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REVIEW ARTICLE

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The therapeutic potential of regulatory T lymphocytes in periodontitis: A systematic review

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This systematic review aimed to: (a) generate a descriptive synthesis of preclinical studies assessing the therapeutic potential of regulatory T lymphocytes (Tregs) to arrest periodontitis, (b) evaluate the methodological heterogeneity of the reviewed animal studies and (c) assess the risk of bias (RoB) of the included studies. The electronic search for animal studies included the MEDLINE, EMBASE, Web of Science and LILACS databases. In addition, a manual search assessed the high-ranked scientific journals in "periodontics/immunology" and the references listed in the included studies. There were no language, year or publication status restrictions. Two independent reviewers selected and extracted the data, and Cohen's Kappa coefficient was calculated to determine the inter-examiner agreement. The Systematic Review Center for Laboratory Animal Experimentation's (SYRCLE) tool was used to assess the RoB. A total of 21 of the 425 studies obtained from the database search were included. Treg function was mainly described in *Porphyromonas gingivalis*-induced periodontitis (57.1%) in mice (76.2%), where Treg suppression was strongly related to disease progression and Treg induction was strongly related to immuno-inflammatory response reduction. Of those 21 studies, eight included eight animal experiments using three distinct therapeutic approaches, including: *P. gingivalis*-driven immunization (n = 3), retinoic acid inoculation (n = 2) and anti-inflammatory molecules in polymeric carriers (n = 3), which could modulate the Treg activity through cytokine production (interleukin-10 and transforming growth factor-β1), CC-chemokine- and CC-chemokine receptor-mediated chemoattraction (CCL22 and CCR4) or Th17-associated receptor activator of nuclear factor κB ligand (RANKL) downregulation. However, the studies with animal experiments did not specify the randomization sequences and housing conditions that were used, and therefore, 42.11% of the entries were rated as unclear RoB. Distinct therapeutic strategies involving Tregs could potentially suppress the immuno-inflammatory response and restore alveolar bone homeostasis during periodontitis. Nevertheless, important methodological variability, poor reporting of treatment effect estimates and unclear RoB suggest using caution when assessing the results of these studies.

KEYWORDS
animal experimentation, periodontitis, regulatory T lymphocytes, review

1 | INTRODUCTION

Periodontitis is an inflammatory disease with an important microbial component in which the immuno-inflammatory response against dysbiotic subgingival biofilm leads to alveolar bone resorption, resulting in tooth loss. Although bacterial dysbiosis is the principal cause of periodontitis initiation, periodontal tissue breakdown is mainly determined by the host's immuno-inflammatory response, where the T-lymphocyte subpopulations play an important role in tooth-supporting alveolar bone resorption.[1,2]

Regulatory T lymphocytes (Tregs) play a key role in the homeostatic control of the host's immuno-inflammatory response by suppressing the proliferation and cytokine production of effector T cells, mainly T helper (Th)1 and Th17 lymphocytes.[2] Nonetheless, their role during the pathogenesis of periodontitis has not been fully clarified. In fact, the scarcity of human evidence, as well as the heterogeneity among different animal models assessing the role of Tregs during the pathogenesis of periodontitis, prevents the precise interpretation of their function and their potential to suppress immuno-inflammatory-driven alveolar bone resorption.[2,3] Furthermore, it is unclear the level of risk of bias (RoB) and the comprehensiveness of reporting in many animal experiments.[4,5] This might be the case for animal studies that investigate the role and potential of Tregs to arrest periodontitis. Currently, there is no information on RoB in these experiments. High or unclear RoB could lead to either overestimation or underestimation of the results of animal studies,[5] which could lead to harmful human interventions or unjustified waste of animal resources.

Systematic reviews of animal studies aim to comprehensively review all available evidence in order to understand the quality of this research, which will be pivotal for further development of human clinical trials.[6] To date, there has been no systematic evaluation of animal studies assessing the regulatory capacity of Tregs during periodontitis. Therefore, this systematic review had three objectives: (a) to evaluate whether Treg-aimed interventions could significantly arrest periodontitis in comparison with a control in animal models, (b) to evaluate the methodological heterogeneity of animal studies on Tregs and (c) to assess the RoB of these animal studies.

2 | MATERIALS AND METHODS

2.1 | Protocol

In order to define the pre-established search strategy criteria, an a priori protocol was registered with the Preclinical Systematic Review & Meta-analysis Facility (SyRF), supported by the National Centre for Replacement, Refinement & Reduction of Animals Research (NC3Rs). This protocol and registration can be found at http://syrf.org.uk/protocols. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement and the guideline for the reporting of systematic reviews and meta-analyses of animal experiments proposed by Peters et al. were consulted to guide the search and report on the findings obtained from this systematic review (Table S1).[6,7]

2.2 | Eligibility criteria

Publications that met the following criteria were included: randomized animal studies involving non-human primates, pigs, dogs or rodents (population), which evaluated the therapeutic strategies assessing Tregs, considered to be $CD4^+Foxp3^+$, $CD4^+CD25^+$, $CD4^+CD25^+Foxp3^+$ or $CD4^+CD25^{high}CD127^{low}Foxp3^+$ cells (intervention), and their function/activation/differentiation/quantity/interaction with other immune cells/cytokines during the resolution of experimental periodontitis by means of alveolar bone resorption reduction/arrest (outcome). Experiments were considered randomized when at least two groups were included (comparator), and the authors reported that the animals were randomly assigned to either the control or intervention group. Papers that did not use a randomized design were excluded. There were no language, year or publication status restrictions for inclusion. For eligible studies in languages different from English, Spanish or French, a qualified translator was consulted.

