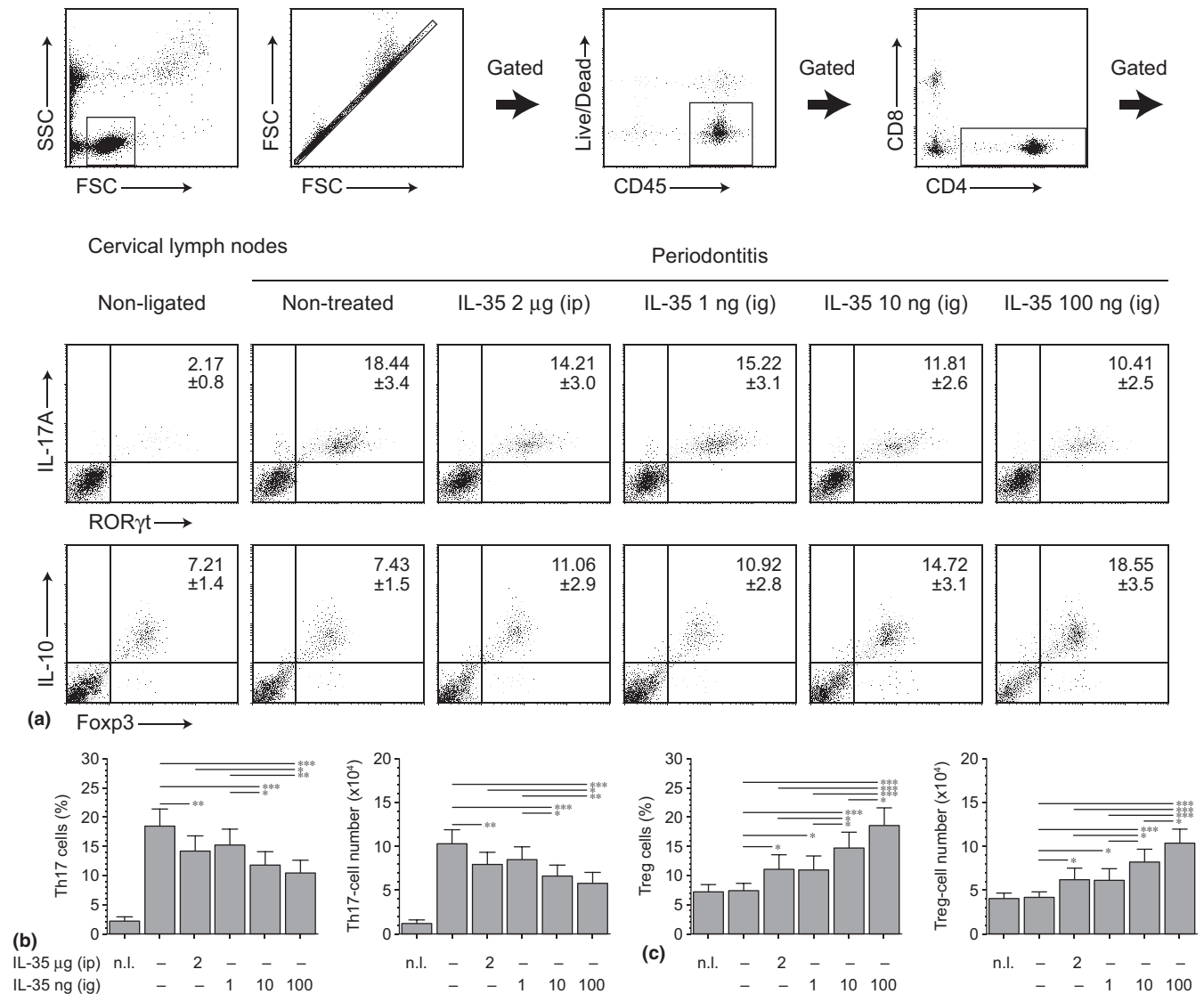
### 3.6 | RANKL<sup>+</sup> Th17-cell detection

