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ORIGINAL ARTICLE

Regulatory B cells induced by interleukin-35 inhibit inflammation and alveolar bone resorption in ligature-induced periodontitis

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Abstract

Background: Regulatory B cells (Bregs) have been reported to suppress immune responses and alveolar bone loss in murine periodontitis models. These cells could be induced by interleukin (IL)-35 which is increased upon periodontal inflammation. Thus, this study aimed to explore the role of Bregs induced by IL-35 in periodontitis.

Methods: Experimental periodontitis was induced in mice by ligature. Two weeks after ligation, the test group was systemically treated with IL-35 for 1 week. Four weeks after ligation, all mice were euthanized, and alveolar bone loss was evaluated by microcomputed tomography. Cytokines associated with periodontitis were analyzed using reverse transcription-quantitative polymerase chain reaction and enzyme-linked immunosorbent assay. Bregs in spleens, cervical lymph nodes, and periodontal tissues were detected by flow cytometry and immunofluorescence staining.

Results: In the mouse model of periodontitis, IL-35 induced the expansion of $CD1d^{hi}CD5^{+}$ B10 cells with increased interleukin-10 (IL-10) and IL-35 production. IL-35 administration also attenuated alveolar bone loss and reduced the levels of proinflammatory cytokines in situ.

Conclusions: Following ligature-induced periodontitis in mice, IL-35 inhibited periodontal inflammation and alveolar bone resorption at least partially through the induction of B10 cells and $IL\text{-}35^{+}$ Bregs.

KEYWORDS
B-lymphocytes, immunity, inflammation, interleukin-35, periodontitis

Shiyi Li and Li Su contributed equally to this work

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1 | INTRODUCTION

Periodontitis is a destructive inflammatory disease caused by interactions between oral microorganisms and the host immune system.[1] Conventional periodontal treatment focuses on the mechanical and chemical removal of etiological microbes[2]; however, immunotherapy has also become increasingly emphasized and investigated.[3]

The proinflammatory role of B cells in periodontitis has long been recognized by researchers.[4] Recently, the contribution of the immunosuppressive subgroup of B cells, known as regulatory B cells (Bregs), to this process has also been highlighted by some studies.[5] Although there is a lack of consistent phenotype or specific lineage, accumulating evidence suggests that Bregs mediate immunoregulation through the production of regulatory cytokines such as interleukin (IL)-10, transforming growth factor-β (TGF-β), and IL-35.[6,7] Additionally, $CD1d^{hi}CD5^{+}$ B10 cells, which are responsible for most IL-10 production by B cells, have been shown to ameliorate the progression of inflammatory and autoimmune diseases in several animal models.[8,9] In experimental models of periodontitis, B10 cells dramatically inhibit periodontal inflammation and alveolar bone loss.[10–14]

IL-35 is a heterodimer made up of IL-12p35 and Epstein Barr virus-induced gene 3 (Ebi3) subunits,[15] and is mainly secreted by regulatory T cells (Tregs) and Bregs.[16] As a novel anti-inflammatory cytokine, IL-35 is not constitutively expressed,[17] but is induced in periodontitis lesions.[18,19] Using inflammatory disease models, including experimental periodontitis models, researchers have demonstrated that IL-35 induces Tregs and simultaneously suppresses T helper 1 (Th1) and T helper 17 (Th17) cells.[20–22] Moreover, evidence suggests that IL-35 is an efficient Bregs inducer. For example, it has been reported that IL-35 conferred protection from uveitis in mice through the augmentation of IL-10-producing B cells.[23] This supports the hypothesis that the differentiation and expansion of Bregs in periodontitis could be affected by IL-35, which is induced in response to periodontal inflammation. In this study, we addressed the participation of B10 cells in ameliorating periodontitis and further explored the underlying modulatory role of IL-35 in B10 cells and other Bregs.

2 | MATERIALS AND METHODS

2.1 | Animals

Six-week-old male C57BL/6J mice were housed under pathogen-free conditions in individual ventilated cages throughout the study. All experimental procedures were approved by the Animal Welfare and Biomedical Ethics Committee of Peking University (LA201406) and conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.[24]

2.2 | Induction of experimental periodontitis and administration of IL-35

Animals were randomly allocated to three groups: control group (Con, $n = 8$), periodontitis group (P, $n = 8$), and IL-35–treated periodontitis group (P + IL-35, $n = 8$). Experimental periodontitis was induced using the ligation method, as previously described.[25] Briefly, 6–0 silk threads were tied around the maxillary second molars and remained in place for 4 weeks. The ligature was checked every 2 days and replaced if not intact. Two weeks after ligation, the IL-35–treated group was administered 100 ng IL-35[*] in 40 μL phosphate-buffered saline (PBS) daily through the tail vein for 7 consecutive days, referring to previous studies,[26,27] and the results of our pilot study (see supplementary Figure S1 in online *Journal of Periodontology*). For comparison, the untreated periodontitis group was administrated in PBS only.

2.3 | Sample collection and preparation

Four weeks after ligation, mice were euthanized by intraperitoneal administration of 150 mg/kg pentobarbital sodium.[†] First, the spleens and cervical lymph nodes (LN) were harvested and placed in ice-cold RPMI 1640. The maxillae were then isolated and divided into two halves at the midline. Gingival tissues surrounding the left maxillary molars were dissected and homogenized individually and stored at −80°C. The left maxilla that were free of gingiva were fixed in 4% paraformaldehyde for 24 h and stored in 70% ethanol at 4°C. The right maxilla were fixed in 4% paraformaldehyde for 24 h, decalcified in 10% ethylenediaminetetraacetic acid (pH 7.4), and embedded in paraffin after dehydration.

2.4 | Microcomputed tomography (micro-CT) analysis

The fixed-left maxilla were scanned using a micro-CT scanner[‡] and analyzed by matched software.[§] The volumetric change of alveolar bone was measured following

[*] CHI-MF-11135, Chimerigen Laboratories, Alston, MA, USA.
[†] Sigma-Aldrich, St Louis, MO, USA.
[‡] Siemens Medical Solutions USA, Inc., Malvern, PA, USA.
[§] Inveon, Siemens Medical Solutions USA, Inc., Malvern, PA, USA.

the protocol described previously.[28] Briefly, the amount of circumferential bone loss was assessed in the axial plane and root inclusion was minimized.

2.5 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was obtained from homogenized gingiva using an RNA extraction kit,[||] and cDNA was generated using a mix reagent.[¶] The expression of mRNA for receptor activator of nuclear factor κB ligand (RANKL), osteoprotegerin (OPG), IL-1β, IL-17A, tumor necrosis factor-α (TNF-α), IL-10, IL-12p35, and Ebi3 was analyzed using a mix reagent[#] in a real-time PCR detection system.[**] The levels of expression of the target gene were normalized to GAPDH and expressed relative to the control group following the $2^{-\Delta\Delta Ct}$ method. The PCR primer sequences are shown in (see supplementary Table S1 in *Journal of Periodontology*).

2.6 | Enzyme-linked immunosorbent assay (ELISA)

The homogenized gingiva from each sample was centrifuged at 12,800 × *g* and the supernatants were collected. The concentrations of RANKL, OPG, and IL-10 in gingival homogenates were measured using commercially available ELISA kits,[††] according to the manufacturer's instructions.