2.3 | Outcome measures

In order to assess the potential of Tregs to arrest periodontitis, the primary outcome measure was the reduction of alveolar bone resorption in the intervention group in comparison with the control. The secondary outcome measures were the modified Tregs characteristics (quantity of local Tregs, Tregs markers' mRNA expression and/or cytokine/chemokine production) and variations in the inflammatory infiltrate (quantity of local pro-inflammatory cells, pro-inflammatory markers mRNA expression and/or pro-inflammatory cytokine/chemokine production) in the intervention group in comparison with the control. The secondary outcome measures were chosen due to the growing evidence supporting the fact that the increase in Tregs, and its related factors, in gingival tissues helps to protect the host from inflammatory-driven bone resorption. Therefore, these outcome measures might be related to treatment success.[8]

2.4 | Literature search

The MEDLINE, EMBASE, Web of Science and LILACS databases were searched up to May 2017. OpenGrey (System for Information on Grey Literature in Europe), PQDT-ProQuest and the Grey Literature Report from the New York Academy of Medicine sources were searched for grey literature. In addition, a manual search was conducted in the following periodontics and immunology-related journals that have the highest impact factor according to the 2016 ISI Thomson Reuters Impact Factor List: *Journal of Dental Research*, *Journal of Clinical Periodontology*, *Journal of Periodontology*, *Journal of Periodontal Research*, *Journal of Periodontal and Implant Science*, *Immunity*, *The Journal of Immunology*, *Nature Immunology*, *Journal*

of Allergy and Clinical Immunology, and *Journal of Experimental Medicine*. The references listed in the included papers were also checked for additional studies. Two reviewers (EAC and AJ) performed the electronic database survey independently and in duplicate. The search strategies used in this review are included in Appendix S1.

2.5 | Data selection and extraction

Two reviewers (EAC and AJ), independently and in duplicate, assessed the titles and abstracts to determine their initial potential inclusion. Then, full-text articles were analysed to decide whether the studies met the inclusion criteria. When disagreements occurred, article selection was discussed until consensus was reached. Excluded studies, and the reasons for their exclusion, were recorded. The following information was extracted from each study: first author and year of publication (for reference), animal model (number/groups/race/species/strain), experimental periodontitis induction (pathogen/administration via), Treg assessment (sample/method of analysis/cells, transcription factors and cytokines assessed) and results (when applied, reported as means and standard deviations or medians and ranges or interquartile ranges, regarding bone resorption or inflammatory infiltrate reduction). Disagreements were resolved by discussion, and a third reviewer (RV) examined all the extracted data, in order to capture all relevant information.

2.6 | Risk of bias assessment

Two independent and calibrated reviewers (EAC and GM) used the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) risk of bias tool[9] to rate the RoB of the experiments reported in the studies. Disagreements about rating RoB were resolved by a third reviewer (RV). SYRCLE is derived from the Cochrane's Risk of Bias tool[10] for clinical studies, and it was adapted to be applied to animal studies. The resulting tool consists of 10 main questions related to selection bias, performance bias, detection bias, attrition bias, reporting bias and other biases.[8] Signalling questions were used to support the main questions in order to determine the RoB. The responses to the tool's questions were answered as Yes (question adequately answered), No (question not answered) or Unclear (not enough information to answer yes or no). Based on the answers to the signalling questions, the RoB domains were classified as low, high or unclear RoB. An overall RoB was not evaluated due to the difficulty of assigning weights for the distinct domains.

2.7 | Calibration of the reviewers

The reviewers were calibrated by assessing 10% of the titles and abstracts obtained in the initial MEDLINE database search. Inter-rater agreement was assessed until the reviewers achieved a Cohen's Kappa (κ) coefficient $\kappa > 0.5$ for every step of the review.