In order to ratify the associated effect of IL-35 on RANKL-mediated alveolar bone loss and periodontal immune response, the expression of RANKL was analysed by flow cytometry in live/single/CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>T lymphocytes obtained from the cervical lymph nodes (Figure 6a) and periodontal lesions (Figure 6b). In periodontitis-affected mice, RANKL expression was restricted to CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>ROR $\gamma$ t<sup>+</sup> Th17 lymphocytes. When IL-35 was administrated, significantly lower frequency and number of CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>ROR $\gamma$ t<sup>+</sup>RANKL<sup>+</sup> Th17 lymphocytes were detected in the cervical lymph nodes of periodontitis mice as

compared with mice without treatment (Figure 6c). Similarly, a significant reduction in the frequency and number of CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>ROR $\gamma$ t<sup>+</sup>RANKL<sup>+</sup> Th17 lymphocytes was detected in the periodontal lesions of IL-35-treated periodontitis mice compared with non-treated mice (Figure 6d).

## 4 | DISCUSSION

The hallmark of periodontitis pathogenesis is alveolar bone resorption caused by the increased activity of osteoclasts, and this

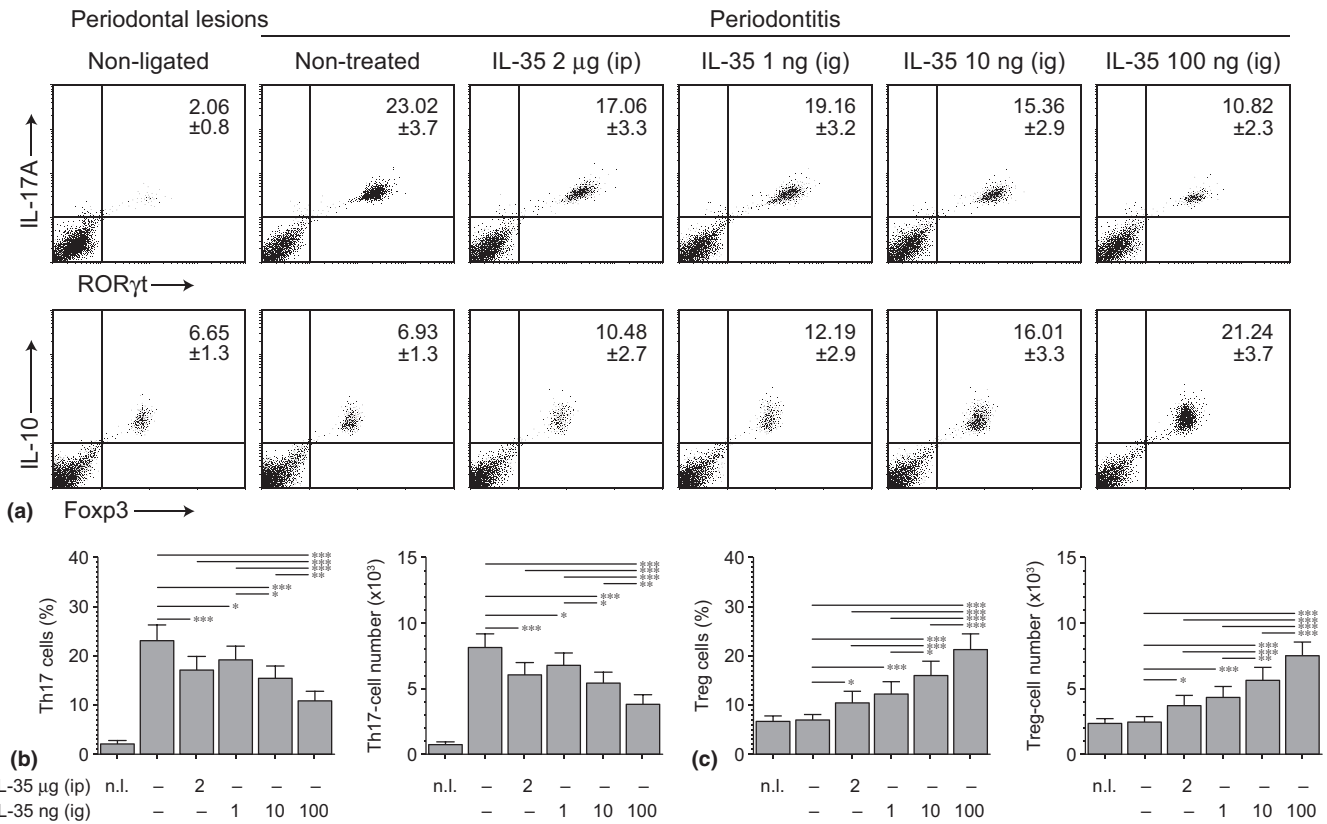


**FIGURE 3** Th17 and Treg-cell detection within the cervical lymph nodes. (a) Flow cytometry analysis demonstrating the presence of CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>RORγt<sup>+</sup>IL-17A<sup>+</sup> Th17 lymphocytes and CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>Foxp3<sup>+</sup>IL-10<sup>+</sup> Treg lymphocytes within the cervical lymph nodes of mice with ligature-induced periodontitis treated with IL-35. Frequency and number of (b) CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>RORγt<sup>+</sup>IL-17A<sup>+</sup> Th17 lymphocytes and (c) CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>Foxp3<sup>+</sup>IL-10<sup>+</sup> Treg