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Community series in unveiling immunological mechanisms of periodontal diseases, volume II

Edited by
Pedro Paulo Chaves de Souza and Teun J. De Vries

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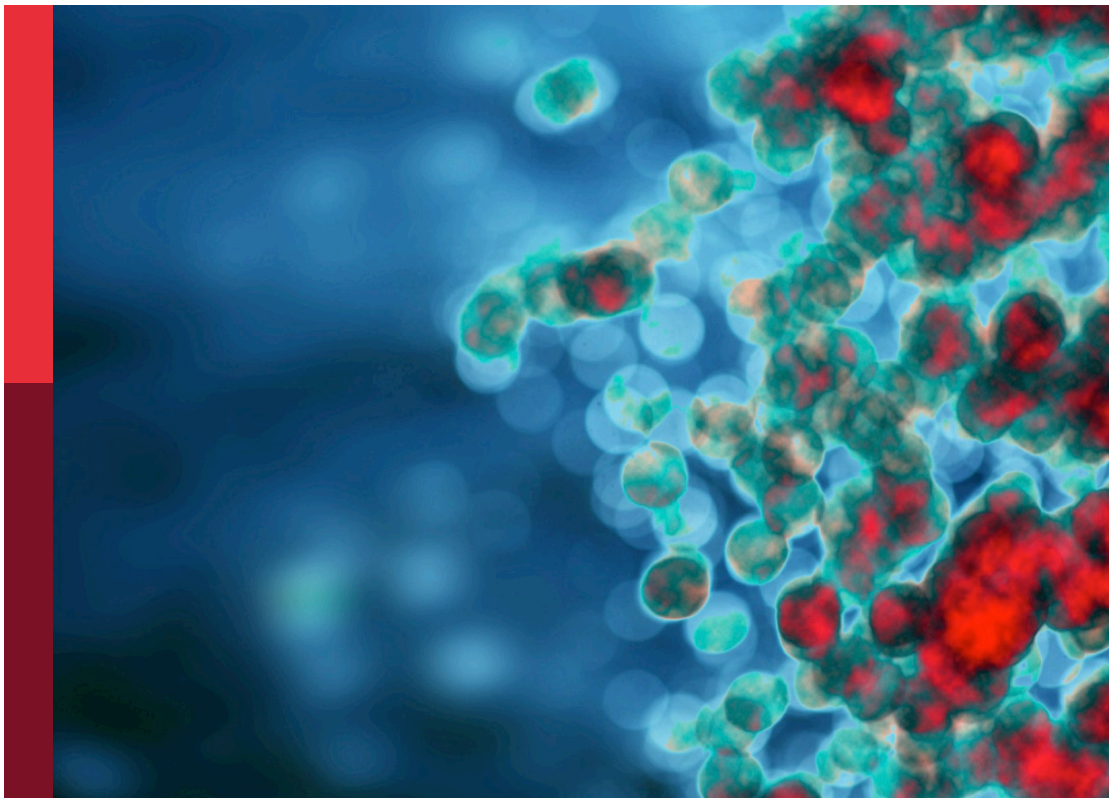

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Community series in unveiling immunological mechanisms of periodontal diseases, volume II

Topic editors

Pedro Paulo Chaves de Souza — Universidade Federal de Goiás, Brazil

Teun J. De Vries — Academic Centre for Dentistry Amsterdam, VU Amsterdam, Netherlands

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EDITED AND REVIEWED BY
Pietro Ghezzi,
Brighton and Sussex Medical School,
United Kingdom

*CORRESPONDENCE
Pedro Paulo Chaves de Souza
pedrosouza@ufg.br
Teun J. de Vries
teun.devries@acta.nl

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Editorial: Community series in unveiling immunological mechanisms of periodontal diseases, volume II

Pedro Paulo Chaves de Souza[1]* and Teun J. de Vries[2]*

[1]The Innovation in Biomaterials Laboratory, School of Dentistry, Federal University of Goiás, Goiânia, Brazil, [2]Department of Periodontology, Academic Centre for Dentistry Amsterdam (ACTA), Amsterdam, Netherlands

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periodontitis, Mendelian randomization, scRNAseq, inflammation, bone resorption

Editorial on the Research Topic
Community series in unveiling immunological mechanisms of periodontal diseases, volume II

It remains a scientific challenge to study the immunological mechanisms of complex diseases associated with the inflamed periodontium, the tissues surrounding our teeth. Periodontitis, a chronic inflammatory disease that is associated with bone loss, is particularly difficult to investigate due to its alternating active and silent phases. The dysbiotic biofilm that is associated with the disease can vary between patients, disease stages and sample sites. Mouse models can help mechanistically but may have shortcomings in mimicking dysbiosis. Understanding the characteristics of patient cohorts may help to further characterize inflammation-related parameters.

The present series is the continuation of the previous series on "Unveiling Immunological Mechanisms of Periodontal Disease" (1). Compared to the previous series, the present one has shifted to themes that were not addressed three years ago, probably reflecting the novel tools that are available through knowledge of the genome and emerging techniques such as single-cell RNA sequencing (scRNA-seq). This Research Topic has contributed to the following themes.

Cell biology models

Understanding the cellular and molecular mechanisms that drive periodontal inflammation and tissue destruction remains the basis of periodontal research. Advances in systems biology and cutting-edge transcriptomic technologies have enabled researchers to dissect the complex interactions between immune cells, stromal cells, and microbial communities in the periodontium. The following studies exemplify how cell biological models are elucidating the immunomodulatory roles of specific cell populations and pathways, offering new insights into the pathogenesis of periodontitis and potential therapeutic targets.

The study by Kim et al. revealed a pivotal role for ICAM1+ gingival fibroblasts as immunomodulatory sentinels in periodontal inflammation. Through integrative analysis of human scRNA-seq datasets, the authors demonstrated that ICAM1, the cell-cell adhesion molecule that fibroblasts can use to interact with immune cells, marks a fibroblast subset that expresses an inflammatory signature. This population orchestrates macrophage recruitment via CCL2, enabling efferocytosis to resolve neutrophilic inflammation, a process critical for mitigating tissue destruction. These findings redefine stromal-immune crosstalk in the periodontal niche and highlight ICAM1+ fibroblasts as a therapeutic target to modulate inflammation-driven bone loss.

Zhao et al. examined the role of the mechanosensitive ion channel Piezo1 in gingival destruction linked to periodontitis. Piezo1 expression is upregulated in the gingival tissue of periodontitis patients and drives macrophage polarization toward the M1 phenotype, leading to pro-inflammatory cytokine production and activation of MMPs, contributing to tissue destruction. The study suggests that inhibiting Piezo1 may reduce inflammation and collagen degradation, making it a potential therapeutic target for periodontitis.

Hu et al. investigated the role of miR-199a-5p in bone regeneration during apical periodontitis (AP), a disease marked by periapical inflammation and alveolar bone loss. Using transcriptomic analysis of clinical samples, the authors identified miR-199a-5p as significantly downregulated in AP tissues. Functional studies revealed that miR-199a-5p overexpression enhanced the proliferation and osteogenic differentiation of human stem cells from the apical papilla (hSCAPs), while its inhibition suppressed these processes. Mechanistically, miR-199a-5p targets *IFIT2*, a gene linked to type I interferon signaling, thereby alleviating its suppressive effects on osteogenesis. Furthermore, *in vivo* experiments demonstrated that hSCAPs overexpressing miR-199a-5p, when loaded onto β-tricalcium phosphate scaffolds, significantly enhanced ectopic bone formation in mice. These findings underscore miR-199a-5p as a critical regulator of bone repair in AP.

Taken together, these studies highlight the multifaceted roles of fibroblasts, mechanosensitive pathways, and miRNAs in periodontal inflammation and bone remodeling, offering new avenues for therapeutic intervention.

Periodontal pathogens and periodontitis

Periodontitis can elicit antibody production against proteins of periodontal pathogens such as *Porphyromonis gingivalis*. A large, well-characterized cohort study such as the PerioGene North case-control study could determine whether antibodies against periodontal pathogens such as anti- arginine gingipains (Rgp), are associated with disease progression. Serum-Rgp IgG levels were clearly elevated in periodontitis patients compared to controls, and were even higher in patients with a high degree of inflammation and with alveolar bone loss Kindstedt et al.

To further explore the interactions of periodontal pathogens with the human immune system, Irwandi et al. proposed the use of the skin blister model to study the immunopathogenesis of periodontal disease. This model offers a controlled environment to explore localized host-pathogen interactions, bridging the gap between ex vivo studies and clinical observations, further advancing our understanding of the systemic links to periodontal inflammation.

Mendelian randomization

Mendelian randomization is a relatively novel method that allows access to causal relationships between risk factors and health outcomes using genetic variants as instruments to infer causal effects (2). The current Research topic includes a study on the description of the role of tumor necrosis factor-receptor 1 TNF-α by Alayash et al., an article on interleukin-6 signaling by Nolde et al. and one on telomere length by Hu et al. While the first study did not find an association between TNF-receptor inhibition and periodontits., the interleukin study demonstrated that downregulation of IL-6 signaling based on genetic information was associated with lower odds of periodontitis. As we age, telomeres shorten (3). Since periodontitis increases with age, one would expect that shorter telomere length correlates with periodontal disease. Hu et al. showed indeed a reverse causal relationship, with shorter telomeres being linked to a higher risk of periodontitis, but no additional effect of telomere length and periodontitis when corrected for age.

Reviews

Finally, this Research Topic also contains two state-of-the-art reviews. Novel research shows that chewing is beneficial to the aging periodontium and may help individuals maintain their teeth into old age (4). Mechanical forces can affect periodontal health through multiple mechanisms. Mechanical forces can influence soft and hard tissue metabolism. Excessive forces can damage the periodontium or result in irreversible inflammation. In their review, Wang et al., described the effect of mechanical forces on the parameters of the periodontium.

The review by Zhang et al. highlighted how emerging omics tools, such as RNAseq are revealing dynamic shifts in epithelial, stromal, and immune cell populations that drive inflammation and bone resorption, revolutionizing our understanding of immunopathology. However, as exemplified throughout this series, these discoveries require rigorous experimental validation. Integrating multi-omics approaches with mechanistic models and clinical cohorts will be critical to unravel the heterogeneity of dysbiotic biofilms and host responses. Together, this synergy of technologies and validation frameworks promises to advance precision therapies that address both the oral and systemic dimensions of periodontal disease.

Author contributions

PS: Writing – original draft, Writing – review & editing. TV: Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Teun J. De Vries,
Academic Centre for Dentistry Amsterdam, VU Amsterdam, Netherlands

REVIEWED BY
Jiwei Sun,
Huazhong University of Science and Technology, China
Jeffrey Ebersole,
University of Nevada, Las Vegas, United States

*CORRESPONDENCE
Yi Luo
medoryi18.27@163.com
Liming Chen
15180711430@163.com

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share senior authorship

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Reverse causal relationship between periodontitis and shortened telomere length: Bidirectional two-sample Mendelian random analysis

Jiaxin Hu[1,2†], Jukun Song[3†], Zhu Chen[1], Jing Yang[1], Qianhui Shi[1], Fuqian Jin[1,2], Qiyuan Pang[1], Xingtao Chang[2], Yuan Tian[4], Yi Luo[1,2*‡] and Liming Chen[1,2*‡]

[1]Guiyang Hospital of Stomatology, Stomatology Hospital of Guizhou University, Guiyang, Guizhou, China, [2]School of Stomatology, Zunyi Medical University, Zunyi, Guizhou, China, [3]The Affiliated Stomatological Hospital & Stomatology of Guizhou Medical University, Guizhou Medical University, Guiyang, China, [4]Guizhou Provincial People's Hospital, Guiyang, Guizhou, China

Background: Observational studies have demonstrated a link between shortened telomere lengths(TL) and chronic periodontitis. However, whether the shortened TL is the cause or the result of periodontitis is unknown.Therefore, our objective was to investigate a bidirectional causal relationship between periodontitis and TL using a two-sample Mendel randomized (MR) study.

Methods: A two-sample bidirectional MR analysis using publicly available genome-wide association study (GWAS) data was used. As the primary analysis, inverse variance weighting (IVW) was employed. To identify pleiotropy, we used leave-one-out analysis, MR-Egger, Weighted median, Simple mode, Weighted mode, and MR pleiotropy residual sum and outlier (MR-PRESSO).

Results: In reverse MR results, a genetic prediction of short TL was causally associated with a higher risk of periodontitis (IVW: odds ratio [OR]: 1.0601, 95% confidence interval [CI]: 1.0213 to 1.1002; P =0.0021) and other complementary MR methods. In the forward MR analysis, periodontitis was shown to have no significant effect on TL (IVW: p = 0.7242), with consistent results for the remaining complementary MR. No pleiotropy was detected in sensitivity analysis (all P>0.05).

Conclusion: Our MR studies showed a reverse causal relationship, with shorten TL being linked to a higher risk of periodontitis, rather than periodontitis shorten that TL. Future research is needed to investigate the relationship between cell senescence and the disease.

KEYWORDS

telomere length, periodontal disease, Mendelian randomization, causality, genome-wide association studies, biological aging, inflammation

1 Introduction

Periodontitis is an inflammatory immune condition brought on by problems with mouth microbes (1). If periodontitis is not control in time, with the continuous development of inflammation, it may eventually destroy the tooth supporting tissue, leading to tooth loss (2, 3). Being a very common non-communicable disease, it has negatively impacted people's quality of life and added to society's financial burden (4).It is the sixth most common diseased in the globe. A person with many missing teeth may appear older, lose their ability to chew and have pronunciation issues. Periodontitis has significant systemic effects in addition to local ones, and prior studies have demonstrated a relationship between periodontitis and cardiovascular, respiratory, hypertensive, and diabetes disorders (5–7). Treatment of periodontitis is made more challenging by the interaction of multiple systemic diseases, and vice versa.

Under the accumulation of plaque, inflammation, and a bad lifestyle, the occurrence of periodontitis seems to be closely related to age (8). According to figures from epidemiological surveys, periodontitis affects 50% of adults in varying degrees, and incidence and severity have both sharply increased in the group over 65 (9). Periodontal attachment loss has been consistently observed to increase with age in various prospective cohort studies. However, the potentially nagetive impact of aging on periodontitis is still debatable, maybe as a result of the true risk factors of ongoing and cumulative periodontitis exposure (10, 11). This indicates that confounding and reverse causality may impair cohort studies and epidemiological research, making causal inference challenging. In conclusion, it is unclear at this time if age has a role in periodontitis risk.

The length of the telomere, which is a TTAGGG nucleotide repeat at the end of a chromosome that protects DNA and maintains chromosomal stability, can be used to measure the biological aging process (12, 13). Telomeres gradually shorten with each cell division due to the DNA replication mechanism's inability to fully copy the 5' end of the lagging DNA chain (14). This process eventually results in cell senescence, which can be accelerated by oxidative stress and inflammation.

Even though prior research on the mechanism of telomere erosion has demonstrated that inflammation plays a significant role in TL shortening (15). However, it is uncertain if periodontitis and TL shortening are connected. Previous research has found that individuals with periodontitis have lower TL values than non-periodontitis patients, and that measured TL values are associated with the severity of oxidative stress and periodontitis, but only in chronic instances (16). A recent study with 3,478 participants demonstrated a link between periodontitis and short TLs; However, cross-sectional studies cannot differentiate the two (17). Furthermore, the TL variation pattern is more consistent with a continuous dynamic fluctuation condition than a monotonous linear increase or drop (18). In observational studies, it may be challenging to control all confounding variables, which causes incorrect conclusions to be drawn about age and the relationship between TL and periodontitis. As a result, there are many conflicting findings about the relationship between periodontitis and TL.

The initial investigation in Mendelian randomization (MR) is genomic sequence analysis (19). Genetic information is difficult to misinterpret and independent of illness state, preventing reverse causation bias, hence it overcomes some limitations of observational investigations. The design of a random allocation trial is comparable to the natural random allocation that takes place during the development of each individual's genetic makeup (20). The MR approach employs genetic markers as a tool variable (IV) for determining causality. Because confounders are frequently unrelated to genetic variation, disparities in outcomes between those who have the variant and those who do not can be attributed to differences in risk factors or susceptibility. As a result, unlike traditional observational studies, which are susceptible to confounding or reversing the causal relationship, MR gives a credible explanation for the varied exposure to the trait of interest (21). To present, no research has been undertaken employing MR approaches to explain the relationship between periodontitis and TL. We performed a bi-directional MR study and a series of sensitivity analyses to validate our hypothesis: (1) whether TL can be used as an effective means of identifying the occurrence of periodontitis. (2) whether shortened TL increases the susceptibility to periodontitis.

2 Material and method

2.1 Mendelian randomization

To explore potential causal linkages between exposure and outcomes of interest, MR analyses will use genetic changes that are closely related to exposure as instrumental variables (IVs) (22). The MR estimate technique was unaffected by measurement mistakes, reverse causality, or confounding since genetic variations were randomly assigned at conception. A genetic variant must satisfy three essential conditions to be a legitimate IV (23) (1): There is a strong relationship between the instruments and exposure ("association") (2); The tool influences outcomes through exposure ("exclusion limit"); (3) Genetic variables were unrelated to confounding variables of exposure outcomes ("exchangeability"); The bi-directional MR design flow between periodontitis and TL can be seen (Figure 1).

Abbreviations: TL, telomere length; MR, mendelian randomization; SNP, single nucleotide polymorphism; GWAS, Genome-Wide Association Studies; MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier; CDC, Centers for Disease Control; AAP, American Academy of Periodontal.

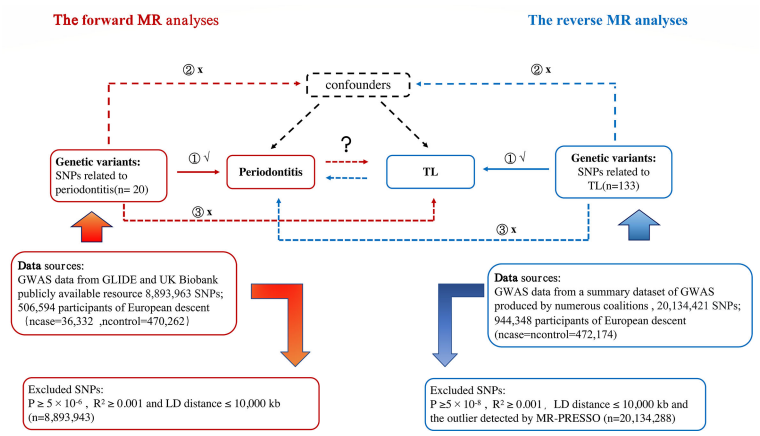

FIGURE 1
Study Design Sketch: Periodontitis is exposed in red in the forward MR analysis, and TL is the outcome. Exposure is represented by the color blue in the reverse MR analysis, periodontitis is the outcome. Abbreviation: TL, telomere length; MR, mendelian randomization; SNP, single nucleotide polymorphism.

2.2 Data source

The largest meta-analyses, which included data from the UK Biobank (UKB) and the Gene-Lifestyle Interactions in Dental Endpoints (GLIDE) consortium, were used to create summary statistics for periodontitis (24). The SNPs were linked to the composite phenotype, which included self-reported loose teeth from UKB and clinically diagnosed periodontitis from GLIDE (Ncases = 18,979, NControls = 442,052). The Centers for Disease Control and Prevention (CDC)/American Academy of Periodontology definition(AAP), comparable standards assessed by probing depth, or self-reporting were used to categorize periodontitis cases (25). And 944,348 participants had TL phenotypic data from a summary dataset of GWAS produced by numerous coalitions (round 2, 2021), with a 1:1 case-control ratio. The GWAS summary data of TL were obtained from the IEU open GWAS project (https://gwas.mrcieu.ac.uk/). All the above populations are of European origin to minimize potential bias due to demographic heterogeneity.

2.3 Selection of the genetic instruments

At $p<5\times10^{-8}$ for TL as the significance threshold, we chose SNPs related to the exposure (26). To expand the statistical effect, the indicative association threshold for periodontitis was set to $p < 5 \times 10^{-6}$. By calculating pair-wise linkage disequilibrium and excluding SNPs with $r^2 \geq 0.001$ and LD distance ≤ 10,000 kb from this set of SNPs, we chose only independent instruments with the lowest p-value (27). The flowchart of the study is presented in Figure 1. F statistic needs to be higher than 10 to get enough strength to limit the deviation from weak tool variables (28). F to calculate statistics, we use: $[(R^2\times (N-2))/(1-R^2)]$.

The impacts of several genetic variations were coordinated for the analysis, and SNPs made up of one base and its complementary base were disregarded. We only used variations appropriate for all observed traits and did not employ stand-ins for the missing variants to preserve the consistency of SNPs used as IVs in various analyses (23).

2.4 Statistical analyses

Six different MR inverse variance weighting (IVW) of random effects, MR Egger, Weighted median, Simple mode, MR-PRESSO and Weighted mode approaches were performed to address variability heterogeneity and pleiotropic effects (29). MR analysis was repeated if significant horizontal pleiotropy was detected in the MR-PRESSO analysis (with a P-value lower than the threshold in the MR-PRESSO outlier test). The inverse variance weighted (IVW) model is used as the main analysis, because it is a meta-method, which combines the Wald estimation of each SNP and effectively regards each SNP as an effective natural experiment. Importantly, it forces the intercept in the regression slope to be zero, so if any IV is invalid, the result may be biased (30). Then MR-Egger and weighted medians were used to enhance IVW estimates since they could offer a more accurate estimate in a wider range of circumstances, although being less efficient (broader CIs) (31). While pleiotropy is permitted for all genetic variations under MR-Egger, it must be independent of variation exposure (32). At the Same Time, the simple and weighted modes were used to evaluate the robustness of the IVW method results. Finally, the MR-Egger intercept test and leave-one-out analysis were used to evaluate horizontal pleiotropy

further. Heterogeneity was also identified using Cochran's Q test and I^2. I^2 statistics may indicate dilution in MR-Egger estimates, which may mean that MR-Egger results may be inaccurate. Several plots were used to evaluate the significant SNPs (leave-one-out, funnel plot, forest plot, and scatter plot) (33).

The study made use of de-identified data from participating studies that were made publically accessible and had their use of human subjects approved by the ethical standards board. This study doesn't need any additional ethical approval.

The R (version 3.6.1) package's Two Sample MR (version 0.4.25) and MR-PRESSO (version 1.0) packages were used for all studies.

3 Results

3.1 Effect of periodontitis on TL

After screening, 20 independent SNPs related to periodontitis were identified. The minimum f statistics of these IV's are all greater than 10 (ranging from 89 to 185), which ensures the hypothesis of "correlation", that is, weak instrument bias is unlikely to affect the estimation of causal effects. Meanwhile, no pleiotropy was found based on Egger intercept (intercept =0.0016, P =0.401), Table 1; And MR-PRESSO did not detect heterogeneity (Q =15.7, $I^{2\ =}$ 0.11, P =0.330), Table 2.

The MR estimation values of different methods are listed in Figure 2. Overall, in the main result IVW, there is no causal relationship between the genetically predicted periodontitis and TL (beta=0.0092, 95% CI:-0.0418 to 0.0602, P = 0.7242). In addition, MR Egger, weighted median, weighted mode method and simple mode method show consistent results. The scatter plot of SNP effect of periodontitis and TL shows the Supplementary Graph S1. According to the heterogeneity test, there is no heterogeneity among individual SNPs. One-way analysis showed that the causal estimation of periodontitis was not driven by any single SNP. One-way analysis diagram, forest diagram and funnel diagram are shown in Supplementary Diagrams S2-S4.

3.2 Effect of TL on periodontitis

After the initial screening, 134 independent SNPs associated with TL were identified. The minimum F statistics of these IV are all greater than 10 (ranging from 57 to 3086), which ensures the hypothesis of "correlation", that is, weak instrumental bias is unlikely to affect the estimation of causal effect. However, MR-PRESSO found that rs10773176 was an extremely abnormal variation. After removing this outlier, heterogeneity is no longer detected (Q =130.3,$I^{2\ =}$ 0.13 ^{P} =0.120), Table 2; At the same time, no pleiotropy was found based on Egger intercept (intercept=0.0006, P =0.532), Table 1. So the finally determined effective IVs are 133 SNPs. The characteristics of all SNPs included in IVs as TL are detailed in Supplementary Table 2.

Overall, in the main analysis using IVW combined with multiple genetic variations, shorter TL has a significant causal relationship with higher odds of periodontitis (OR: 1.0601, 95% CI: 1.0213 to 1.1002, P = 0.0021). For every standard deviation decrease of TL, The odds ratio of periodontitis was 1.0601 (Figure 2). The weighted median (OR: 1.0629, 95% CI: 1.0056-1.1236, P = 0.0310), MR-Egger (OR:1.0677, 95% CI: 1.0113-1.10) were used. 95% CI: 1.0078-1.1312, P =0.0282) The methods have the same direction, and have comparable point estimates and confidence intervals. These causal estimates are further shown in the scatter plot and the funnel plot (Figures 3, 4).

One-way analysis diagram and forest diagram are shown in Supplementary Diagrams S5-S6.

4 Discussion

In this study, we used the largest GWAS summary-level data set accessible to date to conduct a two-sample MR analysis to completely evaluate the causal effect of periodontitis on TL. This is the first study to investigate the bidirectional causal relationship between periodontitis and TL by performing multiple complementary MR approaches. No evidence of periodontitis and TL associations supporting genetic prediction was observed in our two-sample MR forward analysis. However, reverse MR analysis showed evidence that TL were related to periodontitis, and shortened TL increased the risk of periodontitis.

Various studies have examined whether there is a link between TL and periodontitis in recent years (34). According to one theory, systemic inflammation and oxidative stress brought on by chronic periodontitis may shortened TL, as evidenced by previous case-control studies (16, 35). At the same time, consistent results from two large cross-sectional studies conducted based on the National Health and Nutrition Examination Survey (NHANES) confirmed the association of periodontitis with TL (17, 36). The study examined the depth of probing, loss of adhesion, and probing bleeding from the

TABLE 1 MR-Egger test for directional pleiotropy.

exposure	outcome	intercept	SE	p-value
Periodontitis	TL	0.0016	0.0019	0.401
TL	periodontitis	0.0006	0.0009	0.532

df, degree of freedom; MR, Mendelian randomization; Q, heterogeneity statistic Q.

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TABLE 2 Heterogeneity of wald ratios.

exposure	outcome	Q	df	I^2	p-value
periodontitis	TL	15.7	14	0.11	0.330
TL	periodontitis	130.3	113	0.13	0.120

df, degree of freedom; MR, Mendelian randomization; Q, heterogeneity statistic Q.

distal, mesial, or midfacial region of each tooth in two randomly selected quadrants. And the severity of periodontitis was defined according to the CDC/AAP Sciences guidelines to ensure a thorough periodontal evaluation. However, due to the drawbacks of cross-sectional studies, it is still unclear whether periodontitis causes shortened TL or whether shortened TL increases susceptibility to periodontitis, even if there is a potential inflammatory mechanism to explain the effect of periodontitis on TL.

Although different results have been obtained in the past two prospective studies that there was no significant association between TL and periodontitis (10, 37). This result may be limited by several reasons, such as a small study span, younger age of the covered population, and a periodontal examination result without full mouth examination (38). Most importantly, insufficient numbers of people are likely to result in insufficient statistical capacity. Researchers have long questioned whether populations with shortened TL are more prone to periodontitis because a reverse causal relationship cannot be ruled out, even if a cross-sectional study confirms an association between the two (36). However, ethical and moral limitations make large-scale randomized controlled trial (RCT) research challenging. To fully reveal these causal relationships, it is more practical to collect evidence using the MR method. We investigated the potential causal effects of periodontitis on TL using MR methods.

The following possible mechanisms underlie the reverse causal relationship that we found in MR studies between periodontitis and shortened TL. First, shortened TL have been found to affect the proliferation, migration, and mesodermal lineage differentiation of periodontal ligament stem cells, and these properties are essential for periodontal tissue regeneration in periodontitis (39). Another explanation may be that telomere dysfunction can activate the production and secretion of inflammatory factors such as IL-6 and TNF-α (40), leading to the development of periodontitis. Additionally, many cellular functions start to malfunction as TL decrease (34). Therefore, in people with periodontitis, immune cells with shorten telomeres may indicate immune system dysfunction, encourage the growth of local gingival bacteria, and favor the development of oral diseases. This is probably a potential

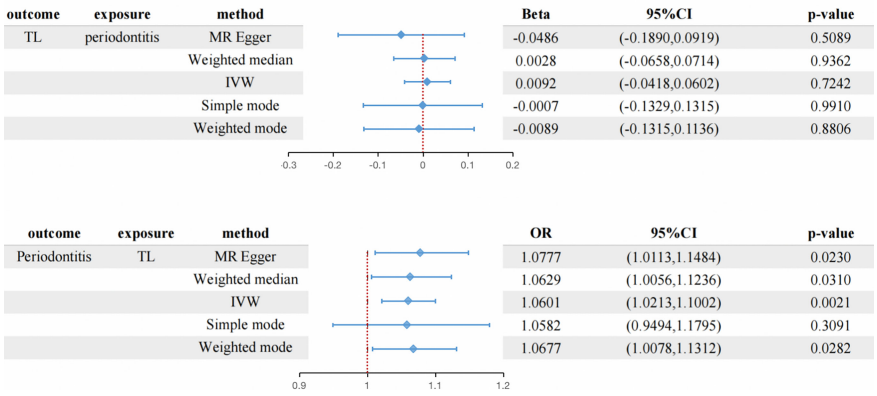

FIGURE 2
The connection between genetically instrumented periodontitis and TL, and vice versa is estimated using MR(the table at the bottom shows the OR of TL shortening to periodontitis) CI, confidence interval; IVW, inverse variance-weighted; MR, Mendelian randomization; OR, odds ratio.

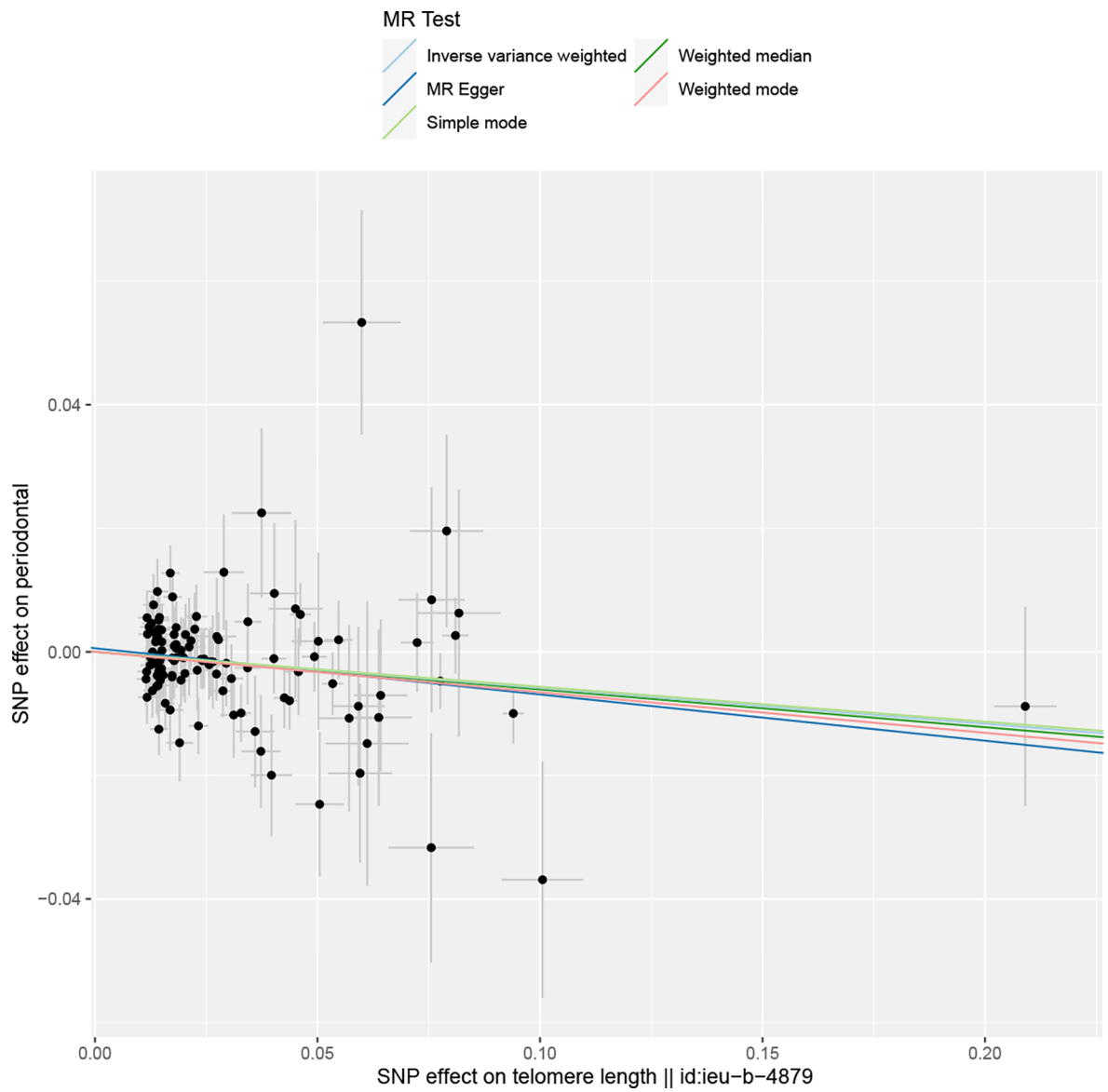

FIGURE 3
The reverse MR: Scatter plot of the effects of SNPs on TL and periodontitis. The horizontal and vertical axes represent the effect of each genetic variation on telomere length and periodontitis. The gray line around the black solid point indicates the corresponding 95% CI for the effect. The slopes of the solid lines show the effect estimates of the five MR methods. MR, mendelian randomization; SNP, single nucleotide polymorphism; CI, confidence interval.

mechanism by which we arrived at this conclusion, and more research is required to determine the mechanism by which shortened telomeres affect periodontitis in the future.

It is worth noting that physiological age is often more accurate than chronological age at predicting healthy aging and is clinically more relevant (41). TL is regarded as a reliable indicator of biological age because telomeres have a significant impact on the rate of cell senescence and death. Our results showed that a shorter TL may increase the odds of periodontitis, but it is likely to be unrelated to actual age (42). At present, the correlation between TL and physiological age has been confirmed by many studies, and it is found that physiological age is affected by many lifestyle factors, including diet, smoking, exercise and sleep habits, which are also found to be related to the disease factors of periodontitis (43). And

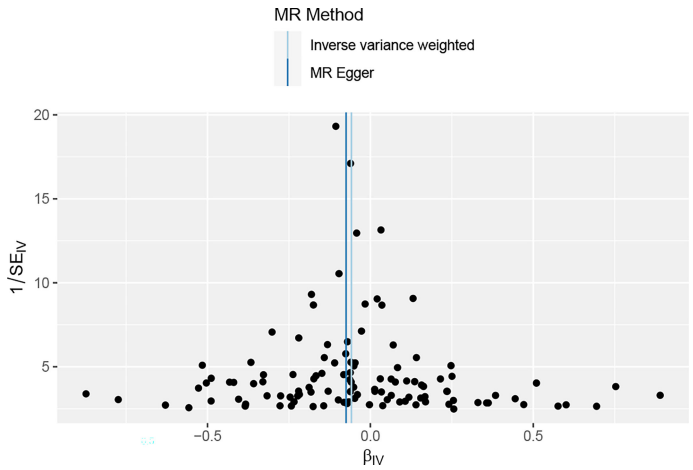

FIGURE 4
The funnel plot of the association between periodontal and TL, reverse MR analysis;It is used to determine whether the associations observed are noticeably heterogeneous.

other illnesses linked to shorter telomeres, like as atherosclerosis, diabetes and obesity, have also been linked to periodontitis (24, 44). Based on our research results, whether TL plays an intermediary role is a potential direction for future research (45).

Our current findings suggest that TL may be a targetable factor in the development of new preventive measures to address periodontitis risk. Although practical interventions that directly alter TL are not yet available (46), they may be possible through a healthy lifestyle (47). Reverse MR supports the view that shorter telomeres may lead to a higher risk of periodontitis, but the clinical significance of MR estimation should be interpreted with caution (23). It must be pointed out that, as with all other MR investigations, it should be better interpreted as test statistics for causal hypotheses, providing alternative etiological evidence supporting a causal relationship between TL and periodontitis.

Our research offers several significant advantages. The large sample size of the two-sample MR study design, as well as the inclusion of genetically predicted phenotypes as exposures of interest in MR studies, decreases the potential for reverse causation and confounding bias when compared to observational studies. Furthermore, the study was developed with only the European population in mind, avoiding aberrations owing to demographic variability.

Our study has the following limitations. First, while our study avoided population abnormality by controlling for the participants' ethnicity, whether our findings were universal for other groups remains to be determined. Second, due to the low heritability of periodontitis, the estimation of the genetic relationship between TL and periodontitis may be skewed. However, because TL is impacted by hereditary factors, the effect of bias on the results should be insignificant. Furthermore, the periodontitis GWAS data we used did not stratify disease severity (48). As a result, when more thorough GWAS data becomes available in the future, more studies will be required to validate our findings.

5 Conclusion

Periodontitis did not significantly affect TL in our forward MR study, but in reverse MR, we discovered an inverse causal relationship between the two, meaning that a shortened TL is linked to a higher risk of developing periodontitis. Further investigation into the connection between cell senescence and periodontitis as well as validation of TL as a biomarker for predicting the development of periodontitis are necessary given that this may suggest that telomere biology is a potential pathway involved in the occurrence and development of periodontitis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

JH designed the research, contributed to data interpretation, and drafted the manuscript. ZC, JY, FJ, QP, XC, QS and YT contributed to data interpretation and the manuscript. YL, LC, and JS reviewed this article. JH and JS contributed equally to this work and should be considered co-first authors. YL and LC

contributed equally to this work and share senior authorship. All authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1057602/full#supplementary-material

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EDITED BY
Teun J. De Vries,
VU Amsterdam, Netherlands

REVIEWED BY
Mia Rakic,
(UCM), Spain
Gaetano Isola,
University of Catania, Italy

*CORRESPONDENCE
Zoheir Alayash
alayash@uni-muenster.de

†These authors share last authorship

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Inhibition of tumor necrosis factor receptor 1 and the risk of periodontitis

Zoheir Alayash[1*], Sebastian-Edgar Baumeister[1], Birte Holtfreter[2], Thomas Kocher[2], Hansjörg Baurecht[3], Benjamin Ehmke[4], Stefan Lars Reckelkamm[1†] and Michael Nolde[1†]

[1]Institute of Health Services Research in Dentistry, University of Münster, Münster, Germany, [2]Department of Restorative Dentistry, Periodontology, Endodontology, and Preventive and Pediatric Dentistry, University Medicine Greifswald, Greifswald, Germany, [3]Department of Epidemiology and Preventive Medicine, University of Regensburg, Regensburg, Germany, [4]Clinic for Periodontology and Conservative Dentistry, University of Münster, Münster, Germany

Aim: To investigate the effect of genetically proxied inhibition of tumor necrosis factor receptor 1 (TNFR1) on the risk of periodontitis.

Materials and methods: Genetic instruments were selected from the vicinity of TNFR superfamily member 1A (TNFRSF1A) gene (chromosome 12; base pairs 6,437,923–6,451,280 as per GRCh37 assembly) based on their association with C-reactive protein (N= 575,531). Summary statistics of these variants were obtained from a genome-wide association study (GWAS) of 17,353 periodontitis cases and 28,210 controls to estimate the effect of TNFR1 inhibition on periodontitis using a fixed-effects inverse method.

Results: Considering rs1800693 as an instrument, we found no effect of TNFR1 inhibition on periodontitis risk (Odds ratio (OR) scaled per standard deviation increment in CRP: 1.57, 95% confidence interval (CI): 0.38;6.46). Similar results were derived from a secondary analysis that used three variants (rs767455, rs4149570, and rs4149577) to index TNFR1 inhibition.

Conclusions: We found no evidence of a potential efficacy of TNFR1 inhibition on periodontitis risk.

KEYWORDS

tumor necrosis factor (TNF), periodonditis, cis-Mendelian randomization analysis, genetics, proinflammatory cytokine

Introduction

Periodontitis is a chronic inflammatory disease of the oral cavity that is linked to an imbalanced relationship between a dysbiotic microbiome and the host inflammatory response, progressively leading to the loss of periodontal ligaments and alveolar bone (1). Tumor necrosis factor (TNF) belongs to a superfamily of cytokines with a broad spectrum of

physiological and pathological effects (2). TNF is first translated as a transmembrane molecule (mTNF) and then cleaved by a metalloproteinase (TNF converting enzyme) to produce a soluble form of TNF (sTNF). Both mTNF and sTNF induce cellular responses through binding to the two receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (3). While the TNF-mediated signaling pathway of the former promotes cell survival, apoptosis, or necrosis, agonism of the latter induces homeostatic signaling (e.g., tissue regeneration, cell proliferation, and cell survival) (4). In periodontal diseases, TNF plays a major role as an osteoclast-stimulating hormone that mediates bone resorption *via* two distinct pathways: receptor activator of NF-κB ligand (RANKL) dependent and RANKL independent (5). During a bacterial infection, a variety of cell types that reside in the periodontium produce and secrete TNF (e.g., macrophages, neutrophils, keratinocytes, fibroblasts, natural killer cells, and T and B cells). Consequently, TNF enhances the expression of RANKL on the cell surface of several cell types, which in turn binds to a RANK receptor on the cell membrane of osteoclast precursors to promote the formation of osteoclasts. In addition, TNF directly binds to its own receptors to induce the differentiation of precursor cells into osteoclasts (6). TNF is considered a key biomarker that reveals insights about diagnosis, prognosis, and treatment in periodontal disease. During inflammatory conditions, such as periodontitis, TNF levels increase in the gingival crevicular fluid to promote the degeneration of inflamed periodontal tissues (7). Several clinical trials have shown an association between salivary TNF and periodontal diseases. TNF was elevated in patients who had clinical indicators of periodontitis, suggesting that this biomarker may be useful in a panel of salivary biomarkers that could facilitate the screening, diagnosis, and management of periodontal diseases (8). Also, TNF levels were reduced in response to periodontitis treatment (9, 10). The most recent case definition system of periodontitis recommended the incorporation of biomarkers, measured from saliva and gingival crevicular fluid, to better assess the prognosis of the disease (11). The update of the classification system is still ongoing to incorporate more robust biomarkers in the future guidelines (12). To date, five licensed TNF inhibitors have been used in treating a set of inflammatory diseases: etanercept, infliximab, adalimumab, golimumab, and certolizumab. These molecules inhibit the formation of the ligand-receptor complex between TNF and its receptors TNFR1/2 *via* different mechanisms (13). The use of TNF inhibitors in animal studies has revealed their potential effectiveness in alleviating periodontal inflammation and reducing alveolar bone loss (14). Similarly, human studies suggested that these medications might improve periodontal clinical parameters in patients with periodontal diseases (10, 15, 16). Findings from systematic reviews are controversial where there is no general agreement on whether TNF inhibitors improve periodontal parameters. Some studies have suggested that treating rheumatoid arthritis (RA) with TNF inhibitors not only alleviates joint inflammation and damage but might also improve periodontal disease outcomes (17). However, other studies reported inconsistent results regarding the efficacy of TNF inhibitors in periodontal diseases (18).

Human genetics helped in identifying the role of genes and genetic polymorphisms in the onset and development of periodontitis (19). The causal pathway from genetic components to periodontitis is often dependent on cytokine gene polymorphisms (20, 21). In addition to discovering genetic components that are directly involved in the etiology of diseases, human genetic data has been extensively used in Mendelian randomization (MR) studies to assess the causal relationship between environmental and biological risk factors and disease susceptibility. MR is an instrumental variable (IV) approach that employs single nucleotide polymorphisms (SNPs) as instruments to index a certain risk factor (22). The random allocation of genetic variants and balancing of environmental factors at conception make an MR design less susceptible to confounding. In addition, these variants are assumed to remain unchanged throughout the lifetime, so they are not affected by the outcome. Thus, the inference drawn through an MR analysis will be less likely due to reverse causation (23). Drug target MR is a cis-MR approach that is an extension to the classical MR method and uses druggable protein expression as an exposure (24). Recent years have witnessed a growing research application of this study design in pharmacoepidemiology to reveal drug-repurposing opportunities (25). The application of MR in this context is considered analogous to in-silico trials that provide virtual randomization to study the therapeutic effect of an intervention in a certain population. For instance, the potential efficacy of IL-6 receptor blocker in preventing coronary heart disease was first discovered utilizing the drug target MR approach (26) then validated in clinical trials leading to the development of ziltivekimab, a novel IL-6R inhibiting drug specifically for use in atherosclerotic disease (27). Considering the growing interest in the potential efficacy of anti-rheumatic agents (e.g., TNF inhibitors) in patients with periodontitis (28) the present study aimed to test if blocking TNFR1 would reduce the risk of periodontitis using a drug target MR approach.

Materials and methods

Study design

We applied a cis-MR design based on summary statistics from genome-wide association studies (GWAS) to assess the causal effect of TNFR1 inhibition on periodontitis risk. To ensure the validity of the causal inference derived from an MR approach, IVs must fulfill three key assumptions. 1) The relevance assumption: genetic variants, extracted as IVs, should be associated with the exposure; 2) exchangeability assumption: IVs are independent of confounders of the IV-outcome relation; 3) and the exclusion restriction assumption: IVs affect the outcome only through the exposure and not *via* other biological pathways (i.e., no horizontal pleiotropic effect) (24). In a classical MR study, genetic variants are selected from throughout the genome, while in drug target MR, cis-acting variants are chosen from the vicinity of a specific gene known to encode a target of interest, typically a protein. Cis-variants most likely influence the biological effects of their corresponding gene, hence, strengthening the validity of the genotype-phenotype association and the relevance assumption. In addition, these variants control the expression of their genes rather than the expression of genes that are located outside the protein-encoding region. Thus, the risk of horizontal pleiotropy would be diminished, minimizing the possibility of violating the exchangeability and exclusion restriction assumptions (26). Finally, the choice of cis-variants nullifies the possibility of reverse causation because the

likelihood of the direction of causality from the encoded gene towards the phenotype (e.g., disease) is favored over the direction from the phenotype to the encoded protein (24).

Indexing TNF inhibition

Selective inhibition of TNFR1 guarantees a targeted intervention, halting the pro-inflammatory cascade of events mediated by TNF while keeping the homeostatic signaling, contributed by TNFR2, untouched (4). It has been found that the blockage of TNFR1 inhibits the production and secretion of inflammatory markers like C-reactive protein (CRP), which is often used as a treatment response biomarker of TNF inhibitors (29). In our study, we restricted the pool of potential IVs to SNPs that are in the vicinity of the TNFR superfamily member 1A (TNFRSF1A) gene (chromosome 12; base pairs 6,437,923–6,451,280 as per GRCh37 assembly), the gene that encodes TNFR1, which is a protein embedded in the cell membrane of inflammatory cells. We selected SNPs proximal to the protein-encoding gene (± 1 kb) to minimize the likelihood of selecting SNPs that affect the outcome through an alternative pathway (horizontal pleiotropy) other than the TNF-TNFR1 mediated pathway. Since CRP is a reliable downstream biomarker for the binding of TNF to TNFR1, we retained the IVs from the TNFRSF1A gene that are associated with CRP levels based on summary statistics reported in GWAS. We set the genome-wide significance threshold at $<5x10^{-5}$ and linkage disequilibrium (LD) clumping at $r^2 < 0.001$ to select independent SNPs with the strongest evidence of association with systemic inflammation. This algorithm retained a single SNP, rs1800693, which is known to be associated with the increased expression of Δ6-TNFR, a soluble protein that mimics the activity of TNF antagonists (30). Also, we detected 3 other SNPs (rs767455, rs4149570, and rs4149577) in the targeted gene that are associated with CRP levels and had previously been utilized in studies aiming to evaluate the effect of genetically proxied TNFR1 inhibition on several disease outcomes. In these MR studies, the indexing of TNF-TNFR1 signaling inhibition utilizing these 3 SNPs showed protective effects for diseases where TNF inhibitors have been approved for treatment, and unfavorable outcomes where TNF inhibitors are known to exacerbate the symptoms (31, 32).

Treatment indexing GWAS

We used genetic association estimates with serum concentration of CRP from a GWAS meta-analysis (N=575,531) of the UK biobank (N=427,367) and the Cohorts for Heart and Aging Research in Genomic Epidemiology consortium (N=148,164). Data in these GWAS was derived from participants of European descent. CRP serum concentration was measured by the standard immune assay as mg/L (33).

Outcome GWAS

Summary statistics for periodontitis were obtained from the Gene-Lifestyle Interactions in Dental Endpoints consortium. A total of 17,353 participants of European ancestry were classified as clinical periodontitis cases and 28,210 as controls. Periodontitis cases were classified by either the Centers for Disease and Control and Prevention/American Academy of Periodontology (CDC/AAP) or the Community Periodontal Index (CPI) case definition (34).

Statistical analysis

Data for the exposure and outcome were harmonized to ensure that the IV–outcome association and the IV–exposure association refer to the same effect allele. We tested the relevance assumption *via* the F statistic for the IVs' association with CRP. We set a cutoff of >10, which is generally acceptable to rule out weak instrument bias (35). First, we utilized the only SNP retained from the statistical selection (rs1800693) in our primary analysis and calculated the Wald ratio to estimate periodontitis risk per a standard deviation decrease in CRP mediated *via* TNFR1-blockade. Second, we considered rs767455, rs4149570, and rs4149577 as IVs for our secondary analysis. These SNPs were in LD with $r^2 \approx 0.7$ in the European 1000 version 3 genomes data. Using more genetic variants will plausibly increase the power of the analysis at the cost of including correlated SNPs through LD and consequently exaggerate the precision of causal effects (36). Thus, we derived causal estimates from a fixed-effects inverse variance weighted (IVW) model accounting for the correlation between genetic variants (37). In an alternative way to compensate for correlation, we extended the IVW method by using principle component analysis (PCA). This method guarantees that all variants contribute to the analysis (38). Principal components in the IVW estimator explained over 99% of the variance in the weighted correlation matrix. All analyses were performed in R version 4.1.2 using TwoSampleMR and MendelianRandomization packages.

Ethics

All analyses were based on publicly available summary statistics without accessing individual-level data; hence, ethical approval was not required. The included GWAS received informed consent from the study participants and have been approved by pertinent local ethical review boards.