2.7 | Flow cytometric analysis

The presence of Bregs in the spleen and cervical LN was investigated using a flow cytometer[‡‡] and the FlowJo software.[§§] First, samples were mashed through a 70-μm cell strainer using a syringe plunger, treated with 1× Red Blood Cell Lysis Buffer,[||||] filtered through another 70-μm cell strainer, and a single cell suspension in PBS was then prepared. For cell surface staining, monoclonal antibodies against CD19-APC,[¶¶] CD5-PE/Cyanine7,[##] and CD1d-PerCP/Cyanine5.5[***] were used. Cells were fixed with a fixation buffer[†††] and permeabilized with 0.1% TritonX-100.[‡‡‡] Subsequently, cells were intracellularly stained with anti-IL-10-P-phycoerythrin antibody.[§§§]

2.8 | Immunofluorescence staining

Immunofluorescence staining of CD19, IL-10, p35, and Ebi3 was performed in periodontal tissues. Paraffin-embedded specimens were sliced into 5-μm sections along the long axis of the molars. After heat-induced antigen retrieval, sections were blocked with 1% goat serum and incubated with premixed primary antibodies, including rabbit anti-mouse CD19,[|||||] mouse IL-10,[¶¶¶] mouse p35,[###] and rat anti-mouse EBI3/IL-27B,[****] and then incubated with a mixture of secondary fluorescent antibodies, including FITC-labeled goat anti-rabbit immunoglobulin G (IgG) (H+L),[††††] Alexa Fluor 647-conjugated goat anti-mouse IgG (H+L)[‡‡‡‡] and Alexa Fluor 594-conjugated goat anti-rat IgG (H+L).[§§§§] Finally, sections were treated with an anti-fluorescence quenching agent containing 4′,6-diamidino-2-phenylindole (DAPI)[|||||||] and sealed with coverslips. Images of periodontal tissues between the first and second molars were captured and analyzed using a laser-scanning confocal microscope.[¶¶¶¶] For each section, four rectangles of equal size were drawn at nearly the same location, the number of positive cells in each rectangle was counted and the mean was calculated.

2.9 | Statistical analysis

Data are presented as the mean ± standard deviation (SD) and analyzed using SPSS 19.0 software. The normality of the data distribution was established using the Kolmogorov–Smirnov test, and independent-sample *t*-tests was used to evaluate differences. Statistical significance was set at a p value of <0.05.

[||] TaKaRa Mini BEST Universal RNA Extraction Kit, TaKaRa Bio Inc., Shiga, Japan.
[¶] Prime Script RT Master Mix, TaKaRa Bio Inc., Shiga, Japan.
[#] Power SYBR Green PCR Master Mix, Roche, Indianapolis, IN, USA.
[**] 7500 Real-Time PCR Detection System, Applied Biosystems, Foster City, CA, USA.
[††] Meimian Inc., Ltd., Jiangsu, China.
[‡‡] Gallios, Beckman Coulter, Inc., Brea, CA, USA.
[§§] Tree Star, Inc., San Carlos, CA, USA.
[||||] BioLegend, San Diego, CA, USA.
[¶¶] BioLegend, San Diego, CA, USA.
[##] BioLegend, San Diego, CA, USA.
[***] BioLegend, San Diego, CA, USA.
[†††] Cytofix Fixation Buffer, BD Biosciences, San Diego, CA, USA.
[‡‡‡] Solarbio, Beijing, China.
[§§§] BioLegend, San Diego, CA, USA.
[|||||] Abcam, Cambridge, MA, USA.
[¶¶¶] Santa Cruz Biotechnology, Santa Cruz, CA, USA.
[###] Santa Cruz Biotechnology, Santa Cruz, CA, USA.
[****] Lifespan Biosciences, Seattle, WA, USA.
[††††] Zhong Shan Golden Bridge Biotechnology (Zs Bio), Beijing, China.
[‡‡‡‡] Beyotime Institute of Biotechnology, Jiangsu, China.
[§§§§] Abcam, Cambridge, MA, USA.
[|||||||] Solarbio, Beijing, China.
[¶¶¶¶] LSM900, Zeiss, Jena, Germany.

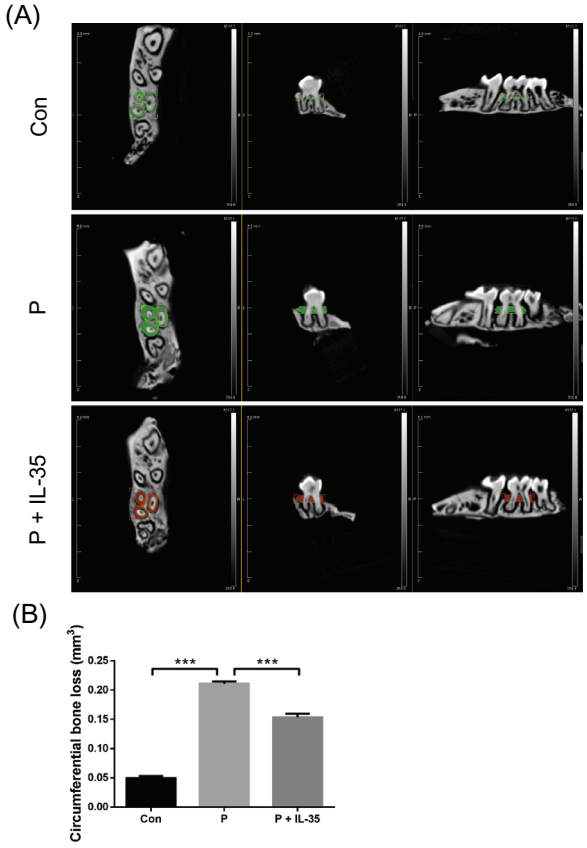

FIGURE 1 Alveolar bone loss. (A) Representative images of orientation and location of volumetric measurement of alveolar bone loss in different groups. From left to right are axial images, coronal images, and sagittal images. The colored area represents the area considered in for volumetric measurements. (B) The volumes of the circumferential bone loss are shown as mean ± standard deviation. ***$p < 0.001$.

3 | RESULTS

3.1 | Alveolar bone loss

The volume of circumferential bone loss was determined from the micro-CT images (Figure 1). No significant bone resorption was observed in the control group at the end of the study, while the volume of bone loss increased significantly in the untreated periodontitis group (0.21 mm^3 vs. 0.05 mm^3, $p < 0.001$), indicating that periodontitis was successfully induced. When compared with the periodontitis group, alveolar bone resorption was markedly suppressed in the IL-35–treated periodontitis group (0.15 mm^3 vs. 0.21 mm^3, $p < 0.001$).

3.2 | mRNA expression of periodontitis-associated cytokines

The transcription levels of cytokines involved in the pathogenesis of periodontitis were evaluated using RT-qPCR. Compared with the control group, there was an increase in the mRNA expression of RANKL in the untreated periodontitis group (4.59-fold), which was mitigated by the administration of IL-35 (1.39-fold) (Figure 2A). The mRNA expression of OPG was also increased in the untreated group (1.36-fold) and the increase was even higher in the IL-35–treated group (2.51-fold) (Figure 2B). Thus, the ratio of RANKL/OPG mRNA increased in the periodontitis group (3.44-fold), but the trend was abrogated after IL-35 administration (0.60-fold) (Figure 2C).

The mRNA levels of the proinflammatory cytokines IL-1β, IL-17A, and TNF-α, and the anti-inflammatory cytokines IL-10 and IL-35 (IL-12p35 and Ebi3) were all elevated in the untreated periodontitis group (5.57-, 6.68-, 1.35-, 1.55-, 1.91-, and 2.15-fold, respectively). However, following treatment with IL-35, the mRNA levels of proinflammatory cytokines (IL-1β, IL-17A, TNF-α) were markedly suppressed (1.84-, 3.14-, and 0.69-fold, respectively) (Figure 2D–F), while the mRNA levels of IL-10, IL-12p35, and Ebi3 were elevated further (3.36-, 4.61-, and 5.64-fold, respectively) (Figure 2G–I).