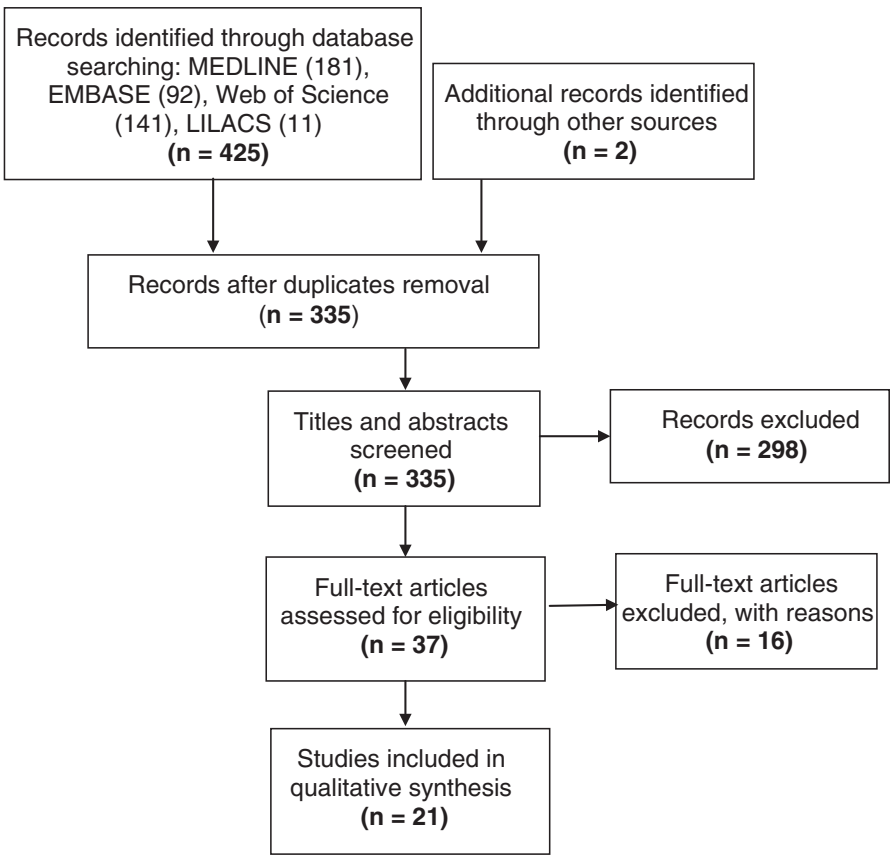

FIGURE 1 PRISMA Flow Diagram of the study selection process

Disagreements were resolved by discussion and approved by a third reviewer (RV). Cohen's κ was calculated as follows: κ = 1 in the case of complete concordance and κ = 0 if no match could be found.[11]

3 | RESULTS

Initially, 425 studies were identified in the electronic databases: MEDLINE (n = 181), EMBASE (n = 92), Web of Science (n = 141) and LILACS (n = 11). No further literature was found in the other sources (Figure 1). After removal of duplicates (κ = 0.74), 335 records were assessed by title and abstract, and 37 studies were selected for full-text eligibility (κ = 0.95). Finally, 21[3,8,12-30] articles met the inclusion criteria and eight[12,13,16-19,21,29] of them included interventions for the immuno-modulation of Tregs (κ = 1). The main characteristics of the included studies and the list of excluded studies, with the rationale for their exclusion, are reported in Tables S2 and S3, respectively.

3.1 | Interventions for immuno-modulation targeting Tregs

Among the included studies, eight reported an interventional approach to treat periodontitis through distinct strategies to increase the number of Tregs or the levels of the anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β1, which could be, at least in part, produced by Tregs (Table 1).[12,13,16-19,21,29] *Porphyromonas gingivalis*-derived immunization was attempted in three studies,[12,16,19] retinoic acid compounds were used in two studies,[17,18] controlled release of anti-inflammatory molecules by polymeric carriers was utilized in two studies,[13,21] and an immunomodulatory agent derived from fungi was used in one study.[29] Overall, studies reported resolution of periodontitis when alveolar bone loss was arrested[12,13,17,18,21,29] and inflammatory cell infiltration decreased.[12,17,18] Furthermore, these results were positively correlated with the expression and production of anti-inflammatory cytokines such as IL-10[12,17-19] and TGF-β1,[12,17,18,21,29] and with the reduction of IL-17.[12,13,17-19,22] Although changes regarding the Treg

TABLE 1 Interventional studies targeting Tregs in experimental periodontitis

		Study	Periodontitis model	Intervention	Results
1	Wang (2015)[12]	Mice	Oral inoculation with *P. gingivalis*	Formalin-killed *P. gingivalis* vaccination	A diminished alveolar bone resorption and inflammatory cell infiltration in periodontal lesions, attributed to the downregulation of Th17 cell response (decrease in $CD4^+ROR\gamma t^+$, IL-17, $RANKL^+CD4^+$ cells and mRNA expression) and upregulation of Treg response ($CD4^+Foxp3^+$, IL-10 and TGF-β1 expression)
2	Napimoga (2012)[13]	Mice	Oral inoculation with *A. actinomycetemcomitans*	15d-PGJ_2 in PLGA nanocapsules	15d-PGJ_2 was able to inhibit periodontal bone loss by reducing the expression of RANKL and lymphocyte infiltration. IL-17, IL-6, IL-15, Foxp3, CCL22, IL-10 and TGF-β1 expressions were also diminished
3	Jeong (2015)[16]	Mice	Oral infection with *P. gingivalis*	*P. gingivalis* HSP60 protein immunization	Only Pg14 peptide from HSP60 induction showed an increase in the number of $CD4^+CD25^+$ Tregs, with a 50% reduction of Th1 cells. Also, a higher concentration of TGF-β1 and an increment in Foxp3 and Nr4a mRNA expression from Tregs
4	Wang (2014)[17]	Mice	Oral inoculation with *P. gingivalis*	All-*trans* retinoic acid (ATRA)	ATRA inhibited periodontal bone resorption and inflammatory cell infiltration, decreasing the number of $CD4^+ROR\gamma t^+$ Th17 cells and downregulation of IL-17 and RANKL, while increasing the percentage of $CD4^+$ Foxp3 Tregs and upregulation of IL-10 and TGF-β1
5	Jin (2014)[18]	Mice	Oral infection with *P. gingivalis*	Tamibarotene (Am80)	Am80 ameliorates periodontal bone loss, suppressing Th17-related cytokines and enhancing Treg-related cytokines with a downregulation of RORγt expression and an upregulation of IL-10, TGF-β1 and Foxp3
6	Hagiwara (2014)[19]	Mice	Intravenous injection with *P. gingivalis*	*P. gingivalis* HSP60 (rGroEL) immunization	rGroEL immunization increased IFN-γ–producing $CD4^+Foxp3^+$ T cells, IL-10–producing $CD4^+Foxp3^+$ Tregs and $CD4^+Foxp3^-$ Tregs could control inflammation induced by *P. gingivalis*
7	Glowacki (2013)[21]	Mice	Oral inoculation with *A. actinomycetemcomitans*	CCL22 in PLGA microparticles	CCL22 release fully restored Treg migration to periodontal tissues along with the production of IL-10, TGF-β1 and OPG
8	Breivik (2005)[29]	Rats	Silk ligature induction	Orally administrated β-1,3/1,6-glucan	β-1,3/1,6-glucan enhanced resistance to ligature-induced periodontitis, showing an increased HPA axis, TGF-β1 and IL-10 response