Results

The F-Statistic for the IVs used in our primary and secondary analysis ranged between 54 and 118, indicating no weak instrument bias (Table 1). The Wald estimator from our primary analysis failed to show an effect of TNFR1 inhibition on periodontitis risk (Odds ratio (OR): 1.57, 95% confidence interval (CI): 0.38;6.46) (Table 2). In our secondary analysis, using three SNPs as IVs (rs767455, rs4149570, and rs4149577), results from the IVW and the PCA method did not show an association between genetically indexed inhibition of TNF-TNFR1 and risk of periodontitis (OR: 0.57, 95% CI: 0.12;2.57, and OR: 0.57, 95% CI: 0.13;2.63, respectively).

TABLE 1 Descriptive information on the TNFRSF1A variants analyzed in the study and their associations with inflammatory markers and disease outcomes.

Instrumental variables	rs1800693[a]	rs767455[b]	rs4149570[b]	rs4149577[b]
Frequency of EA[c]	0.574	0.571	0.374	0.528
F-statistic	118.805	98.010	54.020	81.996
Associations with the inflammatory marker, β (SE), p-value				
C-reactive protein	0.022 (0.002), 9.56e-27	0.020 (0.002), 2.39e-22	0.015 (0.002), 5.23e-13	0.018 (0.002), 9.60e-19
Associations with the outcome, β (SE), p-value				
Periodontitis	0.010 (0.016), 0.530	0.013 (0.016), 0.405	0.004 (0.016), 0.799	0.006 (0.015), 0.685

EA, effect allele; NEA, non-effect allele.
[a]SNP used in our primary analysis for the Wald ratio estimation.
[b]SNPs used in our secondary analysis for causal estimation from the multiplicative fixed-effects model and the principle component analysis method.
[c]Based on allele frequency reported by the genome-wide association studies of C-reactive protein. Single nucleotide polymorphisms were labeled with respect to GRCh37 reference coordinates.

TABLE 2 Mendelian randomization estimates for the effect of tumor necrosis factor receptor 1 inhibition on periodontitis risk.

Periodontitis				
Exposure		OR	95% CI	P-value
CRP	Wald ratio[a]	1.57	(0.38;6.46)	0.53
	IVW[b]	0.57	(0.12;2.57)	0.46
	IVW using PCA[c]	0.57	(0.13;2.63)	0.55

CI, confidence interval; CRP, C-reactive protein; IVW, inverse variance weighted; MPV, mean platelet volume; OR, odds ratio; PCA, principal component analysis.
[a]Wald ratio of a single SNP, rs1800693.
[b]IVW for correlated single nucleotide polymorphisms (SNPs) utilized Wald ratios of 3 SNPs (rs767455, rs4149570, and rs4149577).
[c]IVW using principal components analyses assumed correlated SNPs and are based on Mendelian randomization models of 2 principal components derived from the genetic associations of 3 correlated SNPs (rs767455, rs4149570, and rs4149577).

Discussion

In this study, we addressed whether genetically proxied TNFR1 inhibition reduces the risk of periodontitis using a drug target MR approach. Our current findings are derived from a robust study design using the largest GWAS summary data but failed to show an effect of TNFR1 inhibition on reducing periodontitis risk.

During periodontal inflammation the periodontal pathogens or their products translocate into the blood circulation to induce the bone marrow resulting in innate immune training of hematopoietic stem and progenitor cells and leading to a hyper-responsiveness of neutrophils. Studies have shown that therapeutic modulation of neutrophil-mediated inflammation represent a plausible therapeutic target for periodontitis treatment (39). Our analysis proxied the inhibition of neutrophils' since neutrophil-released mediators of inflammatory tissue damage (e.g. elastase, proteinase 3, myeloperoxidase, and matrix metalloproteinases) modulate the activity of proinflammatory cytokines such as TNF, and interleukins (IL-1β, IL-6, and IL-23). A recent drug target MR method found that the downregulation of IL-6 signaling was associated with reduced odds of periodontitis (40), but our analysis did not find a similar effect for TNFR1 inhibition.

Evidence from our MR study is inconsistent with results from cohort studies that aimed to study if TNF inhibitors improve periodontal clinical outcomes (10, 15, 16). Patients diagnosed with periodontal diseases who administered 200 mg of infliximab showed less bleeding on probing (BOP), lower gingival index (GI) and clinical attachment loss (CAL), and shallower pocket depth (PD) than matched patients and healthy controls (10). Another study that included 40 patients experiencing severe periodontitis showed that subjects assigned to TNF inhibitors showed improvements in BOP, PD, and CAL (15).

It is important to bear in mind that comparing our findings with those from conventional observational studies must be done with caution. First, TNF inhibitors used in the clinical setting inhibit the binding of mTNF and sTNF to both receptors, TNFR1 and TNFR2 (10, 15), but our study indexed the selective inhibition of TNFR1. Although it is documented that TNF promotes bone resorption and periodontal disease progression *via* TNFR1, the non-selective inhibition of TNFR might block additional inflammatory pathways that contribute to the efficacy seen in observational studies (5). Second, there is still limited evidence of the therapeutic efficacy of TNF inhibitors in patients diagnosed with periodontitis without other comorbidities. In fact, observational studies that investigated the association between TNF inhibitors and periodontal clinical parameters included patients diagnosed with periodontitis along with inflammatory diseases (10, 15, 16, 41, 42). However, our MR design infers causal estimates across the general population, not in a specific subpopulation of patients diagnosed with inflammatory diseases (e.g., RA). Third, for the treatment of RA, TNF inhibitors are usually prescribed in combination with disease-modifying anti-rheumatic drugs that might also have a favorable impact on the periodontal clinical parameters (43). In addition, there is a biologically plausible interaction between the two agents (44). Ignoring this phenomenon, as in the case of previous observational studies, may bias the results.

Additionally, similar to our findings, results from a systematic review failed to demonstrate convincing protective effects of TNF inhibitors on periodontal parameters (18). TNF inhibitors are usually prescribed for a specific time period, whereas our study aimed to reveal long-term effect of the blockage of TNFR1 on the risk of periodontitis. Thus, our null results could be either a true lack of efficacy or an average of opposing effects. This phenomenon is of clinical relevance since the treatment duration of TNF inhibitors can be a significant factor that influences the clinical outcomes in periodontal diseases. A systematic review showed contradictory periodontal clinical outcomes depending on the duration of treatment of TNF inhibitors (17). At a follow-up of 6 weeks, patients receiving (infliximab, etanercept, or adalimumab) showed improvements in BOP, GI, and CAL (15, 41). Additional periodontal parameters were improved in a longitudinal study when longer treatment duration was observed (up to 6 months) (16). However, treatment for more than 9 months was associated with higher GI and BOP values (42). This discrepancy can be attributed to patient's noncompliance or secondary loss of response. The latter scenario is a common observation during the treatment with TNF inhibitors, where the host produces antidrug antibodies, thus halting the impact of the medication (45).

Given the disproportionate burden of periodontitis, a growing body of literature investigates the potential efficacy of immunomodulatory treatments, including TNF inhibitors, in periodontal diseases (46); however, drug development remains hindered by the high cost and failure rates. The creation of drug repurposing methods, like cis-MR methods, helps in selecting promising therapeutic candidates for further study in clinical trials. Limitations to this study need to be acknowledged. First, our analysis was based on few genetic IVs. Second, GWAS utilized in our study were conducted on participants of European ancestry; thus, our null results may not be generalizable to other ethnicities.

In conclusion, this instrumental variable analysis failed to find evidence to support the clinical efficacy of TNF inhibitors in reducing the risk of periodontitis. We anticipate that triangulation of evidence from genetics, observational research and clinical trials will elucidate the role of TNF inhibitors in periodontitis. Future work on alternative cytokines or targets of the neutrophil-mediated inflammation may propose drug candidates that alleviate periodontal diseases and prevent periodontitis associated comorbidities.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: The CRP GWAS was obtained through https://gwas.mrcieu.ac.uk/datasets/ieu-b-35/. The periodontitis summary data are available at https://data.bris.ac.uk/data/dataset/2j2rqgzedxlq02oqbb4vmycnc2.

Author contributions

Conception and design, ZA and MN. Development of methodology, ZA, S-EB, HB, and MN. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis), ZA, S-EB, SR, and MN. Writing, review, and/or revision of the manuscript, ZA, S-EB, SR, BH, TK, HB, BE, and MN. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases), ZA. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Teun J. De Vries,
VU Amsterdam, Netherlands

REVIEWED BY
Gaetano Isola,
University of Catania, Italy
Olivier Huck,
Université de Strasbourg, France
Wei Qiu,
Southern Medical University, China

*CORRESPONDENCE
Hongmei Zhang
hmzhang@hospital.cqmu.edu.cn
Xiaoxiao Pang
pangxxhi@hospital.cqmu.edu.cn

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MiR-199a-5P promotes osteogenic differentiation of human stem cells from apical papilla *via* targeting IFIT2 in apical periodontitis

Jing Hu[1,2], Xia Huang[1], Liwen Zheng[1,2], Yuxin Zhang[1,2], Huan Zeng[1,2], Li Nie[1], Xiaoxiao Pang[1,3]* and Hongmei Zhang[1,2]*

[1]Chongqing Key Laboratory for Oral Diseases and Biomedical Sciences, the Affiliated Hospital of Stomatology of Chongqing Medical University, Chongqing, China, [2]Department of Pediatric Dentistry, the Affiliated Stomatology Hospital, Chongqing Medical University, Chongqing, China, [3]Chongqing Municipal Key Laboratory of Oral Biomedical Engineering of Higher Education, College of Stomatology, Chongqing Medical University, Chongqing, China

Introduction: Periapical alveolar bone loss is the common consequence of apical periodontitis (AP) caused by persistent local inflammation around the apical area. Human stem cells from apical papilla (hSCAPs) play a crucial role in the restoration of bone lesions during AP. Studies have recently identified the critical role of microRNAs (miRNAs) involved in AP pathogenesis, but little is known about their function and potential molecular mechanism, especially in the osteogenesis of hSCAPs during AP. Here, we investigated the role of clinical sample-based specific miRNAs in the osteogenesis of hSCAPs.

Methods: Differential expression of miRNAs were detected in the periapical tissues of normal and patients with AP via transcriptomic analysis, and the expression of miR-199a-5p was confirmed by qRT-PCR. Treatment of hSCAPs with miR-199a-5p mimics while loaded onto beta-tricalcium phosphate (β-TCP) ceramic particle scaffold to explore its effect on osteogenesis *in vivo*. RNA binding protein immunoprecipitation (RIP) and Luciferase reporter assay were conducted to identify the target gene of miR-199a-5p.

Results: The expression of miR-199a-5p was decreased in the periapical tissues of AP patients, and miR-199a-5p mimics markedly enhanced cell proliferation and osteogenic differentiation of hSCAPs, while miR-199a-5p antagomir dramatically attenuated hSCAPs osteogenesis. Moreover, we identified and confirmed Interferon Induced Protein with Tetratricopeptide Repeats 2 (IFIT2) as a specific target of miR-199a-5p, and silencing endogenous IFIT2 expression alleviated the inhibitory effect of miR-199a-5p antagomir on the osteogenic differentiation of hSCAPs. Furthermore, miR-199a-5p mimics transfected hSCAPs loaded onto beta-tricalcium phosphate (β-TCP) scaffolds induced robust subcutaneous ectopic bone formation *in vivo*.

Discussion: These results strengthen our understanding of predictors and facilitators of the key AP miRNAs (miR-199a-5p) in bone lesion repair under

periapical inflammatory conditions. And the regulatory networks will be instrumental in exploring the underlying mechanisms of AP and lay the foundation for future regenerative medicine based on dental mesenchymal stem cells.

KEYWORDS

apical periodontitis, microRNA, miR-199a-5p, human stem cells from apical papilla (hSCAPs), osteogenic differentiation, bone regeneration

1 Introduction

Apical periodontitis (AP) is a common oral disease characterized by alveolar bone destruction and inflammatory disorder of periapical tissues, which often cause the severe arrest of root development, resulting in masticatory dysfunction and even loose or lost teeth that reduce the quality of life of individuals (1, 2). In the acute stages of AP, serous exudation, tissue edema, dilatation, and hyperemia of periodontal vascular are the main manifestations, while patients with AP in the chronic inflammatory stage usually exhibit a pathological condition with the formation of inflammatory granulation tissue in the apical area, finally leading to periapical bone destruction (3, 4). However, the exact mechanisms that contributed to these clinical and pathological manifestations remain unclear.

As a subgroup of dental mesenchymal stem cells (MSCs), human stem cells from apical papilla (hSCAPs) can be obtained from the apical tissue of underdeveloped permanent teeth and have been identified as promising seed cells in tissue engineering due to their self-renewal and multi-lineage differentiation potential (5–7). Owing to the capacity to diverge into distinct cell lineages, such as odontogenic, neurogenic, chondrogenic and osteogenic, hSCAPs play a vital role in the development of the root, pulp-dentin complex, and alveolar bone (8, 9). In particular, it has been reported that SCAPs may have superior osteogenic differentiation capacity compared to bone marrow mesenchymal stem cells (BMSCs) (10). Nonetheless, it was also reported that an inflammatory microenvironment could alter the hallmarks of SCAPs, leading to an inhibitory or increasing effect on osteogenic differentiation (11, 12). Effective osteogenic differentiation of MSCs is regulated by numerous factors, including physical, chemical, and biological factors, which may stimulate different signaling pathways, transcription factors, and microRNAs (miRNAs), to direct MSCs differentiated toward osteoblast lineage (13–16). However, the exact role of miRNAs in promoting osteogenic differentiation has yet to be fully understood.

MiRNAs are an evolutionarily conserved set of small non-coding RNAs of approximately 18-22 nucleotides, and display their functions mainly *via* binding to the 3′ untranslated regions leading to degradation or post-transcriptional repression of the mRNA targets (17). Studies have previously implicated the essential regulatory roles of miRNAs in diverse biological or pathological processes, including tumor metastasis, cellular differentiation, proliferation, apoptosis, and tissue development (18–20). The pivotal roles of miRNAs in osteogenesis, such as osteoblast differentiation, angiogenesis, and intra-chondral bone formation have also been identified (21–23). Additionally, previous studies have revealed that miRNAs may play crucial roles in the development and progression of oral diseases, such as apical periodontitis, pulpitis and periodontitis (24–27). However, among more than 2,000 miRNAs identified in humans, only a few were reportedly involved in apical periodontitis (28–30) although their role in regulating osteogenesis of hSCAPs and in clinical apical periodontitis samples have not been validated. Therefore, it is of significance to determine the important roles of miRNAs in promoting osteogenesis in inflammatory periapical tissues as this line of investigation is essential to expanding our current endodontics knowledge and exploring new treatment strategies.

Here, to explore the role of miRNAs in the osteogenesis of hSCAPs during AP, we conducted high-throughput microRNA RNA-seq and validation in extensive clinical samples. We found a novel profile of miRNA in periapical tissues with AP patients, which is helpful to identify the impact of miRNAs which exert significant predictors and facilitators of bone lesion repair in apical periodontitis. Importantly, we demonstrated that miR-199a-5p effectively promoted the osteogenic activity of hSCAPs both *in vitro* and *in vivo via* directly regulating *IFIT2* expression, which suggests its possibility to be potentially utilized to facilitate bone regeneration during apical periodontitis.

2 Materials and methods

2.1 Collection and high-throughput RNA-Seq analysis of periapical tissue samples

The use of patient samples was approved by the Ethics Committee of the Affiliated Stomatological Hospital of Chongqing Medical University. Samples of periapical tissue were acquired from each patient with informed written consent. All relevant procedures were performed following the approved guidelines. As is recommended by the clinical guidelines for

endodontics, the diagnosis of apical periodontitis is based on the history, clinical examination, and periapical radiographic images (31). According to the correct clinical diagnosis, the periapical tissue samples were obtained from patients with severe periapical infection requiring extraction and whose roots had not yet been subjected to physiological resorption. The health control tissue samples were obtained from retained deciduous anterior teeth with a remaining root length greater than 2/3 and without carious. Furthermore, non-peer patients with systemic diseases such as diabetes, heart disease, asthma, etc., and with root resorption greater than 1/3 were excluded. A specific list of the inclusion and exclusion criteria for apical periodontitis is described in Table S1. The extracted teeth were placed in pre-cooled RNALater™ reagent (Beyotime, Shanghai, China) immediately and then rinse with cooling phosphate-buffered saline (PBS, Hyclone, UT, USA), the periapical tissue was quickly scraped (complete within 5 min on ice) and chilled in liquid nitrogen for 15 min, then kept at -80°C.

Periapical tissues from AP and healthy controls were subjected to high-throughput RNA-seq analysis by BMKCloud Biotechnology (Wuhan, China) to screen for differentially expressed miRNAs and mRNAs. Additionally, due to the small amount of periapical tissue in healthy controls, tissues from three different participants were pooled in each sample for sequencing. Potential target genes of miRNA were also predicted by bioinformatics analysis of the ENCORI database (https://starbase.sysu.edu.cn/), and relevant pathway enrichment analysis was performed by the DAVID website.

2.2 hSCAPs isolation, identification, and osteogenic differentiation

The apical papillae of teeth with underdeveloped roots were gently separated according to approved guidelines by the Stomatological Hospital Affiliated with Chongqing Medical University. The papilla tissues were digested with type I collagenase (Sigma, MO, USA) solution, and then maintained in Dulbecco's Modified Eagle's Medium with low glucose (L-DMEM, Hyclone, UT, USA) containing 10% fetal bovine serum (FBS, Hyclone, UT, USA), 1% penicillin-streptomycin at 37°C with 5% CO2, and change the medium at 3-day intervals. Three passages of hSCAPs were used in subsequent experiments.

Surface markers of hSCAPs were analyzed by flow cytometry on a BD Accuri C6 flow cytometer (BD Biosciences, CA, USA). Briefly, Cells were stained with FITC rabbit anti-CD90 (Sino-Biological, Beijing, China), anti-CD29 (Sino-Biological, Beijing, China), and anti-CD45 (Sino-Biological, Beijing, China). FlowJoTM software (Tree Star, Inc., Ashland, OR, USA) was applied to analyze the results with statistical calculations of the percentage of positive cells for visualization in histograms.

For osteogenic differentiation induction, hSCAPs were incubated in an osteogenic medium containing L-DMEM with 10% FBS, 100nM dexamethasone (Sigma, MO, USA), 10 mM β-glycerophosphate (Sigma, MO, USA), and 50 ug/ml ascorbic acid (Sigma, MO, USA).

2.3 Transient transfection of miRNA mimic, antagomir, NC, and siRNA

The miRNA mimics, antagomir and siRNAs were generated by Tsingke Biotechnology Co., Ltd. (Beijing, China), and then they were transfected into hSCAPs with HiPerFect transfection reagents (QIAGEN, Duesseldorf, Germany) according to the producer's instructions. MiRNA mimics and NC were transfected at a concentration of 20 nM, while miR-199a-5p antagomir was used at 50 nM and incubated for 24 h. And then, the transfection medium was replaced with a normal growth medium or osteogenic induction medium to terminate the transfection according to the experimental needs. The specific sequences of miR-199a-5p mimics and antagomir are shown in Table S2.

2.4 Alkaline phosphatase assays and alizarin red S staining

ALP staining and ALP activity quantification assays were conducted after osteogenic induction at 3 or 7 days according to the instructions of an NBT/BCIP staining kit (Beyotime, Shanghai, China) and Alkaline Phosphatase Assay Kit (Nanjing Jiancheng Bioengineering Institute, China), respectively.

Alizarin red S staining was performed to detect mineralized nodules. Briefly, hSCAPs were first rinsed with PBS, fixed in 4% paraformaldehyde (Solarbio, Beijing, China), and subsequently dyed in 1% alizarin red solution (Solarbio, Beijing, China). For semi-quantitative analysis, Image J software will be utilized to analyze the ARs stained images, calculating the percentage of positive areas, three different stained images of ARs will be included in each group.

2.5 Total RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction

The RNAeasy™ Plus Animal RNA Isolation Kit (Beyotime, Shanghai, China) was utilized to extract total RNA. Reverse transcription was performed by using random hexamer primers or miRNA-specific stem-loop RT primers followed by the PrimeScript® RT reagent kit instructions (TaKaRa, Tokyo, Japan), and cDNA generated from mRNA or miRNA are used as templates for amplified with TB Green Premix Ex Taq II (TaKaRa, Tokyo, Japan). In addition, Poly(A) was added to the 3' end of the miRNAs, followed by a reverse transcription reaction with Oligo (dT)-Universal Tag reverse transcription primers to generate the first strand of cDNA corresponding to the miRNA and then measured by qPCR with specific forward primers and commercially accessible reverse primers according to the producer's instructions (TIANGEN, Beijing, China). The relative expression of mRNA or miRNA was assessed by standardizing with those of GAPDH or U6, respectively. The primer sequences which were used in this research are shown in Tables S3, S4.

2.6 CCK-8 assay

Approximately 3×10^3 cells/well were incubated in a 96-well plate and subsequently transfected with miR-199a-5p mimics, antagomir, NC, and siRNA. After transfection, replaced the original medium with a fresh complete medium containing 10 μL CCK8 reagent at specific time points, and incubated with cells for 1 h, then measure the absorbance at 450nm in a microplate reader (Perkin Elmer, Waltham, USA) and experiments were carried out in triplicates.

2.7 Crystal violet staining assay

Seed the hSCAPs in 35-mm dishes and after the cells are plastered, transfected with NC, miR-199a-5p antagomir, and mimics. Crystal violet (Beyotime, Shanghai, China) staining assays were conducted on these transfected cells at different indicated time points following the reagent instructions. Next, dissolve the stained cells in 33% acetic acid at room temperature, and measure the OD value at 570-590 nm for quantitative measurements.

2.8 RNA immunoprecipitation and RNA sequencing

RIP was performed with the Imprint® RNA immunoprecipitation Kit (Sigma, MO, USA). Ten million cells were harvested and lysed in mild lysis buffer (B0314, Sigma, MO, USA) with protease inhibitors and ribonuclease inhibitors, and 5% of each cell lysate was removed as input. Protein A magnetic Beads (B0689, Sigma, MO, USA) were pre-incubated with anti-IgG (I5006, Sigma, MO, USA) or anti-AGO2 (ac186733, Abcam, UK) at room temperature with rotation for 30 min, and then cell lysate was added for further incubation with rotation overnight at 4 °C. The precipitated RNA was extracted by using an RNA Isolation Kit with Spin Column (Beyotime, Shanghai, China) following the producer's instructions.

The rRNA was removed from the immunoprecipitated RNA, and then the products were subjected to high-throughput sequencing by Huada (BGI) Medical Laboratory Co., LTD (Wuhan, China). Filter the sequencing data with SOAPnuke26 (32) to obtain clean data, which were then stored and mapped to the reference genome using HISAT2. Dr. Tom's multi-omics data mining system (https://biosys.bgi.com) was then applied to conduct data mining and analysis.

2.9 Western blot

Extraction of the total protein from cells in RIPA lysis buffer (Beyotime, Shanghai, China) containing the cocktail. Approximately 25 μg of protein was detached *via* a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel, and further transmitted to 0.45 μm PVDF membranes (Millipore, MA, USA) and blocked in TBST containing 5% fat-free milk for about 1 hour. Later, incubate the membranes with antibodies. Visualization of immunological assays was performed by using a chemiluminescent ECL reagent (Beyotime, Shanghai, China). Primary antibodies were used as follows: anti-AGO2 antibody (Abcam, Cambridge, UK), anti-IFIT2 antibody (Proteintech, Wuhan, China), anti-RUNX2 antibody (Abcam, Cambridge, UK), anti-ALP antibody (Abcam, Cambridge, UK), anti-OPN antibody (Abcam, Cambridge, UK), and anti-GAPDH antibody (ZenBioscience, Chengdu, China).

2.10 Dual luciferase reporter analysis

293T cells are used for reporter gene assay. A wild-type reporter vector (IFIT2-3′-UTR- WT) was produced by fusing the IFIT2 3′-UTR sequence with the binding site of miR-199a-5p to the pmirGLO luciferase reporter vector (Promega). In addition, the mutant reporter vector (IFIT2-3′-UTR-MUT) was derived by inserting sentinel mutagenesis of the miR-199a-5p binding site from the IFIT2 3′-UTR sequence into the luciferase reporter vector. These reporter vectors were then cotransfected with miR-199a-5p mimics along with 293T cells by using Hieff TransTM Liposomal Transfection Reagent (YEASEN, Shanghai, China). Eventually, a dual luciferase reporter system (YEASEN, Shanghai, China) was applied to determine the luciferase activity 24 hours post-transfection. Light intensities were standardized with renilla luciferase.

2.11 *In vivo* ectopic bone formation and histological evaluation

All animal experiments were conducted under the ethical committee guidelines of the Affiliated Stomatological Hospital of Chongqing Medical University. Cells from each group (approximately 3×10^6 cells per group) were loaded on β-tricalcium phosphate (β-TCP) porcelain granules (Bio-lu Biomaterials, Shanghai, China) and subcutaneously implanted the mixture into the flanks of 6-week-old BALB/c nude mice. 8 weeks later, these implants were obtained, fixed with paraformaldehyde (4%), decalcified in EDTA decalcification solution (Servicebio, Wuhan, China), and embedded in paraffin. Tissues embedded were serially sliced (5 μm) and processed for hematoxylin and eosin (H&E; Solarbio, Beijing, China) staining and Masson trichrome (Solarbio, Beijing, China) staining. Furthermore, the immunohistochemistry (IHC) staining was also carried out with anti-OCN antibodies (Abcam, Cambridge, UK) as previously described (33). The images were acquired by digital section scanner VS200 (Olympus, Japan).

2.12 SEM imaging and energy dispersive spectrometry analysis

The surface morphologies of β-TCP were observed and imaged by scanning electron microscopy (SEM, ZEISS, Sigma 300, Germany), and the particle sizes were evaluated by Nano Measurer 1.2 software based on its morphology map. An energy

dispersive X-ray spectrometer (EDS, Oxford Instruments, Xplore, UK) was used to detect the elemental composition and distribution of the scaffolds.

2.13 Micro-CT analysis

Micro-CT scans were undertaken by Chongqing Key Laboratory of Oral Diseases and Biology. Micro-CT images were processed by Mimics Research 21.0 and 3 Matic Research 13.0 software to conduct 3D reconstruction and volumetric quantification. The eligible areas within the scaffold were picked to measure total volume (CT threshold above 4000HU) and bone volume (CT threshold between 4000HU-5500HU), for calculating the percentage of occupation of ectopic osteogenesis.

2.14 Statistical analysis

The studies were conducted independently three times at least, and differences in variables between groups were assessed with Graphpad 8.0 software using a student t-test or one-way ANOVA. $p < 0.05$ is regarded as statistically significant. Data were presented as the mean ± SD.

3 Results

3.1 Differential miRNAs expression identified by high-throughput microRNA RNA-seq analysis of periapical tissue isolated from teeth with normal and chronic apical periodontitis

To explore the role of miRNA in the osteogenic differentiation of inflamed periapical tissue, conventional RNA-seq and microRNA RNA-seq were conducted to determine the distinct expression patterns of miRNA and mRNA between periapical tissues from AP patients (S) and health controls (C). Based on the correlation analysis between AP and control groups (Figure 1A), two samples from each group were selected to be further analyzed. Differential expression analysis based on |log2FC| (fold change) >1 and p-value<0.05 identified 1869 up-regulated mRNAs and 1566 down-regulated mRNAs (Figure 1B), as well as 89 up-regulated miRNAs and 67 down-regulated miRNAs. (Figure 1C). Then, with further parameters setting the basal expression level (counts>10), 12 up-regulated and 6 down-regulated miRNAs were detected in AP tissues compared to healthy controls, including miR-335-5p and miR-455-3p (Figure 1D), which were previously reported to

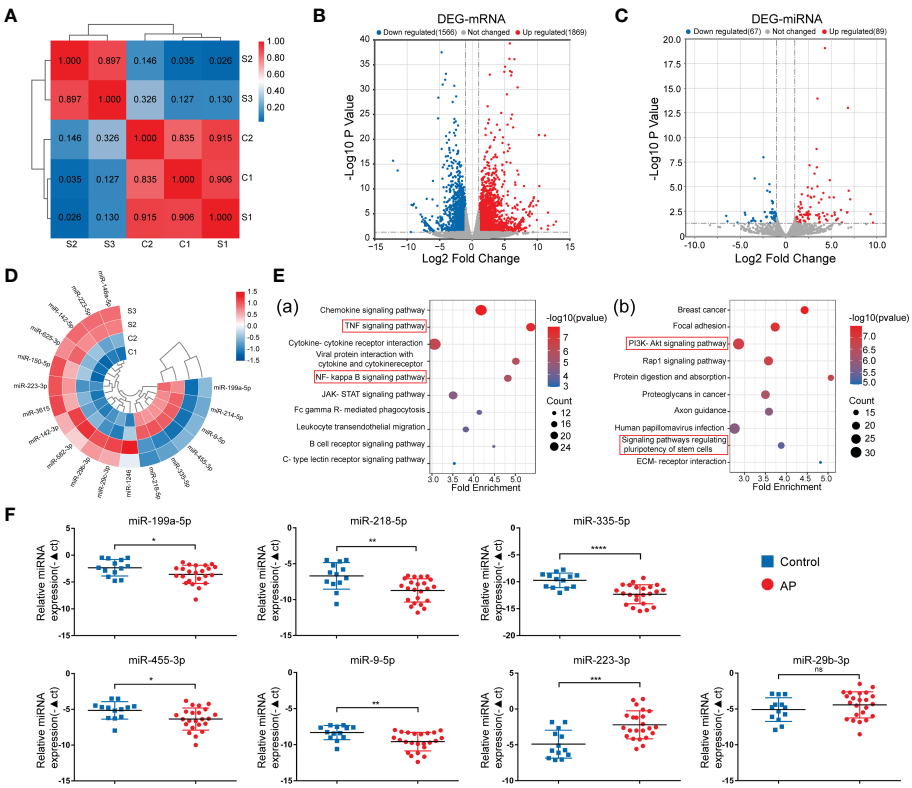

FIGURE 1
Identification of DE-miRNAs in periapical tissue. **(A)** Correlation analysis of the sequenced samples. **(B)** Volcano diagram of DE-mRNAs. **(C)** Volcano diagram of DE-miRNAs. **(D)** Circular heatmap of 12 up-regulated and 6 down-regulated miRNAs. **(E)** KEGG pathway enrichment analysis of the intersection genes between the DE-mRNAs and the predicted target genes of DE-miRNA. **(F)** Relative expression of the seven miRNAs in samples of periapical tissues (AP/Control:23/13). Data were presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, ns, no significance.

enhance osteogenic differentiation of MSCs (34, 35), and were chosen for subsequent bioinformatics analyses.

First, *via* intersecting the target genes of the 18 DE-miRNAs predicted by databases with the DE-mRNAs identified by RNA-seq in our study, we found 445 up-regulated mRNAs and 633 down-regulated mRNAs, respectively (Figures S1A, B). Second, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was used to classify potential functions of these genes (Figure 1E), and we found that the majority of enriched pathways were pointed to the classic inflammation-related signaling pathways, such as TNF and NF-KB signaling pathways (36–38), or pathways regulating stem cell pluripotency and PI3K-Akt signaling pathways associated with osteogenic differentiation (39). By exploring the upstream miRNAs of the genes included in these enriched pathways, seven miRNAs, including upregulated miR-29b-3p and miR-223-3p, as well as down-regulated miR-335-5p, miR-9-5p, miR-218-5p, miR-455-3p, and miR-199a-5p were identified, indicating that these differentially expressed miRNAs may exert osteogenesis effects in apical periodontitis. Lastly, the expression of these miRNAs was validated by qPCR analysis of the periapical tissues from 23 AP patients and 13 healthy controls (Figure 1F). Consequently, the above seven miRNAs were chosen as candidates for further study.

3.2 7 Validation of expression pattern of the seven differentially expressed miRNAs during osteogenic differentiation of hSCAPs

Since the target genes of 7 miRNAs were most enriched in the osteogenic-related signaling pathways, we isolated hSCAPs from young permanent teeth with underdeveloped roots first (Figures S2A, B). The expressions of surface markers were detected by FACS, and results showed that MSC surface markers CD90 and CD29 were positive in hSCAPs but negative for hematopoietic stem cell marker CD45 (Figure S2C). We also detected the expression levels of these candidate miRNAs at different time points of osteogenic differentiation of hSCAPs (Figure 2A) and found that the expression of miR-199a-5p and miR-455-3P gradually elevated, while miR-9-5p, miR-335-5p, and miR-223-3p expression gradually decreased during the osteogenic differentiation of hSCAPs.

We further compared the miRNA expression profiles in hSCAPs with that in periapical tissues to determine the candidate miRNAs involved in periapical osteogenesis. Four miRNAs, including miR-199a-5p, miR-9-5P, miR-455-3P and miR-223-3P, were selected and validated by early osteogenic phenotype, Alkaline phosphatase (ALP) staining. As demonstrated by ALP staining and ALP activity assay, the osteogenic differentiation of hSCAPs was significantly promoted by miR-199a-5p mimics (Figures 2B, C), which was more pronounced than other miRNA overexpression groups. Furthermore, only the miR-199a-5p overexpression group significantly upregulated the expression of osteogenic marker genes *RUNX2*, *ALP*, and *OCN* in hSCAPs (Figure 2D). Collectively, these data suggest that miR-199a-5p may modulate the osteogenesis of hSCAPs.

3.3 miR-199a-5p positively modulates the proliferation and osteoblast differentiation of hSCAPs

To confirm the biological function of miR-199a-5p in cell proliferation and osteogenic differentiation of hSCAPs, we first transfected hSCAPs with miR-199a-5p mimics, antagomir, and negative control (NC), respectively. We found that the accumulation level of mature miRNAs still maintained several hundred-fold increases after transfection of miRNA mimics into hSCAPs for several days (Figure S3). The CCK-8 assay revealed that hSCAPs transfected with miR-199a-5p mimics exhibited increased proliferation as compared to NC, while decreased when miR-199a-5p was knocked down in hSCAPs (Figure 3A). Crystal violet staining revealed statistically significant increased numbers of cells in the miR-199a-5p overexpression group than those transfected with NC or miR-199a-5p antagomir after seeding at the same initial density (Figures 3B). These results reveal that miR-199a-5p may promote the proliferation of hSCAPs and enhance their self-renewal capacity.

Furthermore, we assessed the function of miR-199a-5p in regulating the osteogenic differentiation of hSCAPs. The hSCAPs transfected with miR-199a-5p mimics, antagomirs, and NC for 24h were incubated in osteogenic induction media for 3 or 7 days. Both the expression and activity of ALP were markedly increased in the miR-199a-5p overexpression group (Figures 3C, D). Moreover, we determined the expression levels of the osteoblast-relevant markers by qPCR and found that *RUNX2, OSX, ALP, and OCN* expression were significantly increased in the miR-199a-5p overexpressing group on 3 and 7 days of osteogenic induction (Figure 3E). As expected, we observed a significant decrease in ALP staining and activity with inhibition of miR-199a-5p in hSCAPs. Meanwhile, miR-199a-5p knockdown in hSCAPs led to *RUNX2* and *ALP* inhibition, while *OSX* and *OCN* expression were not affected. Alizarin red staining assay and semi-quantitative analysis revealed that miR-199a-5p overexpression remarkably enhanced calcium nodule deposition (Figures 3F, G). The qPCR analysis showed that miR-199a-5p expressing hSCAPs exhibited a dramatically increased expression of *ALP*, *OCN*, OSX, and RUNX2 than those in the control cells (Figure 3H). Taking together, we demonstrate that miR-199a-5p may facilitate the osteogenic differentiation of hSCAPs *in vitro*.

3.4 miR-199a-5p directly targets IFIT2 in hSCAPs

To identify the target mRNAs post-transcriptionally modulated by miR-199a-5p involved in osteogenic differentiation of hSCAPs, we performed an Ago2 RIP-sequencing. Notably, it was determined that miR-199a-5p was enriched in the immunoprecipitates of the anti-AGO2 group when compared with the IgG group by qPCR, and miR-199a-5p enrichment was remarkably higher in the overexpression group than that in the controls (Figure 4A). Next, we performed RNA-seq analysis of differential expression genes

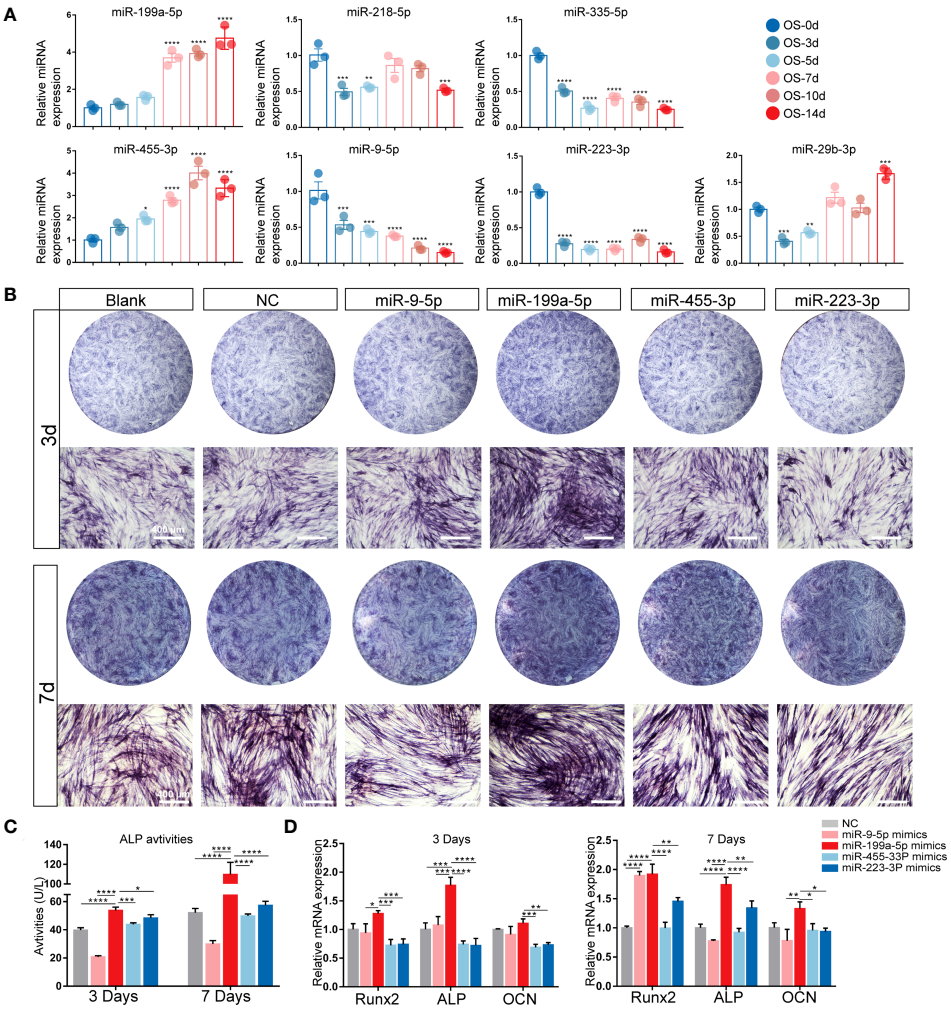

FIGURE 2
Expression patterns of the candidate miRNAs during osteogenic differentiation of hSCAPs. **(A)** Relative expression of the 7 screened miRNAs. **(B)** ALP staining of different miRNA overexpression groups after 3 and 7 days of osteogenic induction (scale bar = 400 μm). **(C)** ALP activity in the different groups. **(D)** The relative mRNA expression level of osteogenic marker genes RUNX2, ALP, and OCN. The data were shown as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

enriched in AGO2 between the controls and the miR-199a-5p overexpression group. Based on *p*-value (<0.05), and fold change (>2), 135 up-regulated mRNAs were collected (Figure 4B), which were subsequently intersected with the predicted target genes of miR-199a-5p (Figure 4D). Consequently, 9 potential target genes were obtained and confirmed by qPCR in RIP products (Figure S4A). Among them, *IFIT2* was the most significantly elevated one.

Furthermore, Protein-Protein Interaction networks (PPI) analysis (Figure 4C) of the up-regulated genes in RIP-sequencing revealed multiple IFIT family genes that are closely linked and enriched (Figure S4B). Additionally, we conducted Gene Ontology (GO), Reactome pathway, and KEGG pathway enrichment analysis on these 135 significantly up-regulated genes, in which type 1 interferon signaling pathway was found to be involved (Figures 4E-G). Moreover, overexpression of miR-199a-5p in hSCAPs revealed a significant downregulation of IFIT2 expression at the protein level, while slightly declining expression of *IFIT2* at the mRNA level. Conversely, miR-199a-5p expression inhibition in hSCAPs enhanced IFIT2 expression at both mRNA and protein levels (Figures 4H, I). A luciferase reporter assay was conducted to confirm the direct association between the 3'UTR of IFIT2 and miR-199a-5p (Figures 4J, K). Collectively, these results demonstrate that miR-199a-5p binds directly to *IFIT2* and acts as a negative regulator of *IFIT2* in hSCAPs.

3.5 Knockdown of IFIT2 can rescue the impact of endogenous miR-199a-5p reduction on osteogenesis

To investigate whether miR-199a-5p functionally targets *IFIT2* in modulating hSCAPs osteogenic differentiation, we first repressed *IFIT2* expression by transfecting hSCAPs with siRNAs against *IFIT2*. Three pairs of siRNA against *IFIT2* were tested and *IFIT2* expression was remarkably repressed by siIFIT2-1 at mRNA levels (Figure 5A). Consequently, siIFIT2-1 was chosen in subsequent

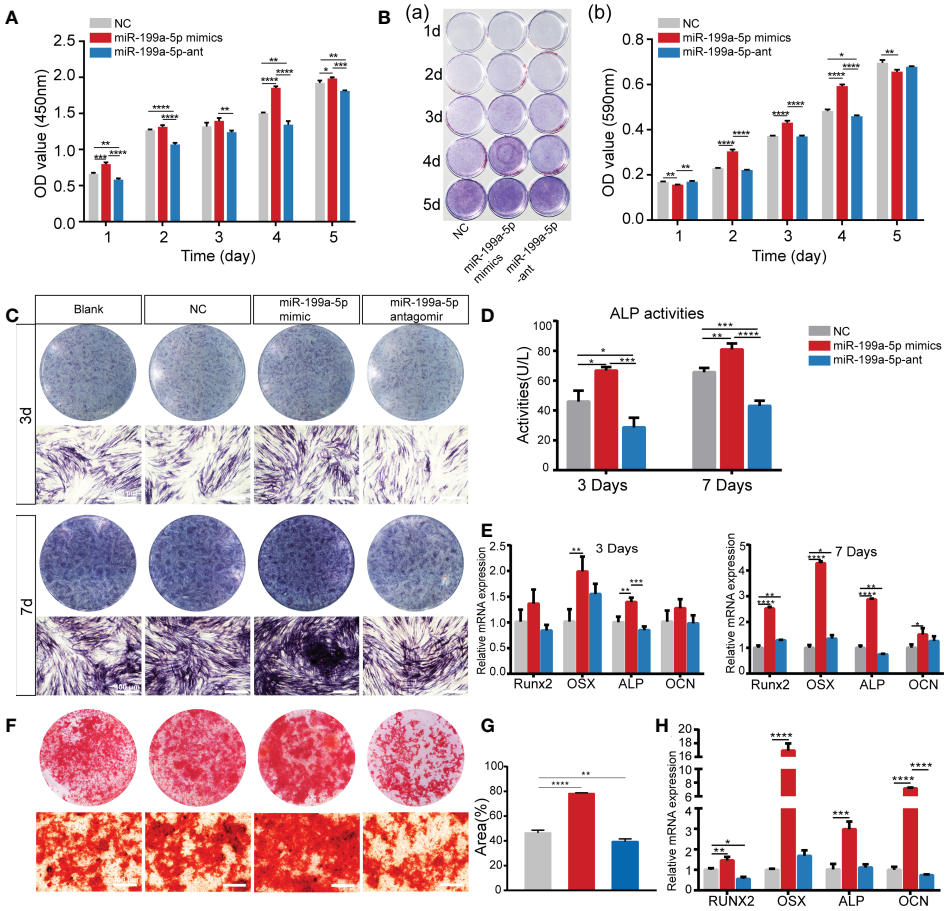

FIGURE 3
miR-199a-5p positively regulates the proliferation and osteogenic differentiation of hSCAPs *in vitro*. **(A)** The CCK8 assay. **(B)** The Crystal violet staining and quantification assay. **(C)** ALP staining of different groups after 3 and 7 days of osteogenic induction (scale bar = 400 μm). **(D)** ALP activity assay. **(E)** The relative mRNA expression level of osteogenic marker genes RUNX2, OSX, ALP, and OCN. **(F)** ARs staining assay (scale bar = 400 μm). **(G)** Semi-quantitative analysis of ARs stained images. **(H)** The relative mRNA expression level of osteogenic marker genes RUNX2, OSX, ALP, and OCN. The data were shown as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

functional experiments. By using *IFIT2*-specific siRNAs, the results revealed that the osteogenic differentiation of hSCAPs was upregulated after *IFIT2* knockdown as demonstrated by increased osteogenesis makers, including RUNX2, ALP, and OPN at the protein levels (Figure 5D) and *RUNX2*, *OSX*, *ALP*, and *OCN* at the mRNA levels (Figure 5E). Furthermore, ALP staining and quantification assay showed that *IFIT2* downregulation remarkably enhanced ALP activities of hSCAPs in the induction of osteogenesis (Figures 5B, C). Importantly, *IFIT2* silencing markedly reversed the reduced ALP activities induced by miR-199a-5p inhibition (Figures 5F, G). The altered expression of RUNX2, ALP at the protein levels and *RUNX2*, *OSX*, *ALP*, and *OCN* at the mRNA levels further reinforced a resemble rescue effect of IFIT2 inhibition. (Figures 5H, I). Since miR-199a-5p transfected hSCAPs showed increased proliferation, we conduct the CCK8 assay to determine if *IFIT2* knockdown could rescue the decreased proliferation of hSCAPs transfected with miR-199a-5p antagomir. The results showed that *IFIT2* silencing accelerated the proliferation of hSCAPs transfected with antagomir (Figure 5J). Taken together, the above results suggest that miR-199a-5p may regulate osteogenic differentiation of hSCAPs *via* targeting *IFIT2*.

3.6 β-TCP ceramic particles loaded with miR-199a-5p expression hSCAPs effectively promote bone formation *in vivo*

We conducted an ectopic osteogenesis assay in BALB/c nude mice to further investigate the impact of miR-199a-5p on bone formation *in vivo*. Briefly, hSCAPs were transfected with NC or miR-199a-5p mimics and cultured for two days in osteogenic induction media. The transfected hSCAPs were then collected and loaded onto β-tricalcium phosphate (β-TCP) scaffolds, which subsequently were implanted subcutaneously into the flanks of mice (n=3).

We first analyzed the characteristics of the scaffolds. SEM imaging (Figure S5A) showed the surface morphology of the scaffolds, and the diameter of the ceramic particles was measured to be approximately 1.48 μm (Figure S5B). Furthermore, the elemental analysis indicated that the scaffold was composed of calcium, phosphate, and oxygen (Figure S5C), which contented 38.20%, 19.56%, and 41.53% by weight, respectively (Figure S5D).

The representative three-dimensional (3D) reconstruction and micro-CT images of sagittal profiles of the retrieved scaffolds revealed that the miR-199a-5p overexpression group had a much

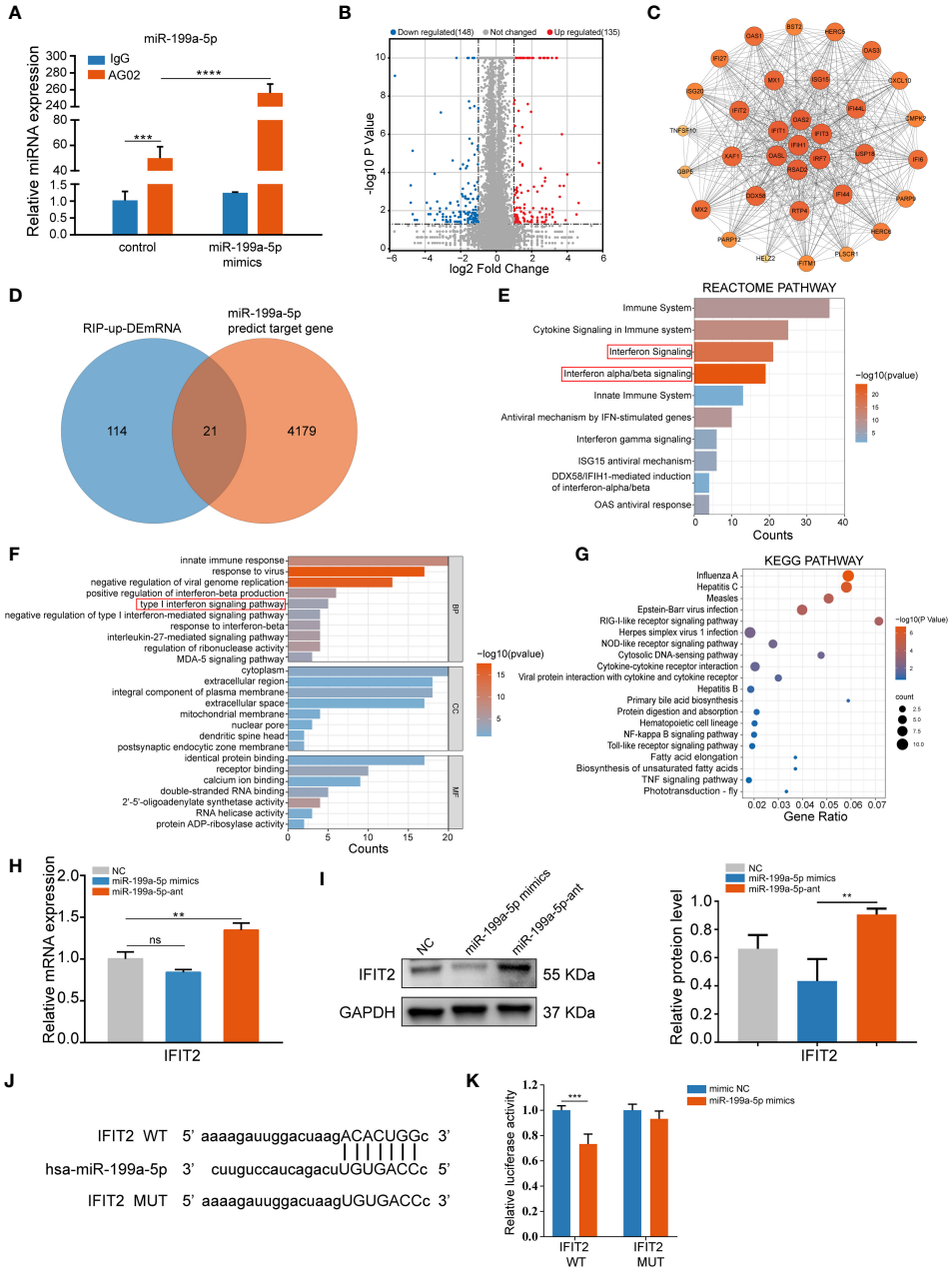

FIGURE 4
miR-199a-5p directly targets *IFIT2* in hSCAPs. **(A)** The relative expression level of miR-199a-5p in immunoprecipitates. **(B)** The volcano plot of differentially enriched mRNAs in the Ago2 immunoprecipitation complex between the miR-199a-5p overexpression and the control group. **(C)** Protein-Protein Interaction (PPI) analysis of the up-regulated genes. **(D)** Venn diagram of the intersection genes between the significantly up-regulated mRNAs (blue) and the predicted target genes of miR-199a-5p (orange). **(E)** Reactome pathway analysis of the up-regulated mRNAs. **(F)** GO analysis of the up-regulated mRNAs. **(G)** KEGG pathway enrichment analysis of the up-regulated mRNAs. **(H)** The relative expression level of IFIT2 at mRNA levels. **(I)** The protein expression levels of IFIT2. **(J)** Potential binding sites between IFIT2 and miR-199a-5p. **(K)** Dual-luciferase reporter assay. The data were shown as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, ns, no significance.

higher density of bone formation, as well as a greater BV/TV ratio (Figure 6A). H & E staining showed a significant increase of bone mass on scaffolds loaded with miR-199a-5p overexpressing hSCAPs and more collagenous tissue in Masson trichrome staining. Furthermore, immunohistochemistry analysis showed that the miR-199a-5p overexpression group had more OCN-positive cells in the bone fragments, as compared to the controls (Figure 6B). Taken together, these results indicate that miR-199a-5p overexpression promotes the osteogenesis of hSCAPs *in vivo* and β-TCP ceramic particles loaded with miR-199a-5p expressing hSCAPs display effective osteogenic capacity *in vivo*.