lymphocytes within the cervical lymph nodes of mice with ligature-induced periodontitis treated with IL-35. Data are represented as Th17 or Treg-cell percentage and absolute number and shown as mean ± SD from 8 independent experiments. \**p* < .05, \*\**p* < .01, and \*\*\**p* < .001

increase in resorptive activity occurs by the enhanced production of RANKL elicited by Th17 lymphocytes, as a consequence of the local Th17/Treg imbalance (Alvarez et al., 2019; Campbell et al., 2016). In the present study, IL-35 inhibited the alveolar bone resorption in periodontitis mice and this inhibition was associated with diminished osteoclast detection and downregulated RANKL expression in the periodontal lesions. Besides, IL-35 caused changes in the detection and function of Treg and Th17 lymphocytes, with an upregulation of Treg-related cytokines and downregulation of Th17-related cytokines. Particularly, IL-35 triggered a decreased detection of RANKL<sup>+</sup> Th17 lymphocytes in periodontal tissues, thus demonstrating a role of IL-35 in the modulation of the immune response during periodontitis.

In periodontitis-affected patients, IL-35 has been detected in the GCF, saliva, serum, and periodontal tissues (Jin et al., 2017; Jing et al., 2019; Kalburgi et al., 2013; Köseoğlu et al., 2015; Mitani et al., 2015; Raj et al., 2018). Recent studies, in which the concentration of IL-35 in the GCF was analysed, revealed a negative correlation with the IL-17A levels (Mitani et al., 2015) and with the periodontal clinical parameters: pocket depth and clinical attachment loss (Jin et al., 2017), which led the authors to suggest the possibility of a protective role of IL-35 during periodontitis. The protective capacity of IL-35 during periodontitis was demonstrated in the present study since the administration of IL-35 successfully inhibited the alveolar bone resorption by modulating the Th17/Treg imbalance.