3.3 | Gingival RANKL, OPG, and IL-10 production

Consistent with the trend shown by the RT-qPCR results, RANKL production in the periodontitis group increased to three times that of the control group, while IL-35 treatment mitigated the upregulation, reducing the levels to approximately 1.7-times that of the control group ($p < 0.001$) (Figure 3A). OPG production in gingiva increased to 1.4 times that of the control levels in the periodontitis group and further increased to almost three times in the IL-35–treated group ($p < 0.001$) (Figure 3B). As an important index of bone metabolism, the RANKL/OPG ratio increased in periodontitis mice, but was dramatically reversed following IL-35 treatment ($p < 0.001$) (Figure 3C).

Additionally, the expression of IL-10 in gingiva from the periodontitis group was 40% higher than that of the control group, while the administration of IL-35 resulted in an increase of 100% of the control group ($p < 0.001$) (Figure 3D).

3.4 | Frequencies of B10 and IL-10^+ B cells in the spleen

To identify the influence of systemic administration of IL-35 on Breg induction, we first quantified the frequency of $CD19^+CD1d^{hi}CD5^+$ B10 cells in the spleen. Compared with the untreated group, the IL-35–treated group had a significantly higher proportion of B10 cells (6.3% vs.

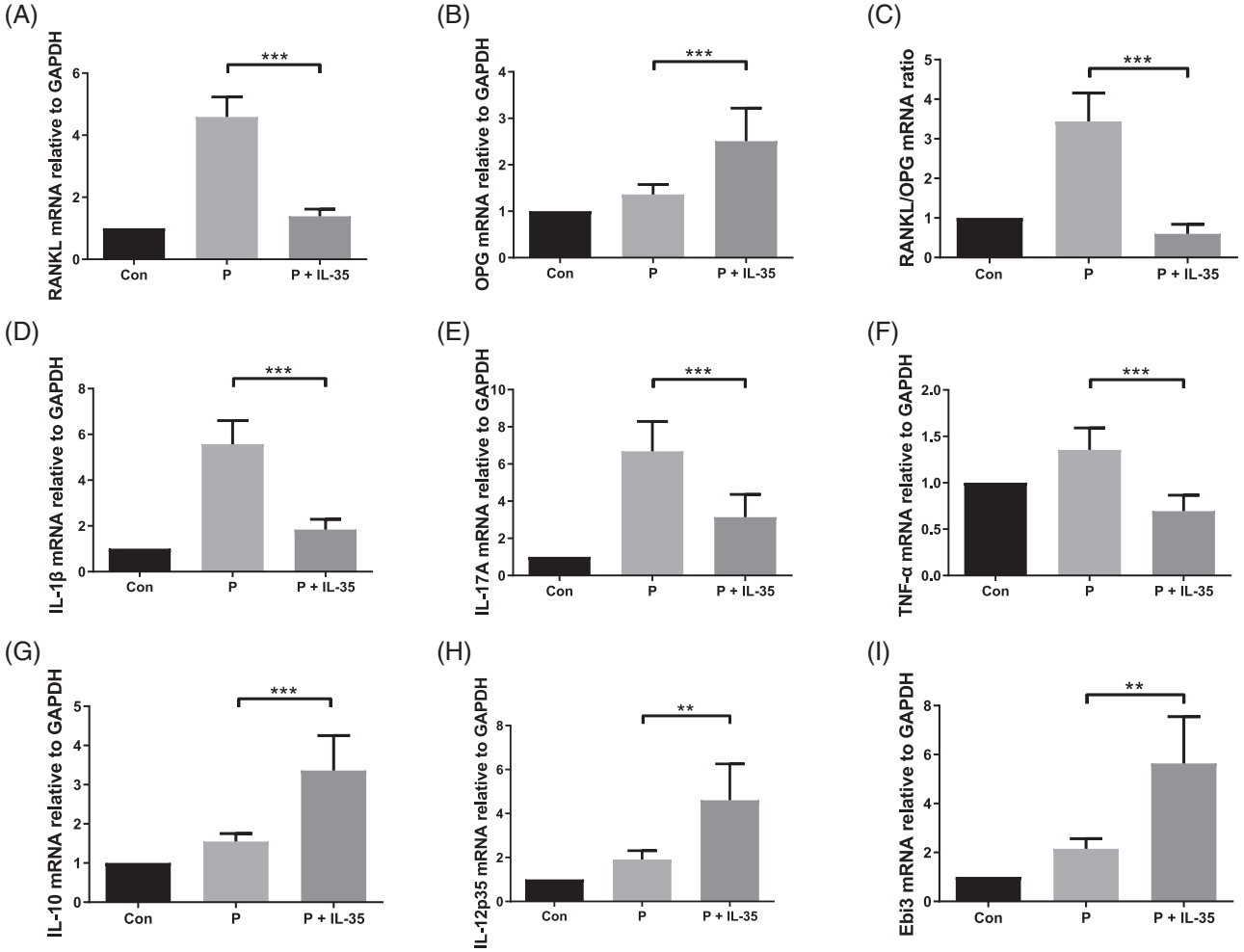

FIGURE 2 The mRNA expression of periodontitis-associated cytokines. The mRNA expression of RANKL (A), osteoprotegerin (B), IL-1β (D), IL-17A (E), tumor necrosis factor-α (F), IL-10 (G), IL-12p35 (H), and Epstein Barr virus-induced gene 3 (Ebi3) (I). (C) Ratio between the RANKL and OPG mRNA levels. For relative expression, the transcription factor and cytokine mRNA expression in control group were considered as 1, as a reference for fold change in expression. Data are shown as mean ± standard deviation. Ebi3, Epstein Barr virus-induced gene 3; IL, interleukin; OPG, osteoprotegerin; TNF-α, tumor necrosis factor-α. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.0%, $p < 0.001$) (Figure 4A,B). The proportion of IL-10$^+$ cells in the B10 subset was also increased (11.0% vs. 5.4%, $p < 0.01$) (Figure 4C,D). Further analysis revealed that the frequency of IL-10$^+$ B cells increased following stimulation with IL-35 (1.0% vs. 0.4%, $p < 0.01$) (Figure 4E,F), the proportion of B10 subset in the IL-10$^+$ B cell population was also elevated in the IL-35–treated group (64.4% vs. 35.9%, $p < 0.01$) (Figure 4G,H). The statistical analysis revealed that the expansion of the B10 subset as well as the increased frequency of IL-10$^+$ B cells in the B10 subset led to augmentation of the overall IL-10$^+$ B cell population in the spleen following stimulation with IL-35. Specifically, IL-10$^+$ B10 cells in the IL-35–treated group increased dramatically compared with the untreated group (0.63% vs. 0.15%, $p < 0.01$), while the IL-10$^+$ non-B10 cells were not significantly increased ($p > 0.05$) (Figure 4I).