4 Discussion

In this study, to investigate the role of miRNAs during AP, microRNA and mRNA sequencing were performed on the periodical tissue samples from AP patients and healthy controls.

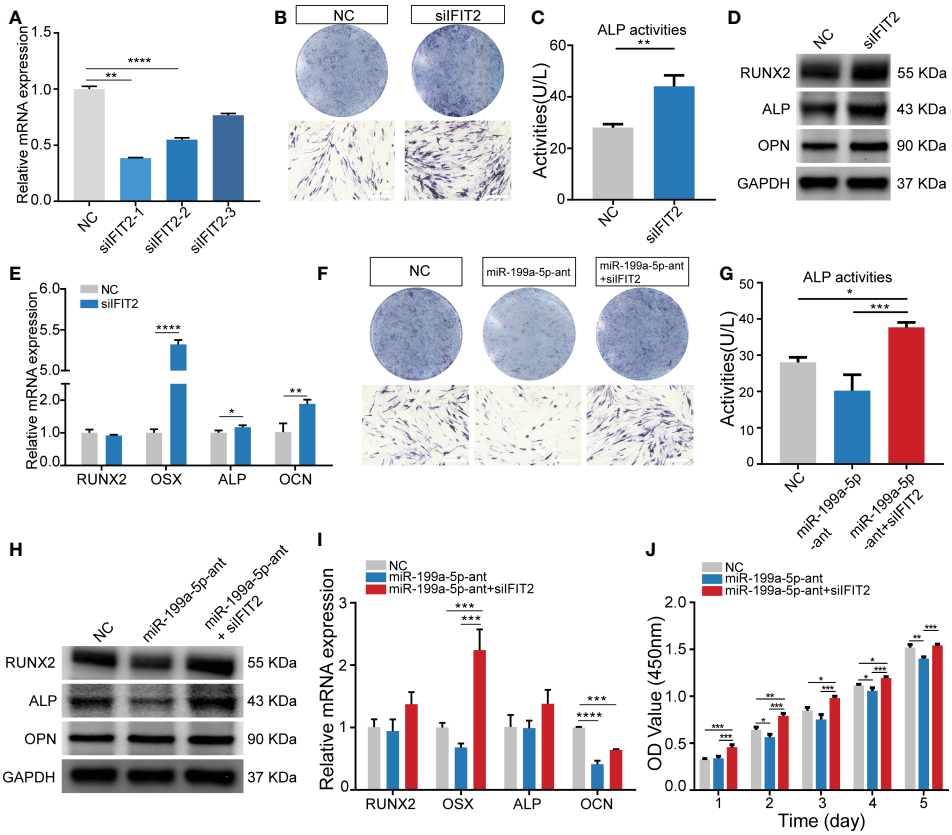

FIGURE 5
Knockdown of *IFIT2* can reverse the effect of endogenous miR-199a-5p reduction on osteogenesis. **(A)** The knockdown efficiency of the three pairs of IFIT2 siRNAs. **(B)** ALP staining after 3 days of osteogenic induction (scale bar = 400 μm). **(C)** ALP activity assays. **(D)** The protein expression levels of RUNX2, ALP, and OPN. **(E)** The relative mRNA expression level of osteogenic marker genes *RUNX2*, *OSX*, *ALP*, and *OCN*. **(F)** ALP staining after 3 days of osteogenic induction (scale bar = 400 μm). **(G)** ALP activity assay. **(H)** The protein expression levels of RUNX2, ALP, and OPN. **(I)** The relative mRNA expression level of osteogenic marker genes *RUNX2*, *OSX*, *ALP*, and *OCN*. **(J)** The CCK-8 assay. The data were shown as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

By validation in extensive clinical samples and functional experiments, we identify a differentially expressed key miRNA (miR-199a-5p), which plays a critical role in the osteogenesis of hSCAPs during AP. We found that overexpression of miR-199a-5p could promote osteogenic differentiation of hSCAPs *in vivo* and *in vitro* by directly targeting IFIT2.

Inflammation of periapical tissues commonly presents with the destruction of the alveolar bone, which is generally treated by controlling the infection and eliminating the inflammation to promote the repair of the periapical bone defects (40–43). Currently, miRNAs, transglutaminases, and other agents have been found to be involved in the development and progression of oral diseases (25, 44, 45). Most importantly, miRNAs have been implicated as valid regulators that can modulate multiple biological processes, including bone regeneration and anti-inflammatory response (46, 47). As Shen and Silva (25) have reported that over 100 miRNAs were differentially expressed in AP and the highest expression of miR-10a-5p appeared to be involved in triggering anti-inflammatory signaling and promoting healing. It is noteworthy that different phases of inflammatory conditions may have different effects on the bone regenerative capacity of stem cells, as Liu et al. (2016) (48) showed that long-term exposure to pro-inflammatory cytokines inhibited the osteogenic differentiation of SCAPs. However, Hess et al. (2009) (49) revealed that TNF-α promoted osteogenic differentiation of human MSCs by triggering the NF-κB signaling pathway. The above results indicate that there are complex signals modulating the differentiation of stem cells under inflammatory conditions. Therefore, we further performed functional enrichment analysis and phenotypic validation of both up-regulated and down-regulated miRNAs-mRNAs networks to comprehensively identify key miRNAs that impact AP bone repair.

Previously, the role of miRNAs in apical periodontitis and pulp diseases has been explored by microarray studies, which identified several miRNAs that were significantly differentially expressed in AP, including miR-181 family, miR-10a-5p, and also miR-199a-5p (28, 30). However, the AP and control samples were not probed in the same chip, which decreased the reliabilities, while miR-199a-5p was not further validated in extensive clinical samples. Interestingly, in our study, we performed miRNA and mRNA sequencing of AP and control samples in the same batch, and we found that miR-199a-5p was one of the most differentially expressed miRNAs, which was further validated in adequate clinical samples to guarantee that the screened miRNAs display a stronger correlation with bone defect repair in AP. Meanwhile, we summarized the characteristics of the

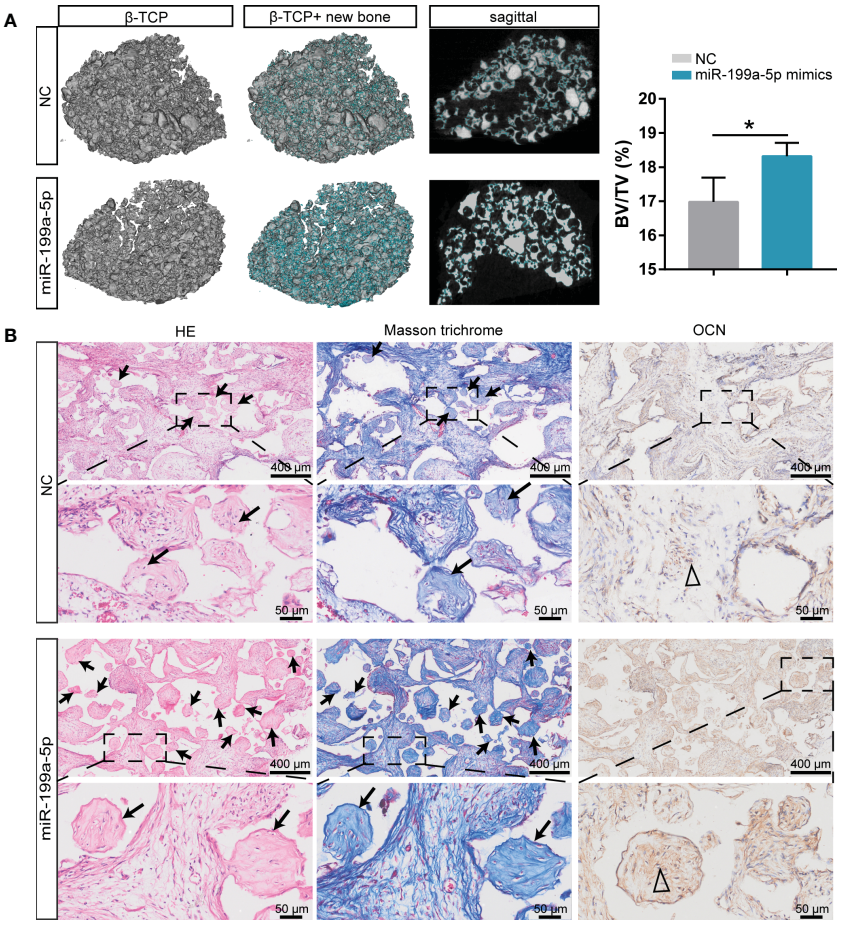

FIGURE 6
Overexpression of miR-199a-5p enhances hSCAPs osteogenic differentiation *in vivo*. **(A)** Left: Representative micro-CT images of 3D reconstruction and sagittal profiles of bone formation in *β*-TCP scaffolds of miR-199a-5p mimics (n = 3) and NC groups (n = 3). The blue areas: the region of new bone formation. Right: Quantitative analysis of BV/TV (%). The data were expressed as mean ± SD. **p* < 0.05. **(B)** Representative images of H&E staining, Masson's trichrome staining and immunohistochemical staining of OCN. The black arrows: bone formation. The triangle: positive cells.

patients (Figure S6A) and conducted a correlation analysis between miR-199a-5p expression and periapical lesion status based on the periapical index (PAI) (Figure S6B), which is the most classic scoring system for the evaluation of AP (50). The results showed a negative correlation between miR-199a-5p expression and periapical lesion status (R=-0.5429) (Figures S6C–E). Subsequently, the functional analysis indicated that miR-199a-5p enhanced osteogenesis of hSCAPs, which may play a pivotal role in bone lesion repairment during AP. Besides, the pathway enrichment analysis of mRNA sequencing in clinical samples and AGO2-RIP sequencing in hSCAPs all pointed to various inflammatory pathways, including TNF signaling pathway and NF-kappa B signaling pathway, indicating that the miRNA identified in our study, miR-199a-5p, may exhibit great potency of promoting osteogenesis even in the scenario of inflammation, which needs to be further studied, such as the role of this key miRNA for bone defect repair under the inflammatory microenvironment and the impact of the immunological components, including inflammatory factors and biological responses on bone formation.

miRNAs are considered as 'junk' RNA to regulate most cellular events through identifying multiple target genes. A previous study has shown that miR-199a-5p overexpression could enhance osteoblast differentiation of human MSCs through regulation of the HIF1a-Twist1 pathway (51). However, we failed to find the genes enriched in AGO2-RIP samples involved in the HIF1a-Twist1 pathway in our study, which indicates that miR-199a-5p could facilitate osteogenic differentiations in different stem cells, but the specific mechanism may be different. Another aspect, miR-199a-5p was shown to positively regulate osteoclast differentiation by targeting Mafb protein (52), which suggests that miR-199a-5p may have a vital role in regulating bone homeostasis *via* simultaneously mediating osteoblast and osteoclast differentiation, but its specific role and underly mechanism needs to be further investigated.

IFIT2, belonging to the interferon-stimulated gene (ISG) family, is widely expressed in mammalian tissues, including bone marrow (53). Previous studies showed that the endogenous Interferon beta (IFN*β*, type-1 IFN) activity represses osteoblast differentiation *in vivo and in vitro* (54, 55) and that low levels of type I IFN-induced cellular IFN activity are commonly mirrored by ISG expression (56, 57). Interestingly, except for *IFIT2*, we found that *IFIT1*, *IFIT3*, and *IFI44L* were also enriched in AGO2 protein transfected with miR-

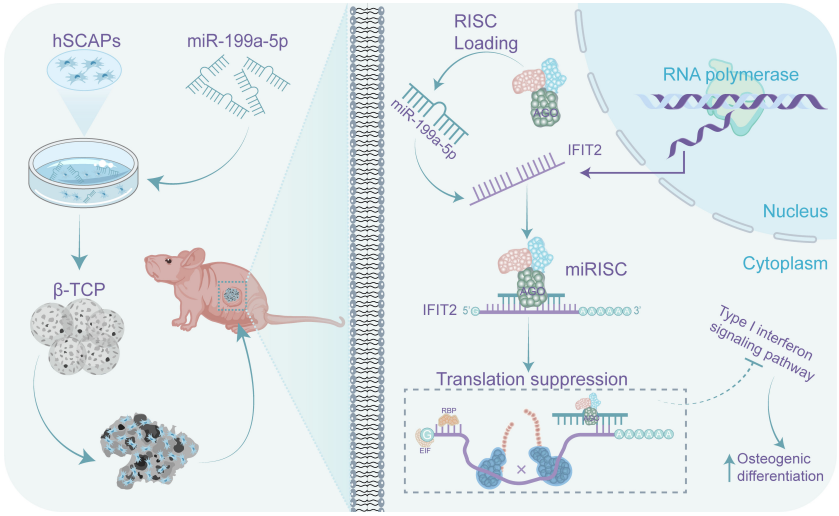

FIGURE 7
A schematic representation on how miR-199a-5p regulates the osteogenic differentiation of hSCAPs. miR-199a-5p promotes the osteogenic differentiation of hSCAPs *via* directly suppressing IFIT2 expression *in vitro* and *in vivo*, leading to blocking the type-1 interferon signaling.

199a-5p mimics, which strongly indicated that type-1 IFN response was blocked in the miR-199a-5p transfected hSCAPs, that may be the critical factor why miR-199a-5p promotes osteogenesis of hSCAPs, but the exact mechanism needs to be fully explored in the future. Additionally, overexpression of IFIT2 correlated with the diminished proliferative capacity of various cells (58, 59), which is consistent with our results that miR-199a-5p inhibiting *IFIT2* expression resulted in increased proliferation of hSCAPs. Collectively, these results reveal that miR-199a-5p promotes osteogenesis of hSCAPs *via* targeting *IFIT2* and blocking the endogenous type-1 IFN response.

For the regeneration of periapical bone tissue defects, SCAPs are valid candidates with excellent osteogenic capacity. According to the classical strategies for biomaterial substitutes used for bone tissue engineering (60), β-TCP ceramic particles were chosen as the scaffold, which displays excellent biocompatibility and osteoinductive capacities (61, 62) and is extensively used in clinical (63). Not surprisingly, β-TCP ceramic particles loaded with miR-199a-5p overexpressed hSCAPs exhibited more ectopic bone formation, which suggests that miR-199a-5p overexpressed hSCAPs hold potential for bone defects repairment and may be promising strategies for bone tissue regeneration. Furthermore, deploying an appropriate drug-delivery system for miR-199a-5p is needed to address its potential in orthotopic periapical tissue repair.

In summary, in this study, we revealed the profile of miRNAs in periapical tissues of AP patients and healthy controls by miRNA sequencing. We found that the most differentially expressed key miRNA (miR-199a-5p) enhanced osteogenic differentiation of hSCAP *in vivo* and *in vitro* by targeting IFIT2. Furthermore, immunoprecipitates significantly enriched in the anti-AGO2 group of hSCAP overexpressing miR-199a-5p were highly correlated with the type-1 IFN signaling pathway. Taken together, these findings suggest that increasing miR-199a-5p expression promotes bone regeneration during AP, which may be partly through the regulation of IFIT2 expression and type-1 IFN signaling (Figure 7). Thus, miR-199a-5p may be potentially utilized as a therapeutic target to facilitate bone defect repair in AP and to be identified as a diagnostic maker for AP.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: the miRNA and mRNA sequencing data are available in the SRA database with the BioProject ID PRJNA931286 and PRJNA930996, respectively. Our RIP-seq data can be accessed in the SRA database with the BioProject ID PRJNA933173.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of The Affiliated Hospital of Stomatology, Chongqing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by the Ethics Committee of The Affiliated Hospital of Stomatology, Chongqing Medical University.

Author contributions

JH: Study design, conducting experiments, data acquisition and collation, figure drawing, and manuscript draft. XH: Sample collection and data analysis. LZ and YZ: Data acquisition and analysis. HuZ and LN: Data analysis and collation. XP and HoZ contributed to the conception, design, and data interpretation of the study and critically revised the manuscript. All authors provided final approval and agree to be accountable for the content of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Teun J. De Vries,
VU Amsterdam, Netherlands

REVIEWED BY
Vivek Thumbigere Math,
University of Maryland, United States
Adina Bianca Bosca,
University of Medicine and Pharmacy Iuliu Hatieganu, Romania

*CORRESPONDENCE
Chunbo Tang
cbtang@njmu.edu.Cn

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Macrophages induce gingival destruction via Piezo1-mediated MMPs-degrading collagens in periodontitis

Tong Zhao[1,2], Zhuangzhuang Chu[1,2], Catherine Huihan Chu[2,3], Shuo Dong[1,2], Guoqing Li[1,2], Jin Wu[1,2] and Chunbo Tang[1,2]*

[1]Department of Dental Implantology and Prosthodontics, Affiliated Hospital of Stomatology, Nanjing Medical University, Nanjing, Jiangsu, China, [2]Key Laboratory of Oral Diseases, Jiangsu Province Engineering Research Center of Stomatological Translational Medicine, Nanjing, China, [3]Department of Dental Orthodontic, Affiliated Hospital of Stomatology, Nanjing Medical University, Nanjing, Jiangsu, China

Macrophages are an integral part of the innate immune response in periodontal tissue and play a crucial role in the progression of periodontitis. Here we reported that macrophages also provoke periodontitis-induced gingival destruction through Piezol-mediated collagen degradation. We discovered that the *PIEZO1* expression was markedly elevated in patients with periodontitis through transcriptomic profiling. Moreover, Piezo1 promoted macrophage polarization toward the M1 type in response to lipopolysaccharide (LPS) and induced production of proinflammatory cytokines, which in turn stimulated production of matrix metalloproteinases (MMPs) leading to collagen degradation. Our study suggests that Piezol might be a potential therapeutic target for treating periodontitis-induced gingival destruction.

KEYWORDS

periodontitis, Piezo1, gingival destruction, macrophage, matrix metalloproteinases

1 Introduction

Periodontitis is a common infectious periodontal disease caused by plaque biofilm, which affects millions of individuals every year and ranks as the sixth most prevalent disease in the world (1). An epidemiological survey reveals that there are hundreds of millions of patients with periodontitis in China, of which 20 - 30% have advanced to the stage of severe periodontitis (2). It is suggested that microbe and their products (e. g. LPS) in periodontal tissues, which include gingiva, periodontal ligament, alveolar bone and cementum, activate innate immunity (3, 4) and trigger periodontitis and subsequent irreversible damage, periodontal attachment loss, gingival atrophy, alveolar bone resorption, and teeth loosening and loss (5). However, the underlying molecular mechanisms are still not well understood.

Macrophages are key components of innate immunity (6). While macrophages accumulate at the site of microbial infection, recognize, and phagocytose microbial pathogens, contributing to the homeostasis of the tissue microenvironment, they also trigger abnormal immune responses to microbe in the gingival tissue causing collagen fibers to deteriorate and consequent gingival atrophy (7, 8). However, how macrophages stimulate the pathogenic process is unclear.

Piezo type mechanosensitive ion Channel component 1 (Piezol) is a recently identified mechanosensitive ion channel that converts mechanical signals into biochemical responses (9, 10). A variety of organs, tissues and cells in the human body can sense mechanical stimuli in the external environment and activate cellular signal transduction pathways through Piezo1 (9, 11). Piezo1 is associated with orthodontic tooth movement to enhance alveolar bone remodeling (12, 13). Studies have also demonstrated an inextricable link between Piezo1 and inflammatory responses (14) (15). Piezo1 is also a mechanosensor of stiffness of macrophages and regulates macrophage function (16)). However, the role of Piezo1 in macrophages-mediated periodontal inflammation remains elusive.

In this study, we used RNA sequencing (RNA-seq) and other approaches (e. g., molecular biology techniques, immunofluorescence techniques) to analyze gene expression profiles of gingival tissues from periodontitis patients and healthy individuals, revealing new insights into the mechanisms underlying macrophage and periodontal tissue destruction in periodontitis.

2 Methods and materials

2.1 Study subjects and tissue samples

A total of 14 subjects participated in this study, including 7 patients with periodontitis and 7 healthy individuals. All subjects were enrolled from the Department of Oral and Maxillofacial Surgery, Nanjing Medical University Oral Hospital. The inclusion criteria for periodontitis group were as follows (1): probing depth (PD) of ≥4mm (2); clinical attachment level (CAL) of ≥5mm (at site of greatest loss) (3); gingival recession of grade I and above according to Miller's classification (4); bleeding on probing (BOP) in the area affected by periodontitis (5); radiographic examination showed loss of alveolar bone. Healthy controls were included for those who required gingival flap surgery to extract their impacted wisdom teeth. All subjects had no history of systemic diseases and inflammatory disorders such as hypertension, diabetes, cerebrovascular disease (CVD), inflammatory bowel disease (IBD), etc. Besides, all subjects were not pregnant or breastfeeding and had no history of smoking. None of the subjects had taken drugs such as antibiotics or immunosuppressants in the previous 3 months. The gingival samples of the periodontitis group were collected from the areas of gingival recession. All gingival samples collected were approximately 0. 5 cm^2 in size and included epithelial and connective tissue. Following rapid washing with phosphate buffer to remove blood, the tissues were immediately transferred into dry 2 mL Eppendorf tubes, temporarily stored in liquid nitrogen, and later stored at -80°C until subsequent procedures. This study was approved by the Medical Ethics Committee of the School of Stomatology, Nanjing Medical University and the Affiliated Stomatology Hospital of Nanjing Medical University with approval number PJ2021-126-001, and informed consent was acquired from each patient prior to the procedure.

2.2 RNA sequencing and sequencing data analysis

Total RNA was extracted from the gingival tissue employing TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol after accurately weighing 30 mg of each sample. Then the RNA purity was determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and the RNA integrity was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The TruSeq Stranded mRNA LTSample Prep Kit (Illumina, USA) was used to undertake cDNA synthesis and library creation according to the manufacturer's protocol. The constructed libraries were quality-checked with Agilent 2100 Bioanalyzer and sequenced by the Illumina HiSeq6000 sequencer after passing quality check. Trimmomatic software was used to quality control the raw data (Raw Reads) to obtain Clean Reads. The acquired Clean Reads were compared with the human genome GRCh38 for sequence comparison by HISAT2 to obtain sample-specific sequence feature information. The gene expression was calculated by the Fragments Per kb Per Million Reads (FPKM) method. Then the differentially expressed genes (DEGs) between periodontitis group and healthy group were identified by the limma package in R software with the threshold of $|\log_2 FC \text{ (fold-change)}| > 1$ and $P < 0.05$. GO enrichment and KEGG pathway enrichment analyses were conducted on DEGs, and the significance of DEGs enrichment was determined by the hypergeometric test. Sequencing and analysis of the transcriptome were performed by OE Biotech Co. (Shanghai, China).

2.3 Cell culture and treatment

RAW264. 7, a mouse-derived mononuclear macrophage leukemia cell line (Cell Bank, Chinese Academy of Sciences, Shanghai, China), was seeded in cell culture flasks and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 1% penicillin and streptomycin (Gibco, USA) and 10% fetal bovine serum (FBS) (Gibco, USA), maintained at 37°C in a humidified atmosphere with 5% CO_2, and passaged every 2 days at a ratio of 1:2.

Human gingival fibroblasts (HGF-1) were purchased from Shanghai Honsun Biological Technology Co., Ltd (Shanghai, China). The culture conditions for HGF-1 cells were the same as those for RAW264. 7 cells. HGF-1 cells were passaged every 3 days at a ratio of 1:3.

RAW264. 7 cells were seeded at a density of 1×10^5 in 6-well plates for 4 days. Porphyromonas gingivalis (P. g) is the main causative agent of periodontitis (17). P. g-LPS (100 ng/mL,

SMB00610, Sigma-Aldrich, USA) induced macrophages to stimulate the periodontal inflammatory environment. Yoda1 and GsMTx4 were purchased from MedChemExpress (HY-18723, USA) and AbMole (M10039, USA). In the presence of LPS, Yoda1 (5μM) or GsMTx4 (4μM) were added to RAW264. 7 cells to induce or inhibit Piezo1 expressions. Negative controls were RAW264. 7 cell culture medium. Groups of cells were cultured for up to 4 days without changing the culture medium during this period. The groups of RAW264. 7 cells were named Control, LPS, Yoda1+LPS and GsMTx4+LPS, respectively. Then each group's medium was collected, and the supernatant rich in macrophage-derived cytokines was obtained after centrifugation. The aforementioned macrophage supernatant was mixed with fresh DMEM medium at a ratio of 1:3 to prepare a conditioned medium for the cultivation of HGF-1 cells to confirm that macrophages would immunomodulate gingival tissue. The groups of HGF-1 cells were named C1D3, L1D3, Y1D3 and G1D3, respectively. The schematic diagram of HGF-1 cell cultured with macrophage conditioned medium was shown in Figure 1.

2.4 Cell viability assay

A cell counting kit-8 assay (CCK8, Dojindo, Japan) was performed to determine the viability of RAW264. 7 cultured with reagents and HGF-1 cultured with conditioned medium. Cells were seeded onto 24-well plates at an initial density of 5×10^4 cells per well. At each assay time point, the medium was replaced with fresh medium containing 10% CCK-8 solution and the cells were incubated for 2 hours at 37°C in the absence of light. The absorbance optical density (OD) values were then measured with a microplate reader at the wavelength of 450nm.

2.5 Immunofluorescence (IF) staining

2.5.1 Tissue staining

Fresh gingival tissue was briefly submerged in PBS to remove residual blood before being preserved in 4% paraformaldehyde overnight at 4°C. The tissues were then washed three times in PBS for 30 minutes each before being embedded in paraffin. Paraffin slices were dewaxed in an environmentally friendly dewaxing solution and subsequently hydrated with anhydrous ethanol and distilled water. Tissue sections were placed in a repair cassette filled with EDTA antigen repair buffer (pH=8. 0) in a microwave oven for antigen repair. The sections were blocked with goat serum for 30 minutes and then incubated overnight with Piezo1 antibody (1:100; NBP1-78446; Novus Biologicals, USA) and CD68 primary antibody (1:50; ab955; Abcam, UK) at 4°C in a wet box. After immersing sections in PBS (pH=7. 4) on a decolorization shaker and rinsing 3 times for 5 minutes each time, the donkey anti-mouse secondary antibody IgG H&L Alexa Fluor 594 (1:200; ab150108; Abcam, UK) and the goat anti-rabbit IgG H&L Alexa Fluor 488 (1:200; ab150077; Abcam, UK) secondary antibodies were added to cover the tissue and incubated for one hour at room temperature under protection from light. Next, add 4',6-diamidino-2-phenylindole (DAPI) (ab228549; Abcam, USA) to stain the nuclei and incubate for 10 minutes at room temperature in the dark. Finally, the slices were sealed with an anti-fluorescence quenching sealer and imaged under a confocal laser scanning microscope (Leica Microsystems, Germany). Image J software was used to analyze the fluorescence data quantitatively.

2.5.2 Cell staining

RAW264. 7 or HGF-1 cells seeded in confocal dishes were fixed with 4% paraformaldehyde at 4°C for 15 minutes after washed by PBS 3 times for 20 minutes each time. The cells were then

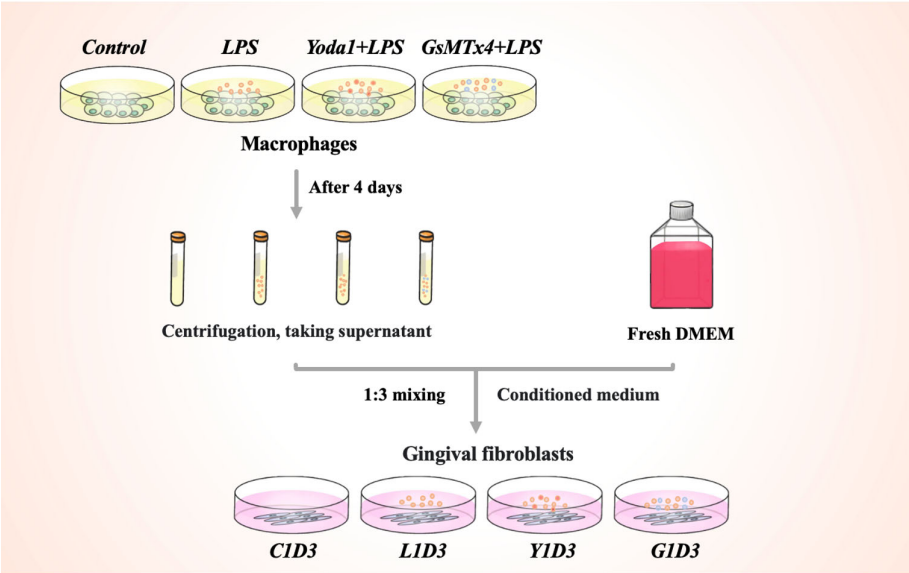

FIGURE 1
Schematic diagram of the conditional culture process.

permeabilized with 0. 2% Triton X-100 for 10 minutes and blocked with 2% BSA for 2 hours, and each step was followed by twice PBS washes to remove residual reagents. After the above steps were completed, primary antibodies were added to the cells and incubated at 4°C overnight. The primary antibodies were added as follows: Piezo1 antibody and CD68 were co-incubated to localize Piezo1 on macrophages; CCR7 (1:200;NB100-712; Novus Biologicals, USA) and CD206 (1:200; #24595; Cell Signaling Technology, USA) were co-incubated to identify macrophage polarization; COL-I (1:200;NB600-408; Novus Biologicals, USA) or COL-III (1:200; NB600-594; Novus Biologicals, USA) was incubated to indicate the existence of collagen in HGF-1. Afterwards, the cells were incubated with donkey anti-goat secondary antibody IgG H&L Alexa Fluor 594 (1:200; ab150132; Abcam, UK) and the goat anti-rabbit IgG H&L Alexa Fluor 488 for one hour at room temperature in the dark. After washing away the secondary antibodies with PBS, DAPI was used to stain the nuclei for 10 minutes in the absence of light. Finally, the images were acquired by the confocal laser scanning microscope and quantitatively analyzed by Image J software.

2.6 Flow cytometry

Flow cytometry was used for the analysis of M1/M2 macrophage. The M1 macrophages were labeled with $F4/80^+$/$iNOS^+$ and the M2 macrophages were labeled with $F4/80^+$/$CD206^+$. RAW264. 7 cells of each group were seeded with a density of 1×10^5 cells per well in a 6-well plate and incubated at 37°C for 4 days. After collected and resuspended with PBS containing 5% BSA, the cells were incubated with phycoerythrin (PE)-conjugated F4/80 monoclonal antibody (2142861, eBiosience™, USA), allophycocyanin (APC)-conjugated CD206 monoclonal antibody (2324767, eBiosience, USA) and Alexa Fluor 488-conjugeted iNOS monoclonal antibody (2365862, eBiosience, USA). The samples were then tested by a FACScan flow cytometer (Becton Dickinson, USA). The data was analyzed using FlowJo software.

2.7 RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

RAW264. 7 cells of each group were seeded with a density of 5×10^4 cells per well in a 24-well plate and incubated at 37°C for 4 days. HGF-1 cells of each group were seeded with a density of 5×10^4 cells per well in a 24-well plate and incubated at 37°C for 7 days. Total RNA was extracted from cells using the TaKaRa MiniBEST Universal RNA Extraction KIT (TaKaRa, Japan) following the manufacturer's protocol. An absorbance ratio of 260:280 was used to evaluate RNA purity, and its concentration was calculated by the absorbance at 260 nm. Total RNA was immediately reverse-transcribed into first-strand cDNA by the TaKaRa PrimeScript™RT Master Mix (TaKaRa, Japan). The obtained cDNA was stored at -20°C and used as soon as possible. As a qRT-PCR reaction template, the cDNA was used SYBR Premix Ex Taq II (TaKaRa, Japan) to amplify target genes on a Q7 system (QuantStudio 7, Applied Biosystems, USA). The reaction was carried out in a program of 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute and 95°C for 15 seconds, 60°C for 1 minute. $2^{-\Delta\Delta Ct}$ analysis method was used for calculating the relative expression of target genes. The target genes expressions were normalized to housekeeping gene β-actin. The primer sequences for qRT-PCR analysis were purchased from BioTNT (Shanghai, China) and were shown in Tables S1, S2.

2.8 Protein extraction and western blotting analysis

RAW264. 7 cells of each group were seeded with a density of 1×10^5 cells per well in a 6-well plate and incubated at 37°C for 4 days. To extract total protein, cells were washed twice with PBS and then lysed in RIPA buffer (Beyotime Biotechnology, China) supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF) (Beyotime Biotechnology, China) on ice. Quantification of protein concentration was then performed by bicinchoninic acid (BCA) protein assay (Beyotime Biotechnology, China). Protein samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel with equal amounts per lane for electrophoresis and then transferred onto 0. 22μm polyvinylidene difluoride (PVDF) membranes (Millipore, USA) with a semi-dry transfer apparatus (BioRad, USA). The membranes were immersed in Tris buffered saline-Tween 20 (TBST) with 5% dehydrated milk, dissolved for 2 hours and then incubated with primary antibodies overnight at 4°C on a transference seesaw shaker. Primary antibodies details are as follows: Piezo1 antibody (NBP1-78446), Anti-TNF-α antibody (ab183218, Abcam, USA), Anti-IL-1β antibody (ab216995, Abcam, USA), Anti-β-actin (ab8226,Abcam, USA). The internal control was β-actin. Afterwards, the membranes were washed with TBST buffer thrice to remove primary antibodies and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, USA) for one hour. After washed in TBST buffer, ECL detection reagents (Thermo Fisher Scientific, USA) were used to visualize the blots, and Image J software was used to analyze the results.

2.9 Intracellular reactive oxygen species (ROS) assay

ROS in macrophage was determined by a ROS Assay Kit (Beyotime Biotechnology, China) according to the manufacturer's protocol. RAW264. 7 cells of each group were seeded with a density of 5×10^4 cells per well in a 24-well plate and incubated at 37°C for 4 days. After two washes with PBS, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) diluted with serum-free culture medium was applied to the cell surface and incubated at 37°C for 20 minutes. An excitation wavelength of 480nm and an emission wavelength of 525nm were chosen to detect fluorescence intensity with a fluorescent microplate reader (SpectraMaxM2e, Molecular

Devices, USA). The test was performed on day 1 and day 4 of RAW264. 7 culture.

2.10 Enzyme-linked immunosorbent assay (ELISA)

RAW264. 7 cells of each group were seeded with a density of 5×10^4 cells per well in a 24-well plate and incubated at 37°C for 4 days. Macrophage culture solution was gathered in accordance with 2. 3 and utilized to quantify the concentrations of macrophage-derived pro-inflammatory cytokines TNF-α and IL-1β by the ELISA kits (D721217, D721017; Sangon Biotech, China) following the manufacturer's protocol. To detect the level of MMPs secreted by HGF-1, HGF-1 cells of each group were seeded with a density of 5×10^4 cells per well in a 24-well plate and incubated at 37°C for 7 days. After collecting cell cultures, the levels of MMP8 and MMP13 were determined using the ELISA assay kits (ELK1172,ELK2185; ELK Biotechnology, China) following the manufacturer's protocol. Standard curves were used to calculate cytokines concentrations. The absorbance OD values were then measured with a microplate reader at the wavelength of 450nm. The standard curve was plotted using ELISACalc software, with the concentration of the standard as the horizontal coordinate and the absorbance OD value as the vertical coordinate.

2.11 Statistical analysis

All data in this study was expressed as mean ± standard deviation (SD). Prism 9. 4 software (GraphPad Software, USA) was employed to conduct the statistical analysis. An analysis of statistical significance was conducted using the Student's *t*-test with a value of $P < 0.05$ being considered statistically significant.

3 Results

3.1 Clinical characteristics

This study included 14 individuals, including 7 patients with severe periodontitis and 7 healthy periodontal controls. The clinical characteristics of the patients in this study were summarized in Table 1. The mean age of periodontitis group and control group was 46. 7 and 39. 6 years, respectively. Patients in the periodontitis group were slightly older than the healthy group, but the differences were not statistically significant ($P > 0.05$). The mean PD of periodontitis group was 5. 57 mm, including a sample with a 7-mm PD (Table S3).

TABLE 1 Clinical characteristics of the study population.

Group	Periodontitis (n=7)	Control (n=7)
Age (years)	46. 7 ± 5. 5	39. 6 ± 3. 7
Females (%)	42. 86	57. 14
PD (mm)	5. 57 ± 0. 84	2. 43 ± 0. 45
CAL (mm)	7. 14 ± 1. 21	0
BOP sites	26%	2%

3.2 RNA sequencing analysis of gene expressions in normal and periodontitis gingival tissues

Total RNA was isolated from seven samples of healthy gingival tissue and seven samples of gingival tissue affected by periodontitis as previously mentioned and cDNAs were then created from the RNA samples of both groups and sequenced by the Illumina HiSeq6000. The principal component analysis (PCA) results depicted the intergroup separation and aggregation tendencies among the control and periodontitis groups (Figure 2A). DESeq (18) was used to analyze gene expression differences between the two groups. A total of 1706 DEGs were detected after differential screening based on the expression of protein-coding genes in different samples. Among them, there were 882 up-regulated genes and 824 down-regulated genes (Figure 2B). The DEGs listed in Supplementary Material 2 included inflammatory cytokines and immune response-related ones (*IL6*, *IL1RL1*, *IL17C*, *CSF3* and *CCL20*) and proteases (*MMP8*, *MMP13*, and *MME*) (Figure S1). Further GO analysis showed that these DEGs were mostly associated with "biological adhesion", "cellular component organization or biogenesis" and "immune system process" in biological process (BP). They were also related to "extracellular matrix" in cellular component (CC) and "channel regulator activity" in molecular function (MF) (Figure 2C). Moreover, KEGG pathway analysis revealed that periodontitis mainly affects cytokine-cytokine receptor, primary immunodeficiency, calcium signaling pathway, and ECM-receptor interaction (Figure 2D). As a second messenger, calcium (Ca^{2+}) is vital in the control of the immune response (19). Intriguingly, we found that Piezo1, a mechanosensitive ion channel, was significantly up-regulated in periodontitis group (Figure 2E). It was well documented that Piezo1 regulates the influx of Ca^{2+} into cells, thus converting mechanical signals into chemical signals (16). Recent studies showed that Piezo1 modulates inflammatory response, indicating Piezo1 might play an important role in immunity (20). Therefore, we chose the *PIEZO1* as the key gene for following research.

3.3 Piezo1 was upregulated in macrophages of periodontitis gingival tissues and induced by LPS

CD68 is a pan macrophage surface marker. To confirm whether Piezo1 existed in macrophages, we carried out IF staining in healthy and periodontitis gingival tissues, as well as in RAW264. 7 stimulated with LPS. IF results revealed Piezo1 located in macrophages (labeled by CD68) of periodontitis gingival tissues (Figure 3A) and in the LPS-stimulated RAW264. 7 (Figure 3B). Semi-quantitative analysis showed higher levels of Piezo1 in periodontal tissues than in healthy gingival tissues, and in LPS-stimulated RAW264. 7 cells than control cells (Figures 3C, D). Taken together, Piezo1 is upregulated in macrophages of periodontitis gingival tissues and is induced by LPS.

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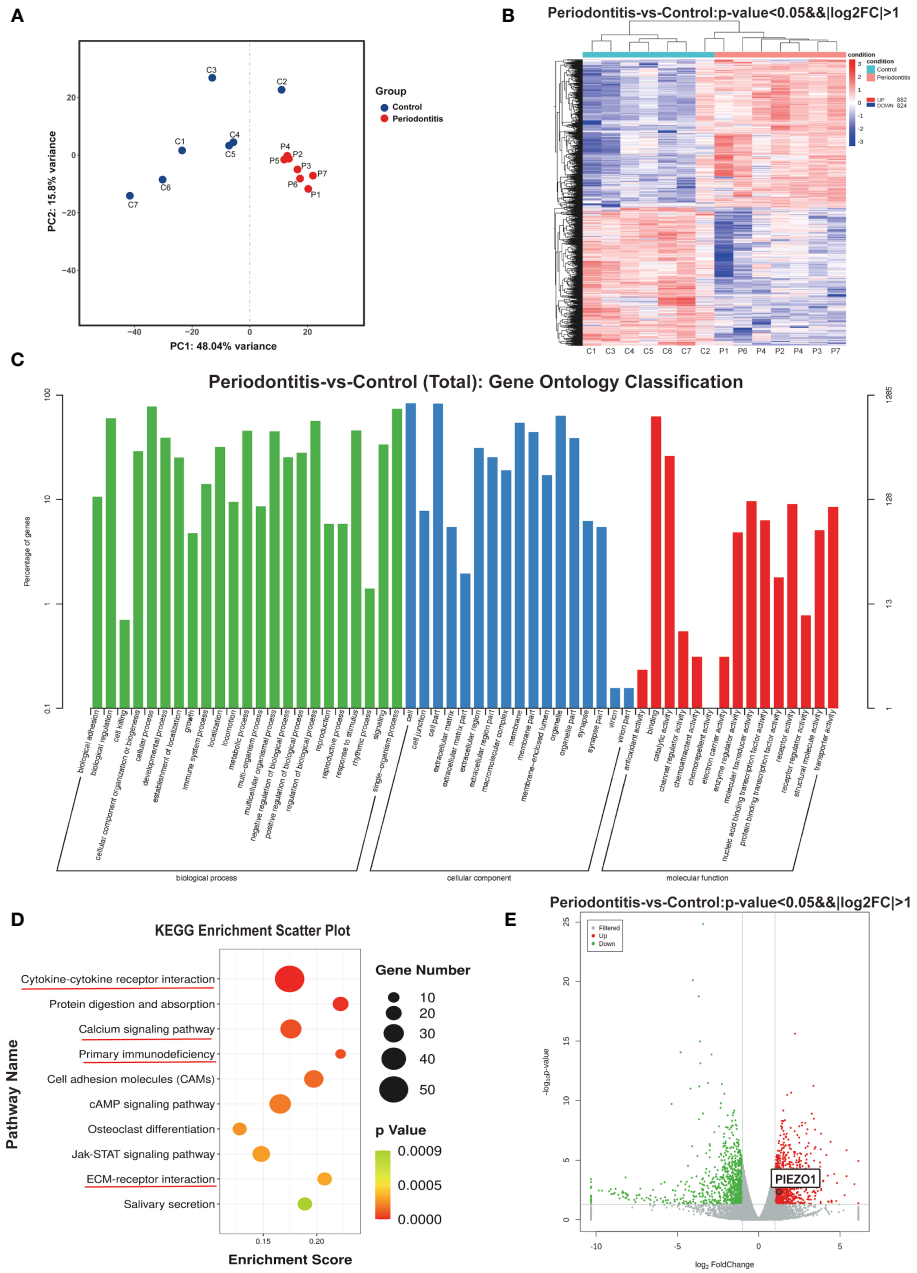

FIGURE 2
RNA sequencing results **(A)** PCA analysis examines the distribution of samples. **(B)** The heat map demonstrates that there are significant differences in gene expression between the two groups. **(C)** GO enrichment analysis results. **(D)** Bubble map of KEGG enrichment analysis of Top10 pathways. **(E)** Volcano plot showing genes differentially expressed between two groups. C, control group; P, periodontitis group; PCA, principal component analysis; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes.

3.4 Piezo1 blockade attenuated M1 macrophage polarization and pro-inflammatory cytokine production in response to LPS

M1 macrophage produce pro-inflammatory cytokines leading to periodontitis. To elucidate whether Piezo1 has an effect on M1 macrophage differentiation and cytokine production, we used GsMTx4, an inactivating non-selective cationic MSC inhibitor known to inhibit Piezo1 activity (21, 22). Although it was reported that GsMTx4 at a low dose (e. g. 0. 5μM) has no apparent effect on the cell viability of astrocytes (23), we determined if it has such an effect in macrophages at a high dose in the presence of LPS. Thus, Raw264. 7 cells were treated with GsMTx4 at 2. 5, 4, or 10μM for up to 4 days. We found that GsMTx4 at the doses up to 4μM had no apparent effect on the cell viability, whereas it at 10μM dramatically reduced the cell viability (Figure S2A). Therefore, GsMTx4 at 4μM was used as the treatment dose in the following experiments.