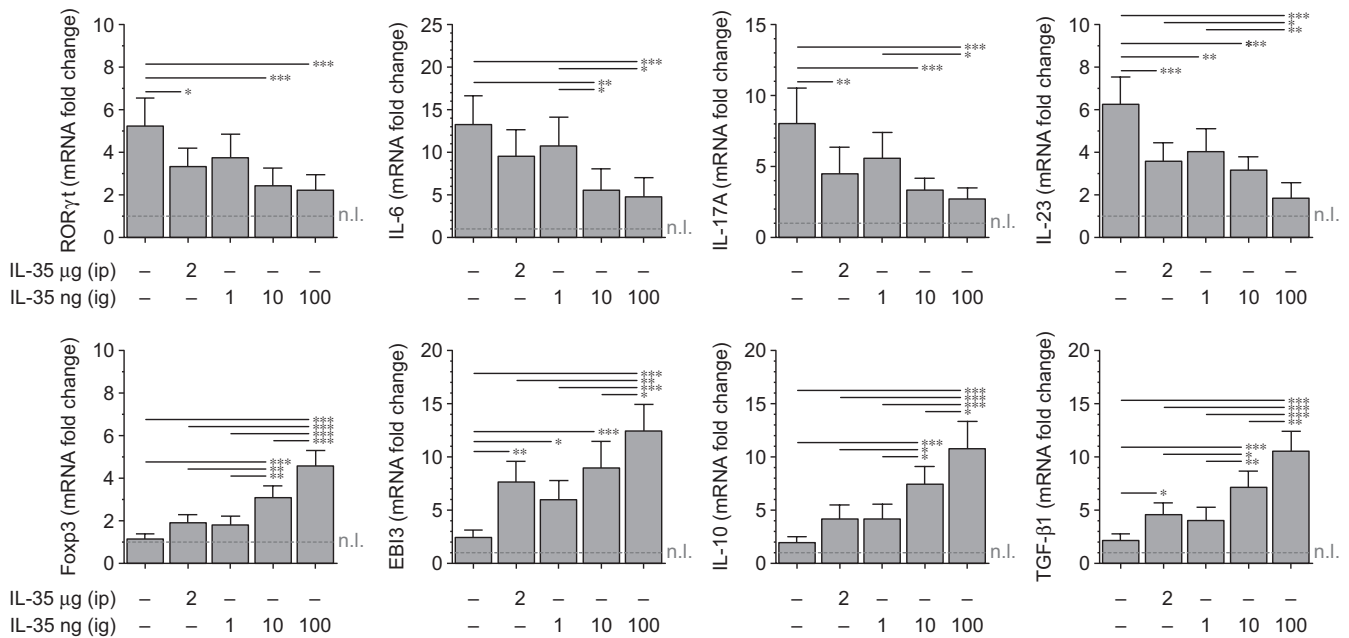
**FIGURE 4** Th17 and Treg-cell detection within the periodontal lesions. (a) Flow cytometry analysis demonstrating the presence of CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>RORγt<sup>+</sup>IL-17A<sup>+</sup> Th17 lymphocytes and CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>Foxp3<sup>+</sup>IL-10<sup>+</sup> Treg lymphocytes within the periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. Frequency and number of (b) CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>RORγt<sup>+</sup>IL-17A<sup>+</sup> Th17 lymphocytes and (c) CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>Foxp3<sup>+</sup>IL-10<sup>+</sup> Treg lymphocytes within the periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. Data are represented as Th17 or Treg-cell percentage and absolute number and shown as mean ± SD from 8 independent experiments. \**p* < .05, \*\**p* < .01, and \*\*\**p* < .001

Loss of IL-35 activity is associated with the progression and worsening of various inflammatory diseases (Egwuagu, Yu, Sun, & Wang, 2015; Zhang, Zhou, Guo, & Tu, 2015); such that, animals lacking functional IL-35 show greater severity of the disease (Liu et al., 2012; Tirota et al., 2013). Conversely, when IL-35 was therapeutically administered, a significant reduction of pathological inflammation has been observed (Gao et al., 2017; Li et al., 2014; Niedbala et al., 2007; Wirtz et al., 2011). Thereby, collagen-induced rheumatoid arthritis-affected mice showed a significant reduction of pathological signs of the disease after IL-35 inoculation (Niedbala et al., 2007).

As is the case with periodontitis, bone resorption is also the hallmark of rheumatoid arthritis. In both diseases, bone loss is positively regulated by the local overexpression of RANKL, which in turn is directly produced by the infiltrating Th17 lymphocytes (Li et al., 2016). The inhibitory effect of IL-35 put forward here for periodontitis has already been demonstrated in rheumatoid arthritis. In fact, a similar treatment based on daily injections of 2 μg IL-35 inhibited the articular bone loss in experimental rheumatoid arthritis, and this inhibition was associated with the suppression of Th17 cells and the expansion of Treg cells (Li et al., 2016; Niedbala et al., 2007). These results are in line with the present study, where IL-35 induced less

detection of RORγt<sup>+</sup>IL-17A<sup>+</sup> Th17 lymphocytes and downregulation of the Th17-related cytokines IL-6, IL-17A, and IL-23; together with, higher detection of Foxp3<sup>+</sup>IL-10<sup>+</sup> Treg lymphocytes and upregulation of Treg-related cytokines IL-10, IL-35, and TGF-β1 in periodontitis-affected tissues. In this context, a diminished detection of RORγt<sup>+</sup>RANKL<sup>+</sup> Th17 lymphocytes was also observed after IL-35 treatment, which is consistent with the reduction of the RANKL/OPG ratio in periodontal lesions (Li et al., 2016; Niedbala et al., 2007) and demonstrates a close relationship between immunological and bone changes induced by IL-35.