15d-PGJ_2, 15-deoxy-delta-12,14-prostaglandin J_2; CCL, CC-chemokine ligand; CD, cluster of differentiation; HPA, hypothalamic-pituitary-adrenal; HSP60, heat-shock protein 60; IFN, interferon; IL, interleukin; PLGA, polylactic-co-glycolic acid; RANKL, receptor activator of nuclear factor kappa B ligand; rGroEL, bacterial chaperonin rGroEL; TGF, transforming growth factor; β-1,3/1,6-glucan, beta-glucan from *Saccharomyces cerevisiae* cell wall.

and Th17 subpopulations were frequently reported, none of the included studies reported data specifically addressing variations in the Treg/Th17 ratio.

3.1.1 | *P. gingivalis*-driven immunization

Alveolar bone resorption

A study on vaccination with killed *P. gingivalis* reported a significant decrease in alveolar bone resorption in the bacteria-vaccinated group in comparison with the sham-vaccinated group ($P < 0.01$).[12] Other strategies using similar approaches[16,19] did not refer to the arrest of alveolar bone resorption as an outcome measure.

Treg characteristics and variations among inflammatory infiltrates

Vaccination with killed *P. gingivalis* therapy also increased the percentage of $CD4^+Foxp3^+$ Tregs in cervical lymph nodes and blood (mean (M) = 10.37, standard deviation (SD) = 0.32 and M = 6.98, SD = 0.34, respectively) in comparison with the sham-vaccinated group (M = 9.09, SD = 0.20, $P < 0.05$).[12] That study also reported significant reductions in the percentages of $CD4^+Foxp3^+ROR\gamma t^+$ cells (M = 0.54, SD = 0.05) in comparison with the sham-vaccinated group (M = 0.74, SD = 0.04, $P < 0.05$). Moreover, the subpopulation of Th17 cells also decreased in the bacteria-vaccinated group (M = 2.57, SD = 0.05) in comparison with sham-vaccinated group (M = 5.26, SD = 0.90, $P < 0.05$), in the cervical lymph nodes. Accordingly, IL-10 and TGF-β1 mRNA expression also increased in the bacteria-vaccinated group in comparison with the sham-vaccinated group ($P < 0.01$).[12] Next, immunization with the peptide 14 from the *P. gingivalis* heat-shock protein (Pg14) resulted in an increase in the percentage of $CD4^+CD25^+Foxp3^+$ Tregs (~2.6-2.9 times) and in a significant increase of Foxp3 expression (1.3 times higher) in the Pg14-vaccinated group compared to all other groups ($P < 0.05$).[12] Nevertheless, the same intervention with Pg19 resulted in a 6.2 times higher increment of $CD4^+IFN\text{-}\gamma^+$ Th1 lymphocytes, 3.3 times higher IL-6 concentration and 4.3 times higher IFN-γ expression in the Pg19-vaccinated group in comparison with the control group ($P < 0.05$), in the spleen.[16] Moreover, immunization with a pathogen-derived heat-shock protein (GroEL) from *P. gingivalis* showed a significant increase in the percentage of IFN-γ–producing $CD4^+Foxp3^+$ cells (M = 3.3, SD = 0.2 vs M = 0.4, SD = 0.2, $P < 0.05$), IL-10–producing $CD4^+Foxp3^+$ cells (M = 3.4, SD = 0.1 vs M = 0.6, SD = 0.2, $P < 0.05$) and IL-10–producing $CD4^+Foxp3^-$ Tregs (M = 0.9, SD = 0.1 vs M = 0.3, SD = 0.1, $P < 0.05$) in the GroEL-vaccinated group in comparison with control, detected in the submandibular gland. However, neither TGF-β1 nor IL-4 were detected in any of the groups.[19]

3.1.2 | Inoculation with retinoic acid-derived compounds

Alveolar bone resorption

All-*trans* retinoic acid (ATRA), a vitamin A active metabolite that plays a role in immune homeostasis, was reported to result in the markedly significant arrest of alveolar bone resorption ($P < 0.001$) in the inoculated group in comparison with the control group.[17] Similarly, an intervention with Tamibarotene (Am80), a synthetic retinoic acid receptor, was also reported to significantly reduce alveolar bone loss ($P < 0.01$).[18]