Next, immunofluorescence (IF) staining was used to analyze macrophage polarization, which was closely related to the

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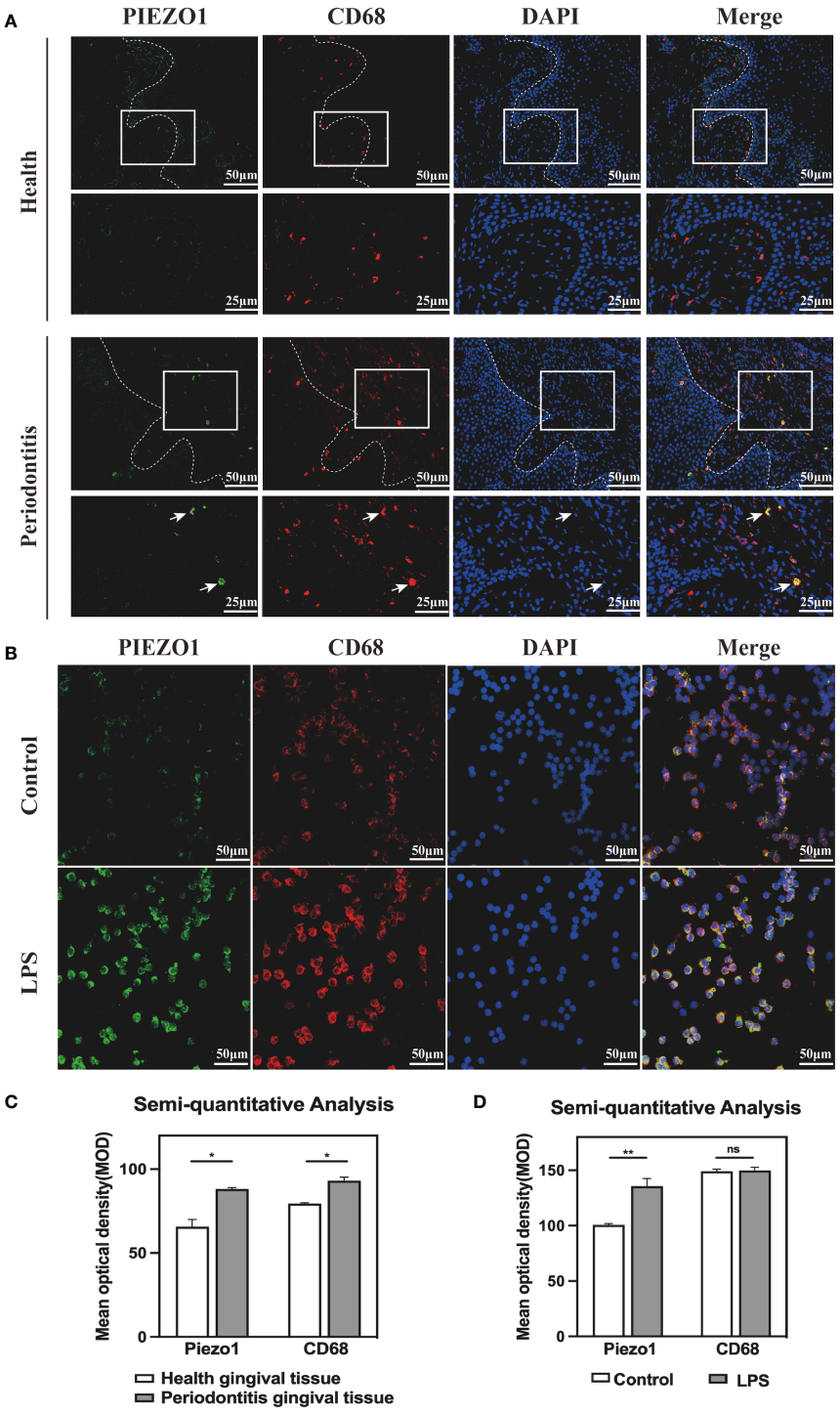

FIGURE 3
Piezo1 was located in macrophages **(A)** IF staining showed co-localization of PIEZO1 and CD68 in gingival tissue. **(B)** IF staining showed co-localization of PIEZO1 and CD68 in RAW264. 7. **(C)** Semi-quantitative analysis of PIEZO1 and CD68 in gingival tissue. **(D)** Semi-quantitative analysis of PIEZO1 and CD68 in RAW264. 7. *P < 0. 05; **P < 0.01; ns, non significance..

inflammatory response. Markers CCR7 and CD206 were respectively used to designate M1 (red color) and M2 (green color). The polarization of stained macrophages cultivated under different conditions was observed (Figure 4A). Compared with the Control and GsMTx4+LPS groups, the expression of CCR7 in the LPS group was noticeably higher, and however, CD206 did not differ significantly among the three groups. Semi-quantitative analysis showed that M2 macrophages had similar polarization ratio on each group, but the polarization trend of M1 macrophages in the LPS group was higher than that in the GsMTx4+LPS group and the Control group (Figure 4B). A further analysis was conducted by flow cytometry to determine the proportions of

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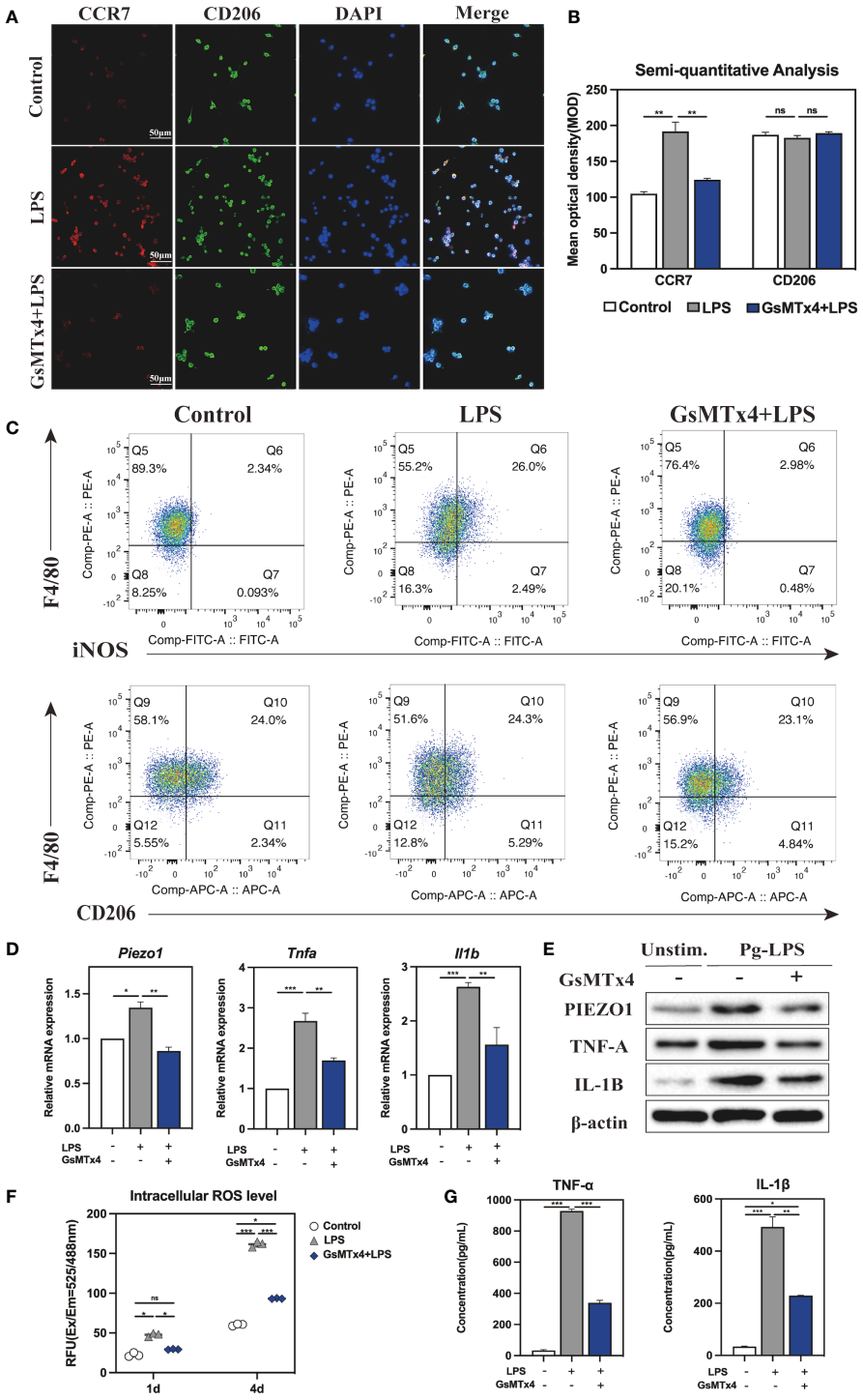

FIGURE 4
Immune response of macrophages after blocking Piezo1. **(A)** IF staining of CCR7 and CD206 in the Control, LPS and GsMTx4+LPS groups after 4-day-cultured. M1 macrophages were marked with CCR7 (red), M2 macrophages with CD206 (green), and nuclei with DAPI (blue). **(B)** Semi-quantitative analysis of CCR7 and CD163 in each group. **(C)** Flow cytometry analysis of RAW264. 7 cells in the Control, LPS and GsMTx4+LPS groups. Q6 represents M1 types ($F4/80^+$/iNOS$^+$) and Q10 represents M2 types ($F4/80^+$/$CD206^+$). **(D)** Expressions of *Piezo1* and inflammation-related genes (*Tnfa* and *Il1b*) in macrophages cultured for 4 days. **(E)** Western blotting analysis of PIEZO1, IL-1B and TNF-A in RAW264. 7 cultured for 4 days. **(F)** Intracellular ROS levels of RAW264. 7 cultured for 4 days. **(G)** Concentration of inflammatory cytokines in macrophage medium detected by ELISA. *P < 0. 05; **P < 0. 01; ***P < 0. 001.

macrophage phenotypes. F4/80, iNOS and CD206 were selected as markers of macrophages, M1 phenotypes and M2 phenotypes, respectively. As shown in Figure 4C, Q6 represents M1 types ($F480^+/iNOS^+$), while Q10 represents M2 types ($F480^+/CD206^+$). The LPS group contained 26. 0% M1, which was much higher than the Control group (2. 34%) and GsMTx4+LPS group (2. 98%) ($P<0.05$). Three groups had nearly accounted M2 phenotypes with a proportion of 21%, 24. 5%, and 24. 8%, respectively, which did not differ statistically ($P>0.05$, Figure S2B). These results demonstrated that inhibition of Piezo1 affected macrophage polarization toward M1 in response to LPS stimulation.

The macrophage inflammatory response was evaluated by qRT-PCR, Western blotting, and intracellular ROS analysis. As for the expression of mRNA, LPS-stimulated macrophage considerably raised, whereas GsMTx4 significantly decreased the expression of *Piezo1* ($P<0.05$) (Figure 4D). TNF-α and IL-1β are potent pro-inflammatory cytokines produced by activated M1 macrophages. The results showed that LPS dramatically enhanced the expression of *Tnfa* and *Il1b* in macrophages, which was markedly inhibited by GsMTx4. The same outcomes were attained for Western blotting analysis (Figure 4E, Figure S2C). Aside from secreting cytokines such as TNF-α and IL-1β, M1 macrophages produce ROS in significant quantities, which is essential to the antimicrobial response. The intracellular ROS analysis showed a significant low level of ROS in the GsMTx4+LPS group compared to the LPS group in 4-day-cultured RAW264. 7 cells (Figure 4F), suggesting that blocking Piezo1 reduced ROS production by LPS in macrophages.

For further illustration, ELISA was utilized to determine the cytokines levels in macrophage 4-day-cultured medium under various culture conditions. The results showed that GsMTx4 markedly inhibited LPS-induced production of TNF-α and IL-1β in RAW264. 7 cells (Figure 4G). Collectively, these data suggest that blocking Piezo1 attenuates macrophage polarization to M1 and decreases the production of inflammatory factors.

3.5 Piezo1 stimulation enhanced M1 polarization and production of pro-inflammatory cytokines in response to LPS

To further understand how Piezol triggers the immune response, we treated macrophages with Yoda1, a Piezo1 selective agonist (24). Similar to processing with GsMTx4, we treated RAW264. 7 with 2, 5, or 10μM of Yoda1 in the presence of LPS and found that Yoda1 had no apparent effect on the cell viability and eventually selected 5μM Yoda1 to stimulate the cells (Figure S3A). Similarly, CCR7 and CD206 were employed to mark M1 and M2 types, respectively, to analyze the polarization of macrophages. The IF staining results visually demonstrated that the red fluorescence intensity representing CCR7 in the LPS group and the Yoda1+LPS group was significantly stronger than that of the Control group, indicating that the first two groups included a higher proportion of M1 macrophages (Figure 5A). Semi-quantitative analysis demonstrated that the mean optical density of red was significantly higher in the LPS and the Yoda1+LPS groups than in the Control group (Figure 5B). Flow cytometry results (Figure 5C) showed that there were 34. 6% M1 types in the Yoda1+LPS group, 24. 6% in the LPS group, and only 1. 49% in the Control group. In contrast, the percentage of M2 types in each group did not differ (Figure S3B). These findings suggest that Piezo1 agonist can enhance LPS-induced M1 polarization but has no apparent effect on M2 polarization.

Moreover, qRT-PCR results revealed that the expressions of *Piezo1*, *Tnfa* and *Il1b* were significantly higher in groups of LPS and Yoda1+LPS than those in the Control group (Figure 5D), and Western blotting analysis yielded similar results (Figure 5E, Figure S3C). As expected, ROS levels in various groups for 4 days rose in the order of Control < LPS < Yoda1+LPS (Figure 5F). ELISA showed that the highest concentrations of TNF-α and IL-1β in macrophage supernatants were reported in the Yoda1+LPS group compared to the Control and LPS groups (Figure 5G). Taken together, boosting Piezo1 can excessively enhance macrophage M1 polarization and the production of inflammatory factors in response to LPS.

3.6 Macrophage-mediated MMPs induced collagen degradation via Piezo1

A major component of periodontal extracellular matrix is type I collagen (COL-I) and type III collagen (COL-III). Our RNA-seq results showed upregulated MMP8 and MMP13 in periodontitis group, which were reported to be involved in the degradation of COL-I, COL-III and extracellular matrix observed in periodontitis (25, 26). It is well known that immunological dysregulation lead to tissue damage (27). Therefore, we hypothesized that Piezo1 stimulated excessive immune response which in turn increased the expression of collagenase (such as MMP8 and MMP13) and led to collagen degradation. To test our hypothesis, we treated HGF-1 cells with the diluted macrophage-derived condition medium (MDCM), which was collected the LPS, the Yoda1+LPS, the GsMTx4+LPS, or the Control groups after cultured for 4 days. The MDCM was combined with fresh DMEM medium in a ratio of 1:3 to create conditioned medium for culturing HGF-1 cells, and each group was named L1D3, Y1D3, G1D3 and C1D3, respectively. The CCK8 assay showed that each MDCM had no effect on HGF-1 cell viability at 1 day. When HGF-1s were conditioned cultured for 4 and 7 days, the C1D3, L1D3, or G1D3 had no significant effect on HGF-1 cell viability, but the Y1D3 produced a mildly inhibitory effect on HGF-1s, suggesting that excessive inflammatory responses affect cell viability (Figure S4).

Macrophages interact strongly with other cells through paracrine secretion. ELISA was employed to measure collagenase MMPs concentration in order to verify whether MDCM alters MMPs secreted from HGF-1s. As shown in Figure 6A, the concentrations of MMP8 and MMP13 were increased in the L1D3 and Y1D3 groups compared to the C1D3 group ($P<0.01$). Compared to the L1D3 group, the Y1D3 group secreted higher levels of MMP8 and MMP13 ($P<0.01$), whereas the G1D3 group secreted less MMPs ($P<0.05$), indicating that MDCM affects the secretion of MMPs from HGF-1. Moreover, qRT-PCR was used to assess MMPs expression level in HGF-1 cells. The results showed that MMP8 and MMP13 were upregulated in the L1D3 and Y1D3 groups, while considerably downregulated in the G1D3 group ($P<0.01$, Figure 6B). The results suggest that LPS-stimulated

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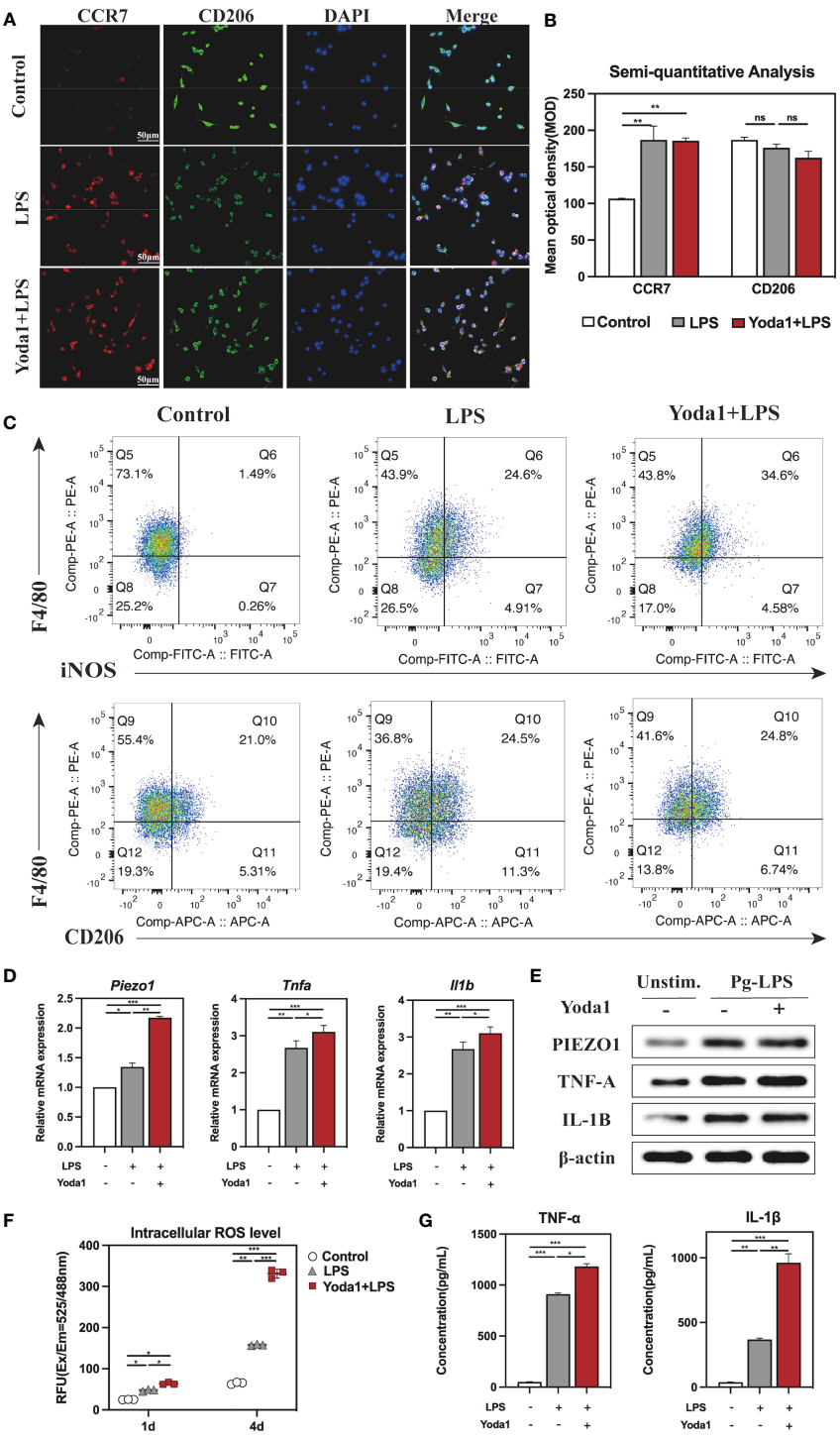

FIGURE 5
Immune response of macrophages after activating Piezo1. **(A)** IF staining of CCR7 and CD206 in the Control, LPS and Yoda1+LPS groups after 4-day-cultured. M1 macrophages were marked with CCR7 (red), M2 macrophages with CD206 (green), and nuclei with DAPI (blue). **(B)** Semi-quantitative analysis of CCR7 and CD206. **(C)** Flow cytometry analysis of RAW264. 7 cells in the Control, LPS and Yoda1+LPS groups. Q6 represents M1 types ($F4/80^+$/$iNOS^+$) and Q10 represents M2 types ($F4/80^+$/$CD206^+$). **(D)** *Piezo1*, *Tnfa* and *Il1b* genes expressions in macrophages cultured for 4 days. **(E)** Western blotting analysis of PIEZO1, TNF-A and IL-1B in RAW264. 7 cultured for 4 days. **(F)** Intracellular ROS levels of RAW264. 7 cultured for 4 days. **(G)** Concentration of inflammatory cytokines in macrophages medium detected by ELISA. *P < 0. 05; **P < 0. 01; ***P < 0. 001.

macrophages or the activation of Piezo1 by Yoda1 both promoted downstream MMPs expression, whereas block of Piezo1 significantly inhibited the LPS-induced MMP expression. Taken together, macrophages affected the expression and secretion of MMPs in HGF-1 through Piezo1. Activating Piezo1 promoted the overexpression and secretion of collagenase MMPs, while inhibiting Piezo1 decreased the level of collagenase MMPs, suggesting that Piezo1 functioned as an upstream regulator of MMPs.

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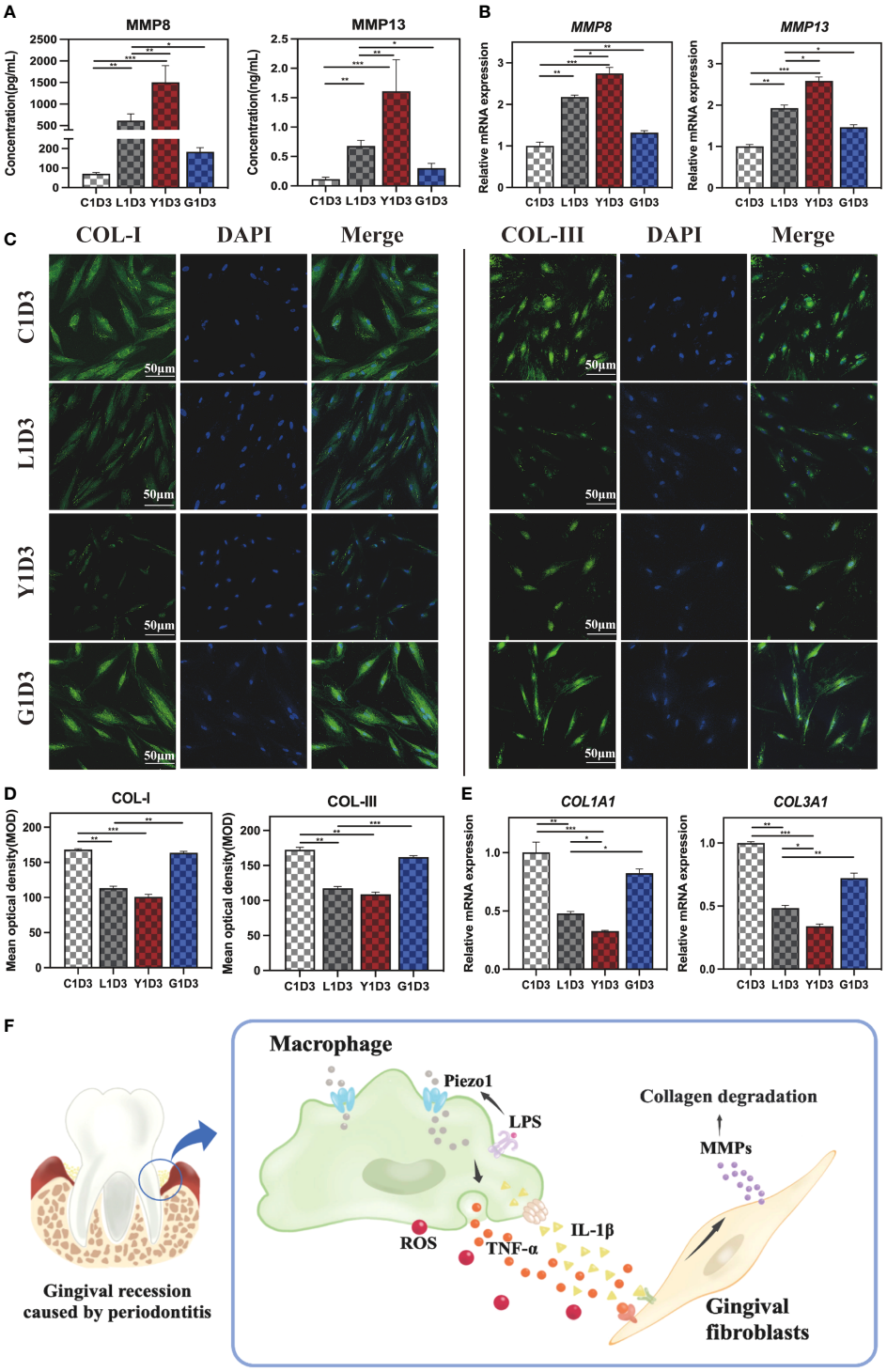

FIGURE 6
Gingival fibroblasts cultured in conditioned medium. **(A)** Concentration of MMP8 and MMP13 in the supernatant of HGF-1 in each group detected by ELISA. **(B)** *MMP8* and *MMP13* gene expressions in HGF-1 cultured in MDCM for 7 days. **(C)** IF staining of COL-I and COL-III in HGF-1 cultured with MDCM for 7days. **(D)** Semi-quantitative analysis of COL-I and COL-III in HGF-1. **(E)** Gene expression of *COL1A1* and *COL3A1* in HGF-1 cultured with MDCM for 7 days. **(F)** Schematic diagram of macrophages mediating MMPs-degrading collagens via Piezo1, thereby destroying periodontal tissue. *P < 0. 05; **P < 0. 01; ***P < 0. 001.

Furthermore, IF staining was used to assess the level of COL-I and COL-III in HGF-1s cultured with MDCM for 7 days. The results demonstrated that the trend of COL-I and COL-III was as follows:*C1D3*≈*G1D3*>*L1D3*≈*Y1D3* (Figure 6C). Semi-quantitative analysis showed the *L1D3* and *Y1D3* groups were much lower than that of the *C1D3* group for both COL-I and COL-III, while there was no obvious difference in the *G1D3* group (Figure 6D). Further validation by qRT-PCR revealed that the expression of *COL1A1* and

COL3A1 in the L1D3 and Y1D3 groups was markedly reduced, while the expression in the G1D3 group was not significantly altered compared with the C1D3 group (Figure 6E). These data suggest that activating Piezo1 in macrophages can exacerbate the downregulation of collagen-related gene and protein expression, thereby promoting collagen degradation, while blocking Piezo1 can counteract this damage.

4 Discussion

Accordingly, we discovered a novel mechanism underlying periodontitis-related gingival destruction mediated by Piezo1 in this research. Our results showed that Piezo1 is substantially expressed in periodontitis tissues, and it may operate on macrophages to modulate the immune response. We found that inhibition or activation of Piezo1 affects macrophage polarization in an inflammatory milieu and modulates MMPs' secretion. We further revealed that macrophages mediate MMPs via Piezo1 to regulate collagen degradation in gingival fibroblasts. The schematic diagram of this research was displayed in Figure 6F. These results offered fresh perceptions into possible mechanisms underlying periodontitis-induced gingival destruction.

Periodontitis is a widely prevalent infectious periodontal disease and is characterized by the destruction of tooth-supporting tissues such as alveolar bone resorption and gingival destruction (28, 29). It is initiated by microbial infection in dental plaque and interacts with the host immune defense, while macrophages play a crucial role in activating the host immune defenses and defending against pathogenic bacterial infections (30). On one hand, macrophages migrate and aggregate to the site of infection, acting as phagocytic bactericides. On the other hand, macrophages secrete numerous cytokines and amplify specific immune responses upon contact with microorganisms (31), thereby inducing inflammation and stimulating an increase in the destruction of collagen fibers in the gingiva, and ultimately leading to gingival atrophy (32). However, the mechanism underlying this double-edged role of macrophages in periodontal inflammation remains elusive. Ca^{2+} concentration affects the inflammatory response by polarizing macrophages toward M1 (33, 34). The nonselective ion channel Piezo1 has been demonstrated to regulate Ca^{2+} influx into macrophages (10, 35), thereby responding to macrophage activation and eliciting an inflammatory response (16, 20, 36). Toll-like receptor 4 (TLR4) and Piezo1 collaborate to mediate Ca^{2+} influx in response to LPS stimulation, enhancing macrophage activity (14). But the role of Piezo1 in modulating periodontal inflammation is undetermined. To bridge this knowledge gap, we determined that Piezo1 functions on macrophages in periodontal inflammatory tissues by IF staining co-localization techniques on infected gingival tissue and macrophages stimulated by Pg-LPS. In periodontitis, activated Piezo1 causes a large extracellular Ca^{2+} influx into macrophages, leading to an increase in M1-type polarization. Then excessive M1 macrophages cause exaggerated inflammatory responses by overproduction of ROS and pro-inflammatory factors (such as TNF-α and IL-1β), in turn resulting in irreversible destruction of periodontal tissues.

It is widely accepted that the component analysis of gingival crevicular fluid (GCF) can shed light on the association between particular metabolic alterations and periodontitis states (25, 37, 38). MMP8 and MMP13 are generally acknowledged to be the most rewarding salivary biomarkers for the diagnosis of periodontitis (25, 39), which are crucial proteases that regulate gingival destruction and alveolar bone destruction in periodontitis (40). Similarly, our transcriptome analysis results from gingival tissue showed that the periodontitis group had considerably greater expression of multiple MMPs, including collagenases (MMP8 and MMP13), than the healthy group, supporting the opinion that these MMPs can be employed as periodontitis biomarkers. An overabundance of MMPs can induce periodontal tissue destruction, fibrosis, and degradation of the extracellular matrix, ultimately leading to loss of connective tissue attachment and gingival destruction (41). Macrophage-derived pro-inflammatory factors are associated with tissue damage in periodontitis, and among them MMP is one of the most strongly associated and extensively researched protease families (42). Our data suggested that macrophages can regulate the secretion of MMP8 and MMP13 *via* activating Piezo1, which in turn affects the collagen level of gingival fibroblasts through the cytokines-rich conditioned medium. However, how the protein hydrolytic activity of intracellular MMPs is regulated remains unclear. Most currently believe that ROS as a signaling molecule may be a possible mechanism to regulate intracellular MMPs activity (43, 44). Oxidative stress can enhance MMP activation and is associated with MMP function. Jing Geng et al. confirmed that Piezo1 can enhance macrophage activity and ROS accumulation (14). Our study yielded the same results. We hold the view that hyperactivated M1 macrophages generate more ROS via Piezo1, causing oxidative stress and hence enhancing MMPs secretion and consequently leading to collagen breakdown. After all, the fundamental mechanism of macrophage control of Piezo1-mediated MMPs needs further investigation.

Using macrophage conditioned medium to culture HGFs for the purpose of evaluating the impact of the former's paracrine factors on the latter can effectively imitate the complicated milieu of periodontitis *in vitro* (45). In this manuscript, collagen expression in HGF was influenced by Piezo1-mediated MMPs through the macrophage conditioned medium. Logically, the degree of collagen degradation is remarkably consistent with the concentration of MMPs. In an inflammatory context, Piezo1 enhancement further diminished fibroblast collagen expression, whereas Piezo1 inhibition noticeably counteracted inflammation-induced collagen degradation.

To summarize, we proposed a novel mechanism underlying periodontitis-induced gingival destruction: macrophages mediate MMPs-degrading collagens via Piezo1, thereby destroying periodontal tissue. Cellular crosstalk between macrophages and fibroblasts plays an essential role in periodontitis pathogenesis. Our findings provide fresh insights into this complicated molecular mechanism, possibly providing a new treatment strategy for periodontitis-induced soft tissue atrophy. Nevertheless, there is still a long way to go before we fully elucidate the biological function and specific mechanism of how Piezo1 mediate MMPs and affects the periodontitis-induced gingival destruction.

Data availability statement

The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA) repository, accession number PRJNA967820 (https://www.ncbi.nlm.nih.gov/sra/PRJNA967820).

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the School of Stomatology, Nanjing Medical University and the Affiliated Stomatology Hospital of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TZ and CT designed the study. ZC and CC collected and analyzed the data. TZ finished the experiments. SD sourced the literature. GL and JW edited the manuscript. CT provided the funding and supervised the whole study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1194662/full#supplementary-material

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EDITED BY
Teun J. De Vries,
VU Amsterdam, Netherlands

REVIEWED BY
Gaetano Isola,
University of Catania, Italy
Raquel Mantuaneli Scarel-Caminaga,
São Paulo State University, Brazil
Rogelio González-González,
Juárez University of the State of Durango, Mexico

*CORRESPONDENCE
Michael Nolde
nolde@uni-muenster.de

†These authors have contributed equally to this work

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Downregulation of interleukin 6 signaling might reduce the risk of periodontitis: a drug target Mendelian randomization study

Michael Nolde[1*], Zoheir Alayash[1], Stefan Lars Reckelkamm[1], Thomas Kocher[2], Benjamin Ehmke[3], Birte Holtfreter[2], Hansjörg Baurecht[4], Marios K. Georgakis[5,6†] and Sebastian-Edgar Baumeister[1†]

[1]Institute of Health Services Research in Dentistry, University of Münster, Münster, Germany, [2]Department of Restorative Dentistry, Periodontology, Endodontology, and Preventive and Pediatric Dentistry, University Medicine Greifswald, Greifswald, Germany, [3]Clinic for Periodontology and Conservative Dentistry, University of Münster, Münster, Germany, [4]Department of Epidemiology and Preventive Medicine, University of Regensburg, Regensburg, Germany, [5]Institute for Stroke and Dementia Research (ISD), University Hospital, Ludwig-Maximilians-University (LMU) Munich, Munich, Germany, [6]Program in Medical and Population Genetics, Broad Institute of Harvard and the Massachusetts Institute of Technology, Cambridge, MA, United States

Aim: Interleukin 6 (IL-6) is considered to play a role in the dysbiotic host response in the development of periodontitis. While the inhibition of the IL-6 receptor using monoclonal antibodies is a well-established therapy for some diseases, so far, its potential benefit in patients with periodontitis has not been examined. We tested the association of genetically proxied downregulation of IL-6 signaling with periodontitis to explore whether downregulation of IL-6 signaling could represent a viable treatment target for periodontitis,

Materials and methods: As proxies for IL-6 signaling downregulation, we selected 52 genetic variants in close vicinity of the gene encoding IL-6 receptor that were associated with lower circulating C-reactive protein (CRP) levels in a genome-wide association study (GWAS) of 575 531 participants of European ancestry from the UK Biobank and the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium. Associations with periodontitis were tested with inverse-variance weighted Mendelian randomization in a study of 17 353 cases and 28 210 controls of European descent in the Gene-Lifestyle Interactions in Dental Endpoints (GLIDE) consortium. In addition, the effect of CRP reduction independent of the IL-6 pathway was assessed.

Results: Genetically proxied downregulation of IL-6 signaling was associated with lower odds of periodontitis (odds ratio (OR) = 0.81 per 1-unit decrement in log-CRP levels; 95% confidence interval (CI): [0.66;0.99]; P = 0.0497). Genetically proxied reduction of CRP independent of the IL-6 pathway had a similar effect (OR = 0.81; 95% CI: [0.68; 0.98]; P = 0.0296).

Conclusion: In conclusion, genetically proxied downregulation of IL-6 signaling was associated with lower odds of periodontitis and CRP might be a causal target for the effect of IL-6 on the risk of periodontitis.

KEYWORDS

periodontal disease(s)/periodontitis, cytokine(s), host modulation therapy, epidemiology, cell signaling

Introduction

Periodontal inflammation is initiated by biofilm formation on the tooth surface and further exacerbated by host inflammatory-immune response to dysbiotic microbiome (1, 2). Hence, inflammation control is a central part of periodontitis treatment (3–5). Drugs targeting host response in addition to subgingival scaling and root planing or surgical periodontal therapy are therefore under active investigation (6).

Cytokine balance plays a central role in inflammation (7). Interleukin 6 (IL-6) is a cytokine involved in the body's innate immune response. It acts on two different signaling pathways: by a specific membrane-bound IL-6 receptor (IL-6R) and by a soluble receptor (sIL-6R), which are called *classic* and *trans*-signaling. The inflammatory role of IL-6 is thereby mediated not by the classic pathway, but rather via the trans-signaling pathway where IL-6 binds to sIL-6R and subsequently interacts with cells via the membrane bound signaling molecule gp130 (8, 9). Here, sIL-6R is released from inflammatory cells such as lymphocytes and macrophages by proteolytic cleavage on the cell surface (10).

Inhibition of both membrane and soluble IL-6R using monoclonal antibodies such as tocilizumab, satralizumab, sarilumab or olokizumab is a standard therapy for patients with rheumatoid arthritis or COVID-19 (11, 12). These agents attenuate all forms of IL-6 signaling, reducing serum levels of C-reactive protein (CRP) and other downstream biomarkers.

In this study, we tested the biological effect of IL-6 signaling downregulation on the risk of periodontitis simulating the effect of monoclonal antibodies that target IL-6R by blocking both IL-6 *classic* and *trans*-signaling (13–16). Mendelian randomization (MR) is a method to study causal effects on disease outcomes that uses randomly allocated germline genetic variants as proxies for exposures (17). This approach is relatively robust to confounding and reverse causality as these variants stay unchanged after conception. Drug-target MR uses variants in the close vicinity of a gene encoding a protein target as proxies to study causal effects on disease outcomes (Figure 1) (19, 20). Accordingly, genetic variants in the vicinity of the *IL6R* gene serve as proxies for the targeted pharmacological agent.

Materials and methods

Drug target MR (or cis-MR) is a study design that leverages large genetic data to estimate the effect of drug therapy in the absence of evidence from randomized clinical trials (RCTs). While estimates from other observational study designs are often biased by unmeasured confounding, MR uses genetic instrumental variables to imitate the randomization of RCTs to yield unconfounded effect estimates and has recently been introduced to the field of periodontology (6, 21). For an unbiased effect estimation, three instrumental variable assumptions must be true: The relevance assumption demands a strong association between genetic instruments and the exposure. This assumption can be tested using statistical approaches such as F statistics. The exchangeability assumption demands that genetic instruments are independent of confounders of the exposure-outcome relation. The exclusion restriction assumption finally demands that genetic instruments affect the outcome only via the exposure.

Genetic instruments and study population

Single nucleotide polymorphisms (SNPs) were used to proxy the effect of IL-6 signaling downregulation and to estimate the downstream effect of drugs for blocking IL-6Rs, such as tocilizumab, on periodontitis risk. We identified SNPs within 300 kilobases on either side of the targeted *IL6R* gene that were associated with CRP serum levels, a reliable downstream biomarker of IL-6 signaling (22) (Table 1, Figure 1). We assumed that these SNPs directly affect *IL6R* leading to a modified version or altered quantities of the protein (Figure 2). Variants were robustly associated with CRP in a genome-wide association study (GWAS) meta-analysis of the combined data of 575 531 participants of European descent of the UK Biobank and the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium (23). All variants had a *p*-value <5e-8 for their association with CRP and low linkage disequilibrium (r^2<0.2). We estimated the effect of CRP-associated variants on the risk of periodontitis using the GWAS of 17 353 cases and 28 210 controls of European descent from the Gene-Lifestyle Interactions in Dental Endpoints (GLIDE) consortium (24). Periodontitis cases were classified by either the Centers for Disease and Control and Prevention/American Academy of Periodontology (CDC/AAP) or the Community Periodontal Index (CPI) case definition.

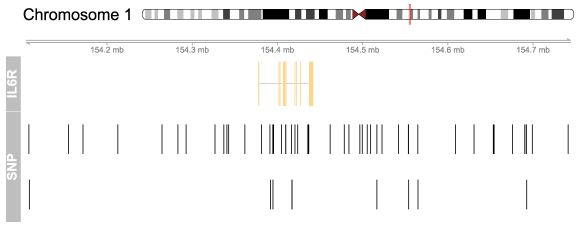

FIGURE 1
Genomic map for the selected 52 SNPs in the vicinity of the IL6R gene. This figure shows the location of the instrumental SNPs around the IL6R gene on chromosome 1. Variants in the close vicinity of the IL6R gene were used to proxy downregulation of IL-6 signaling and to estimate its genetically proxied effect on periodontitis. Variants associated with CRP based on P<5x10-8 within 300 kilobases of the IL6R gene were selected as genetic proxies of IL-6 signaling downregulation. At the top, chromosome 1 is drawn with the subregion of interest marked in red. The 'IL6R' track shows the combined gene model of the alternative transcripts of the IL6R gene. At the bottom, the SNP locations are plotted along the same genomic coordinate. The figure was created using the mapsnp package (18).

Additional analysis

Our primary analysis includes only variants in the vicinity of the *IL6R* gene (cis *IL6R*) acting as a proxy for the target drug effect that contribute to the effect estimate weighted by their effect on CRP. This way we obtain an adequate estimate of *IL6R* function and its effect on downstream IL-6 signaling. To further examine whether CRP is a potential causal target or merely an otherwise uninvolved marker of IL-6R inhibition we performed an additional analysis using four variants located in the *CRP* gene (cis *CRP*) that have previously been selected to fully cover the common variations of this gene in populations of European descent (25). These variants are supposed to alter CRP independent of IL-6 and thus might indicate a causal effect of CRP on the risk of periodontitis.

Statistical analysis

The direction of genetic associations between exposure and outcome was harmonized. We performed the MR analysis using the well-documented multiplicative random-effects inverse variance weighted (IVW) method (26). Odds ratios (ORs) were scaled to reflect the equivalent of a 1-unit reduction in serum CRP levels on the log scale. The instrumental variable relevance assumption was tested by the F statistic (17), and F statistics of at least 10 were regarded as an indication of no weak instrument bias. Leave-one-out analysis was used to assess whether the estimate was biased by a single outlier instrument. The exclusion restriction instrumental variable assumption was probed by searching the PhenoScanner database for associations of SNPs with known risk factors of periodontitis, especially tobacco smoking and diabetes (27). Any association between a genetic instrument and a risk factor for periodontitis would hint at horizontal pleiotropy, a violation of the exclusion restriction assumption (17). In this study the risk of violating the exchangeability or exclusion restriction assumption was substantially reduced as the genetic instruments stemmed from the close vicinity of the coding *IL6R* gene and variants were therefore acting in *cis* (19, 20). We analyzed the data using R version 4.2.1 (R Foundation for Statistical Computing) using the MendelianRandomization and TwoSampleMR packages and designed the study according to STROBE-MR (28).

Results

Characteristics of genetic variants in the *IL6R* gene region used to proxy IL-6R antagonists are presented in Table 1. In brief, 52 SNPs in the vicinity of *IL6R* (cis *IL6R*) were used to proxy IL-6 signaling downregulation. F statistics for the genetic instruments ranged from 31 to 1 802, suggesting no weak instrument bias. Genetically proxied IL-6 signaling downregulation was associated with a reduced odds of periodontitis (OR per 1-unit decrement in log-CRP levels = 0.81; 95% confidence interval (CI): [0.66; 0.99]; P = 0.0497) in the inverse variance weighted (multiplicative random effects) regression (Table 2, Figure 3). No highly influential leverage points were identified in leave-one-out analyses (Appendix Table 1). Searching for associations of SNPs with known risk factors of periodontitis, we found no evidence for an association of any instrument with smoking or diabetes.

The additional analysis, which used variants from the vicinity of the CRP gene (cis *CRP*), yielded a similar effect of CRP reduction on the risk of periodontitis (OR = 0.81; 95% CI: [0.68; 0.98]; P = 0.0296) (Table 2).

Discussion

In this study, we assessed the potential benefit of inhibiting IL-6 signaling to reduce the risk of periodontitis using the principle of instrumental variable estimation. We used genetic variants in the close vicinity of the *IL6R* gene to proxy the effect of inhibiting IL-6R. Our results suggest that intervening on this target through pharmacotherapy might support the prevention and treatment of periodontal disease. While we leveraged CRP as a downstream biomarker of IL-6 signaling downregulation, an additional analysis using genetic variants in the *CRP* gene suggests that CRP might be a substantial causal mediator downstream of IL-6.

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TABLE 1 Summary of CRP associated variants in *IL6R*.

SNP	EA	OA	EAF	BETA	SE	P-value	F statistic
rs10796927	T	C	0.729	0.040	0.002	2.5e-71	330.6
rs111879666	T	C	0.039	-0.055	0.005	1.5e-28	122.3
rs112203594	A	C	0.013	0.038	0.007	2.4e-08	31.3
rs112505856	T	C	0.052	-0.041	0.006	1.2e-11	46.1
rs11264239	A	G	0.057	-0.038	0.005	1.3e-16	67.9
rs113580743	A	G	0.038	0.051	0.005	1.4e-21	90.8
rs116037345	T	C	0.043	0.036	0.006	2.5e-09	35.6
rs116059394	A	G	0.951	-0.046	0.004	2.5e-24	102.7
rs116141616	A	G	0.018	0.037	0.006	9.5e-09	33.1
rs11811450	A	G	0.913	-0.022	0.004	2.7e-10	39.1
rs12083537	A	G	0.807	0.066	0.002	3.1e-159	749.4
rs12406117	A	G	0.587	0.013	0.002	6.5e-11	44.2
rs12726220	A	G	0.927	0.025	0.004	1.3e-08	32.1
rs12735458	A	G	0.987	0.077	0.009	1.1e-18	77.2
rs12750774	A	G	0.308	-0.063	0.002	2.0e-191	902.9
rs139364224	T	C	0.979	-0.060	0.008	5.9e-15	61.5
rs139952834	T	C	0.029	0.064	0.009	3.3e-12	48.1
rs142768042	T	C	0.020	0.040	0.006	4.7e-11	43.4
rs144671207	A	G	0.018	-0.056	0.009	1.6e-09	36.1
rs145262901	A	G	0.018	-0.057	0.010	7.0e-09	33.4
rs145909430	T	C	0.987	0.103	0.008	2.3e-39	174.0
rs147830103	A	G	0.041	0.043	0.007	7.1e-10	38.3
rs16835819	T	C	0.989	0.080	0.009	1.4e-20	85.6
rs1760798	T	C	0.769	-0.018	0.002	2.9e-14	59.9
rs183641528	A	G	0.013	-0.080	0.008	7.3e-26	111.6
rs1889312	A	G	0.428	-0.022	0.002	6.5e-28	124.3
rs3738028	A	C	0.560	0.015	0.002	1.2e-11	47.7
rs3766925	A	T	0.256	-0.015	0.002	6.4e-11	44.3
rs3766926	T	C	0.805	-0.046	0.002	1.0e-78	365.8
rs41269913	T	C	0.045	-0.040	0.005	2.3e-13	53.7
rs4133213	A	C	0.393	-0.085	0.002	1.0e-200	1,802.0
rs4509570	C	G	0.720	-0.048	0.002	9.3e-94	437.4
rs4845626	T	G	0.187	0.052	0.003	1.4e-85	398.5
rs4845663	T	C	0.592	-0.013	0.002	4.5e-10	40.3
rs55676222	A	T	0.914	0.066	0.004	2.0e-71	313.4
rs56100876	A	G	0.018	-0.106	0.008	5.4e-39	172.5
rs61806853	T	C	0.962	0.044	0.005	3.8e-20	84.0
rs61811421	T	C	0.259	0.038	0.002	2.0e-53	245.4
rs67156297	A	G	0.250	0.031	0.002	2.7e-43	197.3

(Continued)

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TABLE 1 Continued

SNP	EA	OA	EAF	BETA	SE	P-value	F statistic
rs72698179	A	C	0.037	0.056	0.006	1.5e-18	77.6
rs7523010	A	T	0.249	0.024	0.003	1.2e-19	85.2
rs7525477	A	G	0.480	0.029	0.002	2.6e-43	197.3
rs75456865	A	T	0.978	-0.044	0.005	6.9e-17	69.5
rs76289529	T	C	0.033	-0.052	0.006	6.4e-20	83.5
rs77994623	T	C	0.136	0.046	0.003	1.6e-61	284.0
rs78739139	A	G	0.062	-0.050	0.005	1.0e-27	118.1
rs79438587	T	C	0.160	-0.018	0.003	1.4e-10	39.8
rs79505546	T	C	0.011	-0.059	0.009	3.9e-11	43.3
rs79778789	A	G	0.986	0.053	0.008	4.4e-11	43.9
rs79794939	T	C	0.073	0.035	0.004	9.2e-20	81.5
rs8192484	A	T	0.973	-0.041	0.007	1.3e-08	32.6
rs9427092	T	C	0.817	-0.029	0.002	1.8e-30	136.4

EA, effect allele. OA, other allele. EAF, effect allele frequency. SE, standard error

Patients with periodontitis have been observed with increased levels of IL-6 in serum (29), saliva (29), and gingival crevicular fluid (30). Similarly, a study found increased concentrations of sIL-6R in the gingival crevicular fluid of periodontitis patients and showed *in vitro* that calprotectin induced sIL-6R production in THP-1 macrophages might be responsible for this association (31). Therefore, patients with periodontitis might be especially susceptible to an excess of IL-6. In contrast, IL-6 secretion in periodontal ligament fibroblasts was suppressed by overexpression of microRNA (miR)-146a in the presence of porphyromonas gingivalis (32) and several studies suggest that expression levels of miR-146a and transglutaminases might similarly be associated with IL-6 expression in inflamed periodontal ligament (33, 34). The continuous observation of IL-6 levels in monkeys with ligature-induced periodontitis revealed that IL-6 occurred as a response to acute initial periodontitis, but remained low in the progression and resolution phases of the disease (35).

Beyond that, the involvement of IL-6 in the development of periodontitis is well recognized (36). IL-6 has pleiotropic effects on lymphocyte promotion and tissue destruction, predominantly mediating B cell activation. Building an IL-6-IL-6R-gp130 complex, IL-6 induces Janus kinase (JAK) to mediate the phosphorylation of signal transducer and activator of transcription 3 (STAT3) and the formation of phosphorylated STAT3 homodimers. Via JAK-STAT3 signaling the expression of IL-6-responsive genes such as suppressor of cytokine signaling 1 (SOCS1) and SOCS3 is upregulated. JAK phosphorylates the cytoplasmic domain of gp130 at tyrosine 759, the binding site of SH2 domain tyrosine phosphatase 2 (SHP2), enforcing the mitogen-activated protein kinase (MAPK) pathway (37). Besides that, IL-6 increased osteocyte-mediated osteoclastic differentiation by activating JAK2 and Receptor activator of nuclear factor-κB ligand (RANKL) *in vitro* (38).

IL-6R is a popular drug target for the treatment of inflammatory diseases. Blockade of IL-6R with monoclonal antibodies such as

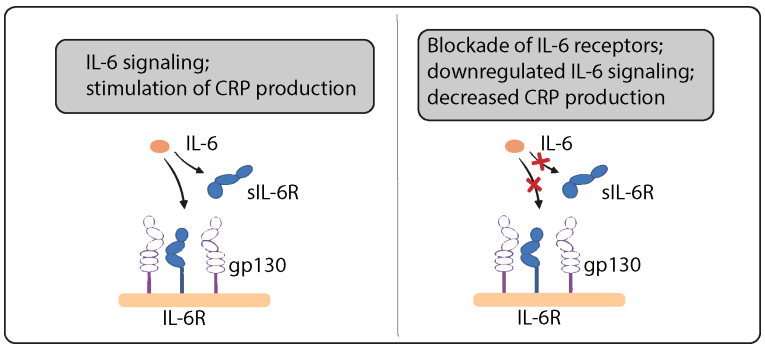

FIGURE 2
IL-6 signaling. Variants in the IL6R gene act as proxies for downregulation of IL-6 signaling. SNPs in this region that are strongly associated with CRP are assumed to act via modifying IL-6 receptors.

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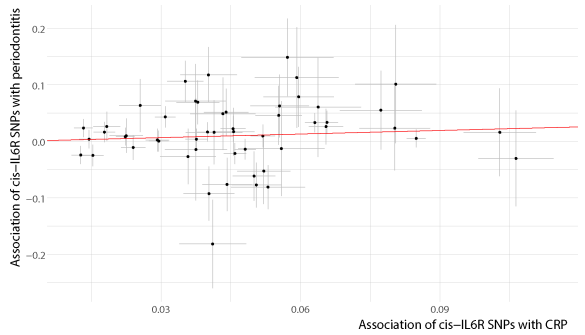

FIGURE 3
Inverse variance weighted (multiplicative random effects) regression. Instrumental SNPs are plotted with error bars indicating the respective standard errors. The slope of the regression line equals the logarithm of the effect estimate. For better graphical display associations were harmonized to lie on the positive x-axis. SNP, single-nucleotide polymorphism; CRP, c-reactive protein.

tocilizumab is a long-established treatment of rheumatoid arthritis to reduce systemic and articular inflammation. Rheumatoid arthritis and periodontitis are known to be directly associated (39) and a study of 55 patients diagnosed with rheumatoid arthritis and chronic periodontitis first showed improved periodontal conditions after 20 months of medication with Il-6R antagonists (40). Similar studies observed improvements of the periodontal status after only 6 months of treatment (41, 42) suggesting potential benefits of even short term intake of IL-6R antagonists. So far, no study examined the effect of IL-6R antagonists on periodontitis in patients without rheumatoid arthritis. This might be caused by fears of potential adverse effects on immunity, which, however, might be less serious when the drugs are administered locally than when administered systemically (4).

Our analytical approach is novel in dentistry, but well-established in other fields of medicine. Notably, the potential benefit of blocking IL-6R for prevention of coronary heart disease was among the first major applications of the drug target Mendelian randomization approach (43) initiating further research and finally leading to the development of ziltivekimab, a novel IL-6R inhibiting drug specifically for use in atherosclerotic disease (44).

We selected genetic instruments based on their association with CRP instead of circulating IL-6. Blockade of IL-6R would inhibit the transfer of IL-6 into cells and lead to an accumulation of IL-6 in the circulatory system. Therefore, genetic variants in the *IL6R* gene region that are associated with increased circulating levels of IL-6 would rather indicate reduced IL-6 signaling, leading to opposite directions of association with disease outcomes to those expected based on serum IL-6 measurements. Instead, CRP is a direct indicator of IL-6 signaling (22), and according to our analysis a possible causal mediator for the risk of periodontitis.