The results presented here are also in line with in vitro reports demonstrating that IL-35 can directly suppress the production of IL-17A in activated Th17 lymphocytes (Okada et al., 2017) and IL-6 in IL-17A-stimulated human periodontal ligament cells (Shindo, Hosokawa, Hosokawa, & Shiba, 2019), as well as the differentiation of osteoclast from monocyte precursors induced by TNF-α or RANKL (Jing et al., 2019; Peng et al., 2018; Yago et al., 2018). Herein, we show that IL-35 downregulated the Th17 response in terms of cell detection and expression of pro-inflammatory cytokines. The reduced RANKL production in the periodontal lesions could also be indicative of a direct effect of IL-35 on the production of RANKL from Th17 cells. Indeed, 10 ng and 100 ng of IL-35 provoked an



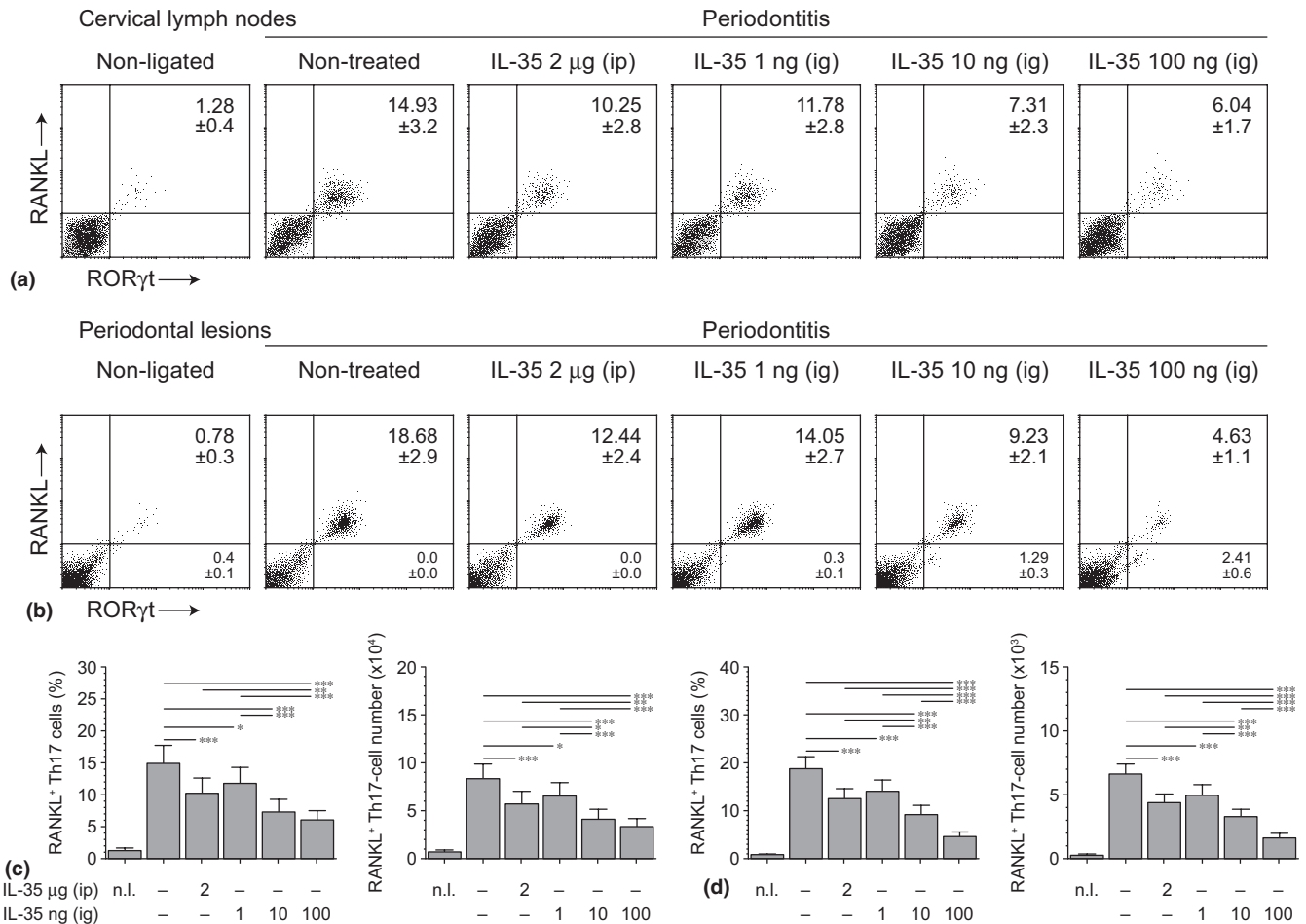
**FIGURE 5** Th17 and Treg-related transcription factor and cytokine expression. mRNA expression of the Th17 and Treg-related transcription factors and cytokines in periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. For relative expression, the transcription factor and cytokine mRNA expression in non-ligated (n.l.) mice were considered as 1, as a reference for fold change in expression. Data are represented as mRNA fold change and shown as mean  $\pm$  SD from 8 independent experiments. \* $p < .05$ , \*\* $p < .01$ , and \*\*\* $p < .001$