3.5 | Frequencies of B10 and IL-10$^+$ B cells in the cervical LN

B10 cells were also induced by IL-35 in the cervical LN. Notably, the systemic administration of IL-35 induced a significant increase in B10 cells compared with that observed in the untreated periodontitis group (1.7% vs. 0.7%, $p < 0.001$) (Figure 5A,B), while the proportion of IL-10$^+$ B cells in the B10 subset was also increased (23.9% vs. 14.5%, $p < 0.05$) (Figure 5C,D). Similarly, the proportion of all IL-10$^+$ B cells (0.7% vs. 0.3%, $p < 0.01$) (Figure 5E,F) and the proportion of B10 subset in the IL-10$^+$ B cell population (49.8% vs. 27.4%, $p < 0.01$) (Figure 5G,H) were also elevated in the IL-35–treated group. Further analysis revealed that the expansion of IL-10$^+$ B cells could be attributed mainly to the increase of IL-10$^+$ B10 cells (0.4% vs. 0.1%, $p < 0.01$)

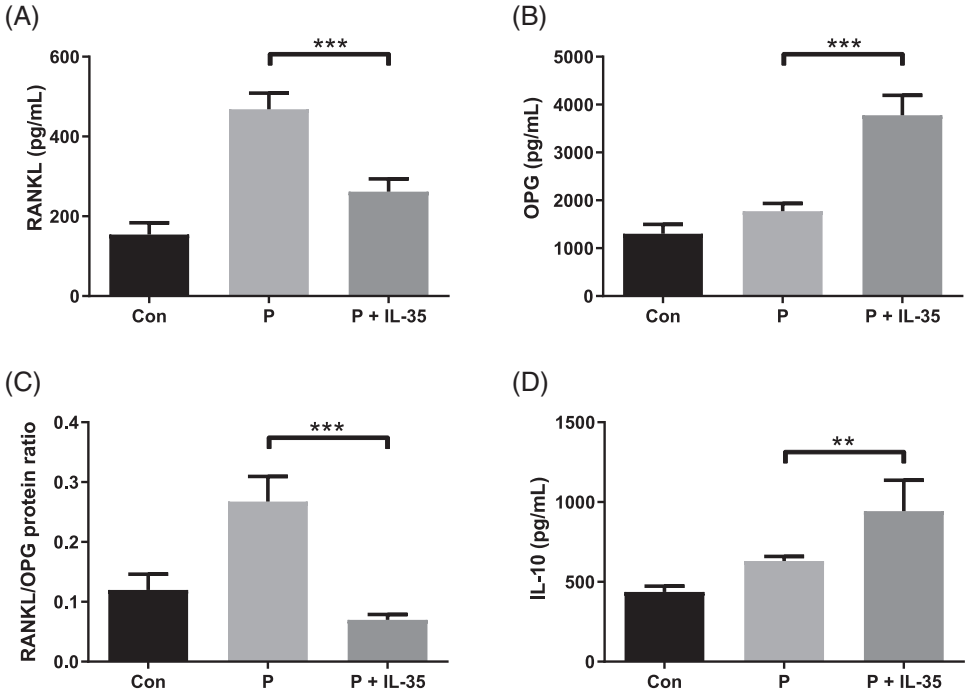

FIGURE 3 Gingival RANKL, osteoprotegerin (OPG), and interleukin (IL)-10 production. The concentrations of RANKL (A), OPG (B), and IL-10 (D) in the gingiva of different groups. (C) Ratio between the RANKL and OPG protein levels. Data are shown as mean ± standard deviation. IL, interleukin; OPG, osteoprotegerin. $^{**}p < 0.01$, $^{***}p < 0.001$.

rather than IL-10$^+$ non-B10 cells (0.3% vs. 0.2%, $p < 0.05$) (Figure 5I).

3.6 | Bregs in periodontal tissues

Immunofluorescent staining was used to identify the localization of Bregs in periodontal tissues (Figure 6). CD19$^+$ B cells and IL-10$^+$ cells were visualized in periodontal lesions, while they were mainly unobservable in healthy gingiva. Additionally, IL-10 and CD19 were not highly coexpressed in situ. In the IL-35–treated group, significantly more CD19$^+$ B cells and IL-10$^+$ cells were observed in the lesions. Although IL-10 was not highly coexpressed with CD19 in situ, the number of CD19$^+$IL-10$^+$ B cells in the IL-35–treated group increased to 1.6 times that of the untreated group ($p < 0.05$).

Similarly, Ebi3$^+$ cells were observed in the region infiltrated by inflammatory cells in the periodontitis group. After systemic administration of IL-35, infiltrated Ebi3$^+$ cells markedly increased. CD19 and Ebi3 were well colocalized, Ebi3$^+$ non-B cells were almost undetectable, and the number of CD19$^+$Ebi3$^+$ B cells increased to 1.9 times in the IL-35–treated group compared with the untreated group ($p < 0.05$). Additionally, p35$^+$ cells were also detected in the IL-35–treated group. Although only a limited number of p35$^+$ cells were detected, p35 was mainly expressed by CD19$^+$Ebi3$^+$ B cells infiltrating the lesions.

4 | DISCUSSION

The CD1d^{hi}CD5$^+$ B10 cell subset is an IL-10$^+$ cell-enriched Breg population and its immunosuppressive function is IL-10 dependent.[9] Previous studies indicated that adoptive transfer of B10 cells alleviated the symptoms of and prevented the development of several inflammatory and autoimmune diseases.[29–31] In periodontitis models, the protective role of adoptively transferred B10 cells has also been confirmed.[10–12] Recently, the induction and augmentation of IL-35 on Bregs has attracted substantial interest.[23,32] Wang et al. showed that IL-35–induced IL-10$^+$ B cells and IL-35$^+$ B cells ameliorated uveitis in mice.[23] Our results revealed that systemic administration of IL-35 significantly suppressed established periodontitis, which was due, in part, to the presence of B10 cells and IL-35$^+$ Bregs induction.

To evaluate the effects of IL-35 on lymphocytes in vivo, previous studies have used various methods, including intraperitoneal injection, intravenous injection, and local application.[22,27,33] There is substantial evidence indicating that Bregs, including B10 cells, are differentiated from B cells at different stages of development, instead of being differentiated from the B1 subgroup alone.[7] As we aimed to investigate the role of Bregs induced by IL-35 in mice with periodontitis, tail vain injection was used to administer IL-35, which increased the frequencies of IL-10$^+$ B cells by 1 to 2 times in the spleen and draining LN.