CRP is mainly synthesized by hepatocytes in the liver in response to inflammation and tissue damage, but it can also be produced locally by arterial tissue. After binding to polysaccharides, CRP activates the classical complement pathway and prepares ligands for phagocytosis. While CRP serum levels are routinely used to indicate systemic inflammation and nonsurgical periodontal treatment consistently lead to reduced serum CRP levels (45), less is known about the causal role of inflammation markers in general, and CRP in specific, in the pathogenesis of periodontitis (46). While our analyses suggest a causal role of CRP in the pathogenesis of periodontitis, this finding was rather unexpected, and it remains to be seen whether CRP is a suitable target to break the vicious feed-forward loop linking periodontitis to systemic low-grade inflammation.

The drug target MR approach has some limitations. First, the method models a linear association of genetically proxied IL6R inhibition around the observed mean of CRP and is not able to estimate the effect at extremes of the distribution. Second, the analyses did not account for anti-inflammatory drug use. Third, the result is foremost to be interpreted as a test of the causal association. The actual size of risk lowering achieved through IL-6R inhibitors cannot reliably be predicted in this kind of analysis. Drug target MR effect estimates rather correspond to continuous long-term modulation of drug targets resembling preventive intake. Drug intake as therapy in an acute phase of periodontitis over a relatively short period of time might therefore yield different treatment effects. Fourth, our analyses used data of patients with European ancestry and may not directly be transferable to populations of different heritage. Fifth, the instrumental variable analysis relies on certain unverifiable assumptions, namely exchangeability and exclusion restriction. However, for studies targeting a protein the biasing effect of horizontal pleiotropy should be minimal. Finally, we recognize that effects on health outcomes are mediated by *classic* and *trans* signaling in differing ways, and that our genetic instruments may not act in the same way as IL-6R antagonists or other approaches to downregulate IL-6, for example by intervening on miR-146a expression in periodontal tissue.

TABLE 2 Genetically proxied downregulation of IL-6 signaling and CRP reduction.

Target	OR	(95% CI)	P-value
cis IL6R	0.81	(0.66;0.99)	0.0497
cis CRP	0.81	(0.68;0.98)	0.0296

OR (odds ratio) representing the change in odds of periodontitis per genetically proxied inhibition of drug target equivalent to 1 unit decrease in serum CRP on the log scale; CI, confidence interval; cis IL6R: variants selected from the vicinity of the IL6R gene; cis CRP: variants selected from the vicinity of the CRP gene

Conclusion

In conclusion, we found genetic evidence for a reduced risk of periodontitis through downregulation of IL-6 signaling. Our results identified IL-6R as a potential drug target to prevent the development of periodontitis and as a host modulating adjunctive periodontitis therapy. While there is some clinical evidence to support our findings, the effect of IL-6R antagonists should be investigated in more detail. Further genetic studies could help dissect the downstream effect of IL-6 signaling on periodontitis. Beyond that, our study highlights the benefit of leveraging genetic data to investigate drug repurposing and adverse effects in dentistry.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: CRP GWAS data are accessible via the GWAS catalog (https://www.ebi.ac.uk/gwas/) under accession ID GCST90029070. Periodontitis GWAS data are available at https://data.bris.ac.uk/data/dataset/2j2rqgzedxlq02oqbb4vmycnc2.

Ethics statement

The individual studies had previously obtained relevant ethical approval and participant consent. This study complied with all relevant ethical regulations, including the Declaration of Helsinki, and ethical approval for data collection and analysis was obtained by each study from local boards as described in the included GWAS.

Author contributions

MN contributed to conception and design, development of methodology, data acquisition, analysis, interpretation of data, drafted, and critically revised the manuscript. ZA and SR contributed to data analysis and interpretation of data and critically revised the manuscript. TK and BE contributed to interpretation of data and critically revised the manuscript. BH contributed to conception, design, and interpretation of data, and critically revised the manuscript. HB contributed to conception, design, development of methodology, and interpretation of data, and critically revised the manuscript. MG contributed to conception and design, data analysis and interpretation of data, and critically revised the manuscript. SEB contributed to conception, design, development of methodology, analysis, and interpretation of data, and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1160148/full#supplementary-material

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EDITED BY
Pedro Paulo Chaves de Souza,
Universidade Federal de Goiás, Brazil

REVIEWED BY
Ton Schoenmaker,
VU Amsterdam, Netherlands
Mario Taba Jr,
University of São Paulo, Brazil

*CORRESPONDENCE
Dana T. Graves
dtgraves@upenn.edu

†These authors have contributed equally to this work

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An update on periodontal inflammation and bone loss

Mingzhu Zhang[1†], Yali Liu[1†], Hamideh Afzali[2†] and Dana T. Graves[2*]

[1]Yunnan Key Laboratory of Stomatology, Kunming Medical University, School of Stomatology, Kunming, China, [2]Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, United States

Periodontal disease is a chronic inflammatory condition that affects the supporting structures of the teeth, including the periodontal ligament and alveolar bone. Periodontal disease is due to an immune response that stimulates gingivitis and periodontitis, and its systemic consequences. This immune response is triggered by bacteria and may be modulated by environmental conditions such as smoking or systemic disease. Recent advances in single cell RNA-seq (scRNA-seq) and *in vivo* animal studies have provided new insight into the immune response triggered by bacteria that causes periodontitis and gingivitis. Dysbiosis, which constitutes a change in the bacterial composition of the microbiome, is a key factor in the initiation and progression of periodontitis. The host immune response to dysbiosis involves the activation of various cell types, including keratinocytes, stromal cells, neutrophils, monocytes/macrophages, dendritic cells and several lymphocyte subsets, which release pro-inflammatory cytokines and chemokines. Periodontal disease has been implicated in contributing to the pathogenesis of several systemic conditions, including diabetes, rheumatoid arthritis, cardiovascular disease and Alzheimer's disease. Understanding the complex interplay between the oral microbiome and the host immune response is critical for the development of new therapeutic strategies for the prevention and treatment of periodontitis and its systemic consequences.

KEYWORDS

periodontal disease, immune response, microbiota, scRNA-seq, innate immunity, adaptive immunity

1 Introduction

Advances in single cell techniques have provided new insight into cell types that are modified in their numbers or activity in subjects with periodontitis. In addition, *in vivo* animal studies have established cause-and-effect relationships through the use of biologic agents or genetically modified mice. Periodontal disease consists of periodontitis and gingivitis, both of which are triggered by bacteria and caused by the host's immune response. While gingivitis causes inflammation without loss of connective tissue

attachment or bone, periodontitis leads to the destruction of the connective tissue attachment and alveolar bone (1–3). Periodontal disease has a significant impact on oral health and has been implicated as contributing to the pathogenesis of several systemic conditions, including diabetes, Alzheimer's disease, rheumatoid arthritis, and cardiovascular disease (2–5).

Periodontitis involves the activation of the inflammatory response caused by a change in bacteria, generally referred to as dysbiosis (6, 7). The nature of dysbiosis is not well defined and represents one of the major challenges in oral health research. Readers are referred to recent reviews examining microbial dysbiosis that precedes the development of periodontitis (5–8). Key cell types in the initial response to bacteria include keratinocytes and stromal fibroblasts, which are not typical immune cells (9, 10). The interaction of these cells with immune cells leads to gingival inflammation and the initiation of pathways that damage connective tissue. In gingivitis, the loss of connective tissue is reversible. In some individuals, gingivitis leads to periodontitis. The factors responsible for this transition have not yet been well defined although recent results provide new information on potential cell-cell communications that are involved.

Single cell RNA sequencing provides transcript level analysis of cells that have been isolated from tissues. This approach is particularly useful because it provides an unbiased examination of hundreds of transcripts in each cell that gives insight into the cell type, cell state, and cell activity. Taken together, the scRNA-seq data has defined key subpopulations of stromal cells, keratinocytes and leukocytes and their potential mechanistic role in periodontitis. However, it is important to consider several key limitations of this approach. The method used to isolate cells from gingival tissue can lead to selective loss or enrichment of certain cell populations, influencing the results. The depth of sequencing can also bias the identification of highly expressed genes over those with lower transcript levels. Furthermore, the arbitrary determination of cell clusters can result in differences in the number of clusters reported by different investigators. Finally, the high cost of scRNA-seq limits the number of biological replicates that can be examined, necessitating confirmation of findings through alternative approaches. Awareness of these caveats is crucial when interpreting scRNA-seq data in the context of periodontitis research.

2 Innate immunity

2.1 Epithelial barrier

The function of epithelial tissues is the protection of the organism from chemical, microbial, and physical challenges which is indispensable for viability (10). Keratinocytes form a barrier through tight junctions, adherens junctions, and gap junctions. Bacteria, in turn, can disrupt the epithelial barrier by inducing leukocytes to produce proteolytic enzymes that degrade inter-epithelial junctions, inflammatory cytokines that downregulate the expression of adhesion molecules and keratinocyte apoptosis that disrupts a continuous barrier (10–12). Bacteria can penetrate epithelial cells and reach the basal layer within 24 hours (13). The severity of periodontitis is positively correlated with the extent of epithelial tissue damage. A reduction of epithelial cells is found in moderate or severe periodontitis (14). Bacterial invasion of the oral epithelium causes increased ROS production, which can lead to mitochondrial damage and accumulation and the production of pro-inflammatory factors (15). *P. gingivalis* can modulate gingival keratinocytes to enhance mRNA levels of inflammatory factors and stimulate apoptosis (16) and degrade the proteins that form intercellular adhesions (17, 18). Other invasive bacteria include *A. actinomycetemcomitans*, *T. denticola*, and *F. alocis* (19). The use of protease inhibitors such as leupeptin has been shown to partially mitigate the loss of barrier function induced by *P. gingivalis*, implicating the involvement of microbial proteolytic enzymes in disrupting the epithelial barrier (16).

In addition to providing a physical barrier to microorganisms as part of the host immune defense, the gingival epithelium also expresses a variety of pattern recognition receptors (PRRs) that enable it to recognize microbiota-associated molecular patterns (MAMPs) and respond by secreting cytokines, chemokines and antibacterial peptides. Keratinocytes in the epithelial barrier play a key role in the initiation of the host immune response.

Single-cell RNA sequencing analysis has identified gingival epithelial subpopulations that contribute to inflammatory signatures, antimicrobial defense and neutrophil recruitment in periodontitis (14, 20). scRNA-seq analysis of gingiva indicates an overall reduction in epithelial cells in subjects with periodontitis (14, 20, 21). Caetano et al. identified ten subpopulations of gingival epithelial cells in periodontitis (14). These subpopulations comprised two basal cell clusters, three epithelial clusters expressing high levels of cell cycle genes, one cluster expressing genes associated with extracellular matrix organization and angiogenesis, and four distinct gingival epithelial subpopulations with transcriptomes linked to immune regulation. The latter express transcripts that encode factors that stimulate B-lymphocyte receptor signaling and neutrophil-mediated immunity. Although the overall population of epithelial cells decreased, there was an increase in immune-related epithelial subpopulations (14). Williams et al. identified three keratinocyte subpopulations including a basal cell cluster, a cluster enriched in genes involved in cornification, and a cluster with a gene expression profile consistent with inflammatory responses (20). The gene expression profiles of these cells indicated a shift towards an inflammatory state, with upregulated pathways related to antimicrobial responses and cytokine biosynthesis in subjects with periodontitis (20). Thus, there is an increase in gingival epithelial subpopulations with pro-inflammatory gene signatures with periodontitis and an overall reduction of non-inflammatory epithelial cells. A distinctive junctional epithelial population was characterized by elevated expression levels of serum amyloid A-proteins (SAA) (21). These proteins were found to trigger the secretion of inflammatory cytokines through interaction with the TLR2 pathway in human gingival fibroblasts.

2.2 Stromal cells in periodontitis

Stromal cells in the gingiva consist of mesenchymal stem cells, pericytes and fibroblasts and contribute to tissue integrity, immune

regulation, and repair processes. They express several receptors needed to recognize microbes and produce cytokines and chemokines in response. Approximately 30 years ago Yu and Graves suggested that gingival fibroblasts through the production of CCL2 could play an important role in recruiting monocytes and macrophages to inflamed gingiva (22). scRNA-seq data supports the concept that fibroblasts are an important part of the host response. Williams et al. characterized five distinct fibroblast subclusters (20). Among these clusters, two exhibited a transcriptome profile linked to matrix synthesis and tissue remodeling, while the remaining three featured gene signatures associated with immune functions, including leukocyte proliferation, granulocyte migration, and complement activation. Notably, individuals with periodontitis showed a general decrease in fibroblast subpopulations but displayed a specific increase in inflammatory fibroblast subpopulations in parallel with findings with epithelial cells (Figure 1). There was an upregulation of genes associated with neutrophil recruitment such as CXCL1 and CXCL8. Caetano et al. identified five distinct fibroblast populations, one pericyte population, and one myofibroblast subpopulation (14). Among the fibroblasts, three subpopulations exhibited enrichment of genes associated with extracellular matrix production, while two other fibroblast subpopulations displayed an inflammatory profile. In the context of periodontitis, a marked reduction was observed in the myofibroblast and pericyte subpopulations, accompanied by an increase in inflammatory fibroblasts, while the other subpopulations remained unchanged. Consequently, both investigations identified a decline in fibroblast numbers, accompanied by an expansion of pro-inflammatory stromal cells in subjects with periodontitis. This data suggests a unique restructuring of the epithelial and stromal compartments in periodontitis, with a specific emphasis on facilitating neutrophil recruitment.

2.3 Innate immune cells

2.3.1 Neutrophils

Neutrophils are abundant in gingiva and the gingival sulcus. They have strong antibacterial activity, making them important in the defense against oral infections. Neutrophils may be drawn to the periodontium and other areas that are infected, inflamed, or injured, promoting periodontitis (23). Inflammation increases when neutrophils die and are not quickly removed (23, 24). Neutrophils have regulatory effects on other cell types such as macrophages through production of chemokines that attract macrophages to sites of inflammation and cytokines that modulate polarization of macrophages to an M1 phenotype (25). Upregulation of genes that promote necroptosis, pyroptosis, and ferroptosis-related processes has been reported in neutrophils in subjects with periodontitis (26). When neutrophil recruitment is reduced there is regression of gingival inflammation (27). In contrast, if neutrophil recruitment is totally blocked there is increased severity of periodontitis in humans and in murine

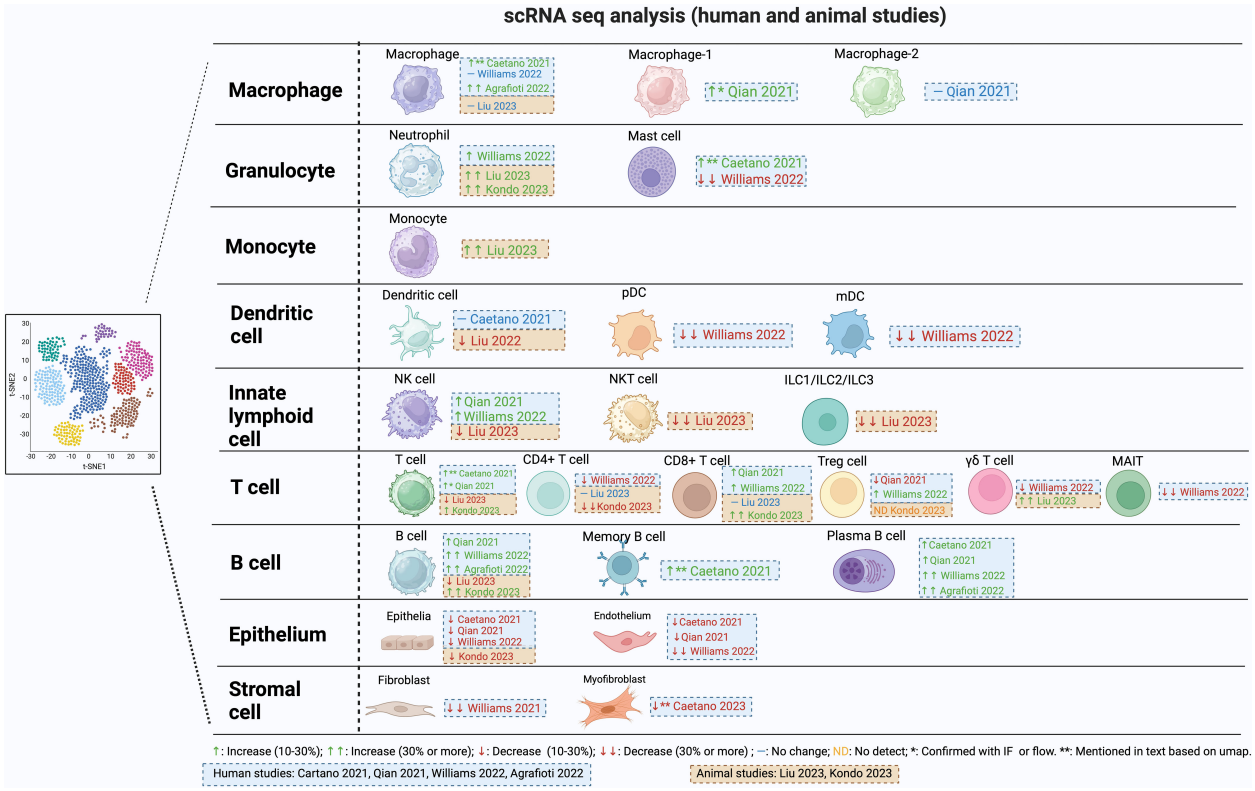

FIGURE 1
Changes in cell population during periodontitis from single cell RNAseq studies.

periodontitis models, demonstrating the importance of these cells in protecting against oral bacteria. Moreover, the absence of neutrophils causes a secondary increase in IL-17 that leads to greater periodontal inflammation and bone loss (28). The study of neutrophils by scRNA-seq is complicated by their high rates of apoptosis during cell isolation (14). However, a significantly higher proportion of neutrophils was documented in subjects with periodontitis (20) and in periodontitis mouse models (29, 30) (Figure 1). It would be helpful if investigators presented differences in actual cell numbers and differences in percentages of cells in supplemental tables, but this has not been done on a consistent basis.

Phagocytosis of pathogens by neutrophils is important in their antibacterial activity (31). Degranulation is one of the ways in which neutrophils exert their anti-microbial and immunomodulatory functions and is important for the progression of periodontitis. The granules are also involved in the inflammatory response and destruction of periodontal tissues through the release of matrix metalloproteinases (MMPs) that break down the extracellular matrix (32) or elastase that disrupts the periodontal epithelial barrier through the cleavage of cell adhesion molecules (33).

Neutrophils produce neutrophil extracellular traps (NETs). Which are a unique DNA structure decorated with antimicrobial peptides (34). Periodontitis is characterized by elevated levels of NETs and delayed NET clearance, compared to healthy gingiva (35). They are produced in response to pathogens and are thought to protect the host by trapping microorganisms, restricting their spread from initial sites of infection, or neutralizing virulence factors (36, 37). Mice that cannot produce NETs are more susceptible to infection (38).

Mast Cells

Mast cells (MCs) are a related granulocyte that can also affect periodontitis negatively (39, 40). Mast cells play a pivotal role in inflammatory responses and can induce bone resorption. They release proteases and histamine from the cytoplasmic granules, as well as cytokines and chemokines. Mast cell counts increase in subjects with chronic periodontitis and in animal studies, mast-cell-deficient mice have significantly reduced alveolar bone loss, demonstrating a cause-and-effect relationship (40, 41). In human gingival tissue, one scRNA-seq publication reported an enrichment of mast cells in subjects with periodontitis compared to non-periodontitis (14), while another report noted a decrease (20) (Figure 1). The basis for this difference is unknown.

2.3.2 Monocytes/Macrophages

Monocytes and macrophages are important in periodontal destruction. Monocytes from the blood reach the tissue microenvironment and develop into macrophages (42). The phenotypic transformation of macrophages plays an important role in the immune response during the onset, development, and regression of periodontitis (43, 44). Macrophages can be polarized to at least two different types with opposite activities: M1-type macrophages and M2-type macrophages. M1 macrophages produce high levels of pro-inflammatory cytokines, and may exacerbate inflammation and tissue damage. M2 macrophages produce anti-inflammatory molecules and growth factors. They participate in processes such as tissue repair, regeneration, and inflammation resolution (43, 44). The onset of periodontitis is linked to the formation of M1 macrophages that are pro-inflammatory and can promote osteoclast differentiation by stimulating the production of RANKL. When M1 polarization is blocked, there is reduced periodontal bone loss (45, 46). M2 macrophages exert anti-inflammatory effects in periodontal tissue by producing IL-10 and TGF-b (47, 48). Controlled release of particles that contain CCL2 induce polarization of M2 macrophages reduce RANKL expression and osteoclast numbers, thereby inhibiting alveolar bone loss (49). Single-cell RNA sequencing data in periodontitis reveals a significant increase in macrophages compared to healthy individuals (14, 50), (Figure 1). Interestingly, macrophages in periodontitis express both pro-inflammatory and anti-inflammatory markers, challenging the notion of exclusive polarization (50).

2.3.3 Dendritic cells (DCs)

DCs connect innate and adaptive immunity by capturing antigens and inducing antigen-specific immune reactions (51). There are two major classes of DC, monocytoid (mDC) that are of monocyte lineage and plasmacytoid (pDC) which are of lymphocytic origin (52). mDC are also known as conventional DC (cDC). cDC are primarily activated in response to bacterial infection and pDC in response to viral infection (51). Human studies show that the number of cDCs decline and pDCs rise in the gingiva of subjects with periodontitis (53). DCs are crucial in guiding naïve T-cells towards T helper cell (Th1, Th2, Th17, Treg, Tfh) differentiation (54) and activating CD4 and CD8 immune responses. They also up-regulate activity in monocytes/macrophages, neutrophils, and NK cells (51, 52).

A reduction in DC function increases susceptibility to periodontitis (55). Without adequate DC activity, the production of antibodies in response to bacterial challenge is significantly reduced (55, 56). Reduced activity of DCs results in a compensatory increase in the expression of inflammatory and pro-osteoclastogenic factors, IL-1β, IL-17, and RANKL (56). Conflicting results have been obtained in scRNA-seq analysis of DC. One publication found there was no clear difference in this cell type between healthy and periodontitis subjects (14) while another (20) reported a notable reduction of ~ 30% in both pDC and cDC in individuals with periodontitis (Figure 1). The reason for this difference is unknown but could involve differences in cell isolation and the parameters of cell clustering.

Langerhans cells (LCs) are a subset of dendritic cells found in the epithelium of mucosa and skin tissues. They respond to both mechanical and bacterial stimulation and play a role in the development of mucosal immunity (57). Depletion of LCs accelerates periodontal bone loss (57, 58) agreeing with increased susceptibility when DC function is compromised (55). Smoking has been found to specifically diminish gingival LCs in healthy individuals, raising the possibility that the loss of LCs may contribute to periodontitis in smokers (53).

3 Innate lymphoid cells

3.1 NK T-cells, γδ T-cells and MAIT cells

Unconventional T-cells include natural killer T (NKT) cells, γδ T-cells and mucosal-associated invariant T (MAIT) cells that express CD3. They are more prevalent in gingiva from subjects with periodontitis (59). NK T-cells are a specialized subset of T-cells with αβ T-cell receptors (TCRs) and NK cell receptors on their surface (59, 60). Gram-negative bacteria possess glycosphingolipids that can activate NK T-cells via antigen presenting cells. NK T-cells can enhance RANKL production, osteoclastogenesis, and alveolar bone loss in mice following oral *P. gingivalis* inoculation and in other models (61, 62).

γδ T-cells express T-cell receptors (TCR) consisting of the gamma and delta chains, with a limited diversity, and do not express CD4 or CD8 (63). These cells are stimulated by a variety of signals, such as direct antigen binding to TCR, stimulation of toll-like receptors or cytokine stimulation. They are found in the epithelium or in the connective tissue adjacent to the epithelium and make up the majority of T-cells in epithelial tissues (64). γδ T-cells are elevated in inflamed human gingiva (64), and are increased to a greater extent than αβ T-cells (64). γδ T-cells stimulate the recruitment of macrophages and neutrophils and produce IL-17A and IFNγ (65). In mice, they are the principal source of IL17A. In the oral inoculation model of periodontitis, γδ T-cells have distinct pathogenic functions, and their reduction significantly reduces loss of alveolar bone. However, this linkage does not exist in the ligature model, pointing out an important difference in the two primary murine models of periodontitis (65). scRNA-seq data reveals an approximately 20% decrease in γδ T-cells among all cell types in individuals with periodontitis compared to healthy individuals (20), contrasting with a 30% increase among immune cells observed in a mouse model of periodontitis compared to the healthy state (29) (Figure 1). Using different reference populations could be a potential reason for the contrasting findings and species differences (64). Another difference may be due to the fact that most human studies represent inflamed tissue that may not exhibit current disease activity, a significant limitation in most human studies, whereas the disease activity is typically progressing in murine models (66).

MAIT have a restricted T-cell receptor (TCR) response (67). Notably, TCR-independent mechanisms such IL-18 signaling can activate MAIT cells. They differ in how they react to various microorganisms, and this diversity may help them discriminate between dangerous pathogens and beneficial commensal species. Germ-free mice have fewer MAIT cells and MAIT cell populations increase during infection, suggesting a protective function against microbial challenge (67). Emerging evidence indicates that MAIT cells may contribute to the development of periodontitis by producing proinflammatory cytokines like IL-17 and TNF when activated by pathogenic microorganisms in the oral cavity. Further research is required to comprehensively elucidate the precise role of MAIT cells in periodontitis (68). A scRNA-seq study reported a significant decline in MAIT cells in subjects with periodontitis (20) The scRNAseq data revealed an upregulation of nucleotide oligomerization domain (NOD)–like receptor signaling pathways, apoptosis, IL-17 signaling, and TNF signaling in MAIT cells from periodontitis subjects (69).

3.2 Innate lymphoid cells-1, -2 and -3.

Lymphoid cells that lack T-cell receptors but are of lymphocyte lineage include innate lymphoid cells (ILC) with three subtypes (ILC1, ILC2, and ILC3). Rather than reacting to antigen, these cells react directly to signals of stress and danger. They possess pattern recognition receptors (TLR2, TLR4, TLR9, NLRP3, RAGE, P2X7 and P2Y2, etc) on their cell surface that respond to danger-associated molecular patterns (LPS, S100 proteins, AGEs, ATP, ROS, etc). ILCs are primarily tissue-resident cells and are classified according to the cytokines they generate (70). ILC1 cells express similar cytokines to Th1 cells such as IFNγ, ILC2 cells produce cytokines similar to Th2 cells such as IL-4, IL-5, IL-9, and IL-13 and ILC3 cells produce IL17A, similar to Th17 cells. According to scRNA-seq data, a mouse model of periodontitis exhibits a reduction of over 30% in ILC cells among immune cells in animals with induced periodontitis (29). ILC1 was the predominant subset, comprising over 60% of ILCs in mice and humans. In humans, the percent ILCs were not significantly altered in the gingiva of subjects with periodontitis. Notably, a small proportion of ILC1 cells expressed RANKL and and ILC3 produced IL17A suggesting they could participate in bone resorption (71). The plasticity, differentiation, tissue-specific migration and accumulation of ILC subpopulations may be an important modulator of the local immune response (72).

3.3 Natural killer (NK) cells

NK cells are lymphocytes belonging to the innate immune system (60). NK cells are cytolytic, killing viral or bacterial-infected, or malignant cells, and can exert pro-inflammatory effects. Through the release of granzymes and perforin, NK cells directly destroy their targets. NK cells produce cytokines like IFN, TNF, IL-5, IL-13, and GM-CSF that upregulate activity in other cells, particularly macrophages and contribute to the control of infections (60, 73) (Figure 1). NK cells have a role in senescent cell clearance. They are stimulated by bacteria through toll-like receptors (TLRs) and cytokines produced by cells such as dendritic cells (73).

NK cells tend to have proinflammatory influences in periodontitis (60). This is manifested through cytokine production, cytotoxic effects, and dendritic-cell crosstalk. Moreover, increased numbers of NK cells in patients wtih periodontitis and decreased numbers after periodontal therapy have been observed (60). Additionally, NK cells are correlated to

the regulation of T-cell proliferation and suppression of B-cells in periodontitis (60). Overall, these findings suggest that NK cells play a role in the pathogenesis of periodontitis, particularly through their proinflammatory influences (60).

4 Adaptive immunity

4.1 CD4+ T-cells

Naive CD4+ T-cells are capable of differentiating into a variety of functional and phenotypical T helper (Th) cell subsets, Th1, Th2, Th17, Treg and Tfh cells (67, 74). Th1 cells are pro-inflammatory and produce IL-1β and IFN-γ to promote inflammation and are associated with tissue damage in periodontitis. Th2 cells play a key role in the production of antibodies. Interleukin-4 (IL-4) and other Th2-cell-derived cytokines are anti-inflammatory and are considered to reduce bone loss. However, antibodies produced by Th2 responses activate complement and could potentially be pro-inflammatory. Th17 cells promote inflammation through IL-17 production. Th17 cells are increased in human periodontitis; reducing Th17 cell numbers reduces alveolar bone resorption in experimental periodontitis (75). In humans, Th17 cells are the principal source of IL17A. IL-17 stimulates osteoblast-lineage cells to secrete RANKL and GM-CSF to enhance osteoclast formation and bone resorption (76). IL-17A also stimulates fibroblasts, epithelial cells, and endothelial cells to produce RANKL, MMP, PGE2, and chemokines to promote the progression of periodontitis (77). IL-17A can affect immune cells such as macrophages, neutrophils, dendritic cells, and B-cells. Regulatory T-cells (Treg) stimulate immunosuppression and resolution of inflammation through production of TGF-b, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), programmed cell death protein 1 (PD1), T-cell immune receptors with Ig and ITIM structural domains (TIGIT), and T-cell immunoglobulin and mucin-containing structural domain 3 (Tim-3) (78). The percentage of these cells increases in the later stages of periodontitis to reduce disease progression and reestablish homeostasis (79). Interestingly, increased RANKL promotes the induction of Tregs and increases formation of M2 macrophages, thus facilitating the resolution of inflammation (79). T follicular helper (Tfh) cells play an important role in the regulation of humoral immunity and germinal center responses, and in periodontitis, may promote local B-cell activation, and maintain a long-term humoral immune response (80). Tfh cells in older individuals may contribute to increased inflammation in periodontitis (74).

Th22 cells are a subpopulation of T-helper cells that produce IL-22 and TNF, which have been linked to the pathogenesis of periodontitis by increasing inflammation (81) and the number of Th17 cells in periodontal lesions (82). Oral inoculation of bacteria in mice stimulates the production of IL-22 through increased numbers of IL-22-expressing CD4+ T-cells in periodontitis-affected tissues (83). This increase is associated with higher levels of RANKL and alveolar bone resorption.

scRNA-seq analysis indicates that T-cells constitute the largest lymphocyte population, followed by B-cells and plasma cells (20) (Figure 1). In human samples with periodontitis there is an overall expansion of T-cells (20, 21). Various studies have identified distinct subclusters of T-cells within single-cell RNA sequencing datasets from both healthy and diseased conditions. These subclusters include CD4+, MAIT, CD8+, γδ T-cells, Treg, TH17, and NK T-cells, which are consistently observed across different studies, albeit with varying proportions in periodontitis (20, 21, 29). Human studies indicate an approximately 25% decrease in $CD4^+$ T-cells in human subjects (20) and a similar reduction of over 30% in mouse periodontitis models (30).

4.2 CD8+ T lymphocytes

CD8+ T-cells kill virally or bacterially infected cells. CD8+ T-cells are fewer in number than CD4+ T-cells in periodontitis lesions (84). CD8+ cytotoxic T lymphocytes produce TNF, IFN-γ and kill cells through expression of Fas ligands, pore-forming proteins (perforins) and proteases (granzyme) (85). CD8+ regulatory T lymphocytes (CD8+ Tregs) produce CTLA4, TGF-b and IL-10 to resolve inflammation. Systemic administration of CTLA-4 reduces alveolar bone resorption in experimental periodontitis (86). Like pro-inflammatory CD4+ T-cells, the pro-inflammatory CD8+ cytotoxic T lymphocytes likely promote periodontitis whereas the pro-resolving CD8+ Tregs help prevent or reduce it. In human gingival tissue, scRNA-seq studies indicate a small ~10% increase in CD8+ T-cells in subjects with periodontitis compared to non-periodontitis subjects (20, 21). Interestingly, periodontitis was associated with an increase in expression of CCL4, CCL4L2, and CCL3L3 in both CD8 T-cells and NK cells. Elevated levels of the CCR5 ligands in cytotoxic CD8+ T-cells underscores their potential role in recruiting inflammatory cells during periodontitis (21).

4.3 B lymphocytes

B-cells are part of the humoral component of the adaptive immune system and are specialized in producing antibodies. B-cells can also present antigens and enhance inflammation through cytokine production, opsonization, and complement fixation mediated by the antibodies they produce. B-cells and plasma cells are increased in periodontitis (87). Hub genes are located at critical nodes in biological processes such as chronic inflammation. Interestingly, a recent study pointed to B-cells as expressing a high number of hub genes that are correlated with inflammation in periodontitis (88). B-cells produce RANKL to promote bone loss (79). Evidence that B-cells contribute to periodontitis was shown in a ligature-induced murine model in which there was significantly less bone loss in B-cell deficient mice (89). On the other hand, B-

cells can potentially reduce periodontitis by limiting bacterial invasion. In support of the latter, reduced dendritic cell activation of B-cells increases periodontitis in an oral inoculation murine model (55). B regulatory cells (Bregs) can reduce inflammation and limit excessive inflammatory responses similar to Tregs. Bregs produce anti-inflammatory cytokines such as IL-10, and inhibit alveolar bone resorption (90, 91). Plasma cells that produce anti-inflammatory cytokines IL-35 and IL-37 also inhibit alveolar bone loss (92). Taken together, evidences suggests that B lymphocytes have a dual role in modulating the progression of periodontitis and can both promote and inhibit alveolar bone resorption depending on the specific conditions.

scRNA-seq studies observed an overall increase in the proportion of B-cells in human subjects with periodontitis compared to healthy controls (21, 50). Three distinct B-cell populations were consistently detected including memory B-cells, IgG-producing plasma B-cells, and follicular B-cells (20). Caetano et al. (14) reported a distinct increase in memory B-cells in moderate periodontitis compared to healthy individuals. The increase is backed up by several publications using alternative approaches showing there is a significant increase in plasma cells in periodontitis compared to healthy individuals (14, 20, 21, 50) (Figure 1).

5 Osteoblast lineage cells and periodontitis

In a periodontally healthy adult, an episode of bone resorption is followed by an equivalent amount of bone formation, which is referred to as coupling. In periodontitis, chronic inflammation inhibits bone coupling after an episode of bone resorption, increasing the size of an osteolytic lesion (2, 93). Thus, osteolytic lesions occur due to bone resorption and inhibition of coupled bone formation. Immune activation significantly reduces coupled bone formation (93). Experimental animal models have demonstrated that oral microbial dysbiosis stimulates inflammation by in bone-lining cells and osteocytes by enhancing nuclear translocation of NF-κB (2). Lineage-specific inhibition of NF-κB in osteoblasts and osteocytes, but not in other cell types, mitigates periodontal bone loss caused by dysbiosis (94). This phenomenon can be attributed to two primary mechanisms. Firstly, inhibition of NF-κB activation reduces RANKL expression in osteocytes and osteoblastic cells, resulting in reduced bone resorption. Osteocyte production of RANKL is significant due to their location within bone. Secondly, the activation of NF-κB in cells of the osteoblast lineage blocks coupled bone formation. The reduction in coupled bone formation is due to NF-κB's role in limiting osteoblast differentiation, indirectly inducing apoptosis in osteoblastic cells and through reducing the synthesis of bone osteoid (94, 95). Such increased apoptosis is significant, as treatments targeting apoptosis have been shown to reduce periodontal bone loss by promoting increased coupled bone formation (96). Inflammation also inhibits mesenchymal stem cell (MSC) differentiation into osteoblasts by blocking the upregulation of transcription factors, runt-related transcription factor 2 (Runx2) and osterix (Osx) (3). Therefore, the activation of NF-κB in osteoblast precursors, osteoblasts, and osteocytes play an essential role in periodontitis, contributing to enhanced bone resorption and limiting the process of coupled bone formation.

6 Periodontitis and systemic diseases

There is a relationship between periodontitis and systemic conditions such psoriasis, rheumatoid arthritis, inflammatory bowel disease, type-2 diabetes, osteoporosis, non-alcoholic fatty liver disease, Alzheimer's disease, pre-term birth, cancer progression and cardiovascular disease (97). In some cases there may be an association through co-morbidities and in others a causal relationship. The relationship is often two-way. Periodontal disease may worsen glycemic control and diabetes may enhance periodontal disease progression (4, 5). The latter may be due to bacteremia entering the bloodstream through invasion of the oral epithelial barrier, which may impact systemic disease a distant sites including an effect on hematopoiesis (4, 5, 98).

Periodontal inflammation and bone loss is enhanced by diabetes (99). The diabetic condition promotes the inflammatory response to bacteria (100) and alters the microbial composition to render it more pathogenic (98, 101). Clinical evidence shows that effective periodontal treatment improves blood glucose levels in patients with type 2 diabetes, suggesting a causal relationship between periodontal disease and glycemic control (102, 103). Similar to the link with diabetes, the intersection between rheumatoid arthritis and periodontitis is thought to be bidirectional. Rheumatoid arthritis subjects have greater loss of attachment, and increased expression of inflammatory mediators (IL-17, IL-2, TNF, and IFN-γ) that is linked to an increase in bacterial load and an increase in periodontal pathogens (104). Periodontal disease may contribute to the etiology of rheumatoid arthritis by increasing exposure of subjects to enzymes that citrullinate proteins to stimulate an auto-immune response (105, 106). In addition to the effect of systemic disease on periodontal tissues, bacteremia caused by periodontal disease may cause epigenetic changes in the bone marrow that affect hematopoiesis. Maladaptive bone marrow (BM)-mediated trained innate immunity (TII) has been proposed as a co-morbidity between periodontitis and arthritis (107, 108). In this scenario, chronic inflammation causes epigenetic changes in the bone marrow to increase the inflammatory response at a distant site.

7 Summary

Periodontal disease is one of the most common causes of oral inflammation and periodontitis is one of the most common osteolytic diseases found in adults (2). They are triggered by bacteria, although the sequelae are due to the impact of bacteria-induced innate and acquired immune responses. New approaches such as scRNA-seq have provided a new understanding of how immune and non-immune cells have bi-directional communication to initiate and amplify the inflammatory response triggered by bacteria. For example, there are

distinct epithelial cell subpopulations that contribute to antimicrobial defense and are likely to play an important role in neutrophil recruitment (14, 20). Subjects with periodontal inflammation experience an overall loss of epithelial cells, but there is an increase in epithelial cells with an inflammatory signature that stimulates neutrophil recruitment. Stromal cells also consist of subtypes that have inflammatory signatures, such as a fibroblast subtype that produces chemokines to stimulate leukocyte recruitment (20, 22). Subjects with periodontitis have a shift towards more pro-inflammatory fibroblast phenotypes, accompanied by a decline in matrix-producing subsets.

Examination of the innate immune landscape reveals complex changes in myeloid cell populations, including neutrophils, macrophages, and dendritic cells. These cells exhibit a balance of protective and destructive functions, depending on their precise activation state. Neutrophils play a crucial role in the pathogenesis of periodontitis, and can be both protective and destructive functions depending on their numbers and activation state (23, 24). While the presence of neutrophils is essential for combating bacterial infection (109) and initiating the inflammatory response, an excessive or dysregulated neutrophil response can contribute to tissue damage and disease progression (26, 27). A substantial reduction in neutrophils, as observed in certain genetic disorders like leukocyte adhesion deficiency, leads to an increased severity of periodontitis (25). Conversely, a large, persistent neutrophil infiltration in periodontal tissues can also exacerbate inflammation and connective tissue breakdown (20). Neutrophils release proteolytic enzymes, such as matrix metalloproteinases (MMPs) and elastase, which degrade the extracellular matrix and disrupt the epithelial barrier. Additionally, the formation of neutrophil extracellular traps (NETs), while initially beneficial for trapping pathogens, can cause collateral tissue damage if not properly regulated and cleared. Therefore, a balanced neutrophil response is critical for maintaining periodontal health, as both the absence and the excessive or chronic presence of these cells can contribute to the initiation and progression of periodontitis.

Macrophages can polarize into distinct phenotypes (43, 44). M1 macrophages promote inflammation, tissue destruction and bone resorption by producing pro-inflammatory cytokines. The onset and progression of periodontitis is closely linked to the formation of M1 macrophages. Conversely, M2 macrophages exhibit an anti-inflammatory phenotype and participate in resolving inflammation and tissue repair by releasing anti-inflammatory cytokines. Single-cell RNA sequencing data from periodontitis lesions reveal a significant increase in macrophages expressing both pro-inflammatory (M1) and anti-inflammatory (M2) markers (45–48). M1 macrophages may drive inflammation and bone loss in the early stages, while M2 macrophages may play a protective role in later stages by resolving inflammation and promoting tissue regeneration.

A number of cell types, particularly lymphocytes with innate immune properties, have recently been identified as contributing to periodontal inflammation and bone loss, particularly. NKT-cells, γδ T-cells, and mucosal-associated invariant T (MAIT) cells (59). In periodontitis, NKT-cells may enhance inflammation, RANKL production, osteoclastogenesis, and alveolar bone loss. γδ T-cells express a distinct TCR composed of gamma and delta chains and play critical roles in barrier surveillance (63, 64). In periodontitis, γδ T-cells are elevated and stimulate the recruitment of macrophages and neutrophils, as well as the production of pro-inflammatory cytokines like IL-17A and IFN-γ. Their absence has been shown to significantly reduce alveolar bone loss in animal models of periodontitis. MAIT-cells are known to increase during infections, suggesting a protective role. Their numbers are reduced in periodontitis, suggesting the loss of a key protective cell type (20, 69). However, this has not yet been functionally demonstrated. Conventional lymphocytes expressing αβ receptors have also been implicated in periodontitis. Anti-inflammatory T-cells (T-regs and B-regs) limit periodontal inflammation and bone loss, while Th1 and Th17 T-cells have been implicated in stimulating inflammation and periodontal bone loss (73, 74, 77). The role of Th2 cells is more complicated as the production of antibodies may be protective or by activating complement, may lead to inflammation-induced tissue damage.

Periodontal ligament fibroblasts and osteoblast lineage cells consisting of osteoblasts and osteocytes are strongly affected by inflammation that leads to periodontitis. One hypothesis of periodontitis links the proximity of periodontal inflammation to bone as a key event that distinguishes gingivitis from periodontitis (92, 93). The onset of periodontitis activates NF-κB signaling, which induces the expression of RANKL in osteocytes, bone lining cells and PDL fibroblasts (110). Interestingly, deletion of RANKL in osteocytes has a dramatic effect on reducing bone resorption stimulated by oral inoculation of P. gingivalis and F. nucleatum. In addition to stimulating bone resorption, inflammation suppresses expression of bone matrix proteins and causes osteoblast cell death leading to disruption of bone coupling (95). Collectively, these inflammatory processes affect osteocytes, osteoblasts, and PDL fibroblasts to induce periodontitis by simulating osteoclast formation and activity and inhibiting repair of osteolytic lesions.

The research presented highlights several important implications for clinical practice in managing periodontitis. The findings related to the involvement of specific cell types and their states at different disease stages could guide the development of stage-specific targeted therapies. Modulating the balance of macrophage polarization towards an anti-inflammatory M2 phenotype, or regulating the recruitment and activation of neutrophils, may help resolve the destructive inflammatory response. The scRNA-seq data could identify valuable biomarkers for monitoring disease progression, predicting treatment responses, and guiding personalized management of periodontitis. Additionally, the insights into cell populations involved in tissue repair and the adaptive immune response could pave the way for

developing regenerative therapies. In summary, the insights gained from research highlight the importance of targeting specific cellular and molecular mechanisms involved in periodontitis to develop more effective prevention and treatment strategies, with the potential to also impact various systemic diseases associated with chronic inflammation.

Author contributions

MZ: Writing – original draft. YL: Writing – original draft. HA: Writing – original draft. DG: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Teun J. De Vries,
VU Amsterdam, Netherlands

REVIEWED BY
Sasanka Chukkapalli,
Texas A&M University College Station, United States
Jianfeng Jin,
VU Amsterdam, Netherlands

*CORRESPONDENCE
Liang Hao
hxkqhl@foxmail.com
Jinle Li
Jinle.li@foxmail.com

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Mechanisms of mechanical force in periodontal homeostasis: a review

Tianqi Wang[1], Xinran Liu[1], Jiaxin Li[1], Yuan Yue[1], Jinle Li[2]*, Min Wang[1], Na Wei[3] and Liang Hao[1]*

[1]The State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases, Department of Prosthodontics, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan, China, [2]The State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases, Department of General Clinic, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan, China, [3]The State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases, Department of Oral Implantology, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan, China

Mechanical forces affect periodontal health through multiple mechanisms. Normally, mechanical forces can boost soft and hard tissue metabolism. However, excessive forces may damage the periodontium or result in irreversible inflammation, whereas absence of occlusion forces also leads to tissue atrophy and bone resorption. We systemically searched the PubMed and Web of Science databases and found certain mechanisms of mechanical forces on immune defence, extracellular matrix (ECM) metabolism, specific proteins, bone metabolism, characteristic periodontal ligament stem cells (PDLSCs) and non-coding RNAs (ncRNAs) as these factors contribute to periodontal homeostasis. The immune defence functions change under forces; genes, signalling pathways and proteinases are altered under forces to regulate ECM metabolism; several specific proteins are separately discussed due to their important functions in mechanotransduction and tissue metabolism. Functions of osteocytes, osteoblasts, and osteoclasts are activated to maintain bone homeostasis. Additionally, ncRNAs have the potential to influence gene expression and thereby, modify tissue metabolism. This review summarizes all these mechanisms of mechanical forces on periodontal homeostasis. Identifying the underlying causes, this review provides a new perspective of the mechanisms of force on periodontal health and guides for some new research directions of periodontal homeostasis.

KEYWORDS

mechanical force, periodontal homeostasis, immune defence, extracellular matrix metabolism, specific proteins, bone metabolism, characteristic periodontal ligament stem cells, non-coding RNAs

1 Introduction

The concept of homeostatic medicine was proposed in 2022 (1). It focuses on the root causes of diseases by exploring the mechanisms and regulation strategies for homeostasis at multiple levels including molecules, cells, organs and systems (2). Under physiological conditions, the periodontal tissue is continuously subjected to environmental stimuli like mechanical forces, salivary flow, and flora stimulation while the internal tissues constantly undergo remodelling to maintain homeostasis (3). The first barrier to the external stimulus is the oral mucosa, which exhibits a strong ability of immune defence (4). Microbiologically, the oral microbiota colonising the mucosal surfaces stimulate a mild immune response and resist invasion of pathogens (4, 5). Next, the extracellular matrix (ECM) metabolism contributes to tissue reconstruction; meanwhile, some mechanosensitive proteins perform functions in response to mechanical stimuli (6, 7). At the cellular level, differentiation between PDL stem cells (PDLSCs) and progenitors could promote tissue maintenance, repair, and regeneration (8, 9). Osteocytes act as an important mechanosensor under mechanical forces (10). Osteoblasts and osteoclasts mediate bone formation and resorption shaping the alveolar bone under mechanical forces (11). At the epigenetic level, some mechanosensing non-coding RNAs, such as microRNAs, could interfere with the expression of related genes (12). In addition, many signal pathways are activated under forces to regulate bone and tissue metabolism (13, 14). These factors help regulate gingiva, periodontal ligament (PDL), and bone homeostasis that form the periodontal homeostasis under mechanical forces.

Mechanical forces including mastication, occlusion, or other forms of mechanical loading exerted on the teeth are transmitted through PDL to the alveolar bone (15). Koivumaa, Mäkilä (16) classified these forces into physiological and non-physiological (pathological and therapeutic) forces. Physiological forces are vital for soft and hard tissue metabolism, which helps maintain periodontal homeostasis. Pathological forces, such as occlusal trauma and bruxism, may damage the periodontium, teeth, or temporomandibular joint. The therapeutic force mainly refers to orthodontic forces that promote tooth movement through bone regeneration and resorption (17). However, absence of physiological forces would result in bone resorption and loss of periodontal tissues (15, 18, 19). Therefore, a balance between proper mechanical forces exerted on the periodontium is quite essential for maintaining tissue homeostasis, whereas improper forces would lead to tissue destruction and breakdown of periodontal homeostasis. There are a variety of mechanosensors on the surface of the cell membrane. After being stimulated by mechanical forces, the mechanosensors undergo configuration changes to activate the cascade signal pathways, and then, biological signals are transmitted to the nucleus to regulate gene transcription (20).

This review illustrates the role of forces on periodontal health (Figure 1) as well as potential new targets and for homeostatic remodeling which puts forward a new research direction of periodontal homeostasis under forces.

2 Aspects of mechanical forces influence periodontal homeostasis

2.1 Immune response under mechanical forces

The gingival epithelium acts as a mucosal barrier against external stimuli while commensal microbiota colonising the epithelium resist invasions of pathogens. The microbiota-gingival epithelium barrier contributes to gingiva homeostasis (4, 5). Pathological forces could disturb the balance between microbial communities resulting in breakdown of homeostasis (21). Apart from that, immunocytes in the gingiva also play an important role in maintaining periodontal homeostasis in healthy state (22). Forces have been found to have the potential to affect functions of neutrophils, macrophages, and T cells (23–25). Some improper forces lead to cell death. Specific mechanisms will be illustrated next.

2.1.1 Microbiota - gingival epithelium barrier

Since the gingival epithelium is the first barrier against invasions of pathogens and other stimulations, its integrity and functionality are critical. A previous study has found an ion channel TRPV2 expressed in gingival tissues to sense mechanical stimuli. The ion channel could detect physical and chemical changes to ensure defence mechanisms in gingival tissues (26). Another study also reports the physiological shear stress produced by salivary flow belongs to essential elements of the gingiva. The physiological force is an important guarantee for gingival epithelium to perform immune defence functions (27). Moreover, pathological forces such as occlusal trauma may disrupt balance of microbiological flora and induce infection. Inchingolo et al. found that treating occlusal trauma could relieve the damage caused by the disorganisation of the microenvironment and an increase in pathogenic microorganisms (21). Once the external factors change, such as removal of stress, microenvironment-specific factors, such as temperature, osmotic pressure, and concentrations of metabolites (iron, calcium, and magnesium), will be altered (5). Thus, appropriate forces are essential for the microbiota-gingival epithelium barrier to maintain immune homeostasis.