increase in the detection of ROR $\gamma$ t<sup>+</sup>RANKL<sup>-</sup> Th17 lymphocytes in periodontal tissues (Figure 6), suggesting an inhibitory effect on the RANKL production in the activated Th17 cells. On the other hand, the detected changes in the RANKL/OPG imbalance could also involve an inhibitory effect of IL-35 on other RANKL-producing cells such as osteoblasts and fibroblasts, which are critical RANKL producers in several diseases whose pathogenesis is associated with the Th17/Treg imbalance (Alvarez et al., 2019; Dong, 2006; Fumoto, Takeshita, Ito, & Ikeda, 2014). In this context, an inflammatory milieu enriched in Th17-related cytokines, such as IL-6 and IL-17A, may exert osteoclastogenic activity by inducing RANKL expression on osteoblasts and fibroblasts, as well as favour the recruitment and activation of other immune cells and the net increment of pro-inflammatory and RANKL-inducing cytokines in the periodontitis-affected tissues (Alvarez et al., 2019; Sato et al., 2006). Interestingly, recently reported data have suggested that IL-35 could also favour bone regeneration by inducing OPG production and osteoblast differentiation through Wnt/ $\beta$ -catenin signalling (Li et al., 2019). In the present study, OPG expression was significantly incremented in periodontal lesions in response to IL-35, which could imply an osteoblastic effect via changing the RANKL/OPG ratio, suggestive of a potential role of IL-35 in alveolar bone regeneration that requires further investigations.

Together with IL-10 and TGF- $\beta$ 1, IL-35 is an anti-inflammatory cytokine mainly produced by Treg lymphocytes. According to the new proposal for categorizing anti-inflammatory cytokines, IL-10 and TGF- $\beta$ 1 are housekeeping anti-inflammatory cytokines more related to permanently preventing the chronification of inflammation;

instead, IL-35 is a responsive anti-inflammatory cytokine induced in response to an overload of inflammatory stimuli, thus is more related to suppressing already established inflammatory processes (Li et al., 2012). IL-35 is a heterodimeric protein composed of IL-12p35 and EBI3, where IL-12p35 is constitutively expressed in most tissues, whereas EBI3 is selectively expressed and highly induced (Collison et al., 2010; Olson et al., 2013), the reason why EBI3 was analysed in the present study instead of IL-12p35. In this context, various immune cell types have been shown to express IL-35 mainly in the course of inflammation; however, in all of these cell types, including non-activated T cells, it has been noted that IL-35 expression is minimal, unlike when these T cells become activated by IL-35 itself (Collison et al., 2007, 2010). In this study, IL-35 inoculation induced the increased expression of Foxp3 and EBI3 in periodontal tissues, and EBI3 is a downstream target of Foxp3 (Collison et al., 2007); thus, the increased Foxp3 expression would lead to the expression of EBI3 in Treg cells and consequently to the higher production of IL-35. In other words, IL-35 inoculation could generate positive feedback in the periodontal Treg response by inducing the differentiation and activation of IL-35-producing Tregs, defined as iTr35 cells (Collison et al., 2007, 2010). The iTr35 cells have been described as a potent IL-35 producer (Collison et al., 2010); hence, they may contribute more to the Th17-cell suppression. Therefore, inoculated IL-35 could have a direct effect on periodontally infiltrating Th17 lymphocytes, as well as an indirect effect by inducing IL-35 by iTr35 cells (Figure 7).