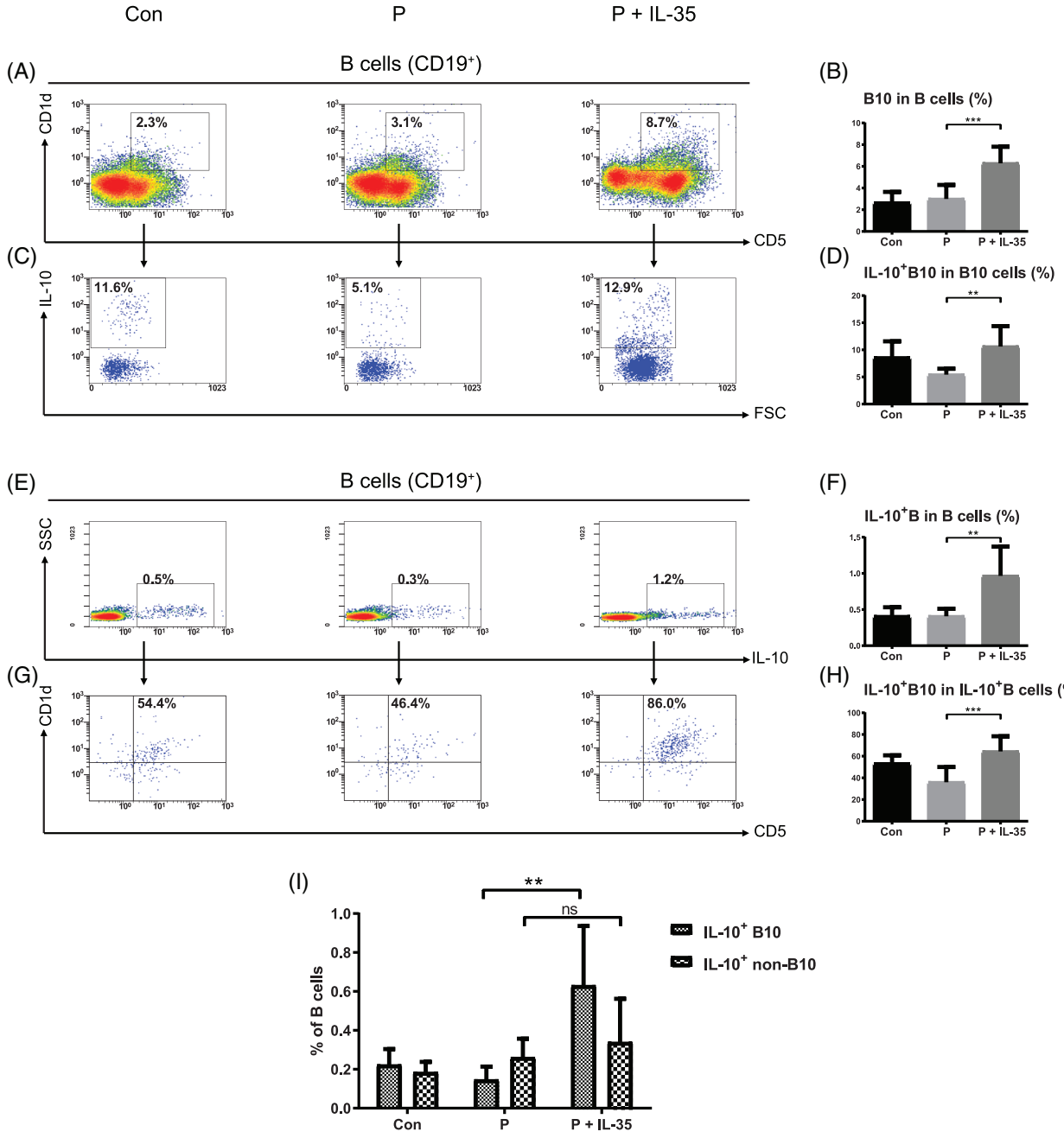

FIGURE 4 B10 and interleukin (IL)-10$^+$ B cells detection in the spleen. Single-cell suspensions were collected and labeled with designated antibodies. B cells were identified within the CD19$^+$ cell population. (A) Representative flow cytometric plots of CD1d and CD5 expression by CD19$^+$ B cells. (B) The frequencies of B10 cells among total B cells within different groups. (C) Representative flow cytometric plots of IL-10 expression by B10 cells. (D) The percentages of IL-10$^+$ B10 cells of B10 cells in different groups. (E) Representative flow cytometric plots of IL-10 expression by CD19$^+$ B cells. (F) The frequencies of IL-10$^+$ B cells among total B cells within different groups. (G) Representative flow cytometric plots of CD1d and CD5 expression by IL-10$^+$ B cells. (H) The percentages of IL-10$^+$ B10 cells of IL-10$^+$ B cells in different groups. (I) The frequencies of IL-10$^+$ B10 cells and IL-10$^+$ non-B10 cells among total B cells within different groups. Data are shown as mean ± standard deviation. FSC, forward scatter; IL, interleukin; SSC: side scatter. $^{**}p < 0.01$, $^{***}p < 0.001$.

Further analyses indicated that such expansion primarily came from the augmentation of the B10 cell subset, as well as the increased percentage of IL-10$^+$ cells in this subset. Furthermore, the mRNA and protein levels of IL-10 in gingiva were elevated, with a concomitant increase in the number of infiltrated IL-10$^+$ B cells in periodontal lesions. Such findings were consistent with those of a study focused on IL-35–induced Bregs in an experimental autoimmune uveitis (EAU) model in which the frequency of IL-10$^+$ B cells in the spleen and draining LN increased

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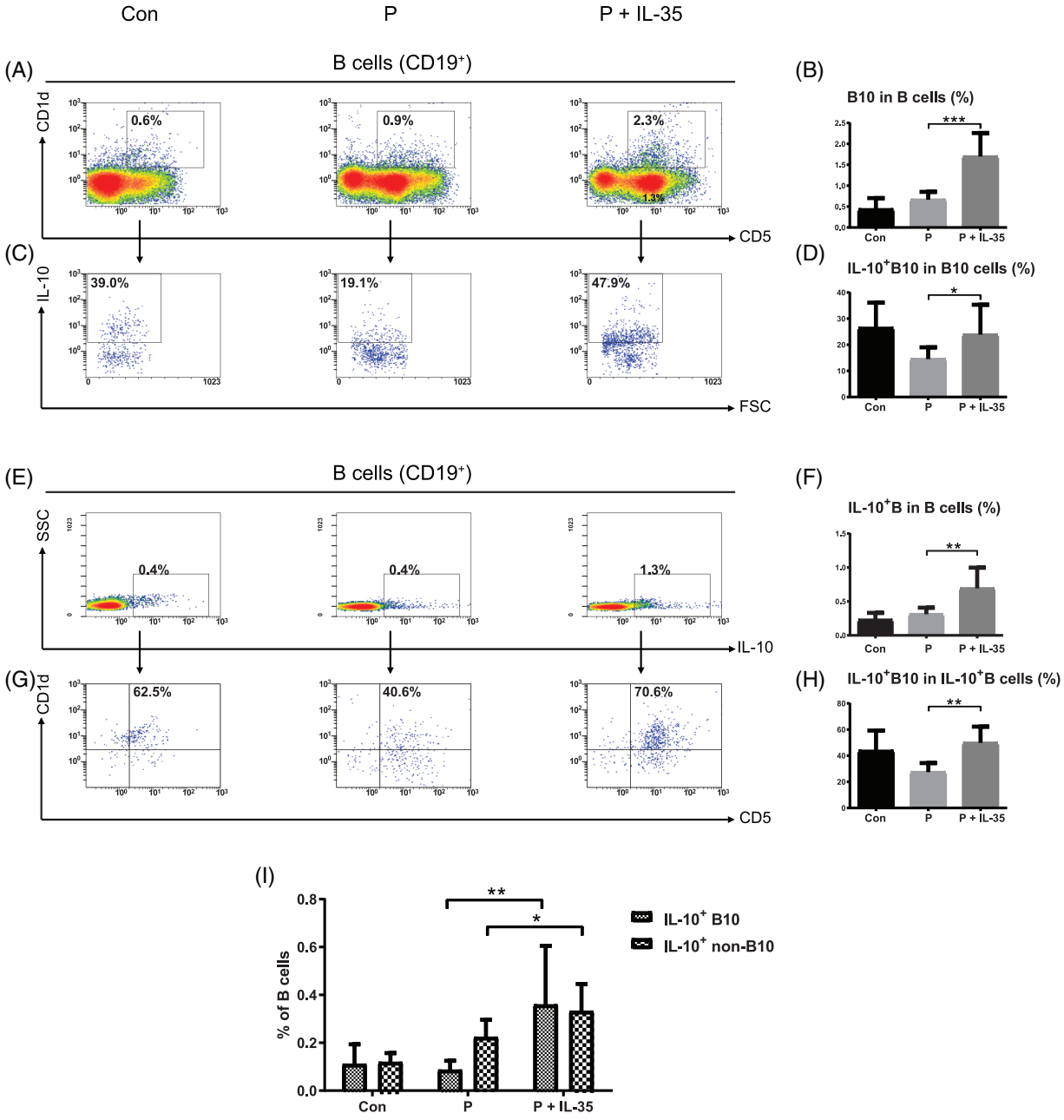