Treg characteristics and variations among inflammatory infiltrates

Treatment with ATRA successfully induced the expression of IL-10 (1.5-fold, $P < 0.01$) and TGF-β1 (0.6-fold, $P < 0.05$) in the inoculated group in comparison with the control group.[17] In addition, treatment with ATRA further increased the percentage of $CD4^+Foxp3^+$ Tregs ($P < 0.001$) and decreased the percentage of $CD4^+ROR\gamma t^+$ Th17 lymphocytes ($P < 0.05$) in the inoculated group in comparison with the control group, detected in the cervical lymph nodes.[17] Treatment with Am80 further increased the percentage of $CD4^+Foxp3^+$ Tregs and decreased the percentage of $CD4^+ROR\gamma t^+$ Th17 lymphocytes in the inoculated group in comparison with the control group ($P < 0.05$), detected in the cervical lymph nodes or gingival mucosa.[18]

3.1.3 | Anti-inflammatory molecules in polymeric carriers

Alveolar bone resorption

Treatment with chemokine ligand 22-polylactic-co-glycolic acid microparticles (CCL22-PLGA) successfully resulted in less bone resorption compared to the control group (M = 78.0, SD = 7.18 vs M = 115.4, SD = 12.07 μm^2, $P < 0.05$).[21] Additionally, the same intervention performed in canines also prevented alveolar bone resorption (M = 47.07, SD = 3.77 vs M = 65.71, SD = 3.77 mm, $P = 0.03$).[21] Treatment with 15-deoxy-prostaglandin-J_2 (10 μg/kg), a cyclopentanone-type prostaglandin loaded in PLGA nanocapsules (15d-PGJ_2-NC), also showed a significant decrease in alveolar bone loss in the treated group in comparison with the control group (M = 65.18, SD = 25.12 vs M = 114.10, SD = 43.13 μm, $P < 0.05$).[13] Apart from that, treatment with soluble β-1,3/1,6-glucan from *Saccharomyces cerevisiae* significantly inhibited bone resorption in rats in comparison with the control group (M = 0.92, SD = 0.10 vs M = 1.03, SD = 0.09 mm, $P = 0.016$).[29]

Treg characteristics and variations among inflammatory infiltrate

Treatment with CCL22-PLGA selectively induced Treg chemoattraction towards the infected lesions, accompanied by an increase in the expression and production of IL-10 and TGF-β1 ($P < 0.05$), and a decrease in the expression and production of IL-17, IFN-γ, TNF-α and RANKL ($P < 0.05$).[21] However, treatment with 15d-PGJ_2-NC (10 μg/kg) resulted in a reduction of the number of $CD4^+CD25^+Foxp3^+$ Tregs.[13] Moreover, β-1,3/1,6-glucan treatment increased the production of TGF-β1 in the infected group in comparison with the control group (M = 34.04, SD = 5.83 vs M = 27.78, SD = 8.02 pg/mL, $P = 0.032$).[29]

3.2 | Methodological heterogeneity

Distinct animal experimentation protocols were used in the reviewed studies. The murine experimental models were used in 100% of the studies,[3,8,12-30] from which the mouse model was the most frequently used animal model (76.2%).[3,12-23,25,26,28,30] In the studies using mice, 10 studies used the C57BL/6 strain,[3,12,14-17,21-23,26] four studies used the BALB/c strain,[13,18,21,25] and three studies did not specify the animal strain used.[19,28,30] In addition, seven studies used only wild-type mice,[12,13,17,18,22,25,26] nine studies used both wild-type and knockout mice,[3,14-16,19,22,23,28,30] and one study used wild-type, knockout and the 10BiT mice,[22] corresponding to a IL-10 reporter animal. From them, only eight studies[3,12,13,16,17,19,28,30] reported the number of mice that were analysed (M = 22.6, SD = 8.30, total = 181 mice). In the studies using rats, three studies used the Wistar strain,[24,27,29] two studies used the Sprague Dawley strain,[8,30] and one study used the Zucker Diabetic Fat strain.[20] All the studies reported the number of rats that were analysed (M = 33.3, SD = 7.76, total = 200 rats). Only one study[21] used also beagle dogs, without reporting the number of animals used (Tables S2 and S4).

Moreover, there was substantial heterogeneity among the protocols to induce experimental periodontitis (Table 1 and Table S4). *P. gingivalis* was chosen in 57.1% of the studies,[3,8,12,14,16-19,21-23,25,28] including distinct bacterial serotypes, lipopolysaccharide (LPS), heat-shock proteins (HSP) and fimbriae. On the other hand, *Aggregatibacter actinomycetemcomitans* strains were used in five studies.[13,15,21,24,26] In addition, three studies opted to use LPS from *Escherichia coli*[17,19,20] and others induced periodontitis by ligature accompanied by or compared to bacterial infection.[8,20,21,27] Different terms were used to refer to infection protocols, such as oral inoculation, oral infection and oral gavage, with concentrated suspensions ranging from 1×10^7 to 1×10^{10} CFU/mL. Nevertheless, regarding the intervention studies,[12,13,16-19,21,29] time for intervention was the most variable feature among the reviewed studies; for the most part, there were no repeated protocols among the studies. Experimental periodontitis was confirmed in all the studies that assessed alveolar bone resorption. Size estimates for the alveolar bone resorption assessment were reported exclusively using bar graphs in seven of eight interventions, and *P*-values were provided; however, measures of uncertainty, such as confidence intervals, were not reported. After contacting the authors, more detailed information was obtained including individual data from each animal experiment, which allowed us to report and calculate size estimates (M and SD) and not only *P*-values, as was originally reported in the articles.[13,15,21] Methodological variations among the infection protocols and interventions, as well as the absence of the exact size effects reported in the results, hindered the ability to conduct a meta-analysis (Figure 2 and Table S4).