Several strategies have been assayed in order to modulate the Th17/Treg imbalance during periodontitis and arrest the alveolar bone loss, such as subcutaneous vaccination with *Porphyromonas*

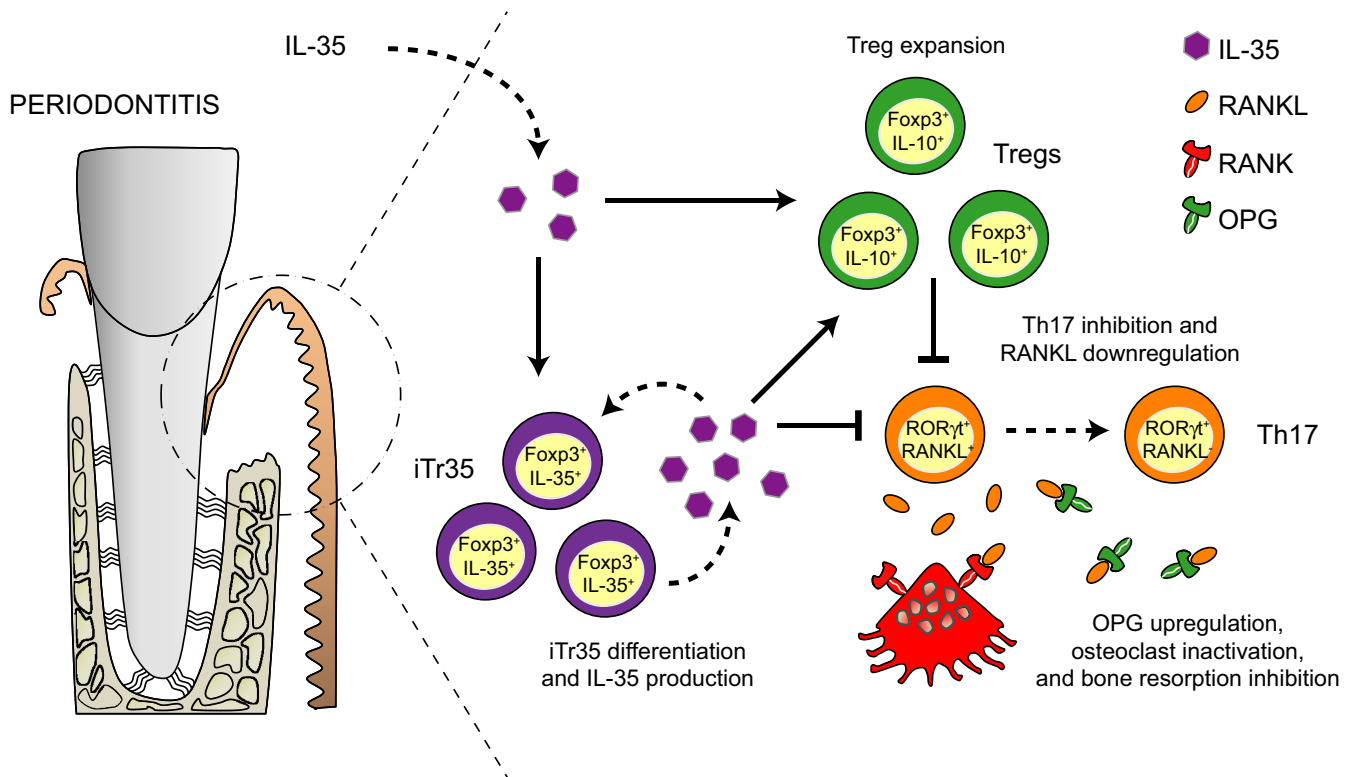


**FIGURE 6** RANKL<sup>+</sup> Th17-cell detection. Flow cytometry analysis demonstrating the presence of CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>ROR $\gamma$ t<sup>+</sup>RANKL<sup>+</sup> Th17 lymphocytes within the (a) cervical lymph nodes and (b) periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. Frequency and number of CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>ROR $\gamma$ t<sup>+</sup>RANKL<sup>+</sup> Th17 lymphocytes within the (c) cervical lymph nodes and (d) periodontal lesions of mice with ligature-induced periodontitis treated with IL-35. Data are represented as RANKL<sup>+</sup> Th17-cell percentage and absolute number and shown as mean  $\pm$  SD from 8 independent experiments. \* $p$  < .05, \*\* $p$  < .01, and \*\*\* $p$  < .001

*gingivalis*, gastric feeding of colchicine, and oral administration of retinoic acid, tamibarotene or oleuropein, with promising results (Jin, Wang, Liu, & Lin, 2014; Taskan, Balci Yuce, Karatas, Gevrek, & Toker, 2019; Toker, Yuce, Yildirim, Tekin, & Gevrek, 2019; Wang, Guan, Jin, Lin, & Gao, 2015; Wang, Wang, Jin, Gao, & Lin, 2014). To the best of the authors' knowledge, this study provides the first evidence that IL-35 administration suppresses periodontitis. The potential advantage of IL-35-based therapy is that its suppressor effect would depend not only on directly administered IL-35 but also on the induction of iTr35 cells responsible for the endogenous production of IL-35, such that the periodontal availability of IL-35 would be even higher.

As in the aforementioned studies, this study involved several inoculations of the drug to ensure an effective periodontal concentration, limiting the clinical projection of the proposal. Several gingival injections could cause local trauma and low-grade inflammation, affecting the immune homeostasis of periodontal tissues. Indeed, mechanical damage, as the one produced during mastication, may promote the IL-6-mediated generation of Th17