FIGURE 5 B10 and interleukin (IL)-10$^+$ B cells detection in the cervical lymph node. Single-cell suspensions were collected and labeled with designated antibodies. B cells were identified within the CD19$^+$ cell population. (A) Representative flow cytometric plots of CD1d and CD5 expression by CD19$^+$ B cells. (B) The frequencies of B10 cells among total B cells within different groups. (C) Representative flow cytometric plots of IL-10 expression by B10 cells. (D) The percentages of IL-10$^+$ B10 cells of B10 cells in different groups. (E) Representative flow cytometric plots of IL-10 expression by CD19$^+$ B cells. (F) The frequencies of IL-10$^+$ B cells among total B cells within different groups. (G) Representative flow cytometric plots of CD1d and CD5 expression by IL-10$^+$ B cells. (H) The percentages of IL-10$^+$ B10 cells of IL-10$^+$ B cells in different groups. (I) The frequencies of IL-10$^+$ B10 cells and IL-10$^+$ non-B10 cells among total B cells within different groups. Data are shown as mean ± standard deviation. FSC, forward scatter; IL, interleukin; SSC, side scatter. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

more than 4-fold after administration of 100 ng IL-35.[23] The much more significant B10 induction in that study could be attributed to the IL-35 they used, in which the peptide linking the two subunits is differed from the one used in the current study, which may affect the efficiency of the p35-Ebi3 fusion protein.

In the EAU model, one study showed that IL-35$^+$ Bregs were also contained within the Breg population of draining LN, suggesting that IL-35 induces B10 and IL-35$^+$ Breg cells in vivo.[23] Similarly, we found that gingival IL-12p35 and Ebi3 mRNA levels were upregulated following tail vein administration of IL-35. Moreover, Ebi3$^+$p35$^+$ B cells were

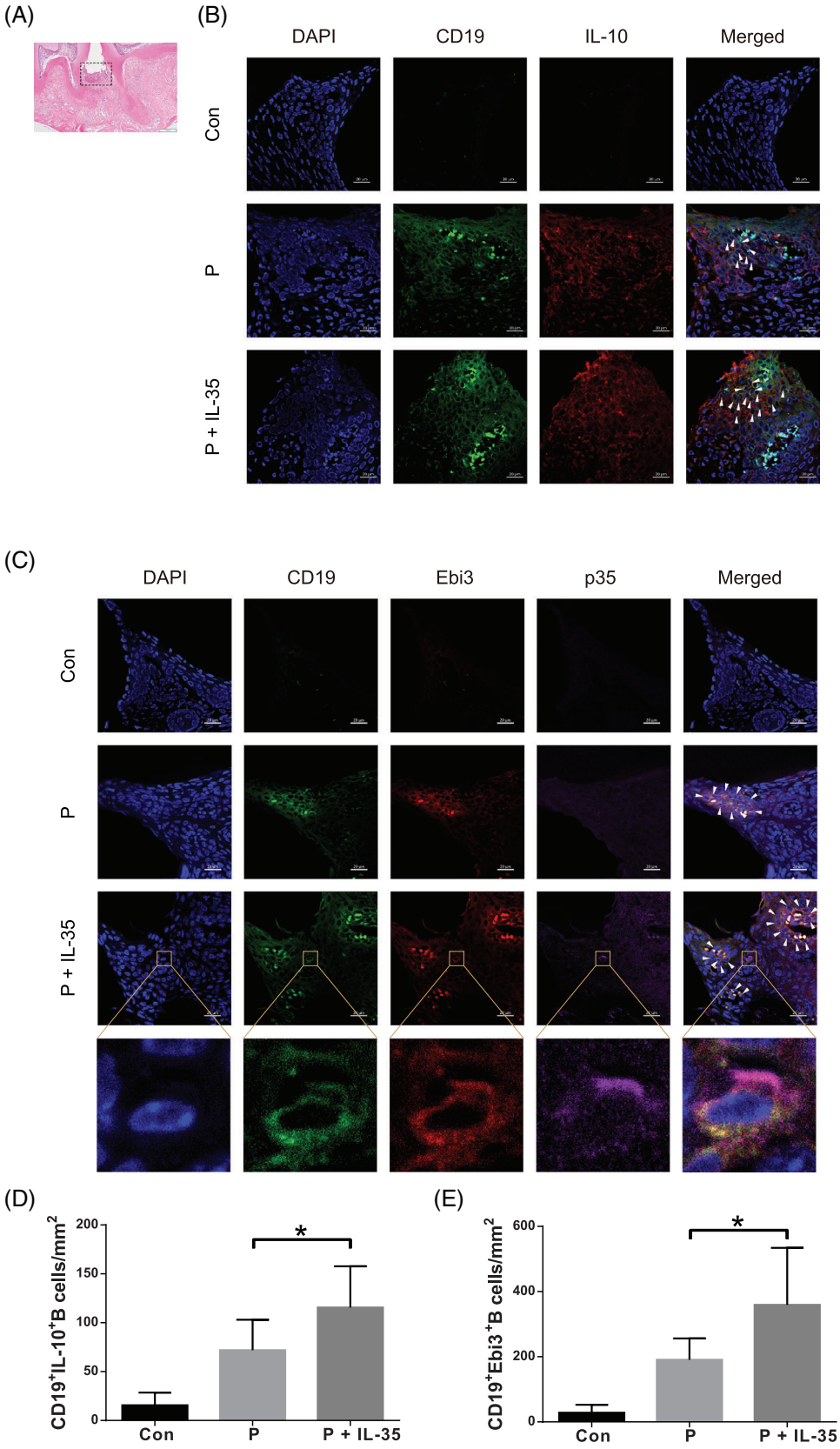

FIGURE 6 Detection of regulatory B cells (Bregs) in periodontal tissues. (A) Hematoxylin and eosin staining showed the infiltration of inflammatory cells in periodontal tissues between the maxillary first molars and the maxillary second molars, immunofluorescence staining was performed in this area to localize Bregs. (B) The colocalization of CD19 and interleukin (IL)-10 in different groups. White arrows indicate representative $CD19^+IL\text{-}10^+$ B cells in the slides. Blue indicates 4′,6-diamidino-2-phenylindole (DAPI), green indicates CD19, red indicates

observed in the periodontal inflammatory region after IL-35 treatment, although only a few were detected. However, this finding may not reflect the exact status of IL-35$^+$ Breg cell infiltration. IL-35 is a heterodimer formed extracellularly from two weakly associated subunits (IL-12p35 and Ebi3) that are secreted independently.[16] The combination of the two subunits is not only dependent on their production in which the output of Ebi3 is significantly more than its counterpart, but also on the local environment.[34] This complicates the detection of IL-35$^+$ cells, which has caused their frequencies to be underestimated.

IL-35$^+$ Bregs produce IL-35 and IL-10 directly, and expand the number of IL-10$^+$ Breg in vivo; ≈ 64% of the IL-10$^+$ Breg subset coexpress IL-10 and IL-35.[23] Because IL-35 expression in gingiva is elevated during periodontitis and positively correlated with the severity of the disease,[18,19] the existence of IL-35–induced IL-35$^+$ Bregs in periodontitis lesions indicated that there might be a positive feedback loop that negatively regulates the periodontal inflammatory immune response. In support of this hypothesis, a recent clinical study indicated that IL-35/IL-37–producing CD138$^+$CD38$^+$ plasma cells in gingiva exert immunosuppressive roles in periodontitis, and might be differentiated from precursor IL-35$^+$ Bregs.[35] Considering that the other cytokine containing Ebi3, IL-27, is also a potent antagonist of inflammation, acting through IL-10 induction and T cell regulation,[36,37] Ebi3$^+$ cells in periodontal lesions were also analyzed in the present study. We found that Ebi3$^+$ cells were mainly restricted to B cells in periodontitis lesions and increased dramatically in response to IL-35 treatment. This could partly compensate for the shortage of IL-35 immunofluorescence detection, but the results should be interpreted cautiously because of the possible role of IL-27 in this process.