3.3 | Risk of bias for the included studies

Risk of bias Assessment using SYRCLE's tool led to a total of 672 entries (Tables S5 and S6). Of them, 42.26% of the entries were answered as Yes, 11.63% as No and the remaining 42.11% as Unclear. Most of the SYRCLE's items (#1, #3, #5, #6, #7 and #8) were marked as Unclear.

None of the studies using immuno-modulatory intervention scored Yes in Item #1 (items reported in full in Table 2). Most of the other items were rated as Unclear due to the lack of reporting of randomization methods during the allocation sequence, although in three of the studies the randomization method was mentioned and consequently rated as Yes*[12,18,19] (Table 2). Only one study[17] was marked as Unclear in Item #2 because it did not report the animal group distribution. Moreover, Item #3 was marked as Unclear for all the studies due to the total absence of reporting on randomization while assigning animals to the intervention/control groups. Item #4 was marked as Yes in almost all cases, due to the unlikeliness of the results being influenced by not randomly housing the animals. Items #5, #6 and #7 were about blinding during the intervention or when assessing the study's outcome. Most of the studies indicated that the researchers were blinded about the interventions;[12,13,17,19,27] nevertheless, the blinded outcome assessment (Items #6 and #7) was frequently marked as Unclear. Missing outcome data (Item #8) was frequently mentioned either as supplemental material or as not

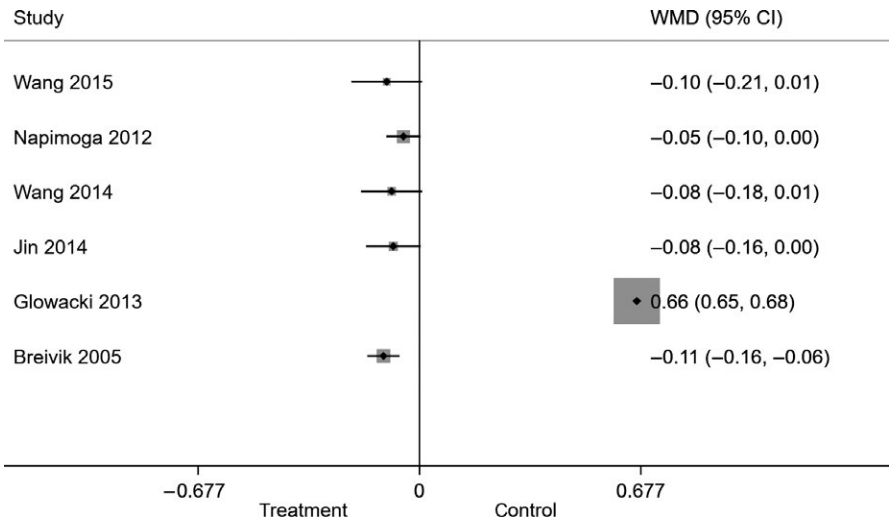

FIGURE 2 Forest plot showing heterogeneity between potential studies for meta-analysis regarding alveolar bone resorption

TABLE 2 Risk of bias of animal interventions targeting Tregs

		Studies							
	SYRCLE's Tool	Wang[12]	Napimoga[13]	Jeong[16]	Jin[18]	Wang[17]	Hagiwara[19]	Glowacki[21]	Breivik[29]
1	Was the allocation sequence adequately generated and applied?	Yes*	Unclear	No	Unclear	Yes*	Yes*	Unclear	Unclear
	*Did the investigators describe a random component in the sequence generation process such as: Referring to a random number table; Using a computer random number generator.	Unclear	Unclear	No	Unclear	Unclear	Unclear	Unclear	Unclear
2	Were the groups similar at baseline or were they adjusted for confounders in the analysis?	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes
	*Was the distribution of relevant baseline characteristics balanced for the intervention and control groups?	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes
	*If relevant, did the investigators adequately adjust for unequal distribution of some relevant baseline characteristics in the analysis?	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes
	*Was the timing of disease induction adequate?	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
3	Was the allocation to the different groups adequately concealed during?	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
	*Could the investigator allocating the animals to intervention or control group not foresee assignment due to one of the following or equivalent methods? Third-party coding of experimental and control group allocation central randomization by a third party Sequentially numbered opaque, sealed envelopes	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
4	Were the animals randomly housed during the experiment?	Yes	Yes	Yes	Yes	Yes	Yes	No	Unclear
	*Did the authors randomly place the cages or animals within the animal room/facility? Animals were selected at random during outcome assessment	Yes	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
	*Is it unlikely that the outcome or the outcome measurement was influenced by not randomly housing the animals?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
5	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?	Yes*	Yes	Unclear	Yes	Unclear	Yes*	Unclear	Yes
	*Was blinding of caregivers and investigators ensured, and was it unlikely that their blinding could have been broken?	Yes	Yes	Unclear	Yes	Unclear	Yes	Unclear	Yes
6	Were animals selected at random for outcome assessment?	Yes	Unclear	Unclear	Unclear	Unclear	Unclear	No	Unclear
	*Did the investigators randomly pick an animal during outcome assessment, or did they use a random component in the sequence generation for outcome assessment?	Yes	Unclear	Unclear	Unclear	Unclear	Unclear	No	Unclear

(Continues)

TABLE 2 (Continued)