lymphocytes and define the immune tone of the gingiva (Dutzan et al., 2017). In this context, mechanical damage may contribute to the Th17-cell-driven alveolar bone resorption during periodontitis (Dutzan et al., 2017). Nowadays, advanced delivery systems have been developed to be locally used in order to prolong the permanence and effectiveness of drugs, which allow to reduce the necessity of multiple inoculations and avoid their potential cytotoxicity. Among these systems, nanocarriers composed of different polymers such as polylactic-co-glycolic acid (PLGA), chitosan, gelatin, or silica have been proposed for the controlled delivery of immunomodulatory therapies to deal with periodontitis (Cafferata et al., 2018). Thus, it would be interesting to analyse the effect of nanocarriers loaded with IL-35 in the suppression of periodontitis in order to propose a more translational strategy that contributes to the immunomodulation of periodontitis. Although studies on IL-35 are relatively few and the signal transduction mechanisms involved in its functions are not elucidated yet, IL-35 therapy shows promising potential for controlling alveolar bone resorption during periodontitis.



**FIGURE 7** The protective role of IL-35 during periodontitis. In response to the inflammatory stimuli during periodontitis, IL-35 promotes the expansion of Treg lymphocytes and the differentiation of an exclusive IL-35-producing subpopulation, termed iTr35. The ability of IL-35 to promote its own expression induces the amplification of an immune-regulatory response capable of inhibiting the bone-resorptive Th17 response, by downregulating the expression of RANKL and Th17-related cytokines. Additionally, IL-35 induces the production of OPG, which could be an indicator of a possible role in bone regeneration during periodontitis. Thus, the administration of IL-35 combined with periodontal therapy could be potentially effective for the treatment of periodontitis

## 5 | CONCLUSION

Taken together, these results show that the increase of the availability of IL-35 in the periodontitis-affected tissues results in the inhibition of the alveolar bone resorption by decreasing osteoclast activation, in the context of changes in the RANKL/OPG ratio as a consequence of Th17/Treg imbalance modulation.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this study.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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