The IL-12 family comprises IL-12 (IL-12p35/IL-12p40), IL-23 (IL-23p19/IL-12p40), IL-27 (IL-27p28/Ebi3), and IL-39 (IL-23p19/Ebi3) besides IL-35. IL-12 and IL-23 are proinflammatory cytokines, facilitating Th1 and Th17 polarizations, respectively. In the context of periodontitis, the expressions of IL-23 and IL-12 are both increased in periodontal lesions, whereas further study has indicated that the IL-23–induced Th17 pathway is stimulated in inflammatory periodontal lesions.[38] Like IL-35, IL-27 is also an immune suppressive member, but was not proven to be increased in periodontitis.[19] As a novel proinflammatory cytokine, increased levels of IL-39 were detected in the gingival crevicular fluid of patients with periodontitis indicating that it might also contribute to the development of periodontitis.[39] The activity of IL-35 is mediated by IL-12Rβ2/IL-27Rα in B cells,[23] and gp130/IL-12Rβ2 or homodimers of gp130 or IL-12Rβ2 in T cells.[40,41] These subunits of receptors are also shared among IL-12 family members.[40] IL-27 may compete for gp130 and IL-27Rα, while IL-12 compete for IL-12Rβ2 with IL-35.[42] In other words, the biological activity of IL-35 could be affected by other IL-12 family members as well as cell type.[43]

Through B10 and IL-35$^+$ Breg induction, systemic IL-35 administration effectively improved the established ligature-induced experimental periodontitis. The frequency of IL-10$^+$ B cells in gingiva increased approximately one-fold, which was consistent with the results of a previous study using adoptively transferred B10 cells to treat *P. gingivalis* and ligature-induced periodontitis.[12] It is worth noting that the local application of Toll-like receptor ligands and costimulatory molecules failed to expand B10 cells in gingiva, although IL-10 production was increased.[13,14] In this study, IL-35 treatment markedly reduced the expression of proinflammatory cytokines in gingiva, which were significantly increased in experimental periodontitis, and also increased the expression of immunosuppressive cytokines. These findings agreed with the results of the adoptively transferred B10 cell study. Compared with B10 cell transfer, IL-35 administration was more effective at reducing the RANKL/OPG ratio and alveolar bone resorption. This finding could be attributed to the induction of IL-35$^+$ Bregs, which support a long-lasting B10 subset via B10 expansion, and orchestrate immunosuppression in periodontitis lesions with B10 at the same time.

Additionally, the effects of IL-35 on other immune cells should also be considered. IL-35 has been confirmed to be expressed in several distinct cell types such as Tregs, B cells, dendritic cells, myeloid cells, and tumor cells.[44] It has been demonstrated that IL-35 favors the differentiation and expansion of Tregs,[45] and exert a protective effect in several disease models.[22,32] A recent study also revealed that IL-35 administration could impede experimental periodontitis through the regulation of Th cell polarization, which reduced the bone resorption by 50% to 62%.[20] However, Bregs were preferentially activated

IL-10. All images are presented at a magnification of ×400. (C) The colocalization of CD19, Epstein Barr virus-induced gene 3 (Ebi3) and p35 in different groups, images are presented at a magnification of ×400. White arrows indicate representative CD19$^+$Ebi3$^+$ B cells in the slides. Typical CD19$^+$Ebi3$^+$p35$^+$ B cell in the IL-35–treated periodontitis group is shown in the lower panel. Blue indicates DAPI, green indicates CD19, red indicates Ebi3, purple indicates p35. (D) CD19$^+$IL-10$^+$ B cell numbers per mm^2. (E) CD19$^+$Ebi3$^+$ B cell numbers per mm^2. DAPI, 4′,6-diamidino-2-phenylindole; Ebi3, Epstein Barr virus-induced gene 3; IL, interleukin. Data are shown as mean ± standard deviation.
*$p < 0.05$.

and expanded by IL-35 in other disease model. In an obese mouse model, gut microbiota–derived metabolite 3-idoleacetic acid and lipopolysaccharide administered in combination induced IL-35$^+$ B cells, but not IL-35$^+$CD4$^+$ T cells, while the latter were derived from the IL-35 produced by the former.[46] To identify the roles of a specific regulatory lymphocyte subgroup in periodontitis, overall analysis of regulatory lymphocytes in different stages of periodontitis development are needed in the future.

The present study verified the suppressive effects of IL-35–induced Bregs on established experimental periodontitis. IL-35 induced B10 cells and IL-35$^+$ Bregs, which affected the local immunoregulation in periodontitis, and thus, suggested a potential novel method of treating periodontal inflammation and alveolar bone loss. The present study, for the first time, established that systemic administration of IL-35 may induce B10 cells and IL-35$^+$ Bregs, which are engaged in local inflammation and regulate the local immune response during periodontitis.

This study also increases our understanding of the role of elevated IL-35 in periodontitis, as well as highlights a promising target for periodontal immunotherapy. Nevertheless, the pathological significance of Bregs at different stages of periodontitis may be different, and it is believed that there are overlaps and conversion between IL-35$^+$ Bregs and IL-10$^+$ Bregs. Thus, dynamic examinations and analyses of different Breg subsets are needed to fully understand their functions in the process of periodontitis. Meanwhile, the immunosuppressive effect of Bregs is a double-edged sword, and their pathogen clearance ability might be compromised.[47,48] Therefore, the characters and the functions of Bregs in the pathogenesis of periodontitis need further detailed investigations.

5 | CONCLUSIONS

The study suggested that IL-35 induced the expansion of B10 cells and IL-35$^+$ Bregs, which inhibited alveolar bone loss and regulated inflammatory and bone metabolic cytokines in periodontitis. IL-35–induced Bregs may be a novel therapeutic target for the treatment of periodontitis and provide insights into the signals involved in the generation and induction of Bregs.

AUTHOR CONTRIBUTIONS

Shiyi Li and Xiaoqian Yu contributed to the design of the study. Shiyi Li and Wenmin Zeng contributed to the establishment of the animal model. Shiyi Li, Li Su, Guojing Liu, Wenmin Zeng, and Xiaoqian Yu contributed to the sample collection and preparation. Shiyi Li, Qingxian Luan, Guojing Liu, and Xiaoqian Yu contributed to the data acquisition and analysis. Shiyi Li and Xiaoqian Yu contributed to the writing and revision of the manuscript. All the authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest related to this study.

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REFERENCES

1. Hajishengallis G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol*. 2014;35:3-11.
2. Mombelli A. Microbial colonization of the periodontal pocket and its significance for periodontal therapy. *Periodontology 2000*. 2018;76:85-96.
3. Golub LM, Lee HM. Periodontal therapeutics: current host-modulation agents and future directions. *Periodontology 2000*. 2020;82:186-204.
4. Abe T, AlSarhan M, Benakanakere MR, et al. The B cell-stimulatory cytokines BLyS and APRIL are elevated in human periodontitis and are required for B cell-dependent bone loss in experimental murine periodontitis. *J Immunol*. 2015;195:1427-1435.
5. Dai J, Bi L, Lin J, Qi F. Evaluation of interleukin-10 producing CD19(+) B cells in human gingival tissue. *Arch Oral Biol*. 2017;84:112-117.
6. Jansen K, Cevhertas L, Ma S, Satitsuksanoa P, Akdis M, van de Veen W. Regulatory B cells, A to Z. *Allergy*. 2021;76:2699-2715.
7. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity*. 2015;42:607-612.
8. Candando KM, Lykken JM, Tedder TF. B10 cell regulation of health and disease. *Immunol Rev*. 2014;259:259-272.
9. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity*. 2008;28:639-650.
10. Cao G, Zhang X, Song Y, et al. Local promotion of B10 function alleviates experimental periodontitis bone loss through antagonizing RANKL-expressing neutrophils. *J Periodontol*. 2021;92:907-920.
11. Shi T, Jin Y, Miao Y, Wang Y, Zhou Y, Lin X. IL-10 secreting B cells regulate periodontal immune response during periodontitis. *Odontology*. 2020;108:350-357.
12. Wang Y, Yu X, Lin J, et al. B10 cells alleviate periodontal bone loss in experimental periodontitis. *Infect Immun*. 2017;85:e00335-17.