		Studies							
	SYRCLE's Tool	Wang[12]	Napimoga[13]	Jeong[16]	Jin[18]	Wang[17]	Hagiwara[19]	Glowacki[21]	Breivik[29]
7	Was the outcome assessor blinded?	Yes	Yes	Yes	Yes	Unclear	Unclear	Unclear	Yes
	*Was blinding of the outcome assessor ensured, and was it unlikely that blinding could have been broken?	Unclear	Unclear	Unclear	Yes	Unclear	Unclear	Unclear	Yes
	*Was the outcome assessor not blinded, but do review authors judge that the outcome is not likely to be influenced by lack of blinding?	Yes	Yes	Yes	Yes	No	No	No	Yes
8	Were incomplete outcome data adequately addressed?	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
	*Were all animals included in the analysis?	Unclear	Unclear	Unclear	Yes	Yes	Yes	Unclear	Yes
	*Were the reasons for missing outcome data unlikely to be related to true outcome? (eg, technical failure)	Yes	Yes	Yes	Unclear	Yes	Yes	Unclear	Yes
	*Are missing outcome data balanced in numbers across intervention groups, with similar reasons for missing data across groups?	Unclear	Yes	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
	*Are missing outcome data imputed using appropriate methods?	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
9	Are reports of the study free of selective outcome reporting?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	*Was the study protocol available and were all of the study's pre-specified primary and secondary outcomes reported in the current manuscript?	No	Yes	No	No	No	No	No	No
	*Was the study protocol not available, but was it clear that the published report included all expected outcomes (ie comparing methods and results section)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
10	Was the study apparently free of other problems that could result in high RoB?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	*Was the study free of contamination (pooling drugs)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	*Was the study free of inappropriate influence of funders?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	*Was the study free of unit of analysis errors?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	*Were design-specific risks of bias absent?	No	No	No	No	No	No	No	No
	*Were new animals added to the control and experimental groups to replace drop-outs from the original population?	No	No	No	No	No	No	No	No

Item #1 was answered as Yes, when random allocation was mentioned but no reference to a sequence generation process. In particular, allocation is often referred to a third party generally associated with the maintenance of the animal facility and not directly involved to the investigators or the project itself; hence, allocation is "random" for investigators. Item #2 was answered as Yes or Unclear, if two or more of the signalling questions were answered as Yes or Unclear, respectively. Item #4 was answered as Yes, if one of the signalling questions was answered as Yes, when one of the signalling questions was answered as No and the other as Unclear then the principal item was rated as Unclear. Item #5 was answered as Yes*, when blinding from knowing which animals were in intervention/control groups was mentioned but there is no reference of how it was assured. Item #7 was answered as Yes, if one of the signalling questions was answered as Yes, when one of the signalling questions was answered as No and the other as Unclear then the principal item was rated as Unclear. Item #8 was rated as Unclear, if two or more of the signalling questions were answered as Unclear; if there was a tie between Yes/No and Unclear among the signalling questions, the principal item was rated as Unclear. Item #9 was rated as Yes, if one of the signalling questions was rated as Yes. Item #10 was rated as Yes, if most of the signalling questions were answered as Yes.

likely to influence the final results; nonetheless, there was no reference to the final number of animals used across all studies. Finally, selective outcome reporting or other sources of bias were frequently detected as inconsistencies between material and methods section and the reporting of results in all of the reviewed studies (Items #9 and Item #10).

4 | DISCUSSION

The interventions aiming to increase Treg activity during experimental periodontitis included in this systematic review succeeded in arresting alveolar bone resorption in the experimental groups in comparison with the control animals, with the resulting overall increase in the production of IL-10 and TGF-β1 and detection of $CD4^+CD25^+Foxp3^+$ cells. Although the lack of reporting regarding randomization procedures primarily led to RoB rated as "Unclear", substantial methodological heterogeneity and inadequacy of size estimates that were reported hindered the quantitative synthesis of the results.

4.1 | Interventions for immuno-modulation targeting Tregs

Vaccination targeting key pathogens, such as *P. gingivalis*, can inhibit the progression of periodontitis by eliciting the expression of specific antibodies against *P. gingivalis*, by using derived structures: extracellular capsule, fimbriae, gingipains, hemagglutinin or heat-shock proteins.[12,16,19,31] Promising results show attenuation of alveolar bone resorption and inflammation, attributed to the downregulation in the expression of RORγt, IL-17 and RANKL in Th17 lymphocytes and the upregulation of Treg response by increasing their number and increasing the expression of IL-10 and TGF-β1.[12,31] Conversely, other studies have reported that immunization with *P. gingivalis* favours the polarization of Th17 response and the subsequent alveolar bone loss by inducing the RANKL production.[26,32] These conflicting results are discussed by the authors,[12,33] who argue that concentration of inoculated bacteria and periodontitis induction methods that are used may modify the expected events. For instance, subcutaneous injection with formalin-killed bacteria could have a protective effect in comparison with using live bacteria.[12,33] Nonetheless, it is presumed that antigen exposure could lead to a stronger response of the adaptive immune response and the consequent periodontitis initiation and progression.

Other approaches used to regulate the Th17/Treg imbalance successfully reduced *P. gingivalis*-induced alveolar bone loss by increasing the number of Tregs.[17,18] These approaches were based on the use of ATRA or Am80, which bind in an unspecific way to retinoic acid receptors (RAR)-α, RAR-β or RAR-γ in Th17 lymphocytes to suppress the expression of RORγt. Retinoid-based treatments have already been tested for other inflammatory diseases, such as graft-versus-host disease and rheumatoid arthritis. Nevertheless, conflicting results including the downregulation of TGF-β1 expression and decrease in the number of $Foxp3^+$ cells have also been reported.[34] Different in vivo models, experimental conditions and administration methods seem to affect the metabolism of retinoids.