2.1.2 Immunocytes

As innate immune cells, neutrophils account for the highest proportion of innate immune cells in healthy gingiva, whereas deficiency in neutrophils can increase susceptibility to periodontitis. When exogenous pathogens invade periodontal tissues, neutrophils significantly increase; several steps are required before neutrophils mature and perform functions, including phagocytosis; reactive oxygen species production; and intracellular and extracellular degranulation (22). Granulocyte colony-stimulating factor (G-CSF) may interfere with the CXCR4/SDF-1 axis by reducing chemokine (C-X-C motif)

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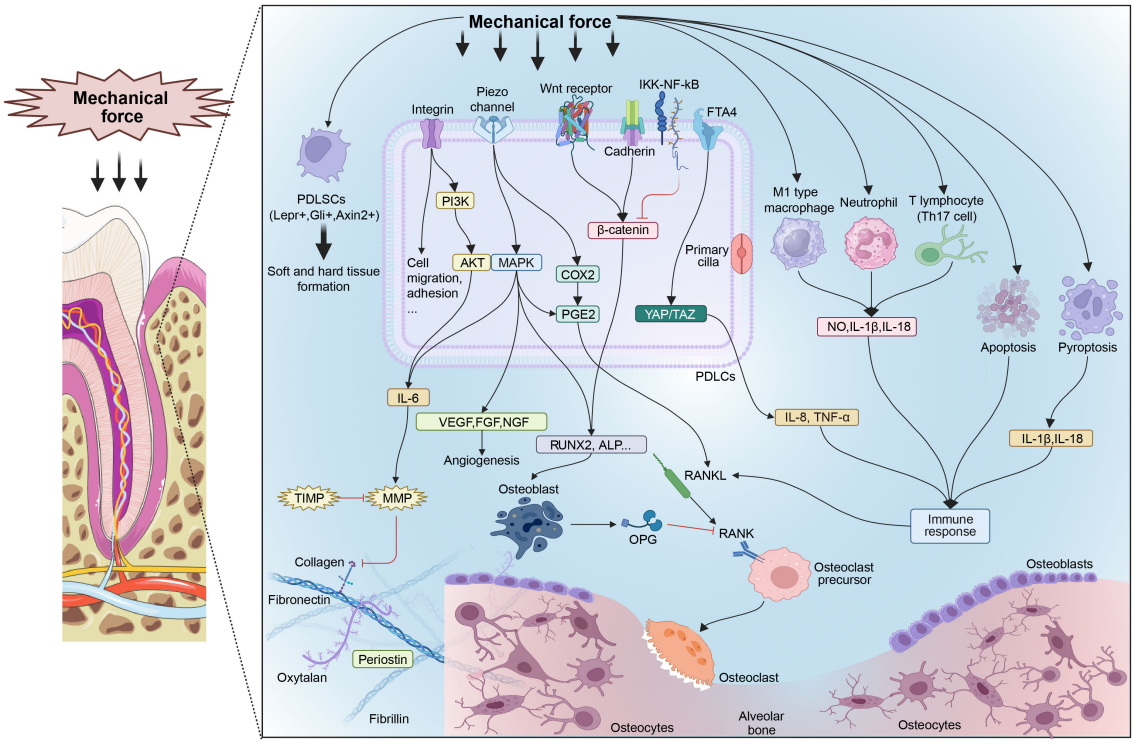

FIGURE 1
Mechanisms of mechanical forces influencing periodontal homeostasis. ECM components such as collagen, fibronectin, and specific proteins reconstruct under mechanical forces. Several mechanosensitive signal pathways are activated to boost tissue metabolism. Characteristic PDLSCs respond to mechanical forces and promote tissue metabolism. Some pathological forces promote immunocyte differentiation and cell death, leading to an inflammatory response. PDLSCs, periodontal ligament stem cells; PDLCs, periodontal ligament cells; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases; PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; MAPK, mitogen-activated protein kinase; IL, interleukin; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor; COX2, cyclooxygenase-2; PGE2, prostaglandin E2; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; RANKL, receptor activator of NF-κB ligand; RANK, receptor activator of NF-κB; OPG, osteoprotegerin; IKK, inhibitor of IκB kinase; NF-κB, nuclear factor-κ-gene binding; YAP, yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; Th17 cell, helper T17 cell; NO, nitric oxide; TNF, tumor necrosis factor.

receptor 4 (CXCR4) expression or increasing the stromal cell-derived factor 1 (SDF-1) cleavage and subsequently regulating neutrophil release (28). Traumatic occlusion up-regulates SDF-1 and CXCR4 expression in PDL tissues to maintain bone metabolism by increasing osteoblast differentiation (23). Notably, chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL5, and CXCL8 are responsible for the recruitment of neutrophils. Furthermore, chemokine (C-C motif) motif ligand 2 (CCL2), CCL5, and CXCL10 are chemoattractants of macrophages and lymphocytes. The aforementioned chemokines are regulated by bacterial and mechanical signals. Thus, changes will influence the recruitment of immune cells toward the infection zone (29).

Although fewer macrophages than neutrophils are found in healthy gingiva, they are involved in homeostasis maintenance by phagocytosing pathogens and secreting inflammatory cytokines (30). According to earlier studies, mechanical forces may increase the number of M1-type macrophages and, thus, mediate the inflammatory response and bone remodelling. He et al. found that H_2S is secreted by PDLSCs under mechanical loads. H_2S, as a gas signalling molecule, can induce M1-type macrophage polarisation via the STAT1 signalling pathway (31). Jiang et al. found that mechanical force induces PDLSC autophagy. Macrophages are polarised toward the M1 phenotype, led by force-induced autophagy via suppression of the AKT signalling pathway (24).

Healthy gingiva consists of the inflammatory infiltration including T cells acting as antigen-presenting cells (APCs) which regulate local immunity. Balances between helper T (Th) cells including Th1/Th2 cells and Th17/Treg cells are important for the periodontal immune microenvironment (32). Th17 cell differentiates under stimulation by transforming growth factor (TGF)-β and Interleukin (IL)-6 and then releases cytokine IL-17, which is relevant to neutrophil recruitment by activating CXCL8. CXCL8 activation is a key element in periodontal bone resorption, as it facilitates receptor activator of nuclear Kappa-B Ligand (RANKL) production. Similar to Th1/Th2 cells, Th17 and Treg cells perform distinct functions during the inflammatory process. Treg cells generate cytokines, IL-10 and TGF-β, which serve as anti-inflammatory mediators and help maintain immunological homeostasis (33) Recent research has focused on the response of Th17 and Treg cells when exposed to various mechanical forces. Heavy pathological forces up-regulate IL-6, while TGF-β and IL-6 cause Th17 differentiation. In addition, heavy forces promote the expression of HIF-1α, which mediates Th17 differentiation.

Consequently, bone resorption and an excessive inflammatory response under heavy force stimulation may be partly attributed to the up-regulation of Th17 cells (25). Mechanisms involved in enhancing the expression of Treg cells and thereby enabling them to perform protective roles in periodontal homeostasis belong to an area of research that remains to be explored.

2.1.3 Cell death

Many reports have suggested that physiological forces influence the metabolism of soft and hard tissues, while pathological forces or improper therapeutic forces result in tissue damage or even cell death (necrosis or programmed cell death). Necrosis refers to the passive death of cells induced by extreme physical or chemical stimuli or severe pathological factors. Once necrosis occurs, membrane permeability increases, resulting in cell swelling; organelle deformation or enlargement; and eventually cell rupture (34).

Programmed cell death is a type of host immune defence that can be disposed due to infected, senescent, or other abnormal cells against infection, radiation, and other harmful situations controlled by genes. Several types of programmed cell death are relevant to periodontal homeostasis under mechanical forces, including apoptosis and pyroptosis. Cysteine-aspartic proteases (caspases) are known to mediate cell death and inflammation during programmed cell death (35, 36).

Apoptosis, a basic physical process, is characterised by cell shrinkage, loss of cell junctions, karyopyknosis, and nuclear fragmentation. The application of mechanical forces can induce PDLSC apoptosis in a time- and force-dependent manner. Regarding fibroblasts, forces from matrix proteins are converted through focal adhesion receptors, which bind to adaptor proteins intracellularly; the adapter proteins link adhesions to the actin cytoskeleton and finally transfer force, thus enabling a cell reaction. Adhesion-associated molecules such as filamin A could prevent cell detachment or detachment-induced cell death under mechanical forces via enhanced formation and maturation of matrix adhesions (37). Piezo1 may transfer force and subsequently activate the p38/ERK1/2 signalling pathway and promote apoptosis in tissue and cells. Furthermore, Piezo1 also acts as a homeostatic sensor that may sense cell crowding, thereby, regulate cell numbers by inducing cell apoptosis or cell division (35). Moreover, the expression of cell caspase-8 and caspase-9, which initiate and execute extrinsic and intrinsic apoptotic pathways, increases under mechanical forces (38).

Pyroptosis, or cell inflammatory death, is an important innate immune process to defend against infection. It is characterised by constant cell swelling and bubbling until the plasma membrane ruptures with the release of cell contents, which induces a strong inflammatory response. Previous studies have found that occlusal trauma and microbial infection can activate the NLRP3 inflammasome and induce pyroptosis in the periodontium, releasing the pro-inflammatory cytokine IL-1β and increasing RANKL expression. In other words, pathological forces cause bone loss and inflammatory responses by inducing pyroptosis. However, physiological forces, such as normal mastication, can induce PDLCs to secrete exosomes, which may interact with macrophages and subsequently reduce IL-1 production. Thus, pyroptosis in macrophages can be reduced by modulating the NF-κB signalling pathway (36). Glyburide, an NLRP3 inhibitor, reduces pyroptosis in periodontal tissues under occlusal trauma and relieves damage. These findings indicate new therapeutic strategies for treating periodontal diseases caused by pathological forces.

2.2 ECM metabolism under mechanical forces

The ECM plays an essential role in maintaining tissue integrity and structural stability. Meanwhile, mechanical force is an important stimulus to regulate ECM remodelling, characterised by the synthesis and degradation of matrix proteins, such as collagen type I and III, fibronectin, and laminin, which are generally controlled by matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) (39). A previous study concluded that mechanical forces significantly impact MMPs and TIMPs in PDL. The precise variation in specific types of MMPs or TIMPs remains elusive, as they are affected by force-applying methods, force-applying time, force magnitude, and other factors. Nevertheless, MMPs generally show a rising trend under mechanical forces, while TIMPs are mainly responsible for controlling MMP activity. For example, TIMPs are up-regulated to reduce MMP activity under tension forces, as the ECM will experience formation and regeneration at this site (6). In addition, a study summarising gene expression changes under mechanical forces showed that genes encoding integrin α5 and αL subunits and their ligands, fibronectin and intracellular cell adhesion molecules, were up-regulated, indicating dynamic mechanotransduction and vigorous cell migration during ECM remodelling (40).

2.2.1 Signalling pathways

Molecularly, mechanical forces could activate some signal pathways or induce cytokine secretion to regulate ECM metabolism. IL-6 is a cytokine up-regulated by mechanical forces and can regulate MMP3 expression through PI3K or mitogen-activated protein kinase (MAPK) signalling pathways (13). Another study found that occlusal forces can activate the sonic hedgehog signalling pathway via Bardet-Biedl syndrome protein-7 and subsequently regulate cell migration and angiogenesis in the PDL (41). Angiogenesis is essential for PDL metabolism, with one study reporting that occlusal forces activate the MAPK signalling cascade to induce the synthesis of vascular endothelial growth factor (VEFG-A), fibroblast growth factor (FGF)-2 and nerve growth factor (NGF), all of which are involved in angiogenesis (22). Another growth factor important for cell proliferation and differentiation, insulin-like growth factor-1, is also induced by mechanical forces via the TGF-β signalling pathway (42).

2.3 Specific proteins function under mechanical forces

2.3.1 Collagen, fibronectin and laminin

As the most abundant protein component in ECM, collagen is involved in many biological functions. Some types of collagen form the fibrous structure of gingiva and PDL, which helps to endure mastication forces (43, 44). Besides, type I collagen binding to integrins then transmit extracellular mechanical signals into intracellular signals to mediate cell attachment, proliferation, differentiation, and other functions (7). Fibronectin is the main component of ECM, which plays an important role in cell adhesions. Similar to collagen, fibronectin also contributes to forming PDL fibres. Under mechanical forces, fibronectin combines with integrins to promote cell activity and involves in the cytoskeletal recombination (43, 45). Laminin presents in the epithelial basement membrane that forms a barrier between tissues. Laminin-5 participates in cell-cell interactions mediated by integrins; Laminin-5 is important for cell adhesion, growth, migration, and differentiation (39). The three proteins are all regulated by MMPs and TIMPs that are affected by forces exerted on the ECM (6).

2.3.2 Periostin

Periostin, an important ECM protein, is expressed in the periosteum and the PDL and is crucial for maintaining PDL integrity and stability of the periodontal structure and function, particularly under mechanical forces. Periostin modulates the distribution of ECM proteins, such as fibronectin. In addition, periostin regulates type I collagen fibrogenesis related to the biochemical properties of the PDL (46). Previous research on periostin-null mice found severe periodontal destruction, including sparse and disordered collagen fibres or even loss of collagen fibrous network structure that deteriorated over time after tooth eruption and subjection to occlusal forces (47). A study concluded that the expression of periostin is responsively high in tissues with high mechanical forces and rich collagen. The expression of periostin is up-regulated with increases in the strength of mechanical forces and significantly down-regulated in the absence of mechanical forces (48). Moreover, force-induced periostin could interact with integrin and activate downstream signalling pathways to regulate cell differentiation and migration related to soft and hard tissue formation (49). Under compressive forces during orthodontic processes or some pathological forces, periostin inhibits cell death by regulating Notch 1 expression (50). As discussed above, periostin plays a protective role in maintaining ECM microenvironment stability under mechanical forces. Besides, inflammation may also alter periostin expression, as bacterial or inflammatory stimuli will cause a deficiency in PDL fibroblasts and subsequently reduce periostin production, thus decreasing periostin during periodontitis. Moreover, a lack of periostin leads to increased susceptibility to bacterial infection. Therefore, a decline in periostin destabilises the PDL and aggravates inflammatory infiltration, bone loss, and PDL destruction (51).

2.3.3 Fibrillin

Fibrillin, secreted by periodontal fibroblasts and distributed in the gingiva and periodontal tissues, is a major microfibrillar fundamental element that contributes to connective tissue elasticity and integrity. Microfibrils constitute and participate in forming oxytalan fibres that play a supporting role during mastication. Fibrillin can regulate TGF-β and bone morphogenetic protein activities via interactions with microfibril-associated proteins, such as latent TGF-β-binding protein 1 and fibronectin (52) Fibrillin-1 is also essential for maintaining periodontal homeostasis under mechanical forces and is involved in regulating periostin under mechanical forces via the TGF-β signalling pathway (53). However, another study found no alteration in fibrillin-1 when the PDL was subjected to external forces; therefore, the specific function of fibrillin in periodontal homeostasis remains under exploration.

2.3.4 Integrins

Cell-ECM interaction is an essential process for signal transmission while integrin plays the central role of this process. The integrins are types of transmembrane receptors presented on the plasma membrane link ECM to the cell cytoskeleton, and thereby mediating cell adhesion, migration, mechanotransduction, and other physiological processes. Periodontal cells mainly express integrinα2β1, α3β1, and α5β1, its extracellular domain can bind to ECM components like collagen and fibronectin with high affinity while its intracellular domain is responsible for the recruitment of adaptor protein and scaffold protein and indirect interaction with microfilament cytoskeleton (54, 55). As a force sensor, force applied to the ECM fibre will induce conformational changes of integrins and their cytoplasmic domain bind to actin-binding adaptor proteins that transmit applied forces from integrins to the actin cytoskeleton. The downstream cell activities respond in several ways. One is the recruitment and activation of signalling proteins like FAK, paxillin, SRC and ERK and then activate some downstream signal pathways like PI3K/AKT. The other way connects ECM and integrins directly to the nucleus, enabling force to be transmitted to the nucleus to further regulate gene expression (56).

2.3.5 Focal adhesions

Focal adhesions(FAs), mechanosensitive macropolymers, anchored at junction between cell and the ECM. The focal adhesions composed of several protein layers including the integrin signalling layer; the intermediate force transduction layer containing talin and vinculin; and the microfilament skeleton regulatory layer (55). According to FAs in periodontal tissues, periodontal cells mainly express integrin α2β1, α3β1, and α5β1 that could combine with ECM components including collagen and fibronectin, and then activate downstream signaling crosstalk via proteins such as vinculin or the actin cytoskeleton and downstream effectors (55). This mechanism is essential for periodontal cells to detect and measure the mechanical properties of their microenvironment, such as periodontal tissue stretching and

shear stresses exerted by salivary flow, and respond appropriately to these cues (20).

2.3.6 Cadherins

Cadherins are a family of transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion. Similar to focal adhesion, cadherins are mechanosensitive that have a similar function with focal adhesions. In response to mechanical forces, cell adhesion receptors such as cadherins can induce actin cytoskeletal recombination, which alter cell activity (55); E-cadherin transmits external forces and activates Adenosine 5'-monophosphate-activated protein kinase (AMPK), showing a protective role against metabolic disturbances (57). Cadherin-11-mediated adherens junctions can alter the mechanical properties of the ECM (58). Another study discovered that cadherin-11 shows decreased expression in a time- and intensity-dependent manner under mechanical force stimulation. Meanwhile, β-catenin expression is altered in conjunction with cadherin-11. Moreover, β-catenin can translocate into the nucleus and induce osteogenesis- and fibrogenesis-related gene expression. In addition, cadherin-11 can mediate ECM collagen synthesis, and cadherin-11 knockdown in PDLCs changes cell shape and suppresses collagen synthesis. Accordingly, a previous study indicated that the cadherin-11/β-catenin pathway in PDLCs is inhibited by mechanical stresses, which may change the shape of the PDLCs and reduce collagen production (59).

2.3.7 Nuclear proteins

Forces are transmitted from ECM via FAs through cytoskeleton to the nucleus from several pathways. First, actin polymerisation directly affects the conformation of nuclear pore complex (NPC), and then the mechanosensitive transcription regulators such as YAP/TAZ (Yes- associated protein/transcriptional coactivator with PDZ- binding motif) and myocardin-related transcription factors (MRTFs) flow inside the nucleus to modulate gene expression (56). Second, stress fibres mechanically connect the ECM and FAs to the nucleus via the linker of nucleoskeleton and cytoskeleton (LINC). LINC is mainly composed of nesprin and SUN-domain protein while nesprin connects outwards to the cytoskeleton such as cytosolic actin, microtubules, and intermediate filaments in the cytoplasm and SUN-domain protein is anchored to the nuclear lamin (60). This yields transcriptional modulation through transcription factors and chromatin changes (61). LINC is mainly composed of nesprin and SUN-domain protein while nesprin connects outwards to the cytoskeleton such as cytosolic actin, microtubules, and intermediate filaments. in the cytoplasm and SUN-domain protein is anchored to the nuclear lamin (60). The interaction between lamins and LINC plays an important role in the regulation of nuclear mechanical properties and mechanotransduction. Lamins could directly regulate chromatin distribution and gene transcription through self-expression, post-translational modification, and structural changes after stress stimulation. Although abnormalities in lamins also impair LINC causing cytoskeleton nucleolysis coupling leading to mechanotransduction defects and downstream chromatin dysfunction (62).

2.4 Bone metabolism under mechanical forces

Since the most obvious clinical feature of periodontal destruction is bone resorption, bone metabolism is one of the most important elements in periodontal homeostasis. Osteocytes, derived from osteoblasts, are the most abundant cells in bone. By secreting regulatory factors, osteocytes could regulate the activities of osteoblasts and osteoclasts (63). Most importantly, osteocytes function as the main cell in bone to sense mechanical stimulation to induce mechanotransduction (64). Regarding mechanosensors on the osteocytes, a previous review has reported some mechanosensors including cytoskeletons (actin filaments, microtubules, and intermediate filament); cell dentrites and cell body; primary cilla; integrin-based FAs; gap junctions(Cx43); ion channels; and glycocalyx (10). The downstream signalling pathways that are activated to regulate bone metabolism will be discussed in the next paragraph. Bone formation and resorption are executed by osteoblasts and osteoclasts to keep alveolar bone homeostasis. Physiological forces, such as mastication and normal occlusion, boost circulation and metabolism of periodontal tissues, while loss of occlusion leads to atrophy of periodontal tissues and bone resorption (19). Simple pathological forces such as traumatic occlusion cause vertical resorption of the alveolar ridge and negatively impact tooth mobility but do not damage gingival tissues by forming periodontal pockets or cause attachment loss. In some circumstances, a tooth subjected to pathological forces can shift or tilt toward the compression side to eliminate applied forces. This mechanism is similar to orthodontic tooth movement. Therapeutic forces such as orthodontic forces exerted on the tooth surface can promote tooth movement through alveolar bone reconstruction. Normally, bone resorption happens on the compression side, and bone formation occurs on the tension side (17).

2.4.1 Signalling pathways

The mechanosensitive signalling pathways may control gene transcription and protein expression, leading to bone formation and resorption. For instance, on the compressive side, compressive forces induce an aseptic inflammatory response to generate inflammatory cytokines, such as IL-1β, IL-6, and tumour necrosis factor (TNF)-α, thus stimulating RANKL expression and osteoclastogenesis. On the tension side, osteoblastogenic factors, such as alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), bone morphogenetic protein (BMP), osteocalcin, and type I collagen, are found to be up-regulated in PDL fibroblasts (14). The canonical Wnt pathway is essential in osteocytes for mechanosensing and regulating bone mass, while its specific role in osteoblastogenesis or osteoclastogenesis depends on the force type, force application time, and force magnitude. Canonical ligands, such as Wnt1, Wnt3a, and Wnt10b, increase under mechanical loading (65). Moreover, the non-canonical Wnt pathway regulates bone metabolism, and Wnt4 promotes bone formation by inhibiting IKK-NF-kB and activating the WNT-PCP-ROCK pathway under conditions of periodontitis and

occlusal trauma (66). Additionally, Wnt pathway inhibitors, such as sclerostin, dickkopf 1, and Wnt inhibitory factor-1, regulate bone homeostasis and serve as treatment targets for bone diseases (65, 67).

MAPK signalling cascades are also activated under mechanical forces and mainly function in bone formation via the up-regulation of many osteogenic genes. Under mechanical stimulus, three major downstream cascades of the MAPK signalling pathway, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 kinase, and c-Jun N-terminal kinase (JNK), are activated, while osteoblast markers, such as BMP-2, ALP, RUNX2, osteopontin, and osterix, demonstrate enhanced expression (68).

Hippo-YAP is a newly discovered signalling pathway in which YAP/TAZ senses cellular microenvironment changes, such as structural and mechanical alterations. External changes, such as cell–cell contact, cell stretching, and cell shape, lead to actin cytoskeleton remodelling, which controls YAP/TAZ activity. WNT5A and FZD4 may be positively regulated by YAP, and the YAP/WNT5A/FZD4 axis contributes to the osteogenesis of PDLCs under stretch forces (69). Moreover, the Hippo-YAP signalling pathway is responsible for the progression of periodontitis under pathological forces such as traumatic occlusion. Crosstalk between Hippo-Yap and JNK pathways during traumatic occlusion and periodontitis leads to the up-regulation of JNK downstream effector activator protein AP-1 and inflammatory cytokines (IL-6, IL-8, TNF-α, etc.) that stimulate RANKL expression, leading to bone resorption. Therefore, inhibition of YAP can be considered a new target for the treatment of periodontitis with occlusal trauma (70, 71).

Prostaglandin (PG) E2/cyclooxygenase (COX)-2 is another force-sensitive signalling pathway that may have a bidirectional regulatory role in bone metabolism. PGE2 is an important downstream target of the mechanosensitive ion channel Piezo1 and some mechanosensitive pathways, such as MAPK and Wnt pathways (72, 73). Many researchers suggest that a low PGE2 concentration promotes fibronectin synthesis by osteoblasts, while a high PGE2 concentration causes bone resorption by stimulating osteoclast differentiation (74).

Apart from the typical signalling pathways described above, other pathways, such as the IKK-NF-κB, Notch, JAK2/STAT3, and PIEZO1/Ca^{2+}/HIF-1α/SLIT3 signalling pathways, are reported to contribute to bone homeostasis under mechanical forces (75–77).

Gasotransmitters, such as H_2S and NO have also been reported to regulate bone homeostasis. Endogenous H_2S can be induced by mechanical forces and secreted by PDLCs to regulate bone metabolism. H_2S tends to promote osteoclastogenesis through its chemoattractant effect on macrophages and regulates osteoblast activity during orthodontic treatment (78). NO, another gas mediator, can regulate multiple cell behaviours in response to mechanical forces. A previous review concluded that NO not only can function alone to regulate osteoclast and osteoblast activity but can also interact with other signalling pathways, such as Wnt/β-catenin, ERK1/2, and PI3K/AKT, to enhance other cell activities (74).

2.5 Characteristic PDLSCs function under mechanical forces

PDLSCs exhibit an essential role in PDL tissue maintenance and regeneration. When they are subjected to mechanical forces, the functions of mechanosensing and mechanotransduction enable them to transfer extracellular forces into biological signals that induce cell proliferation, self-renewal, and differentiation (79). Many studies have reported the potential of PDLSCs to promote soft and hard tissue formation. Regulation of PDLSC subpopulations under mechanical forces with regard to periodontal homeostasis has raised much attention in recent years. With the application of the cell lineage tracing technique, subpopulations of PDLSCs characterised with markers, including leptin receptor (Lepr) and Gli, have been identified as contributors to periodontal homeostasis under mechanical forces. Lepr+ cells may be activated by injury and force stimulation via Piezo 1, while Lepr+ stem cells may induce periodontal regeneration following periodontal damage due to pathological forces (8). Further, mechanical forces are essential for the activation of Gli+ multipotential stem cells to periodontal tissues and promote bone remodelling and injury repair (9, 18). In addition, Axin2+ PDL progenitor cells are highly sensitive to tension forces and play an important role in tension force-induced PDL expansion and alveolar bone formation (80). The discovery of PDLSCs with additional characteristics to promote tissue regeneration under specific conditions remains a promising area of research.

2.6 Non-coding RNAs

ncRNAs, which account for more than 90% of cellular RNAs, do not code for proteins but instead play regulatory roles in many biological processes involved in cell differentiation, metabolism, and function. ncRNAs are usually divided into two subtypes: short ncRNAs (<200 nucleotides) and long ncRNAs (lncRNAs; >200 nucleotides); microRNAs (miRNAs) are short ncRNAs, whereas lncRNAs exhibit a similar biological origin as mRNA. lncRNA functions in transcriptional, post-transcriptional, and epigenetic regulation of gene expression (81). miRNA, circular RNA (circRNA), and some specific lncRNAs have been reported to be mechanosensitive. miRNA regulates bone metabolism and periodontal homeostasis in response to mechanical forces, and its differential expression depends on the intensity and duration of applied forces. circRNA is a structurally stable lncRNA with a single-stranded covalent closure. A large number (2678) of differentially expressed circRNAs are induced in force-stimulated PDLSCs, while specific circRNAs may promote PDLSC osteogenic differentiation (82, 83). Furthermore, some lncRNAs, such as DANCR, p21, and SNHG8, also function by manipulating signalling pathways and cytokines (84).

At the mechanotransduction stage, ncRNA may increase the mechanical sensitivity of mechanoreceptors and ion channels; ncRNA also interacts with downstream signalling pathways to

control force-biology signal conversion. During anabolic and catabolic phases, ncRNA may interact with osteogenesis- and osteoclastogenesis-related transcription factors such as RUNX2 or indirectly change the expression of osteogenic- and osteoclastogenic-associated molecules, such as RANKL-RANK-OPG, Wnt/β-catenin, or TGF-β/BMP to regulate bone metabolism. In addition, ncRNA regulates osteoclast and osteoblast differentiation, maturation, and function through signalling cascades. Apart from bone metabolism, ncRNA is also involved in autophagy, which serves as a kind of adaption in the periodontium under mechanical forces characterised by ECM degradation and reuse. Some therapeutic forces may induce aseptic inflammatory responses in PDLCs; ncRNAs may regulate the inflammatory and immune response by targeting inflammatory cytokines and signal pathways (84).

3 Discussion

The concept of periodontal homeostasis, proposed in recent years, provides a new perspective of periodontal health. Periodontal homeostasis refers to the dynamic equilibrium of periodontal tissue metabolism including gingiva metabolism, PDL metabolism and bone metabolism. Gingiva metabolism contributes to the immune barrier of the periodontium. The microbiota-epithelium barrier is quite essential for the periodontal tissues to defend against external stimuli that are important for maintaining tissue immune homeostasis. Besides, mechanical forces could induce immune cells to differentiate into several types, while the type of immune cell induced by pathological forces is often averse to eliminating pathogens and tissue repair. PDL, acted as a "cushion", could transmit force exerted on teeth to the alveolar bone. Under forces, ECM metabolism involves in the synthesis of PDL fibres as well as angiogenesis that helps maintain PDL integrity. Some certain proteins such as collagen, fibronectin, periostin, fibrillin, and integrin help maintain PDL integrity when pathological or improper therapeutic forces are applied; certain proteins may also participate in repairing damage. Characteristic PDLSCs such as Lepr+, Gli+, and Axin+ cells respond to forces and promote tissue regeneration. Bone metabolism is the ultimate change under applied forces, which manifests as bone formation and resorption. Many mechanosensitive signalling pathways have been found to regulate bone and ECM metabolism while the ncRNAs also function in the soft and hard tissue homeostasis.

Teeth are subjected to physiological or non-physiological mechanical forces. Physiological forces, such as mastication and occlusion, play a protective role in periodontal tissues while the specific mechanisms are concluded above. Mastication not only promotes bone and matrix metabolism but also helps clean teeth. A previous study has found that loss of occlusion forces on teeth resulted in bone resorption and periodontium atrophy (19). Therefore, appropriate forces exerted on teeth are essential for the periodontal tissues. However, there is a particular issue, although teeth are under normal physiological forces, loose teeth caused by periodontitis often experience malocclusion during the occlusal process. Under this circumstance, physiological forces will aggravate periodontitis. Therefore, treating periodontitis in time is essential, fix or extract loose teeth, and adjust occlusion (85). Non-physiological forces include pathological and therapeutic forces. Pathological forces, such as traumatic occlusion and bruxism cause damage to periodontal tissues. When the destructive forces exceed the ability of the periodontal tissue to repair itself, forces would cause cell death through mechanisms, such as apoptosis, pyroptosis, or necrosis, which is characterised by cell membrane rupture and cell content outflow that induces a strong inflammatory response. This implies a breakdown of periodontal homeostasis. However, whether pathological forces alone would cause periodontitis remains to be studied. Normally, proper therapeutic forces such as orthodontic forces promote tooth movement through bone metabolism and PDL metabolism according to the direction of applied force, which influences periodontal homeostasis. The impact of improper therapeutic forces is the same as pathological forces.

In conclusion, we summarise the mechanisms of mechanical forces including physiological forces, pathological forces and therapeutic forces in periodontal homeostasis to help recognise the essential role of force on the periodontal health. We hope that the potential target could be found by clinicians to provide treatment of some periodontal diseases.

Author contributions

TW: Conceptualization, Methodology, Resources, Writing – original draft. XL: Methodology, Resources, Validation, Writing – review & editing. JXL: Methodology, Resources, Validation, Writing – review & editing. YY: Formal analysis, Supervision, Validation, Writing – review & editing. JLL: Formal analysis, Funding acquisition, Supervision, Validation, Writing – review & editing. MW: Formal analysis, Supervision, Validation, Writing – review & editing. NW: Formal analysis, Supervision, Validation, Writing – review & editing. LH: Project administration, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Pedro Paulo Chaves de Souza,
Universidade Federal de Goiás, Brazil

REVIEWED BY
Xiaofei Shen,
Nanjing Drum Tower Hospital, China
Jason Dale Turner,
University of Birmingham, United Kingdom

*CORRESPONDENCE
Kang I. Ko
gank@upenn.edu

†These authors have contributed equally to this work and share first authorship

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ICAM1$^+$ gingival fibroblasts modulate periodontal inflammation to mitigate bone loss

William S. Kim[1†], Kawintip Prasongyuenyong[1,2†], Annette Ko[1], Rahul Debnath[1], Zhaoxu Chen[1], Jonathan X. Zhou[1], Emon Shaaf[1] and Kang I. Ko[1*]

[1]Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, United States, [2]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Prince of Songkla University, Hatyai, Songkhla, Thailand

Tissue-resident fibroblasts are heterogeneous and provide an endogenous source of cytokines that regulate immunologic events in many osteolytic diseases. Identifying distinct inflammatory fibroblast subsets and conducting mechanistic *in vivo* studies are critical for understanding disease pathogenesis and precision therapeutics, which is poorly explored in periodontitis. Here, we surveyed published single-cell datasets for fibroblast-specific analysis and show that Intercellular Adhesion Molecule-1 (ICAM1) expression selectively defines a fibroblast subset that exhibits an inflammatory transcriptional profile associated with nuclear factor-κB (NF-κB) pathway. ICAM1$^+$ fibroblasts expand in both human periodontitis and murine ligature-induced periodontitis model, which have upregulated expression of CCL2 and CXCL1 compared to other fibroblast populations. Using a mouse model to selectively target gingival stromal cells, we further show that disruption of an inflammatory pathway by inhibiting transcriptional activity of NF-κB in these cells accelerated periodontal bone loss. Mechanistically, this was linked to a reduction of CCL2 expression by the ICAM1$^+$ fibroblasts, leading to impaired macrophage recruitment and efferocytosis that was associated with persistent neutrophilic inflammation. These results may have a significant therapeutic implication as ICAM1$^+$ gingival fibroblasts exert a protective response by regulating innate immune responses that are needed for the controlled inflammatory events in early stages of periodontitis.

KEYWORDS

stromal cell, innate immunity, periodontitis, CCL2, macrophage, inflammation, host response, fibroblast

Introduction

Periodontitis is a prevalent oral inflammatory disease characterized by the progressive destruction of the supporting periodontal apparatus, which ultimately leads to tooth loss. A prevailing pathologic mechanism responsible for exacerbated periodontal bone loss is the aberrant inflammation that favors osteoclastogenesis (1). Studies have demonstrated that both innate and adaptive arms of immunity are altered in periodontitis. For example, heightened neutrophilic inflammation in acute and chronic phases of periodontitis causes connective tissue damage by releasing destructive proteolytic enzymes (2). Moreover, T-helper cells and γδT cells that secrete a pro-inflammatory cytokine interleukin-17 are overly active and decouple bone metabolism to mediate alveolar bone loss (3, 4). The hyperactivated immune response is largely absent in steady-state conditions, suggesting that endogenous resident cells may be important initiators for downstream events that lead to osteolytic inflammation in periodontitis. Non-immune tissue resident cells, i.e. mesenchymal cells, have been shown to drive the pathogenesis of other inflammatory diseases such as rheumatoid arthritis and intestinal colitis (5, 6). However, relatively little is known about the functional significance of mesenchymal sentinel cells in periodontal pathogenesis.

Gingival fibroblasts are abundant stromal cells that maintain the structural integrity of the lamina propria within tooth-associated gingiva. Recent advances in single-cell research have highlighted that fibroblasts are comprised of heterogenous populations with distinct molecular signatures (7). In rheumatoid arthritis, which shares many pathologic features with periodontitis (8), a pro-inflammatory fibroblast subset mediates abnormal recruitment of leukocytes that induces destruction of the synovium, which was proven *in vivo* (9). In periodontal literature, emerging single-cell RNA-seq (scRNA-seq) studies unanimously identify a gingival fibroblast subset that uniquely expresses cytokine and chemokine genes responsible for trafficking both innate and adaptive immune cells (10, 11). However, *in vivo* mechanistic studies to determine the functional significance of these fibroblasts are surprisingly lacking. Furthermore, most studies on gingival fibroblasts rely on *in vitro* culture systems, which may not capture complex cellular interactions within the diseased periodontal niche (12, 13). This is particularly important to understand as controlled inflammation is necessary for the antibacterial immune response but can also be destructive when persistently present in the periodontium (14).

A major challenge in studying inflammatory fibroblast population is the lack of a definitive surface marker for cell isolation and limited *in vivo* mouse models that selectively target fibroblast lineage cells. In skin fibrosis, *engrailed-1* lineage cells identify scar-forming fibroblasts, inhibition of which can minimize scar formation (15). In a rheumatoid arthritis model, specific deletion of fibroblast activation protein-α-expressing fibroblasts mitigates synovial inflammation and joint destruction (9). In contrast, we recently reported that lineage cells labeled by the promoter activity of paired related homeobox-1 (Prx1) label immunomodulatory fibroblasts needed for proper cutaneous and oral wound healing to occur (16). Thus, whether inflammatory fibroblasts are protective or destructive may be disease-specific and context-dependent, although a potential role of these fibroblasts in periodontal disease pathogenesis remains unclear.

Here, we combine the survey of published scRNA-seq data, validation assays using human gingival tissues, and a murine experimental periodontitis model to identify ICAM1 (Intercellular Adhesion Molecule 1) to be a cell surface marker that distinguishes inflammatory fibroblasts. *In vivo* studies to disrupt inflammatory transcriptional activity of the canonical NF-κB pathway in fibroblasts demonstrate that these cells are needed to prevent excessive periodontal bone loss. Mechanistically, this was linked to an insufficient production of CCL2 and perturbed modulation of macrophage recruitment and efferocytosis that control excessive neutrophilic damage to periodontium. Together, our study highlights a protective function of inflammatory gingival fibroblasts that mount an appropriate innate immune response in periodontitis to minimize tissue damage. Moreover, it implicates the use of an ICAM1 antibody-based cell sorting method as an effective strategy to study inflammatory gingival fibroblasts.

Materials and methods

Human tissues

Discarded gingival tissues were collected from patients between the ages of 18-70 undergoing resective surgery at the University of Pennsylvania School of Dental Medicine. Marginal gingiva from the clinical health group was derived from patients undergoing crown lengthening or tooth extraction where periodontal probing depth was ≤3mm. Marginal gingiva from the periodontitis group was from patients undergoing osseous surgery, where periodontal probing depth was ≥5mm with a designated periodontitis stage II or III diagnosis according ref (17). Exclusion criteria included history of diabetes and smoking. The collection and use of discarded biospecimen were approved under IRB #844933 at the University of Pennsylvania, and informed consent was obtained from all participants.

In vivo animal model

Animal studies were carried out in compliance with IACUC-approved protocols (#804855 and #807062). Mice were purchased from the Jackson Laboratory as follows: C57BL/6J (B6, #000664), B6.Cg-Tg(Prrx1-cre)1Cjt (Prx1Cre, #005584), B6.Cg-Gt(ROSA) 26Sort9(CAG-tdTomato)Hze/J ($R26R^{tdTomato}$, #007909), and B6.Cg-Cl2tm1.Pame/J ($Ccl2^{mCherry}$, #016849). $Ikbkb^{flox/flox}$ was provided by Dr. Michael Karin (UC San Diego). Control mice were obtained from the same litter unless specified otherwise. All animal experiments were initiated at 6-10-week-old of age. Mice were anesthetized with a 2-4% ketamine solution during surgical procedures and prior to euthanizing via intraperitoneal injection. Mice were euthanized after anesthetization using a cervical dislocation method.

Ligature-induced periodontitis

Mice were anesthetized, and a 6-0 silk ligature was tied around the second maxillary molar as previously described (18). Mice were euthanized at indicated end timepoints, and maxillary tissues were harvested and fixed for downstream analyses including micro-CT analysis, histomorphometric analysis, immunofluorescence, and TRAP staining.

Flow cytometry and sorting

Human tissues were isolated and processed within 30 minutes of dissection. Gingival tissues were minced and digested using 0.15 mg/ml DNase I, 3.2 mg/ml CollagenaseIV and 2.6 mg/ml Dispase II for 1h at 37C under constant agitation. After straining through 70um filter, red blood cells were lysed in ACK buffer, nonspecific binding blocked with antibody against CD16/32, and stained with fluorophore-conjugated antibodies and viability dye. Data was acquired using LSRII (BD Biosciences) and analyzed using FlowJo software (10.8.0). Fluorescence-minus-one (FMO) controls were used to determine gating strategy. In other experiments, fluorescence-based sorting was performed on FACSAria (BD Biosciences), and ~10^4 cells were separated directly into lysis-buffer containing tubes prior to mRNA extraction. For murine studies, ligatured teeth were extracted after euthanasia, and gingival collar ~2mm in width was dissected circumferentially by blunt dissection along the osseous floor of maxilla. Tissue digestion for single-cell preparation, staining, and analyses were carried out as described above. The list of antibodies is available in Appendix Table 1.

Computational analysis

Previously published scRNA-seq datasets (GSE152042, GSE164241, and GSE217720) were downloaded and pooled for integrative analysis. Downstream analyses were performed using R language, and data visualization was performed using Seurat (v.3) (19). Cells that expressed <200 or >5,000 UMI or more than 15% mitochondrial gene expression were excluded. Datasets were integrated via Seurat Integration (Seurat 3). Mutual nearest neighbors (anchors) were found using the SelectIntegrationFeatures and FindIntegrationAnchors functions. The data was integrated using the IntegrateData function with a k.weight of 90. Dimensional plots of the integrated dataset were split by individual sample to evaluate adequate cell type mixing. Additionally, integration was quantitatively analyzed using the local inverse Simpson's Index (LISI) (20). Fibroblasts were selected based on the enriched expression of *COL1A2, COL3A1, DCN, FAP* and *PDGFRA*. Differentially expressed genes were calculated using Findmarker function with default Wilcoxon rank-sum analysis. Gene module score for inflammatory gene set was determined by creating a list of known CCL- and CXCL- genes. Gene expression ratio score was calculated by multiplying average log2(fold-change) and the ratio between gene expression percentage in cluster 0 versus other fibroblast clusters. Signaling pathways of fibroblast subclusters were analyzed by via PROGENy (21).

Micro-CT analysis

Murine maxillae were fixed in a solution of 10% formalin before being washed with distilled water twice and placed in a solution of 1x PBS for uCT imaging. Maxillae were imaged with a Scanco μCT 45 (Scanco Medical AG, Switzerland) at an applied voltage of 55 kVp and a voxel size of 14.6 μm. Images were reconstructed and analyzed with Dragonfly (Windows, Ver. 2022.1.0.1259). μCT sections were used to evaluate bone resorption with a distance and area measurement. Teeth were positioned in the sagittal plane where the apex of all roots was visible, and the dental pulp of all teeth extended to the apex of all buccal roots. CEJ-ABC distance was quantified by measuring vertical distance between CEJ-CEJ to the most coronal aspect of alveolar bone between first and second maxillary molar teeth. For bone area remaining, an arbitrary 700μm area below CEJ-CEJ to the base of the alveolar bone perpendicular to occlusal plane was used to quantify total periodontium area and radiopaque bone remaining percent.

Histomorphometric analysis

Murine tissue samples were fixed in a 10% formalin solution immediately after extraction and incubated at 4 C for 18-24 hours. Samples containing bones or teeth were then decalcified in a solution of 14% EDTA (pH 7.4) for 2 weeks with solution changes every three days. Samples were then dehydrated and embedded in paraffin using standard protocols. Samples were then cut at 5um, rehydrated, and stained with hematoxylin and eosin. To measure bone loss, a line (CEJ-CEJ line) was drawn connecting the cemento-enamel junctions of the first and second molars. Another line was drawn starting from the midpoint of this line down to the alveolar bone crest and was used to measure cementoenamel junction to alveolar bone crest distance (CEJ-ABC distance).

Immunofluorescence

Frozen or paraffin-processed samples were used for immunofluorescence experiments. Briefly, cryosections were cut at 10um and rehydrated in 1X PBS with 0.05% Tween-20, whereas paraffin sections at 5um were deparaffinized, hydrated prior to non-specific blocking in buffer containing 1% BSA, 0.1% Triton-X, 0.05% Tween-20, and 1% serum matching the host species of the secondary antibody. Immunofluorescence staining was performed using primary and secondary antibodies using standard protocols, and detailed antibody information is provided in Appendix Table 1.

TRAP staining

Paraffin-processed sections were rehydrated and stained with a TRAP staining solution (pH = 4.85) for osteoclasts before being washed and counterstained with a solution of 0.08% fast green (CAS: 2353-45-9). TRAP staining solution consisted of 184 mg/ml sodium acetate anhydrous (CAS: 127-09-3), 284 mg/ml L-(+) tartaric acid (CAS: 6106-24-7), 5.6% glacial acetic acid (CAS: 64-19-7), 0.1 mg/ml naphthol (CAS: 1596-56-1), 0.5% ethylene glycol monoethyl ether (CAS: 110-80-5), and 0.6 mg/ml fast red violet LB salt (CAS: 32348-81-5). PH adjustment was made with the addition of sodium hydroxide (Carolina Biological Supply, CAS: 1310-73-2) or glacial acetic acid. All chemicals were from Sigma-Aldrich.

Image analysis

Images of histological and immunofluorescence slides were taken using the Keyence BZ-X800 microscope and analyzed using the associated BZ-X800 Analyzer software. Image analysis was conducted using ImageJ or QuPath software (22) to count positive and double-positive cells in a given area by at least two examiners in a blinded manner.

Quantitative PCR

Cells were directly sorted into cold lysis buffer. RNA content was extracted using RNeasy Micro Kit (Qiagen) using the manufacturer's protocols. The High-capacity CDNA synthesis kit (Applied Biosystems) was used to generate cDNA. Fast power SYBR Green (Applied Biosystems) and the Step-One Plus machine (Thermo Fisher Scientific) were used to perform qPCR. To normalize the data, L32 was used as a housekeeping gene. List of primers can be found in Appendix Table 2.

In vitro immunocytochemistry

8-week-old B6 mice were used to harvest palatal gingiva and digested into a single-cell suspension. Primary oral fibroblasts were plated on a 96-well plate containing DMEM (Gibco, #11885-084) supplemented with 10% FBS and 1X antibiotic-antimycotic and incubated at 37°C in a humidified atmosphere with 5% CO_2 until 90% confluency. Fibroblasts were stimulated with a combination of TNF (10 ng/ml) (R&D: 410-MT-025/CF) and lipopolysaccharide from *P. gingivalis* (LPS-Pg) (1 ug/ml) (Sigma-Aldrich: SMB00610) for 24h. Cells were fixed, blocked in a buffer containing 1% BSA, 0.1% Triton-X, 0.05% and Tween-20, and incubated with primary antibodies against ICAM1 at 4°C overnight. After incubation with the secondary antibodies, cells were washed and stained with DAPI for 5 min. The immunoreactive proteins were then visualized under the fluorescent microscope.

In vitro phagocytosis assay

Bone marrow-derived macrophages (BMMs) were generated following a published protocol (23) using macrophage colony-stimulating factor (20 ng/ml, PeproTech: 315-02) for 5 days. BMMs were then stimulated with low-dose LPS (10 ng/ml) for 24h to induce macrophage phagocytotic phenotype, followed by incubation in conditioned media from the ICAM1$^+$ enriched or control fibroblasts, with or without neutralizing anti-CCL2 monoclonal antibody (20 μg/ml, eBioscience, 16-7096-81). For the phagocytosis assay, latex beads (2 μm diameter, carboxylate-modified polystyrene, fluorescent red; L3030, Sigma) were added to the BMMs at a 10:1 ratio (beads to BMMs) and incubated for 3 hours at 37°C. BMMs were then collected, washed with PBS to remove non-phagocytosed beads, and analyzed by flow cytometry.

In vitro treatment with NF-κB inhibitor

Primary oral fibroblasts were generated and pre-stimulated with or without a combination of LPS and TNF as described above. Cells were washed and incubated in media containing selective NF-κB inhibitor (BMS-345541, 10uM, Cayman Chemical) for 6h. Cells were collected for flow cytometry analysis, and supernatant was used for ELISA assay using mouse MCP-1 ELISA kit (Biolegend, 446207) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was conducted on Graphpad Prism software and Rstudio. All data represent mean ± SEM. Normal distributions were verified with QQplots and homogenous variances were verified using Bartlett's test for homogenous variances. Student's t-test or Welch's t-test were used for comparing two groups to account non-homogenous variances. Mann-Whitney U test was used for nonparametric analyses. One-way ANOVA and Šidák's correction and Brown Forsythe AVOVA with Dunnett's T3 Multiple comparisons were used for pairwise comparisons. Statistical significance was determined with a p-value < 0.05. Experiments were independently replicated at least two times, and each data point represents an individual animal unless specified otherwise.

Results

Identification of ICAM1$^+$ fibroblast subset with inflammatory features in human periodontitis

We first sought to identify a cell surface marker that labels an oral fibroblast subpopulation with inflammatory signatures. We took advantage of publicly available human scRNA-seq atlas

datasets and analyzed oral fibroblast subsets that are found in different locations in the oral cavity such as tooth-associated gingiva from healthy group (gingival margin, GM), from those diagnosed with periodontitis (PD), and non-tooth associated gingiva from the hard palate (anterior palate, AP) (10, 11, 24). The data was pre-processed to remove dead cells with >15% mitochondrial gene expression and doublets with high gene feature counts (Supplementary Figures 1A, B). Dimensional plots juxtaposing the unintegrated and integrated data showed a drastic improvement in mixing across sample and batch groups, and each patient dataset showed sufficient mixing at an individual level (Supplementary Figure 1C). Furthermore, the LISI for unintegrated and integrated were also calculated to quantitatively evaluate batch effects. The integrated data had a median LISI approximately 3-fold greater than the unintegrated data, with an unintegrated median LISI of 2.27 (95% CI [2.25,2.28]) to an integrated median LISI of 6.10 (95% CI [6.09,6.12]) (Supplementary Figure 1D). The integrated atlas was then processed to designate cell types based on enriched expression of canonical genes for each cluster, which generated 11 clusters (Figure 1A). To focus our analysis on oral fibroblasts, we generated subset clusters that expressed fibroblast genes and examined it at a higher granularity to identify those that exhibited elevated cytokine signatures. Among 6 fibroblast subgroups, cluster 0 was the most abundant and highly expressed cytokines such as *CXCL13, CXCL1, CXCL2, CCL19* and *CCL2* but not *CXCL12* which was also expressed in cluster 1 (Figures 1B, C). Given this enriched expression of CCL- and CXCL- cytokines, we further analyzed this inflammatory fibroblast cluster by calculating gene ratio scores, such that the significantly upregulated genes that are highly expressed in cluster 0 but not in other clusters are assigned with top scores. We selectively examined differentially expressed genes that encode for surface proteins and identified *ICAM1* (encoding for CD54) to be upregulated in this inflammatory fibroblast cluster (Figures 1D, E). When the scRNA-seq dataset was further stratified by individual patients, *ICAM1*$^+$ fibroblast percentage was the highest at approximately at 40% in those diagnosed with periodontitis when compared to those with healthy marginal gingiva or palatal gingiva (Figure 1F). PROGENy pathway analysis further confirmed that *ICAM1*$^+$ enriched cluster 0 was highly implicated in active inflammatory pathways associated with TNF and NF-κB signaling pathways (Figure 1G). However, *ICAM1* gene expression was upregulated in non-fibroblast clusters such as endothelial cells and macrophages (Figure 1H), indicating the need for combinatorial inclusion of pan-fibroblast specific markers to isolate these inflammatory gingival fibroblasts.