13. Yu P, Hu Y, Liu Z, et al. Local induction of B cell interleukin-10 competency alleviates inflammation and bone loss in ligature-induced experimental periodontitis in mice. *Infect Immun*. 2017;85:e00645-16.
14. Hu Y, Yu P, Yu X, Hu X, Kawai T, Han X. IL-21/anti-Tim1/CD40 ligand promotes B10 activity in vitro and alleviates bone loss in experimental periodontitis in vivo. *Biochim Biophys Acta Mol Basis Dis*. 2017;1863:2149-2157.
15. Devergne O, Birkenbach M, Kieff E. Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *Proc Natl Acad Sci U S A*. 1997;94:12041-12046.
16. Egwuagu CE, Yu CR, Sun L, Wang R. Interleukin 35: critical regulator of immunity and lymphocyte-mediated diseases. *Cytokine Growth Factor Rev*. 2015;26:587-593.
17. Li X, Mai J, Virtue A, et al. IL-35 is a novel responsive anti-inflammatory cytokine–a new system of categorizing anti-inflammatory cytokines. *PloS One*. 2012;7:e33628.
18. Köseoğlu S, Sağlam M, Pekbağrıyanık T, Savran L, Sütçü R. Level of interleukin-35 in gingival crevicular fluid, saliva, and plasma in periodontal disease and health. *J Periodontol*. 2015;86:964-971.
19. Mitani A, Niedbala W, Fujimura T, et al. Increased expression of interleukin (IL)-35 and IL-17, but not IL-27, in gingival tissues with chronic periodontitis. *J Periodontol*. 2015;86:301-309.
20. Cafferata EA, Terraza-Aguirre C, Barrera R, et al. Interleukin-35 inhibits alveolar bone resorption by modulating the Th17/Treg imbalance during periodontitis. *J Clin Periodontol*. 2020;47:676-688.
21. Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 2007;450:566-569.
22. Niedbala W, Wei XQ, Cai B, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol*. 2007;37:3021-3029.
23. Wang RX, Yu CR, Dambuza IM, et al. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med*. 2014;20:633-641.
24. Percie du Sert N, Hurst V, Ahluwalia A, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLoS Biol*. 2020;18:e3000410.
25. Abe T, Hajishengallis G. Optimization of the ligature-induced periodontitis model in mice. *J Immunol Methods*. 2013;394:49-54.
26. Cai Z, Wong CK, Dong J, et al. Remission of systemic lupus erythematosus disease activity with regulatory cytokine interleukin (IL)-35 in Murphy Roths Large (MRL)/lpr mice. *Clin Exp Immunol*. 2015;181:253-266.
27. Wang Y, Mao Y, Zhang J, et al. IL-35 recombinant protein reverses inflammatory bowel disease and psoriasis through regulation of inflammatory cytokines and immune cells. *J Cell Mole Med*. 2018;22:1014-1025.
28. Hiyari S, Wong RL, Yaghsezian A, et al. Ligature-induced peri-implantitis and periodontitis in mice. *J Clin Periodontol*. 2018;45:89-99.
29. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest*. 2008;118:3420-3430.
30. Yanaba K, Yoshizaki A, Asano Y, Kadono T, Tedder TF, Sato S. IL-10-producing regulatory B10 cells inhibit intestinal injury in a mouse model. *Am J Pathol*. 2011;178:735-743.
31. Yang M, Deng J, Liu Y, et al. IL-10-producing regulatory B10 cells ameliorate collagen-induced arthritis via suppressing Th17 cell generation. *Am J Pathol*. 2012;180:2375-2385.
32. Luo Z, Lundin S, Mejia-Cordova M, et al. Interleukin-35 prevents development of autoimmune diabetes possibly by maintaining the phenotype of regulatory B cells. *Int J Mol Sci*. 2021;22:12988.
33. Suzuki M, Yokota M, Nakamura Y, Ozaki S, Murakami S. Intranasal administration of IL-35 inhibits allergic responses and symptoms in mice with allergic rhinitis. *Allergol Int*. 2017;66:351-356.
34. Collison LW, Vignali DA. Interleukin-35: odd one out or part of the family? *Immunol Rev*. 2008;226:248-262.
35. Jing L, Kim S, Sun L, et al. IL-37- and IL-35/IL-37-producing plasma cells in chronic periodontitis. *J Dent Res*. 2019;98:813-821.
36. Hall AO, Beiting DP, Tato C, et al. The cytokines interleukin 27 and interferon-γ promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity* 2012;37:511-523.
37. Stumhofer JS, Silver JS, Laurence A, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol*. 2007;8:1363-1371.
38. Ohyama H, Kato-Kogoe N, Kuhara A, et al. The involvement of IL-23 and the Th17 pathway in periodontitis. *J Dent Res*. 2009;88:633-638.
39. Sari A, Dogan S, Nibali L, Koseoglu S. Evaluation of IL-23p19/Ebi3 (IL-39) gingival crevicular fluid levels in periodontal health, gingivitis, and periodontitis. *Clin Oral Investig*. 2022;26:7209-7218.
40. Collison LW, Delgoffe GM, Guy CS, et al. The composition and signaling of the IL-35 receptor are unconventional. *Nat Immmunol*. 2012;13:290-299.
41. Garbers C, Hermanns HM, Schaper F, et al. Plasticity and cross-talk of interleukin 6-type cytokines. *Cytokine Growth Factor Rev*. 2012;23:85-97.
42. Ye C, Yano H, Workman CJ, et al. Interleukin-35: structure, function and its impact on immune-related diseases. *J Interferon Cytokine Res*. 2021;41:391-406.
43. Pylayeva-Gupta Y. Molecular pathways: interleukin-35 in autoimmunity and cancer. *Clin Cancer Res*. 2016;22:4973-4978.
44. Huang C, Li N, Li Z, et al. Tumour-derived Interleukin 35 promotes pancreatic ductal adenocarcinoma cell extravasation and metastasis by inducing ICAM1 expression. *Nat Commun*. 2017;8:14035.
45. Collison LW, Chaturvedi V, Henderson AL, et al. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*. 2010;11:1093-1101.
46. Su X, Zhang M, Qi H, et al. Gut microbiota-derived metabolite 3-idoleacetic acid together with LPS induces IL-35(+) B cell generation. *Microbiome*. 2022;10:13.
47. Goenka R, Parent MA, Elzer PH, Baldwin CL. B cell-deficient mice display markedly enhanced resistance to the intracellular bacterium Brucella abortus. *J Infect Dis*. 2011;203:1136-1146.
48. McKay JT, Egan RP, Yammani RD, et al. PD-1 suppresses protective immunity to Streptococcus pneumoniae through a B cell-intrinsic mechanism. *J Immunol*. 2015;194:2289-2299.

SUPPORTING INFORMATION

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

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