4.2 | Methodological heterogeneity

Lack of homogeneity among the interventions for the immunomodulation of Tregs studies hindered the conducting of a meta-analysis. In the analysed studies, methodological heterogeneity was explained by the substantial differences in the types of experimental periodontitis protocols used, the time and duration of the infections, and the type of bacterial strain used. In addition, the authors vaguely described the *modus operandi* to induce periodontitis, referring partially to "oral inoculation", "oral infection", "oral delivery", "oral infection" or simply to "injection", without specifying further detailed information. Although we considered all strategies used to induce periodontitis as "oral gavage", we also found differences regarding the reported time of collecting the samples after infection (Table S4).[12,13,17,19,27] The lack of a complete report makes the reproducibility of these animal experiments challenging, if not impossible.

Furthermore, only a few studies[13,21,29] explicitly reported the size estimates of the treatment effects with measures of variability, such as SD (Table S4); instead, *P*-values within the inequality thresholds were frequently reported. The exact meaning of *P*-values can easily be misinterpreted regarding what they truly mean, when they are selectively reported and when they are not accompanied by estimates of effect size and uncertainty.[35] Inadequate reporting of exact size effects and measures of uncertainty diminishes the chances for the objective understanding of the results and further prevents its quantitative appraisal. Taken together, these findings suggest a lack of adherence to ARRIVE guidelines,[36] making animal experiments more difficult to reproduce, leading to questionable results and a waste of resources.

4.3 | Risk of bias

Evaluation of RoB of the included studies frequently resulted in the RoB being rated as "Unclear", due to the lack or absence of reported information that prevented the adequate judgement of RoB. For example, regarding SYRCLE's item #1, most of the authors reported "allocation concealment" in their experiments, though none of them referred an explicit random component in the sequence generation. Similarly, SYRCLE's item #3 was always rated as "unclear", where none of the studies described any person in charge of randomly allocating animals into the intervention groups. Moreover, most of the "Unclear" ratings were related to the SYRCLE's items that evaluated randomization before intervention or during outcome assessment. These findings acknowledge an important "reporting bias" in studies using animal experimentation to investigate periodontitis, where authors could hypothesize that the use of syngenic animals in controlled environments could make randomization in trials less relevant. In fact, several guidelines for animal studies, including SYRCLE[9] and PREPARE,[37] highlight how allocation conditions or

the time of the intervention could influence the results. These allocation conditions mainly refer to the stress produced by light exposure within cages in a rack causing alterations of behaviour and circadian rhythms, or increase in temperature causing changes in the metabolism and/or pharmacodynamics in the animals.[9] For example, alveolar bone resorption significantly varies depending on the time of the day the orthodontic forces are applied.[37] Another example is the increased alveolar bone loss and gingival inflammation caused by fatigue and sleep deprivation in a rat model of periodontitis.[38] The findings reported above encourage the collective improvement of reporting animal experiments that adhere to ARRIVE guidelines.[36] Thus, special consideration should be given to animal housing facilities, personnel manipulation and randomization protocols, which could eventually homogenize preclinical trials allowing for proper assessment and synthesis of the results.

4.4 | Strength and limitations

Narrative reviews about the potential efficacy of immuno-therapies involving Tregs exist;[31,39] however, the lack of a systematic approach to identify the literature in these reviews might have resulted in some important information being omitted from the analysis. The present study is the first systematic assessment of animal studies targeting the capability of Tregs to treat periodontitis and the first to evaluate their RoB. The exhaustive search for the available literature with a priori defined outcome measures substantially reduced the probability of publication bias and selective outcome reporting.[40] Nevertheless, the lack of meta-analysis in this systematic review hinders its ability to provide a precise, quantitative assessment of the results.

4.5 | Future research directions

Immuno-modulatory therapies are in continuous development in the arena of bone resorptive diseases. Considerable efforts have been made to arrest periodontitis by stimulating the production of anti-inflammatory mediators (IL-10, TGF-β1 and CTLA-4), recruiting peripheral Tregs (CCL22/IL-4) or inhibiting pro-inflammatory cell subsets (Th1 and Th17 lymphocytes) in animal studies. Methodological improvements regarding heterogeneity of studies and RoB are necessary before advancing to a pilot trial in humans. A combination of strategies, such as locally targeting the maintenance and permanent expression of Tregs, could effectively suppress effector T lymphocytes in the long term, preventing the initiation and progression of periodontitis, eventually leading to a safe human intervention.

5 | CONCLUSION

Most of the therapeutic strategies targeting Tregs that were reported in this systematic review could be effective for treating periodontitis. If a biocompatible drug capable of restoring the Th17/Treg balance and arresting the Th17-driven alveolar bone resorption is designed, it could eventually lead to clinical translation. Apart from that, unclear RoB that was found across the reviewed studies, the poor reporting of the estimates and important methodological variability regarding experimental periodontitis indicate the need for a cautious interpretation of the results and a stronger adherence to guidelines for animal studies. The adherence to these guidelines would enhance the reproducibility of the results and favour the clinical translation of the preclinical successful interventions.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

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

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