To validate ICAM1 as an *in vivo* surface marker for an inflammatory fibroblast subset that expands in periodontitis, we performed flow cytometry on cells prepared from freshly discarded tissues in patients with or without a periodontitis diagnosis. We utilized a fibroblast gating strategy to exclude endothelial, leukocyte, and epithelial lineage cells and used fibroblast activation protein (FAP) as a positive fibroblast marker to selectively quantify ICAM1$^+$ inflammatory fibroblasts (Figure 2A). The overall percentage of pan-fibroblasts or pericytes normalized by lineage-negative CD90$^+$ mesenchymal cell count did not differ between the clinical health and periodontitis groups (Figure 2B). When ICAM1$^+$ fibroblasts were specifically examined, we found a significant 2.4-fold increase in the periodontitis group compared to the control group (Figure 2C), consistent with the patterns observed from the scRNA-seq dataset. Furthermore, we found that relative ICAM1 expression is the highest in endothelium, moderate in leukocytes, and low in fibroblasts, which aligned with our transcriptomic analysis (Figures 1H, 2D, E). We further examined the spatial distribution of ICAM1$^+$ fibroblasts by immunofluorescence and found increased numbers of ICAM1$^+$ fibroblasts to be localized around inflammatory foci in periodontitis but not in healthy groups (Figures 2F, G). We considered the patient's chronological age for this phenotype; however, it did not exhibit a positive correlation with increasing ICAM1$^+$ fibroblast count (Figure 2H).

Inflammatory ICAM1$^+$ fibroblasts expand in a murine model of ligature-induced periodontitis

We next used flow cytometry to test if ICAM1 expression in fibroblasts also distinguishes an expanding inflammatory fibroblast subset in murine models of ligature-induced periodontitis (LIP). Consistent with the results from human specimens, there was a significant increase in the percentage of ICAM1$^+$ fibroblasts in ligated gingivae compared to non-ligated sites, without affecting the total percentage of pan-fibroblasts or pericytes when normalized to lineage-negative cell count (Figures 3A–C). Furthermore, immunofluorescence experiments revealed a significant increase in ICAM1$^+$ fibroblastic cell numbers in the connective tissue of LIP group compared to control group (Figures 3D, E). Immunocytochemistry experiments on primary gingival fibroblasts demonstrated that ICAM1$^+$ expression is significantly induced by adding other inflammatory and bacterial factors such as TNF and LPS (Figure 3F). To confirm that murine and human ICAM1$^+$ fibroblasts exhibit a similar inflammatory phenotype, we sorted ICAM1$^+$ and ICAM1$^-$ fibroblasts from the diseased gingivae and compared mRNA expression levels of selected cytokines (Figure 3G) In line with the transcriptomic data, human ICAM1$^+$ fibroblasts had significantly higher expression of cytokines *CXCL13, CXCL1, CXCL2, CCL19* and *CCL2* when compared to ICAM1$^-$ fibroblasts (Figure 3H). In murine LIP models, ICAM1$^+$ fibroblasts had significantly elevated expression of cytokines *Cxcl1* and *Ccl2* compared to ICAM1$^-$ fibroblasts (Figure 3I). Despite the variable magnitudes in cytokine expression between species, these results demonstrate that ICAM1$^+$ fibroblasts exhibit immune-regulatory phenotype when compared to other fibroblast populations in both humans and murine models of periodontitis.

Perturbation of NF-κB pathway in Prx1Cre lineage exacerbates periodontal bone loss

The expansion of ICAM1$^+$ fibroblasts in a diseased state may represent either a protective or detrimental inflammatory

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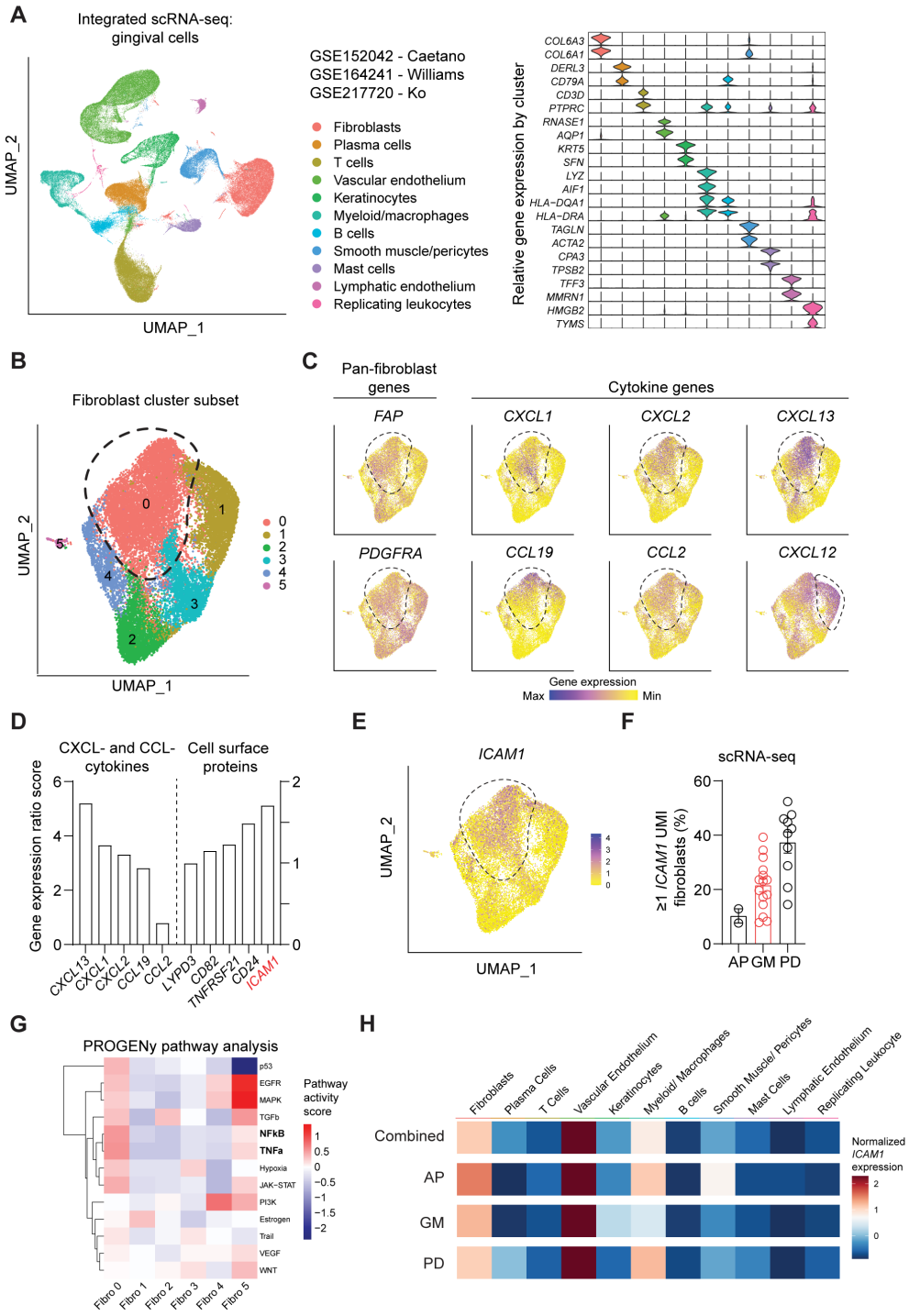

FIGURE 1
Single-cell RNA-seq analysis of public oral tissue atlas identifies ICAM1^{+} inflammatory fibroblasts. **(A)** Left, uniform manifold approximation and projection (UMAP) plot of n = 27 patients with 113,154 single-cells from integrated data set from ref (10, 11, 24), colored by major cell types. Right, stacked violin plot illustrating characteristic marker gene expression across each identified cell population in the integrated dataset. **(B)** UMAP plot of subset gingival fibroblast clusters from integrated public scRNA-seq dataset. **(C)** UMAP of feature plots for pan-fibroblast marker *PDGFRA* and *FAP* and fibroblast-derived *CCL*- and *CXCL*- cytokine genes. Dashed lines demarcate cluster 0, except *CXCL12* plot that designates fibroblast cluster 1. **(D)** Gene expression ratio score of significantly expressed CXCL- and CCL- cytokine genes and cell surface protein genes in cluster 0. **(E)** UMAP of feature plot for *ICAM1* expression across the fibroblast clusters. Dashed line demarcates cluster 0. **(F)** Quantification of fibroblasts that express ≥1 unique molecular identifier (UMI) for *ICAM1*. Each dot represents the percentage value of an individual patient. AP, anterior palate; GM, gingival margin (healthy); and PD, periodontitis. **(G)** Heatmap illustrating the activity of pathway-responsive genes (PROGENy) across different fibroblast clusters. **(H)** Heatmap illustrating *ICAM1* expression across cell populations in datasets that combine all oral tissues, AP, GM or PD. Scale represents centered log-ratio normalized *ICAM1* expression for each condition.

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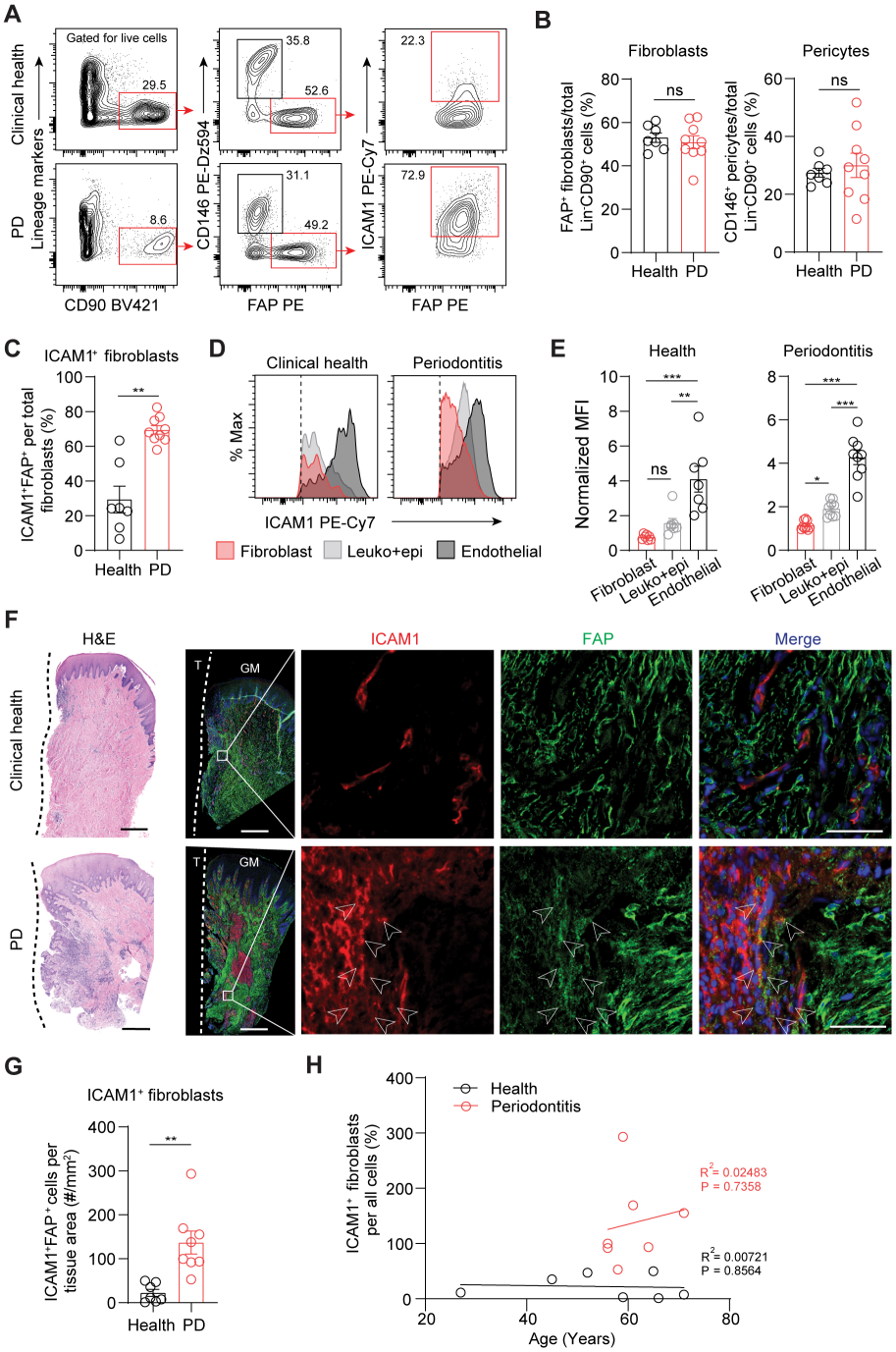

FIGURE 2
Inflammatory ICAM1$^+$ fibroblasts expand in human periodontitis. **(A)** Representative flow cytometry plots of gingival tissues derived from clinical health or periodontitis (PD) patient groups. Gating strategy for quantification of ICAM1$^+$ fibroblasts population (lineage$^-$CD90$^+$CD146$^-$FAP$^+$) is shown; lineage-negative selection was based on CD45 (leukocyte), CD31 (endothelial), and EpCam (epithelial) expression. **(B)** Quantification of percent fibroblasts (Lin$^-$CD90$^+$FAP$^+$) and pericytes (Lin$^-$CD90$^+$CD146$^+$) normalized by mesenchymal cell numbers (Lin$^-$CD90$^+$). **(C)** Quantification of percent ICAM1$^+$ fibroblasts (Lin$^-$CD90$^+$FAP$^+$ICAM1$^+$) comparing healthy vs. PD groups. **(D)** Representative flow cytometry histogram of mean fluorescence intensity (MFI) for ICAM1 expression in fibroblast (Lin$^-$FAP$^+$), leukocyte+epithelial cells (CD45$^+$EpCam$^+$) and endothelial cells (Lin$^+$CD146$^+$) from healthy and PD groups. **(E)** Quantification of ICAM1 expression in fibroblast, leukocyte+epithelial cells and endothelial cells in healthy and PD groups. **(F)** Representative H&E and immunofluorescent images of healthy and PD cryosections stained with antibodies specific against ICAM1 (red) and FAP (green). Arrows point to ICAM1$^+$FAP$^+$ fibroblasts. Scale bar, 500µm; inset scale bar, 50µm. Dashed line demarcates the border between tooth and sulcular/junctional epithelium. **(G)** Quantification of ICAM1 fibroblast numbers normalized by tissue area (mm^2) comparing healthy and periodontitis groups. **(H)** Scatter plot of correlation between patient age and ICAM1 fibroblast numbers in healthy and periodontitis groups. R^2, Pearson's correlation coefficient and, P, p-value. Each dot represents data point from individual patients (N=7-9 per group). Data represent mean ± SEM. **(B, C, G)** Student's t-test comparing health vs. PD; **(E)** Welch's ANOVA with Dunnett's test for multiple comparisons; ns, not significant, *p<0.05, **p<0.01, ***p<0.001.

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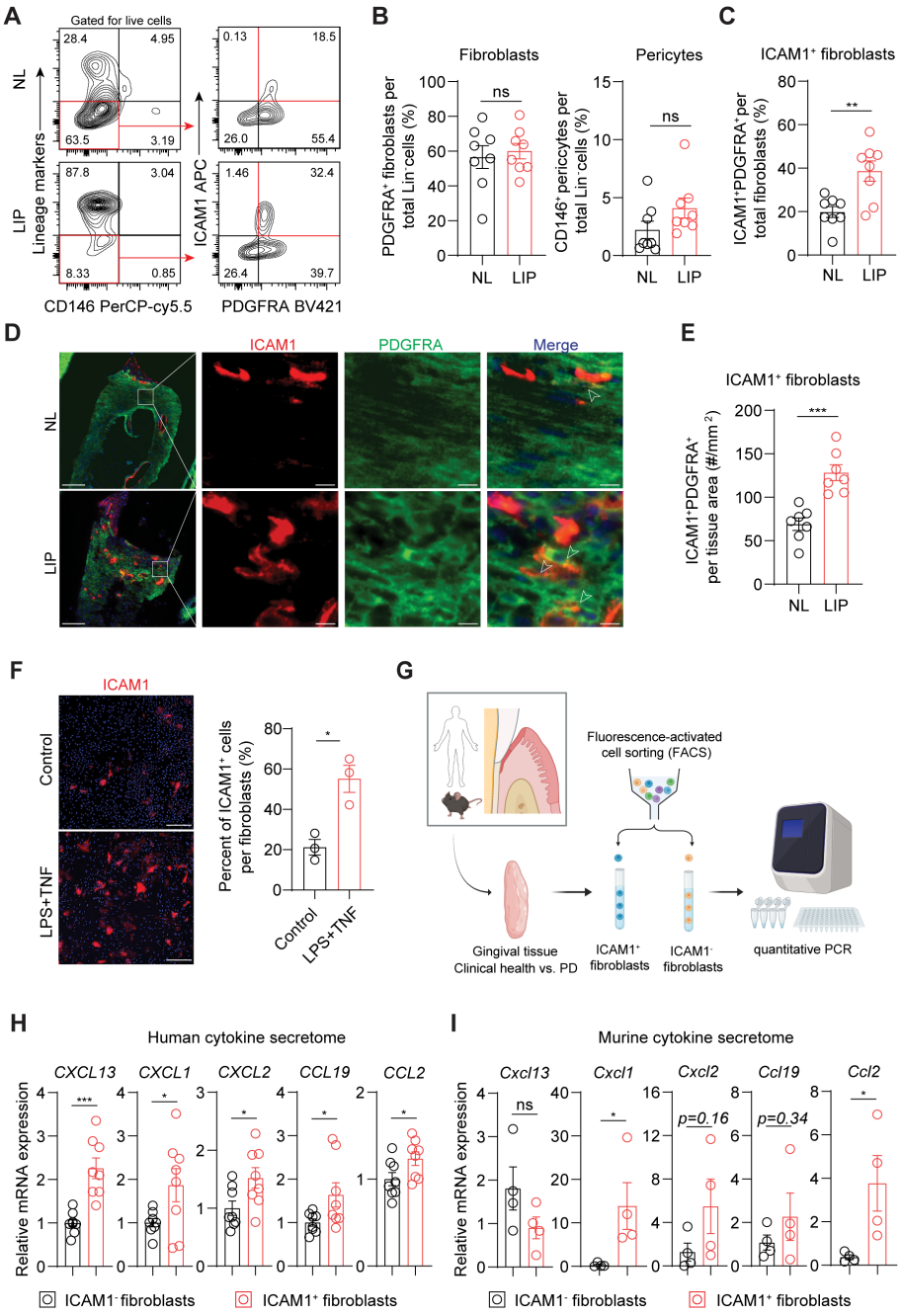

FIGURE 3
Inflammatory ICAM1$^+$ fibroblasts expand in a murine model of ligature-induced periodontitis. **(A)** Flow cytometry gating strategy for analysis of lineage-negative (CD45$^-$ CD31$^-$ EpCam$^-$ Ter119$^-$) pericytes, gingival fibroblasts and ICAM1$^+$ fibroblasts in non-ligated control (NL) and ligature induced periodontitis (LIP) group. **(B)** Quantification of percent fibroblasts (Lin$^-$PDGFRA$^+$) and pericytes (Lin$^-$CD146$^+$) normalized by Lin$^-$ mesenchymal cell numbers in NL and LIP groups. Each dot represents one mouse as a split-mouth design. **(C)** Quantification of percent ICAM1$^+$ fibroblasts (Lin$^-$PDGFRA$^+$ICAM1$^+$) normalized by total fibroblast numbers. Each dot represents one mouse as a split-mouth design. **(D)** Representative immunofluorescent images of NL and LIP paraffin sections stained with antibodies specific against ICAM1 (red) and PDGFRA (green). Arrows point to ICAM1$^+$ PDGFRA$^+$ cells. Scale bar, 100µm; inset scale bar, 10µm. **(E)** Quantification of ICAM1 fibroblast numbers normalized by lamina propria area (mm^2) comparing NL and LIP groups from the immunofluorescence experiments. **(F)** Left, representative immunocytochemistry images of primary gingival fibroblasts stained with ICAM1 antibody comparing control versus stimulated groups. Lipopolysaccharide from *P. gingivalis* (LPS, 1 ug/ml) and tumor necrosis factor alpha (TNF, 10 ng/ml) were used for stimulation. Scale bar, 20 µm. Right, quantification of ICAM1$^+$ fibroblast numbers normalized by total fibroblast cells comparing control and LPS+TNF group. **(G)** Schematic diagram of qPCR for fibroblast-derived cytokines comparing FACS-sorted ICAM1$^-$ and ICAM1$^+$ fibroblasts in human and mouse models of periodontitis. **(H, I)** Quantification of relative mRNA expression by qPCR for CXCL13, CXCL1, CXCL2, CCL19, and CCL2 comparing sorted ICAM1$^-$ and ICAM1$^+$ fibroblasts. **(H)** Gingival tissue specimens from N=8 periodontitis patients; each dot represents individual patient. **(G)** Gingival tissues harvested from mice with LIP; each dot represents pooled samples from 2-3 mice for a total of 4 data points (N=10 mice). Data represent mean ± SEM. Welch's t-test **(B–H)** and Mann-Whitney U test **(I)** comparing control vs. experimental group; *p<0.05, **p<0.01, ***p<0.001, ns, not significant.

response. To determine its functional significance, we selectively targeted inflammatory oral fibroblasts by utilizing a constitutive Prx1Cre mouse model that labels immunomodulatory fibroblasts in the oral mucosa and skin (16, 25). To first confirm that Prx1-lineage fibroblasts exhibit an ICAM1$^+$ phenotype and reside in tooth-associated murine gingivae, we generated *Prx1Cre*$^+$*.R26R*$^{tdTomato/+}$ reporter mice and examined ICAM1 expression under basal and ligated conditions using flow cytometry. We quantified and found a consistently increased percentage of ICAM1$^+$tdTomato$^+$-lineage fibroblasts in LIP group compared to non-ligated control group (Figures 4A, B). Importantly, approximately 80% of ICAM1$^+$ fibroblasts were tdTomato$^+$ (Figure 4C), indicating that Prx1Cre line effectively captures most of the inflammatory ICAM1$^+$ fibroblasts in the gingiva. We next investigated the impact of disrupting inflammatory functions in these fibroblasts on periodontal parameters by specifically deleting *Ikbkb* gene that encodes a kinase necessary for canonical activation of NF-κB, a master inflammatory transcription factor (26). In our integrated human scRNA-seq dataset, *PRRX1* gene was expressed in both fibroblast and pericyte clusters (Supplementary Figure 2A), and murine Prx1Cre model also labels pericytes in oral mucosa (16). However, *PRRX1*$^+$ fibroblasts exhibited much more elevated gene expression associated with inflammatory pathways such as TNF and NF-κB signaling relative to *PRRX1*$^+$ pericyte clusters (Supplementary Figure 2B), indicating that our genetic approach to perturb NF-κB pathway may have a drastic impact on Prx1Cre$^+$ fibroblasts. *Ikbkb* deletion in Prx1Cre$^+$ fibroblasts led to a significantly reduction in crestal bone height on day 7 and a decrease in bone area remaining on day 7 and 14 post-ligature from the μCT images (Figures 4D–F). The detrimental impact of *Ikbkb* deletion was confirmed by histomorphometric analysis which demonstrated significant attachment loss on day 7 LIP compared to control groups (Figure 4G). Furthermore, TRAP staining revealed that the number of osteoclasts along bone lining was significantly increased in the experimental group (Figure 4H). This effect was not a developmental defect on the alveolar bone because there were no changes to the bone level between control and *Ikbkb*-deleted groups that did not receive ligature (Supplementary Figures 2C, D). These results demonstrate that the inflammatory function of gingival fibroblasts is needed to prevent excessive bone loss and therefore play a protective role in the early stages of periodontal disease.

Defective inflammatory fibroblast function is associated with deficit in macrophage recruitment and efferocytosis

We next investigated the dysregulated inflammatory mechanism responsible for the accentuated bone loss by examining immune cell populations that contribute to periodontal pathogenesis such as neutrophils (27), macrophages (28), γδT cells (4) and CD3$^+$ T cells (3). *Ikbkb* deletion in Prx1Cre lineage fibroblasts resulted in a significant 27.4% increase in Ly6g$^+$ neutrophils and a remarkable 60.9% reduction in F4/80$^+$ macrophages, whereas that of γδ and CD3$^+$ T cells did not change (Figures 5A, B). Immunofluorescence on affected gingival tissues confirmed inverse patterns of macrophage and neutrophil numbers between control and experimental groups (Figures 5C, D). Intriguingly, F4/80 immunopositivity was intimately associated with MPO$^+$ signals near the epithelium in the control group but not in the *Ikbkb* deleted group (Figure 5C), suggesting that a failure to clear destructive neutrophilic bodies by macrophages, termed efferocytosis (29, 30), may be affected in the experimental mice. We therefore examined the expression of MerTK, an efferocytosis marker (31, 32) and found a significant reduction in MerTK$^+$F4/80$^+$ macrophage numbers in the *Ikbkb* deleted group compared to control group (Figure 5E). Efferocytosis triggers macrophage polarization towards a pro-resolving (M2) phenotype (33, 34), thus we examined the number and percentage of M2 and found comparatively lower M2 in the experimental group compared to control (Figure 5F). To confirm that these observations were not simply a result of excessive neutrophil recruitment, we examined the expression of a neutrophilic cytokine CXCL1, which is highly expressed in gingival keratinocytes affected by periodontitis (35). We found that epithelium was a major source of CXCL1 and that its expression levels were similar between control and experimental groups (Supplementary Figures 3A, B), indicating that the intrinsic chemoattractant affinity for neutrophils may not be affected in our mouse model.

To understand how inflammatory fibroblasts affect macrophage infiltration to the periodontium during induction phase of periodontitis, we examined CCL2, a monocyte and macrophage chemokine that is largely expressed by these fibroblasts (Figure 3H). We found that the number of ICAM1$^+$CCL2$^+$ fibroblasts was significantly reduced in *Prx1Cre*$^+$*.Ikbkb*$^{f/f}$ mice (Figure 6A), providing key evidence for the reduced macrophage numbers in the experimental groups. In contrast, CCL2$^+$ pericyte percentage remained low and unchanged by the *Ikbkb* deletion in Prx1Cre$^+$ cells. *In vitro*, the enrichment of ICAM1$^+$ fibroblasts with LPS and TNF pre-stimulation as in Figure 3F enhanced CCL2 expression by these cells and increased CCL2 concentration in the media (Figures 6B, C). This was effectively prevented by BMS-345541 that selectively inhibits NF-κB activity (Figures 6B, C), demonstrating that CCL2 expression by the ICAM1$^+$ fibroblasts was dependent on the NF-κB and is consistent with the published literature in other cell types (36, 37). We next explored if fibroblasts are the major cell type that express CCL2 in our LIP model. Utilizing CCL2-mCherry reporter mice, we found that fibroblasts accounted for nearly 60% of CCL2$^+$ cells, whereas leukocytes constituted approximately 20% of CCL2$^+$ cells in day-7 LIP (Figure 6D). Immunofluorescence experiments were carried out to determine spatial distribution of the CCL2$^+$ fibroblasts, and we found these cells to be localized to the lamina propria and not in the periodontal ligament space in LIP mice (Figure 6E). To test if the ICAM1$^+$ fibroblast-derived CCL2 modulates phagocytic activity of the macrophages, we collected conditioned media from primary oral fibroblast cultures that were enriched with ICAM1$^+$ as above and performed a phagocytosis assay using primary bone marrow-derived macrophages in these media

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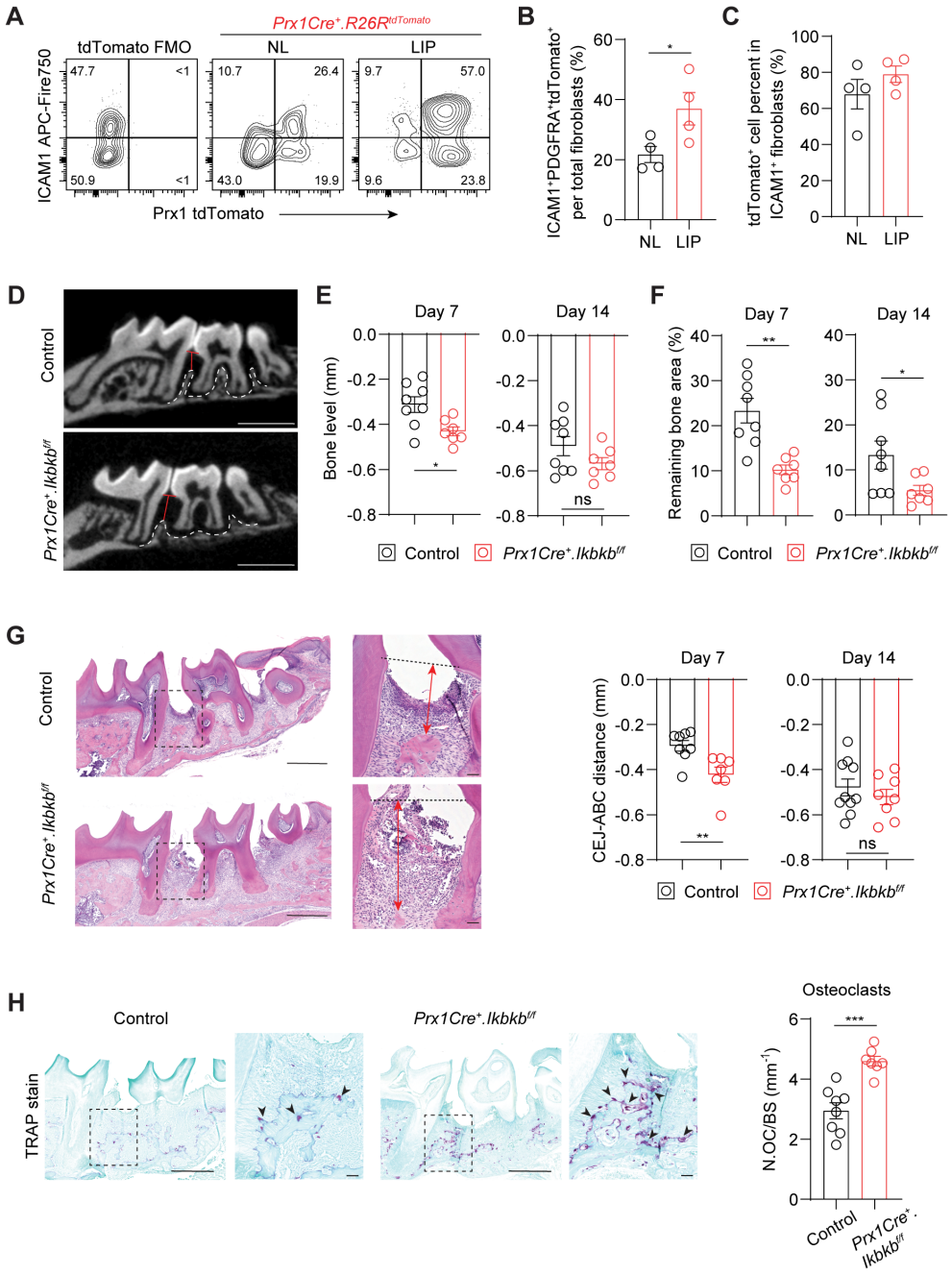

FIGURE 4
Perturbation of canonical NF-κB pathway in Prx1Cre-lineage reduces ICAM1$^+$ fibroblasts and exacerbates periodontal bone loss. **(A)** Representative flow cytometry plots of ICAM1$^+$ and Prx1Cre$^+$ tdTomato$^+$ fibroblasts in the non-ligated (NL) and ligature-induced periodontitis (LIP) groups from *Prx1Cre$^+$.R26R^{tdTomato}* reporter mice. Ligature was in place for 7 days (7d). FMO control from B6 mice is shown. **(B)** Quantification of percent Prx1$^+$ lineage ICAM1$^+$ fibroblasts (PDGFRA$^+$ICAM1$^+$tdTomato$^+$) normalized by total fibroblast numbers comparing NL and LIP groups. **(C)** Quantification of percent Prx1$^+$ lineage ICAM1$^+$ fibroblasts (PDGFRA$^+$ICAM1$^+$tdTomato$^+$) normalized by ICAM1$^+$ fibroblasts (PDGFRA$^+$ICAM1$^+$) in NL and LIP groups. Each dot represents one mouse as a split-mouth design, N=4 mice. **(D)** Representative micro-CT images of maxillae in control and experimental (*Prx1Cre$^+$.Ikbkb$^{f/f}$*) mice that received 7d ligature. Red lines designate the distance from alveolar bone crest (ABC) to cementoenamel junction (CEJ) level, white dashed lines demarcate alveolar bone remaining. Scale bar, 1mm. **(E, F)** Quantification of bone level **(E)** and remaining bone area percent **(F)** in control and *Prx1Cre$^+$.Ikbkb$^{f/f}$* mice on 7d and 14d post-ligature. **(G)** Left, representative hematoxylin and eosin-stained images of ligated control and experimental mice 7d post-ligature. Dashed lines designate CEJ level, red arrows represent distance from CEJ to ABC. Right, quantification of CEJ-ABC distance in mm. Scale bar, 50μm. **(H)** Left, representative TRAP-stained images of ligated control and experimental mice 7d post-ligature. Arrows point to TRAP$^+$ osteoclasts. Scale bar, 50μm. Right, quantification of osteoclast numbers normalized by bone lining perimeter (mm^{-1}). Each dot represents data point from individual mouse (N=7-8 per group). Data represent mean ± SEM. Student's t-test **(B, C, G, H)** or Welch's t-test **(E, F)** comparing control vs. experimental group; ns, not significant, *p<0.05, **p<0.01, ***p<0.001.

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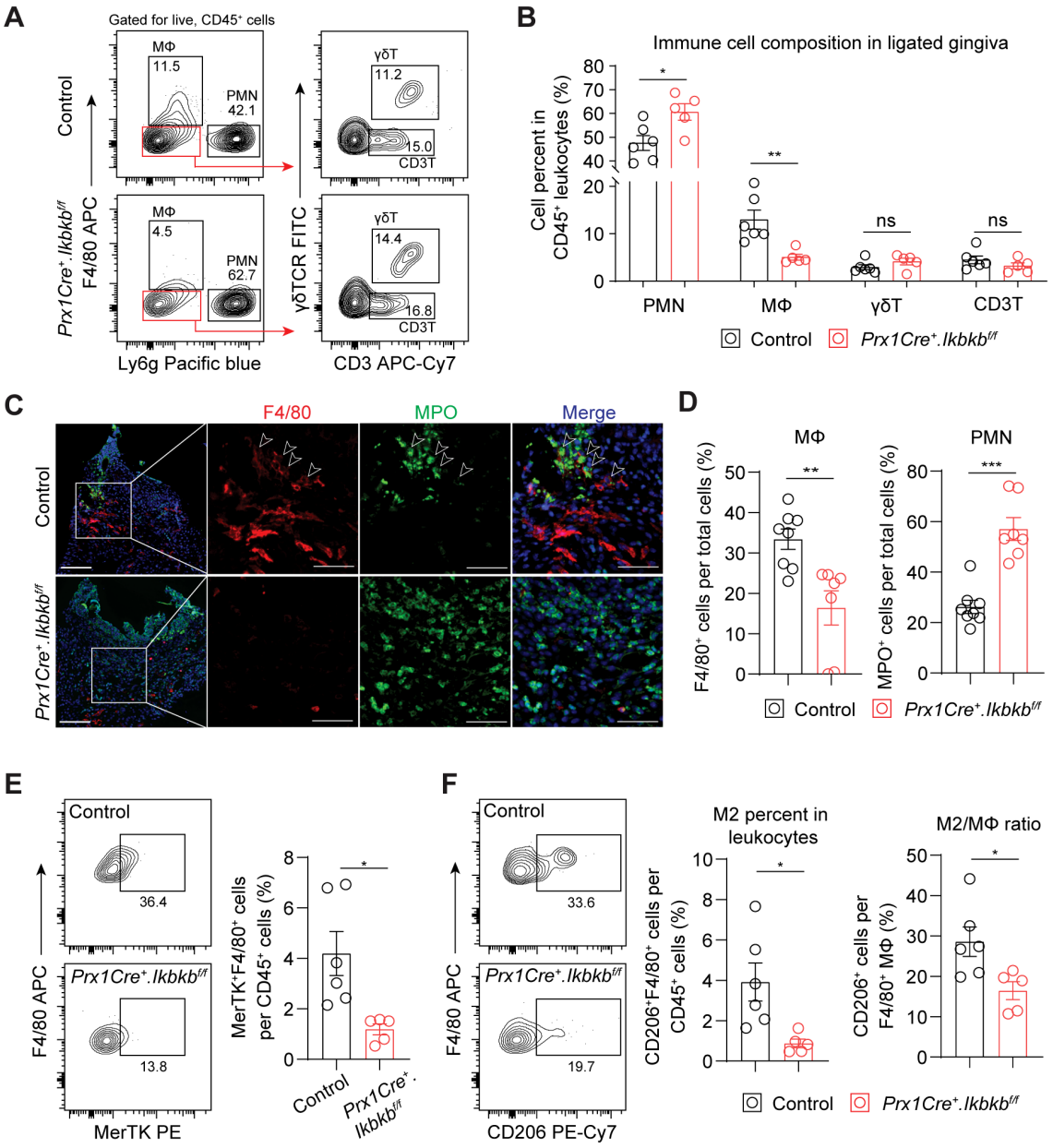

FIGURE 5
Periodontal damage by defective inflammatory fibroblasts is associated with deficit in macrophage recruitment and efferocytosis. **(A)** Representative flow cytometry plots for periodontal immunophenotyping in control and experimental (*Prx1Cre*$^{+}$*.Ikbkb*$^{f/f}$) mice on 7d post-ligature. Pre-gated population for live leukocytes (CD45^{+}) is shown. **(B)** Quantification of percent neutrophils (polymorphonuclear cells, PMN; CD45^{+}Ly6g^{+}), macrophages (MΦ; CD45^{+}F4/80^{+}), CD3^{+} T cells (CD3T; CD45^{+}Ly6g^{-}F4/80^{-}CD3^{+}) and gamma delta T cells (γδT; CD45^{+}Ly6g^{-}F4/80^{-}CD3^{+}γδTCR^{+}) normalized by CD45^{+} leukocytes in ligated control and experimental mice. **(C)** Representative immunofluorescent images of 7d ligated control and experimental mice in paraffin sections stained with antibodies specific against macrophage marker F4/80 (red) and neutrophil marker myeloperoxidase (MPO; green). Arrows point to F4/80 immunopositivity in proximity to MPO signal. Scale bar, 100μm; inset scale bar, 50μm. **(D)** Quantification of percent macrophages (F4/80^{+}) and neutrophils (MPO^{+}) normalized by total cells from immunofluorescence experiments. **(E)** Left, representative flow cytometry plots of macrophages that express MerTK (F4/80^{+} MerTK^{+}), an efferocytosis marker, in 7d ligated control and experimental mice. Right, quantification of percent MerTK^{+}F4/80^{+} cells normalized by CD45^{+} leukocytes. **(F)** Left, representative flow cytometry plots of M2 macrophages (F4/80^{+}CD206^{+}) in 7d ligated control and experimental. Right, quantification of percent M2 macrophage normalized by CD45^{+} leukocytes and by total macrophage numbers. Each dot represents data point from individual mouse (N=5-8 per group). Data represent mean ± SEM. Student's t-test **(B, D, E)** comparing control vs. experimental group.; ns, not significant, *p<0.05, **p<0.01, ***p<0.001.

(Figure 6F). By quantifying double-positive F4/80^{+} and internalized fluorescent bead signals, we found that macrophages phagocytosed significantly more fluorescent beads when incubated in conditioned media collected from the ICAM1^{+} fibroblast-enriched group, and this modulatory effect was diminished when neutralizing CCL2 antibody was added to the conditioned media (Figures 6G, H). Taken together, the results support an immune sentinel role for inflammatory ICAM1^{+}

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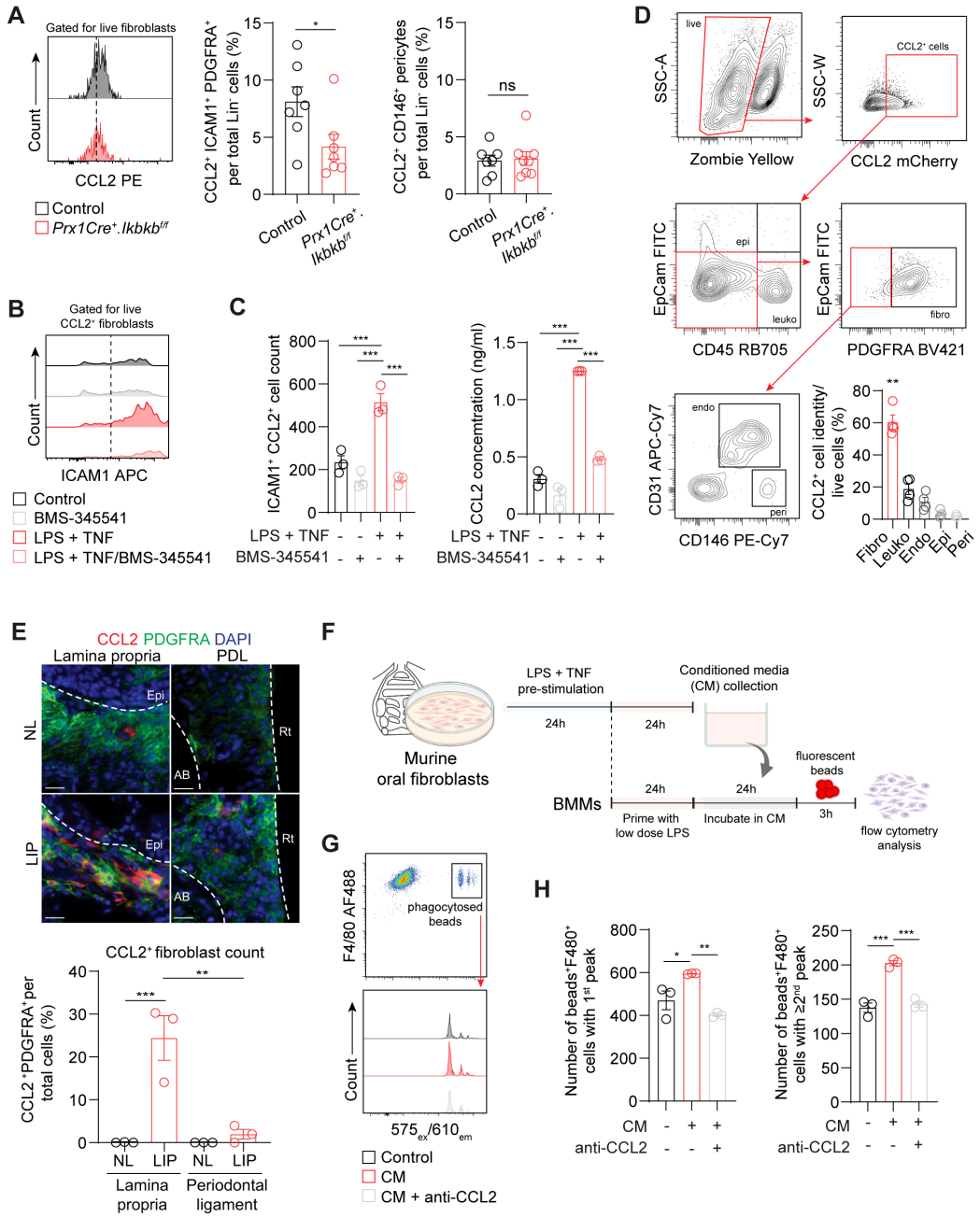

FIGURE 6

(A) Left, representative flow cytometry histogram of CCL2 signal in 7d ligated control and experimental mice. Middle and right, quantification of percent $CCL2^+ICAM1^+$ fibroblasts ($Lin^-PDGFRA^+$) and $CCL2^+$ pericytes (Lin^-CD146^+) normalized by lineage-negative mesenchymal cell numbers. **(B)** Representative flow cytometry histogram of ICAM1 expression in fibroblasts pre-gated for $CCL2^+$ signal in control or $ICAM1^+$ oral fibroblast-enriched conditions (LPS + TNF) treated with or without BMS-345541 *in vitro*. **(C)** Left, quantification of $ICAM1^+CCL2^+$ fibroblasts cell counts by flow cytometry analysis. Right, ELISA analysis of CCL2 concentration in the supernatant of cultured control or $ICAM1^+$ enriched fibroblast conditions with or without BMS-345541. **(D)** Top, representative gating strategy for $CCL2^+$ cell phenotyping by flow cytometry using tissues collected from $CCL2^{mCherry}$ reporter mice that had ligature placed for 7 days. Bottom right, quantification of $CCL2^+$ fibroblasts ($CD45^-EpCAM^-PDGFRA^+$), leukocytes ($CD45^+$), endothelial cells ($CD31^+$), epithelial cells ($EpCAM^+$), and pericytes ($CD31^-CD146^+$) normalized by total $CCL2^+$ cells. Each dot represents one mouse (N=4). **(E)** Top, representative immunofluorescent images of non-ligated control (NL) and ligature induced periodontitis (LIP) from $CCL2^{mCherry}$ reporter mice. Paraffin sections were stained with antibodies specific against PDGFRA (green) and red fluorescent protein (red), and immunopositivity in the lamina propria and periodontal ligament space (PDL) was examined. Scale bar, 20μm.Bottom, quantification of percent $CCL2^+$ fibroblasts ($CCL2^+PDFGRA^+$) normalized by total nucleated cells in field of view. N=3, split mouth design. **(F)** Schematic diagram of *in vitro* phagocytosis assay using conditioned media from $ICAM1^+$ enriched oral fibroblast culture and primary bone marrow-derived macrophages. **(G)** Top, flow cytometry gating strategy for identification of double positive $F4/80^+$ fluorescence $beads^+$ from *in vitro* phagocytosis assay. Bottom, representative flow cytometry histogram of fluorescence beads signals showing three distinct peaks from control, conditioned media (CM), and CM + anti-CCL2 neutralization groups. **(H)** Quantification of fluorescence $beads^+$ $F4/80^+$ macrophage numbers per 10^4 events. Left, number of $beads^+F4/80^+$ with a first peak (one bead) in the histogram; right, number of $beads^+F4/80^+$ with a second or third peak (two or three beads phagocytosed). N=3 each. All *in vitro* experiments were repeated independently twice. Data represents mean ± SEM. For **(A)**, one-way ANOVA test followed by pairwise t-test's with Šidák's correction was performed. For **(C)** Brown Forsythe ANOVA test with Dunnett's T3 Multiple comparison test; ns, not significant, *p<0.05, **p<0.01, ***p<0.001.

fibroblasts that modulate the recruitment and phagocytotic activity of macrophages via CCL2, safeguarding the periodontium from excessive bone loss.

Discussion

Gingival fibroblasts have long been speculated to play an important immune sentinel role in periodontal pathogenesis (38). However, most studies rely on bulk *in vitro* culture systems that do not consider the functional and molecular heterogeneity of fibroblasts *in vivo*, thus our understanding of the role of fibroblasts in periodontitis remains incomplete. Here, we identified ICAM1 as a surface marker that selectively labels fibroblasts with inflammatory signatures in both human periodontitis and experimental mouse LIP models. Genetic perturbation of inflammatory function in fibroblasts by utilizing a Prx1Cre mouse model resulted in accelerated periodontal bone loss, supporting a protective role of these fibroblasts in periodontal disease progression. When immune cells were examined, the most notable changes were a significant reduction in macrophage numbers and an increased neutrophil count. This was attributed to insufficient efferocytosis by macrophages, which are necessary to clear neutrophilic debris (29, 30). This was linked to a reduction in $CCL2^+$ fibroblast numbers in mice that had *Ikbkb* deleted. The observed immunopathology is also consistent with a recent report demonstrating the destructive role of persistent neutrophilic inflammation in experimental periodontitis (27). Our study reveals that $ICAM1^+$ fibroblasts are an important initiator of inflammatory events that involve recruitment of macrophages necessary for debris clearance, thereby halting excessive periodontal bone loss.

We found that $ICAM1^+$ fibroblasts expanded in diseased periodontium compared to healthy control groups in both humans and murine models. These fibroblasts shared similar inflammatory features such as elevated expression of cytokines CCL2 and CXCL1 compared to $ICAM1^-$ fibroblasts. Human $ICAM1^+$ had additional cytokine upregulation such as CXCL13 and CCL19 that were not present in murine $ICAM1^+$ fibroblasts. We interpret this difference to be due to varying disease stage and duration found in human periodontitis that may not be present in murine LIP. In advanced human periodontitis, T and B/plasma cells predominate (39), for which CXCL13 and CCL19 expression from the $ICAM1^+$ fibroblasts may be important as they are chemotactic for B and T cells, respectively (40, 41). In contrast, neutrophils and macrophages are the major immunocyte infiltration in murine experimental periodontitis (Figure 5B), where CCL2 expression by this fibroblast subset may be the most necessary. It is plausible that the $ICAM1^+$ inflammatory fibroblasts shift their secretome profile to accommodate a transition from the innate to adaptive immune response toward the later stages of periodontal disease. This possibility is supported by the studies demonstrating plasticity of fibroblasts that acquire context-dependent inflammatory phenotype in aging (42), fibrotic disease (43), gut inflammation (6), and rheumatoid arthritis (44). Nevertheless, $ICAM1^+$ fibroblasts clearly represent a specific mesenchymal subset that modulates immune responses in periodontitis, which may be useful for distinguishing inflammatory gingival fibroblasts in future studies.

Here, we utilized Prx1Cre based genetic manipulation to target fibroblast lineage cells and inhibit their immunomodulatory function by preventing NF-κB activation. $ICAM1^+$ fibroblasts exhibit a transcriptional profile highly implicated with NF-κB pathways (Figure 1G), and inhibiting *Ikbkb*, an upstream activator of NF-κB in Prx1Cre fibroblasts exacerbated periodontal breakdown. Interestingly, $ICAM1^+$ fibroblasts exhibit elevated expression of CCL2 and CXCL1 (Figure 3H), and these cytokines were also found to be elevated in $Prx1Cre^+$ oral fibroblasts compared to lineage-negative fibroblasts (16). Given the observation that this experimental approach resulted in a drastic innate immune dysregulation, we expect potential off-target effects of *Ikbkb* deletion in other non-immunomodulatory fibroblasts to be minimal. Although Prx1Cre also labels pericytes, our scRNA-seq and pathway analysis and the results demonstrating that $CCL2^+$ pericyte numbers are low and unaffected by the *Ikbkb* deletion indicate that pericytes may have less influence for the observed bone loss phenotype in our model. It is known that pericytes play an important regulatory role for limiting immune infiltration in the brain (45) and are responsive to IL-17 inflammatory pathways in rosacea (46), thus pericyte-leukocyte interaction is likely context and organ-dependent. An optimal murine model to target $ICAM1^+$ inflammatory fibroblasts is not feasible, as ICAM1 is expressed in many other cell types including endothelial and immune cells (47), therefore neither a global knockout nor ICAM1 promoter driven Cre model is adequate for specifically studying $ICAM1^+$ fibroblasts.

CCL2 was upregulated in both human and murine $ICAM1^+$ fibroblasts, which was effectively reduced by lineage-specific deletion of *Ikbkb* thereby disrupting macrophage trafficking in the experimental periodontitis model. Our results support that CCL2-mediated immunomodulation by the fibroblasts is needed for physiologic response in periodontitis. Furthermore, they are supported by the studies demonstrating that CCL2 is essential for proper oral wound healing (24) and that exogenous application of CCL2 alleviates periodontal bone loss by modulating macrophage polarization (48). In cardiac injury, mural fibroblast-derived CCL2 is necessary for normal macrophage recruitment and positioning and thus is athero-protective (49). As $ICAM1^+$ fibroblasts are a major source of CCL2, surgical approaches involving gingival transplantation enriched with these immunomodulatory fibroblasts may be a viable therapeutic approach to prevent aberrant periodontal inflammation and tissue damage.

In summary, we have identified ICAM1 as a cell surface marker to distinguish inflammatory fibroblasts that exert a protective immune function during early periodontal disease process. As the focus of our study was on characterizing the identity and role of these fibroblasts in experimental periodontitis, we did not explore the downstream events of ICAM1 activation via cell-to-cell contact mechanism with other immunocytes which is well characterized (47). $ICAM1^+$ fibroblasts have been shown to directly interact and downregulate T cell responses *in vitro* (50), and similar mechanisms may affect periodontal disease progression, which may be investigated in future studies. Despite the limitations, our *in vivo* work provides strong evidence for the protective immunoregulatory role of $ICAM1^+$

gingival fibroblasts in a periodontal niche. Further, it illustrates new ways to enable live selection of these cells in human and mouse models of periodontitis along with a genetic model to study them *in vivo*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by University of Pennsylvania Institutional Review Board (IRB). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by The University of Pennsylvania's Institutional Animal Care and Use Committee (IACUC). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WK: Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. KP: Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. AK: Data curation, Formal analysis, Investigation, Software, Writing – original draft, Writing – review & editing. RD: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. ZC: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. JZ: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. ES: Formal analysis, Investigation, Writing – review & editing. KK: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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SUPPLEMENTARY FIGURE 1
(A) Violin plot showing number of features expressed by each sample. Dotted red lines indicate thresholds used to filter data. **(B)** Violin plot showing percentage of mitochondrial genes expressed by each sample. Dotted red lines indicate thresholds used to filter data. **(C)** Top Left, UMAP plot of unintegrated data. Top right, UMAP plot of integrated data. Bottom, UMAP plots for individual samples of integrated data. Legend applies to all plots. Samples with the AP- prefix were from GSE217720, samples labeled GM_Healthy1, GM_Healthy2, PD_Mild, and PD_Severe were from GSE164241, and all remaining samples were from GSE164241. **(D)** Juxtaposition of LISI scores calculated for unintegrated and integrated data comparing sample overlap.

SUPPLEMENTARY FIGURE 2
(A) UMAP plot showing *PRRX1* expression across all samples. **(B)** Left, PROGENy analysis for *PRRX1*$^{+}$ fibroblasts or pericytes in all conditions (AP, GM, and PD). Right, PROGENy analysis for *PRRX1*$^{+}$ fibroblasts or pericytes for the PD condition. **(C)** Left, representative micro-CT images of maxillae in 8 weeks old control and experimental *Prx1Cre*$^{+}$.*Ikbkb*$^{f/f}$ mice. **(D)** Quantification of bone level in mm and remaining bone area between first and second molar teeth. Student's t-test, ns, not significant. N=4-5 each group.

SUPPLEMENTARY FIGURE 3
(A) Left, representative H&E and immunofluorescence image of 7d ligated control and *Prx1Cre*$^{+}$.*Ikbkb*$^{f/f}$ mice. Paraffin sections were stained with an antibody specific against CXCL1 and immunopositivity in the epithelium was examined. Epithelial tissue was determined via H&E staining of the same paraffin section. Scale bar, 100 µm. **(B)** Quantification of normalized mean fluorescent intensity (MFI) of CXCL1 in the epithelial area. Welch's t-test comparing control vs. experimental groups; ns, not significant.

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EDITED BY
Pedro Paulo Chaves de Souza,
Universidade Federal de Goiás, Brazil

REVIEWED BY
Olivier Huck,
Université de Strasbourg, France
Guilherme Oliveira,
Federal University of Uberlandia, Brazil

*CORRESPONDENCE
Francesco D'Aiuto
f.daiuto@ucl.ac.uk

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The translational potential of inflammation-induced skin blister human models in exploring the pathogenesis of periodontitis and its systemic health implications

Rizky Aditya Irwandi[1,2], Crystal Marruganti[1,3], George Collins[4,5], Jhonatan de Souza Carvalho[4,6], Derek Gilroy[4] and Francesco D'Aiuto[1]*

[1]Periodontology Unit, UCL Eastman Dental Institute, University College London, London, United Kingdom, [2]Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia, [3]Unit of Periodontology, Endodontology and Restorative Dentistry, Department of Medical Biotechnologies, University of Siena, Siena, Italy, [4]Department of Ageing, Rheumatology and Regenerative Medicine, Division of Medicine, University College London, London, United Kingdom, [5]Department of Cardiology, St Bartholomew's Hospital, Barts Health NHS Trust, London, United Kingdom, [6]Department of Diagnosis and Surgery, São Paulo State University (UNESP), School of Dentistry, Araraquara, Brazil

Periodontitis is a highly prevalent chronic disease. Despite decades of extensive research on the topic, a complete understanding of its immunopathogenesis, especially when linked to other inflammatory comorbidities, is lacking. *Ex vivo* human and *in vivo* animal experiments have shown the host inflammatory response's crucial role in both the disease's onset and its systemic implications. These approaches, however, remain questionable when translating these findings into real-world scenarios linked to periodontitis. A clear need for new *in vivo* human models is discussed, especially within the context of understanding the host response to key pathogens linked to periodontitis, such as *Porphyromonas gingivalis* (*P. gingivalis*). Therefore, a skin blister model was employed to describe the stages of the host immune response in humans after challenges by microbial and/or sterile insults. A novel human challenge model using UV-killed *P. gingivalis* holds promise in producing new evidence and bridging the gap of the host response to periodontitis and its links with other common chronic diseases.

KEYWORDS

human challenge model, periodontitis, *Porphyromonas gingivalis*, skin blister model, periodontal disease, self-resolving inflammation, periodontitis pathogenesis, periodontitis-systemic link

1 Introduction

Periodontitis is a chronic inflammatory disease characterized by an altered homeostasis between the subgingival microbiome and the host gingival inflammatory response in susceptible individuals (1). A recent report from the World Health Organization (WHO) estimates that the severe form of the disease has affected more than a billion cases worldwide and up to 1/5 of the global adult population (2). Periodontitis is characterized by the progressive destruction of hard and soft tissues supporting the tooth (gingiva, periodontal ligament, and alveolar bone) (3). If left untreated, it will inevitably result in tooth loss, masticatory impairment, and a severe impact on individuals' quality of life (4). The global burden of this disease across the United States and Europe is estimated to be over $150 billion (5). Its treatment relies upon self-performed improved dental hygiene practices as well as professional dental biofilm removal, with a small minority of patients requiring more sophisticated procedures aimed either at resolving resistant gingival inflammation (localized gum surgeries) and/or attempting to rebuild in part the lost hard and soft tissues supporting the dentition (6). The relevance of periodontitis as a global health problem has reached greater attention over the last 30 years, confirming firm links with a substantial host response characterized by a sustained low-grade systemic inflammation (7) and an altered circulating inflammatory cell profile (8). Over decades, studies have reported that periodontitis is independently associated with numerous inflammatory disorders, such as cardiovascular disease, type-2 diabetes, and rheumatoid arthritis, amongst others (9).

For decades, researchers have tried to understand the pathogenesis of periodontitis, and its possible causal association with other common inflammatory comorbidities has been heavily researched. The latest efforts have focused on the host-pathogens interplay using genetic susceptibility and single-cell transcriptomic analyses, revealing that polymorphisms of inflammatory response genes (10) and innate immune cell dysfunction (11) confer susceptibility of the host to periodontitis. Despite periodontal tissue inflammation resolution following the periodontitis management, the augmented level of some systemic inflammation markers persists, and circulating inflammatory cells still retain their altered periodontitis-associated phenotypes, including increased counts of peripheral blood-derived immune cells (12) demonstrating enhanced inflammatory responses (13, 14). Collectively, the evidence highlights the crucial role of host inflammatory responses (immune cells and their inflammatory mediators) to periodontitis and the underappreciated potential contribution of host immunoinflammatory status to periodontitis-associated systemic inflammation.

To date, studies exploring human immune responses to a keystone pathogen *Porphyromonas gingivalis* (*P. gingivalis*) focus on *ex vivo* human models using tissue cultures (15–19) and local gingival tissue phenotyping (20, 21). Furthermore, animal models of periodontitis have been used to describe and understand the gingival inflammatory response to the pathogen in rodents (22, 23). However, both approaches have numerous limitations, notably that they might not translate into the complexity of the *in vivo* human host response to *P. gingivalis* due to environmental restrictions in a controlled niche of tissue cultures and different animal immune systems. For instance, neutrophil extracellular trap (NET) formation (NETosis) potentiates periodontal inflammation in mice during experimental periodontitis (24). NETosis is evident in periodontal lesions in mice and NET removal by systemic delivery of DNAse-I protects mice from *in vivo* periodontal bone loss. Mechanistically, NETosis is regulated by peptidylarginine deiminase-4 (PAD4)-mediated histone citrullination and this further confirms the role of NETosis in periodontitis – PAD4 KO mice displayed significant protection from periodontal bone loss compared to wild-type (WT) counterparts and *in vivo* pharmacologic delivery of Cl-amidine to inhibit PAD4-mediated citrullination protected WT mice from ligature-induced periodontitis (25). However, the mechanism of NETosis in mice is different from that in humans, as experiments of human neutrophils have shown that the cells were still capable of performing NETosis following the PAD4 inhibitor, Cl-amidine (26). In terms of DNAse-I treatment, it failed to degrade NETs purified from neutrophils in humans (27). This conflicting evidence highlights that the mechanism shown in mice was not confirmed in humans. Therefore, the generation of new human *in vivo* evidence is crucial in unveiling the immunological mechanisms of periodontitis and its association with inflammatory comorbidities in humans.

The skin blister model is a recognized *in vivo* human inflammation model that is safely and effectively used to study host-pathogen interactions (28). Using this model, researchers have unraveled important mechanisms and pathways of the human immune response to infective agents and chronic inflammatory diseases. This model involves the intradermal injection of a putative agent (infective or not) and the subsequent generation of a local skin blister (using a vacuum machine). The exudate harvested from the artificial blister offers the opportunity to study the kinetics of the onset and resolution of human inflammation, including the innate and adaptive immune responses to the challenge (29, 30). This review discusses how the skin blister model represents a novel opportunity to understand more about the human response to a common pathogen implicated in the pathogenesis of periodontitis (*P. gingivalis*) and its close links with other systemic inflammatory comorbidities. A brief review of the immune responses implicated in the onset of periodontitis and its systemic consequences is followed by a description of the novel *P. gingivalis* human challenge model, outlining its potential benefits and limitations.

2 Host susceptibility to periodontitis and its link to inflammatory comorbidities: the case for immune response

Periodontitis occurs as a result of polymicrobial synergy and dysbiosis in susceptible hosts. A combination of impaired immunity

and systemic and environmental factors could be a vital determinant of the shift within the gingival ecosystem to dysbiosis. The local host inflammatory response is responsible for periodontal tissue destruction (31). For the focus of this review, a prominent role will be given to the trait of immunologic susceptibility which not only affects the onset of periodontitis and its progression but also wound-healing potential during various treatment steps of periodontitis (32).

Studies on understanding the immunologic susceptibility to periodontitis have focused on population-level genetic analyses, including single nucleotide polymorphisms (SNPs) of inflammatory genes – reviewed extensively in (10) and (33). Recent genome-wide association studies (GWAS) have contributed to the identification of novel loci encoding immune response-related genes associated with periodontitis (34–39). The genome studies have expanded further using single-cell RNA sequencing (sc-RNA seq), such as in the experiments of Williams et al. demonstrating the presence of exaggerated responsiveness in stromal cells along with augmented neutrophil and leukocytes infiltration in periodontal tissue of patients suffering from periodontitis (40). Further sub-clustering of inflammatory cells, particularly myeloid cells, also exhibited the amplification of inflammatory response. Specifically, macrophages derived from gingival tissue of periodontitis individuals exhibited pro-inflammatory phenotype by highly expressing NLRP3, an inflammasome-mediating IL-1β production (41). Translating the evidence reported by SNP, GWAS, and sc-RNA seq studies ideally requires *in vivo* human evidence on the exact mechanisms of the pathogenesis of periodontitis.

Refined understanding of the cellular and molecular mechanisms of host-microbe interactions in the pathogenesis of periodontitis, as extensively reviewed elsewhere (42–44), has important implications for the treatment of periodontitis. The current approach of standard periodontal treatments incorporates dental biofilm reduction surrounding the teeth and periodontal tissues (non-surgical and surgical periodontal therapies) (45). However, the mechanical interventions for periodontitis have been unable to improve clinical outcomes in certain patients, not to mention those with systemic inflammatory diseases such as diabetes - uncontrolled disease is detrimental to periodontal health and can boost treatment complications (46, 47). Host modulation therapy, therefore, emerges to overcome the limitation of mechanical debridement and has been proposed as an adjunctive therapy alongside the standard treatments (48, 49). Despite the numerous studies of novel host-modulating agents for periodontitis, the majority of the agents are still in preclinical studies and have yet to move forward to clinical applications (50).

Understanding the immunological basis of periodontitis could also be relevant when assessing the links between the disease and other common chronic inflammatory diseases. Periodontitis is not confined to the gingival tissues but has been consistently associated with increased systemic inflammation and altered circulating inflammatory cell profiles (8, 51). Clinical evidence confirms that patients with periodontitis exhibit elevated levels of pro-inflammatory mediators, including IL-1β, IL-6, TNFα, and C-reactive protein (CRP), as well as increased neutrophil counts in peripheral blood when compared to controls (52–55). This relationship seems reversible as effective treatment of periodontitis results in a normalization of some inflammatory biomarkers, including CRP (56, 57). Some cellular markers, however, do not seem to be affected by the resolution of local gingival inflammation as achieved by conventional dental cleaning sessions (13, 14, 58). Dampening the hepatic systemic response due to a reduction of local inflammatory cytokines is the most plausible mechanism explaining a reduction of CRP after treatment of periodontitis. The lack of resolution of immunologic changes could result from a trained immunity state induced by periodontitis-associated systemic inflammation (59). As previously described, this process could be defined as innate immune memory involving changes in the hematopoietic stem and progenitor cells (HSPCs) in the bone marrow after exposure to certain microorganisms (60).

Both clinical and animal experimental evidence support the notion that trained immunity could be a key mechanism explaining the systemic health implications of periodontitis. A recent clinical study confirmed a possible link between raised periodontal inflammation and femoral bone marrow activity in patients with periodontitis compared to controls (61). Ishai and colleagues provided evidence that increased bone marrow activity could mediate the relationship between periodontitis and arterial inflammation (62). Further, two key animal experiments demonstrated HSPC's involvement when infection with pathogens linked to periodontitis was performed. In mice, continuous release (subcutaneous injection) of a keystone periodontal pathogen, *P. gingivalis*, caused increased osteoclast differentiation in the bone marrow that was IL-6-dependent (63). Indeed, isolated peripheral blood mononuclear cells from patients with periodontitis compared to healthy controls are predisposed to RANKL-induced osteoclastogenesis *ex vivo* (64). Further experimental evidence shows that periodontitis-triggered maladaptive innate immune memory in the bone marrow produces myeloid cells with enhanced inflammatory responses. Li and colleagues further confirmed that either experimental periodontitis or arthritis-induced trained immunity plays a role in the two-way relationship between periodontitis and arthritis (65). In this context, enhanced responses in inflammatory cells (e.g. increased production of pro-inflammatory mediators upon *ex vivo* stimulation) are evident in patients with periodontitis; the immune cells retain these phenotypes following the treatment of periodontitis – extensively discussed in ref (8). This begs the question: could trained immunity be triggered by periodontitis in humans? It remains an important question that warrants further investigation.

3 The translational potential of skin blister model in inflammation research

In vivo human inflammation models can be classified into two categories based on the delivery route of the challenge. The first group relies upon intravenous injection of bacterial endotoxins (66), cytokine administration (67), typhoid vaccine (68), and strenuous exercise (69), for example. Given the potentially robust systemic

inflammatory reaction and possible side effects, most of these models require being conducted in highly controlled research centers. Alternatively, less invasive inflammation models triggered by local intervention have been developed. Our group has previously characterized a human model of treatment of periodontitis (70). A transient systemic inflammatory response of moderate magnitude has been described extensively (70–72). Local administration of inflammatory stimuli, for example, in the skin, has also been proposed as a possible human model to characterize human reaction to infective agents. This localized tissue inflammation model facilitates the investigation of leukocyte migration and accumulation of soluble inflammatory mediators at the site of injection. In a human lung infection model, different encounters (e.g. endotoxin, ozone, and rhinovirus) to induce inflammation have been utilized and can be delivered through inhalation. Sample isolation is needed to understand the temporal inflammatory profile after the challenge. However, it can be more challenging to perform and less comfortable for the participants (sputum collection) (73), limiting its applicability and reliability.

Skin models of inflammation offer instead a contained and practical alternative to these systemic challenge models. Indeed, dermal models are characterized by minimal invasiveness, multiple sites accessible to monitor during the induction of inflammation, and easier access for sample/fluid collection. Several types of skin inflammation models have been established in humans based on the categorization of agents used, including tuberculin purified protein derivative (PPD) (74), BCG (75), cantharidin (28), Candida (candin) antigen (76), varicella-zoster virus (VZV) (77), *Escherichia coli* (*E. coli*) (78), and *Streptococcus pneumoniae* (*S. pneumoniae*) (79) and lipopolysaccharides (LPS) (80). An artificial blister is created at the inoculation site using a vacuum machine secured on the skin at selected time points after intradermal injection. The negative pressure generated by the machine separates the epidermis from the dermis (Figure 1), allowing confinement, collection and drainage of an inflammatory exudate (81). The intradermal injection of the pathogen resulted in localized inflammatory responses with complete local clearance of the pathogen and no systemic dissemination of the pathogen (30, 82). As a self-resolving inflammatory model in humans, these experiments have been replicated by several research groups and have shown good tolerability for participants recruited (including healthy individuals of different ages and patients with chronic diseases) (83–85).

For example, in human immunobiology the cantharidin-induced skin blister model helped to identify impaired efferocytosis in the elderly. Furthermore, using this model researchers identified oral intake of losmapimod – a selective p38 mitogen-activated protein kinase (MAPK) inhibitor – as a therapeutic intervention capable of reversing this inflammatory resolution defect (84). A similar model has also been validated to evaluate the anti-inflammatory properties of anti-TNF (adalimumab) and corticosteroids in reducing innate inflammatory cell recruitment to the injection site (86).

Amplified acute inflammation onset with delayed resolution was evaluated in patients with ulcerative colitis when challenged with UV-killed *E. coli* and *S. pneumoniae* prior to suction skin blister formation (83). Using the same model of UV-killed *E. coli* injection, another study reported prolonged immune alterations following the resolution of inflammation, challenging the notion that homeostasis is achieved after acute inflammation resolves (78). Several endogenous specialized pro-resolving mediators (SPMs) and their receptors have been identified as key regulators of the initial phase of inflammation resolution, and furthermore the administration of exogenous SPMs has been shown to boost this process in humans (87).

The skin blister model has also contributed to a greater understanding of the dynamics of adaptive immune cells in humans. When a tuberculin PPD injection (skin blister model) was utilized to study human T cell recall responses, numerous regulatory mechanisms for maintaining human memory T cells *in vivo* were observed (88). The isolation of antigen-specific memory T

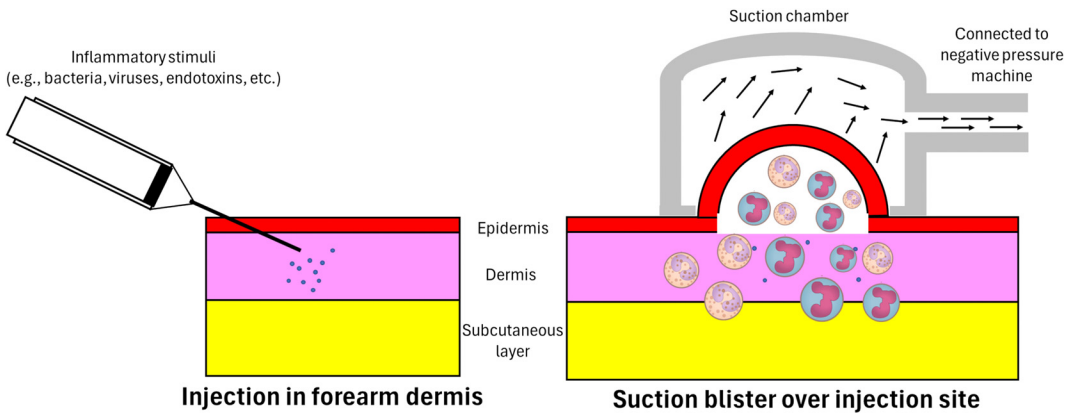

FIGURE 1
Intradermal injection and inflammatory exudate acquisition. A 30 gauge needle attached to a 1-ml syringe is used to administer the inflammatory stimuli into the dermis just under the epidermis. A suction chamber is then positioned and secured over the injection site at a pre-specified time-point. The negative pressure generated by an electronic vacuum machine separates the epidermis from the dermis and draws in the accumulated inflammatory exudate, including inflammatory cells and soluble mediators – in response to the introduced stimulus – at the dermis. As a result, a unilocular space between the dermis and epidermis containing inflammatory exudate is formed (an artificial blister).

cells from the suction blister followed by *in vitro* stimulation to become regulatory T cells (Tregs) was attainable, and this was demonstrated to have a suppressing role in inflammation (89). Synchronized production of IL-2 by CD4+ T cells in blister fluid and skin biopsy was showed in the model, enabling the non-invasive characterization of potential defects in adaptive immune response in the elderly. Blister fluid analyses became a valid alternative to skin biopsy to characterize the dynamic of host response to various triggers (74).

A series of further experiments involving patients with active, latent, or cured tuberculosis (TB) was able to describe the role of IL-17A and Th17 responses in patients with active TB, when compared to those with latent TB (85). When TB treatment was administered in these patients, the same immune changes resolved, with a decrease in IL-17A and Th17 responses. In this case, it was skin biopsy samples following the intradermal injection of tuberculin PPD that were analyzed (85). Subsequent experiments using this model enabled the assessment of the safety and specificity of novel monoclonal antibodies aimed at reducing the recruitment of Th17 during adaptive immunity (90). When candin was used as an inflammatory insult, the skin blister model revealed that restricted immunosurveillance by memory T cells in the ageing population is dependent on the reduction of macrophage-derived TNFα production (76). Finally, when using VZV as an insult, researchers showed how regulatory T cells may be derived from memory T cells during localized inflammatory responses (91).

4 Intradermal injection of UV-killed *P. gingivalis*: an *in vivo* human skin inflammation model to investigate periodontitis and its link to systemic diseases

4.1 Skin inflammation model triggered by *P. gingivalis* in periodontal research

For over three decades, mice studies have utilized a skin inflammation model to assess host response to *P. gingivalis*, a common Gram-negative anaerobe implicated in the development of periodontitis. The most common model is the subcutaneous chamber model, which involves implanting a titanium coil chamber beneath the skin in the dorso-lumbar region of the mouse. After the coil placement has healed, *P. gingivalis* is injected into the chamber, allowing researchers to evaluate the host response to the bacterial challenge (92). When *P. gingivalis* injection was repeated on the model, researchers were able to understand the initiation of adaptive immune response in a time-dependent manner (93, 94). Moreover, studies using this model demonstrated how *P. gingivalis* can evade the host oxidative immune response and survive by escaping host antimicrobial killing (95, 96). Thanks to this model, researchers were able to demonstrate how *P. gingivalis* (through the function of gingipain) stimulated a specific inflammatory response and, at the same time, inhibited the antimicrobial killing and neutrophil phagocytosis resulting in an oral microbial dysbiosis which could ultimately lead to the development of periodontitis (97).

Although skin inflammation models using cantharidin (28), tuberculin PPD (29, 77), varicella zoster virus, *E.* coli (30), or *S. pneumoniae* (79) have been successfully used before, experiments using *P. gingivalis* in humans have not been attempted to date. Cantharidin is a vesicant secreted by blister beetles and 24 hours after its application onto the skin, a blister is formed on the skin and accumulates in the subsequent 72 hours. The 24 hour skin blister represents the acute phase of inflammation while the resolution of inflammation can be observed in the blister 72 hours after dermal cantharidin application (84, 98). When using other inflammatory insults, the induction of an artificial blister on the skin is needed with the help of a small electronic vacuum machine. Specifically, negative pressure generated by this machine is applied over the injection site, generating an artificial blister (Figure 1). The timing of blister formation is dependent upon the inflammatory insult and experimental design of the study (29, 30, 77, 79). For example, artificial blister formation was performed 7 days after the injection of either tuberculin PPD or VZV to investigate T-cell recall profiling and responses in humans (29, 77). When using *E. coli* or *S. pneumoniae* it took 4- and 48-hours post-injection to evaluate the acute response and inflammation resolution (30, 79). Host effects when challenging the skin has been previously reported. For example, when challenging participants with UV-killed *E. coli*, moderate swelling and mild discomfort in the axillary region were reported, but receded after 24 hours, with a complete resolution within 48 hours (30). Furthermore, when adopting the artificial blister creation, skin pigmentation has been reported and resolves within 4-6 weeks in Caucasians participants, although it could persist longer in darker skin types (30).

4.2 The potential benefits and limitations of the *P. gingivalis*-induced skin inflammation model

Research investigating the immunological mechanisms involved in the pathogenesis and progression of periodontitis and its link to other systemic chronic diseases has primarily used human *ex vivo* or animal *in vivo* models. Whilst on the one hand, these *ex vivo* and animal experiments allowed us to understand the changes that occur within the gingival tissue in response to dental biofilm accumulation and its potential systemic implications, the translation of this evidence into humans remains to be confirmed. A specific human model of inflammation in periodontal research could therefore improve our understanding of how humans respond to challenges of individual or multiple micro-organisms implicated with a common oral disease, as well as how it might impact systemic wellbeing.

Mouse models for periodontitis have been comprehensively reviewed by Rojas and colleagues (99). The current limitation of the model encompasses substantial immunological differences between mice and humans (100). Seok et al. showed that the genomic responses to different inflammatory stimuli in humans poorly correlated with mice models (101). Hence, the translational

projection of results - in particular the investigation of the host-microbial interactions in periodontitis – to meet human research frameworks is limited and difficult (99). Moreover, humanized mice models for immune investigations have been developed, but the complications due to xenogeneic transfers are ongoing challenges that researchers need to overcome, not to mention the potential bias of results owing to xenogeneic immune response (102). Therefore, human *in vivo* models could address those issues as experiments, on humans offer the benefits of being relevant to the complexity of human *in vivo* physiology.

Despite the versatility of animal models in biomedical research such as transgenic mice, the successful translation of animal studies for human clinical applications remains questionable. This is particularly true of inflammation-driven disease studies (103). For instance, more than 100 clinical trials have failed to reproduce the successful approach of modulating septic response to infection in animals revealed by sepsis studies (104). Such preclinical research can result in expensive and fruitless clinical implications for patient care. These unreliable findings mean that the overreliance of animals in preclinical research may not contribute to tangible clinical benefit, instead becoming an unnecessary and avoidable research inefficiency (105). Conducting research utilizing human *in vivo* models seems wise to reduce the use of animals in research and that in compliance with the Three Rs principle (replacement, reduction, and refinement) that underlies appropriate involvement of animals in research (humane animal experimental research) (106).

There would be several advantages in developing a human experimental model for *P. gingivalis*. Firstly, it could help compare individuals' host response to various types of micro-organism, including different genetically modified periodontal bacterial strains (i.e. lacking certain virulence factors such as gingipains and/or pathogen-associated molecular patterns such as LPS). Secondly, there is growing interest in understanding the potential impact of periodontitis on the overall host inflammatory burden and development/progression of other common chronic diseases such as diabetes, Alzheimer's and cardiovascular diseases. Gathering information on the host response (both cellular and biochemical) to common pathogens linked to periodontitis such as *P. gingivalis* could confirm a number of hypothetical causal pathways/mechanisms that have been proposed to date. Thirdly, performing these experiments in individuals presenting with healthy gingival tissues or already affected by periodontitis could help understand the different susceptibility traits linked to the onset and development of periodontitis. Finally, the same model could provide evidence to support new strategies for addressing periodontal inflammation and managing patients with periodontitis. This could enhance approaches to reducing damage and improving the chances of regaining soft and hard tissues around teeth (107–110). SPMs in periodontitis are extensively discussed elsewhere (111, 112). The exact SPMs that humans produce endogenously are inflammatory stimulus-dependent, meaning that different microorganisms may trigger distinct patterns of SPM production (113, 114). A human inflammation-induced skin model including *P.gingivalis* could provide insights into which SPM signatures drive the resolution of inflammation.

It is also important to note the limitations of using the skin infection model. First, the use of inactivated bacteria in such a model will not necessarily correlate with the same responses expected when challenged by live micro-organisms. Second, several lines of evidence adapting pathogens to induce skin-inflammation (30, 79) highlighted a substantial variation in outcomes between individuals following the challenge, as well as low numbers of cells retrieved from the blister exudates. Third, when using pre-specified time points, a novel skin blister model using periodontal pathogens would be limited to short-lived exposures to micro-organisms, and researchers could only yield two samples per participant (30, 79). Multiple sites on the skin could be used to perform the same challenge (80), but this will inevitably increase the complexity and potential risk of the model for potential participants, without mentioning the increased ethical concerns/considerations (discussed below).

4.3 Intradermal injection of UV-killed *P. gingivalis*: practical considerations

Although some limitations were previously discussed, the safety and practical aspects of developing a model of *P. gingivalis* skin blister inflammation in humans must be in a full adherence to ethical standards of medical research involving human participants as detailed in the Declaration of Helsinki (115). In order to achieve that, the model must first involve a team of immunologists, microbiologists and experts in clinical pharmacology confirming that the research utilizing the model is scientifically sound and rigorous in design. Supported by the urgency of the need for human models discussed above, research utilizing the inflammation-induced skin blister human model may only be conducted if the importance of the research outcomes outweighs risks and burdens to the research participants. As the model includes the intradermal injection of *P. gingivalis*, measures to minimize risks of the intervention must be implemented, including deliberate selection of bacterial strains and their growth media and tolerable reactions to the participants after the injection (discussed below). Lastly, appropriate compensation for the volunteers who participate in the studies using the model must be ensured because the procedure of intradermal injection and immune reactions that follow are likely to cause participant's discomfort and inconvenience.

P. gingivalis was selected as putative pathogen relevant to periodontitis and able to generate a local inflammatory response. Indeed, it is a keystone pathogen in periodontitis, and its systemic effects have been reported in both pre-clinical and clinical studies (23, 97, 116, 117). The first task to begin the development of the model includes the selection of which *P. gingivalis* strain and growth media to allow safe but effective intradermal injection. The wild-type strain 2561 could be considered a candidate of *P. gingivalis* rather than either strains BAA-308 (W83) or 53978 (W50), in-keeping with the safety concerns of attempting for the first time the intradermal injection of a Gram-negative micro-organism, hence a less virulent strain could be chosen (118–122). Second, the possible growth media for *P. gingivalis*; in particular, past experiments mainly used brain heart infusion (BHI) to culture the bacteria

(23, 123, 124). This media, however, contains bovine brains, and safety concerns were evaluated about the possible risk of cross-contamination during the process of bacterial isolation. Alternative growth media had therefore been evaluated such as PYG broth (125–128). This growth media was chosen to grow the bacterium in anaerobic conditions similar to the BHI media, and it is also free from animal-derived products (128).

The next step in the development of such a model following the establishment of *P. gingivalis* isolates would be to confirm a safe route of administration of *P. gingivalis* for human experimental applications. This could be achieved by generating inactivated, structurally preserved, and measurable micro-organisms. UV exposure is selected as a potent technique to inactivate bacteria without compromising the bacteria's structural integrity. UV light only targets bacterial DNA, particularly inducing pyrimidine dimers between DNA thymine bases. Subsequently, the micro-organism becomes incapable of replicating without losing their protein structures, including virulence factors (129). A set of experiments could then designed to determine the amount of UV-killed *P. gingivalis* for intradermal injection that is safe and sufficient in generating a self-resolving intradermal inflammatory response. Adequate bacterial quantification experiments should be completed to enable the generation of a sufficient number of micro-organisms to use in the model (130). Viable plate counts in combination with turbidity (optical density) measurements could be used to plot bacterial growth (130, 131) and appropriate quantification of UV-killed *P. gingivalis*. After measurable inactivated *P. gingivalis* is established, dose escalation experiments to determine bacterial numbers that humans could tolerate but sufficient to generate a reproducible inflammatory response, are required. Several lines of evidence can be reviewed and used to complete this part of the experiments using different quantities of UV-killed *P. gingivalis* (30, 79, 83).

5 Conclusion

The inflammation-induced skin blister model is a well-established human model of inflammation that successfully captures the immunoinflammatory status of healthy and diseased individuals. Specifically, the model can unveil the complex mechanisms of human immune responses in chronic inflammatory diseases and/or in response to specific inflammatory stimuli/infections. The host laboratory developed for the first time a safe and reproducible UV-killed *P. gingivalis* intradermal injection model followed by artificial suction blister formation and collection of cellular and soluble inflammatory infiltrates.

Periodontal research could use this novel model to enhance our understanding of which immunological mechanisms occur in humans with periodontitis and shed greater light on the potential link between this common chronic disease and other systemic inflammatory disorders.

Author contributions

RI: Writing – original draft, Writing – review & editing. CM: Writing – review & editing. GC: Writing – review & editing. JC: Writing – review & editing. DG: Writing – review & editing. FD'A: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships that could potentially create a conflict of interest.

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EDITED BY
Teun J. De Vries,
VU Amsterdam, Netherlands

REVIEWED BY
Josefine Hirschfeld,
University of Birmingham, United Kingdom
Jeffrey Ebersole,
University of Nevada, Las Vegas, United States

*CORRESPONDENCE
Elin Kindstedt
elin.kindstedt@umu.se

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The PerioGene North study reveals that periodontal inflammation and advanced jawbone loss in periodontitis associate with serum gingipain antibodies but not with systemic autoimmunity

Elin Kindstedt[1,2]*, Charlotte de Vries[3], Magnus Wänman[1], Barbara Aleksandra Potempa[4], Jan Potempa[4,5], Susanne Lindquist[6], Anders Esberg[7], Karin Lundberg[3] and Pernilla Lundberg[1]

[1]Department of Odontology, Section for Molecular Periodontology, Umeå University, Umeå, Sweden, [2]Wallenberg Centre for Molecular Medicine, Umeå University, Umeå, Sweden, [3]Division of Rheumatology, Department of Medicine Solna, Karolinska Institutet and Center for Molecular Medicine, Karolinska University Hospital, Stockholm, Sweden, [4]Department of Oral Immunology & Infectious Diseases, University of Louisville School of Dentistry, Louisville, KY, United States, [5]Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland, [6]LipumAB, Umeå, Sweden, [7]Department of Odontology, Umeå University, Umeå, Sweden

Introduction: Periodontitis is associated with rheumatoid arthritis (RA). One hypothesis posits that this connection arises from the formation of autoantibodies against citrullinated proteins (ACPA) in inflamed gums, possibly triggered by *Porphyromonas gingivalis*. We previously demonstrated an increased antibody response to *P. gingivalis* arginine gingipains (anti-Rgp IgG), not only in individuals with severe periodontitis compared to controls, but in RA versus controls, with an association to ACPA. In the present study, we set out to further explore the relationship between anti-Rgp IgG, ACPA and periodontitis, including clinical periodontal parameters, in the large and well-characterized PerioGene North case-control study.

Methods: We measured serum levels of anti-Rgp and ACPA IgG by enzyme-linked immunosorbent assay (ELISA), in 478 patients with periodontitis and 509 periodontally healthy controls within PerioGene North. Subsequently, anti-Rgp IgG levels and ACPA status were analysed in relation to periodontitis and clinical periodontal parameters.

Results: Serum anti-Rgp IgG levels were elevated in cases versus controls ($p< 0.001$). However, receiver operating characteristic (ROC) curve analysis revealed that anti-Rgp IgG could not efficiently discriminate cases from controls (AUC= 0.63; 95% CI: 0.60 – 0.66). Among cases, increased anti-Rgp IgG levels associated with high periodontal inflammation and advanced alveolar bone loss ($p<0.001$ for both). An ACPA response was detected in 15 (3.1%) cases and

6 (1.2%) controls (p=0.033), but no association to periodontitis was evident after adjustment for age and smoking and anti-Rgp IgG levels did not differ between ACPA-positive and ACPA-negative individuals.

Conclusion: We show that anti-Rgp IgG identifies a subgroup of periodontitis patients with high degree of periodontal inflammation and advanced alveolar bone loss, but we do not find support for a link between periodontitis or anti-Rgp IgG and ACPA status in PerioGene North. Given the association between anti-Rgp and alveolar bone loss, the mechanistic role of gingipains in bone resorption should be experimentally explored.

KEYWORDS

periodontitis, alveolar bone loss, periodontal inflammation, cysteine peptidase gingipain B, anti-citrullinated protein antibodies

1 Introduction

Periodontitis is an oral condition that is characterized by progressive destruction of the tooth supporting tissues, including both collagenous connective tissue of the gingiva and the tooth anchoring jawbone (1). In periodontally healthy states, indigenous polymicrobial communities at mucosal surfaces maintain an ecological balance via inter-microbial and host-microbial interactions. However, genetic and acquired factors, most notably smoking, but also obesity, immune deficiencies, immunoregulatory defects, diabetes mellitus and other systemic diseases may disrupt this homeostatic balance, leading to selective growth of species with the potential for destructive inflammation. This condition, known as dysbiosis, underlies the development of periodontitis in susceptible hosts. The pathogenic process is not linear, but involves a positive-feedback loop between a dysbiotic microbiota on the tooth surface below the gum line and the host inflammatory response (2). The molecular pathways underpinning tissue destruction have not been clarified, and there are currently no biomarkers available to help clinicians predict onset of periodontitis or identify individuals at risk of a more aggressive disease course.

Besides the local and detrimental effects on the periodontium that jeopardize tooth retention, the disease is strongly connected to a number of other non-communicable diseases, including rheumatoid arthritis (RA) (3). Different models for a causal and reciprocal relationship between periodontitis and RA have been described (4). In support of a causal link between periodontitis and RA, results from experimental studies show that periodontitis can exacerbate arthritis (5). Our findings of increased alveolar bone loss in individuals with high levels of anti-citrullinated protein antibodies (ACPA), before RA onset, imply that periodontitis predates the onset of ACPA-positive RA (6). However, it remains elusive whether there is a causative link.

The gram-negative anaerobic oral bacterium *Porphyromonas gingivalis (P. gingivalis)* is frequently detected in subgingival plaque of periodontitis patients (7). *P. gingivalis* expresses multiple virulence factors for its growth, survival, and immune evasion (8), including the arginine gingipains (Rgp), potent extracellular cysteine proteases which efficiently degrade host proteins by cleaving polypeptides C-terminal of arginine (9, 10). Protein degradation by gingipains can facilitate further enzymatic processing by *Porphyromonas* peptidylarginine deaminase, resulting in citrullinated neoepitopes (11). Gingipains have also been described to contribute directly to the inflammatory response by cleavage of protease activated receptor-2 on the neutrophil surface, triggering formation of neutrophil extracellular traps with the release of endogenously citrullinated proteins (12). Notably, citrulline-reactive B cells have been successfully isolated from gingival tissue of periodontitis patients, suggesting that break of tolerance and ACPA production in RA may take place in inflamed gums (13). Moreover, gingipains are highly immunogenic (14), and we have previously shown an increased anti-Rgp antibody response, not only in individuals with periodontitis compared to controls, but also in RA, especially ACPA-positive RA, compared to controls even before RA onset (15, 16). We have previously demonstrated associations between anti-Rgp IgG and alveolar bone loss, as well as presence of ACPA in PAROKRANK, a case-control study including patients hospitalized for a first myocardial infarction. Interestingly, we also found a particularly strong association between anti-Rgp IgG and severe periodontitis in a subfraction of PerioGene North study (17). Therefore, *P. gingivalis* antibodies, more specifically gingipain antibodies, are gaining increased attention as a potential biomarker for individuals with periodontitis and concomitant risk for systemic autoimmunity (13, 16–18).

In summary, accumulating evidence suggests that periodontitis is linked to RA-specific autoimmunity through a loop in which *P. gingivalis* promotes ACPA formation. In the present study, we address this link by analysing the association between periodontal clinical parameters, anti-Rgp IgG and ACPA in 987 individuals within the large and well-characterized PerioGene North case-control study (19).

2 Material and methods

2.1 Study design

PerioGene North is a multicentre case-control study consisting of 526 periodontitis cases and 532 periodontally healthy controls. Study participants were consecutively recruited between 2007 and 2019, from specialist clinics and general dental care within the counties of Västerbotten, Gävleborg, Uppsala and Västmanland in northern Sweden. Cases were examined by senior consultants in periodontology and controls were examined by general dentists. To validate the absence of alveolar bone loss in controls, all radiographs were reviewed by senior consultants in periodontology.

2.2 Clinical data collection

All participants underwent a complete oral and periodontal examination, including registration of periodontal inflammation, measured as bleeding on probing (BoP), and periodontal probing pocket depth (PPD), at six sites per tooth using a PCP-12, 3-6-9-12 (Hu-Friedy) dental probe. Furcation involvement was assessed but not registered in the study protocol. Alveolar bone loss was assessed for each tooth using dental radiographs (bitewing and apical images). Information regarding number of teeth per quadrant with PPD (< 4 mm, 4-6 mm and > 6 mm) and degree of alveolar bone loss (< 1/3, ≥ 1/3 to ≤ 2/3 or > 2/3 of the root length) was registered in the study protocol. Data collection was conducted before the present classification for periodontitis was introduced (20). By using a proposed framework for applying the 2018 periodontal status classification on completed epidemiological studies, we concluded that all cases fulfilled the stage III criteria and 87 cases (18.2%) with <20 teeth could be classified as stage IV (21). This means that all cases presented with severe periodontitis. Cases were further subcategorized based on level of BoP (high ≥20%, low <20%), PPD and alveolar bone loss according to previous studies (22).

Degree of periodontal inflammation: A low degree of periodontal inflammation was defined as having BoP < 20%. High degree of periodontal inflammation was defined as having BoP ≥ 20%.

Level of periodontal pocket depth and alveolar bone loss: Each tooth was given a score of 1-3 depending on the PPD/alveolar bone loss value. A PPD < 4 mm or alveolar bone loss < 1/3 of the root length was given the score 1. A PPD between 4 and 6 mm or alveolar bone loss between ≥ 1/3 to ≤ 2/3 of the root length was given the score 2. A PPD > 6 mm or alveolar bone loss > 2/3 of the root length was given the score 3. The total score for the entire dentition was summed up and then divided by the number of teeth. In the present study, a score between 1.01 - 1.49 was referred to as a low degree of periodontal probing depth or alveolar bone loss, 1.50 – 1.99 as moderate, and over 2.0 as a high level.

Information about sex, birth country, past and/or current tobacco use, education level and awareness of parent with periodontitis (self-reported heredity) was recorded. Information about height and weight was obtained for calculation of body mass index (BMI). Information about general health (diseases according to ICD-10 categories) was obtained from registries of the National Board of Health and Welfare in Sweden.

2.3 Inclusion and exclusion criteria

Inclusion criteria for cases were i) having at least one tooth in each quadrant with alveolar bone loss ≥ 1/3 of the root length and ii) having ≥15 remaining teeth, or ≥8 if teeth were only present in one jaw. Cases with alveolar bone loss that could be explained by local aggravating factors such as root fractures or pulpal infections were excluded. The cases were included after being refered to specialist clinics from generel dental care and had received previous periodontal treatment to various extent. Inclusion criteria for controls were i) no alveolar bone loss, i.e. <3 mm distance from the cementoenamel junction to the bone crest, ii) PPD <4 mm, iii) having ≥24 remaining teeth and iv) being ≥ 34 years of age. Participants with known contagious blood diseases were excluded.

2.4 Blood sampling

A venous blood sample of 3 x 10 mL was collected from all participants at inclusion. The participants were not fasted at the time of sampling. Collection and handling of blood samples, including fractionation into plasma, serum, and buffy coat, and storage at -80°C followed the standardized routines at the Medical Biobank of Northern Sweden, Västerbotten County Council, Sweden.

2.5 Antibody measurements

Anti-Rgp IgG in serum were measured using a previously described *in-house* enzyme-linked immunosorbent assay (ELISA) (15, 17), with purified, recombinant hexahistidine-tagged RgpB protein as coating agent (23). To compare serum samples analysed on different ELISA plates, anti-Rgp IgG levels were presented as arbitrary units (AU) with interquartile ranges (IQR) calculated from a standard curve (a pool of anti-Rgp IgG positive sera in 1:1 x7 serial dilution) included on all plates. Serum samples were analysed in duplicates and blank wells were included on all plates to account for background signal.

Presence ACPA was measured as anti-cyclic citrullinated peptide2 (CCP2) IgG, using the Immunoscan CCPlus® test kit (Svar Life Science, Malmö, Sweden), according to manufacturer's instructions. Serum samples were analysed in single wells, with positive samples (≥25U/mL) re-analysed in duplicates.

2.6 Statistical methods

Descriptive analyses were used for frequency distributions of categorical variables, whereas median values with interquartile range were calculated for continuous variables. Group comparisons were conducted utilizing chi-square tests (categorical variables) or the non-parametric Mann-Whitney U test or Kruskal-Wallis test (continuous variables). Log-transformed anti-Rgp IgG levels were analyzed in relation to periodontitis, periodontal inflammation, periodontal pocket probing depth and alveolar bone loss, using a linear regression model, adjusted for age, age^2, sex and smoking. Results

are presented as Exp(B) = exponential B (interpretable as multiplicative effect), with 95% confidence interval (CI) and p-value. Adjustment for multiple testing was performed with Tukey's method. P-values <0.05 were considered statistically significant. Receiver operating characteristic curves (ROC) was used to evaluate the discriminatory performance of anti-Rgp IgG to distinguish cases from controls and to determine the area under the curve (AUC). Youden's J statistic was used to determine the cutoff with highest sensitivity and specificity.

Statistical Package for Social Sciences (SPSS), version 26 (IBM Corporation, Armonk, NY, USA), and R, version 4.2.3 (R foundation for statistical computing, Vienna, Austria), with packages: *tidyverse, magrittr, haven, psych, cutpointr, pRoc, emmeans, gtsummary*, and *brglm2*, were used for the analyses.

3 Results

3.1 Study population characteristics

Among 1,058 enrolled study participants, 60 were excluded in the present study due to missing clinical data or missing serum, and eleven were excluded due to not fulfilling the inclusion criteria, resulting in 478 study participants with periodontitis (referred to as cases) and 509 periodontally healthy (referred to as controls). The distribution of men and women was similar between cases and controls, but cases were older and had a higher proportion of smokers. Two controls and three cases were diagnosed with rheumatoid arthritis (Table 1). A comprehensive description of the study population characteristics has been published previously (19).

3.2 High anti-Rgp IgG levels are associated with periodontitis, but show poor potential to discriminate between cases and controls

An antibody response towards *P.gingivalis* Rgp was evident in both periodontitis cases and controls, with higher median anti-Rgp IgG values for cases (237.2 AU; IQR= 125.3-611.9) than controls (154.2 AU; IQR= 79.4-300.8), p<0.001 (Table 1 and Figure 1A). The average increase of anti-Rgp IgG levels in cases versus controls was 71% (p<0.001) when adjusted for age, age^2, sex and smoking (Table 2). A ROC curve analysis showed that anti-Rgp IgG levels could separate cases from controls with a sensitivity of 37.5% and specificity of 83.1% (AUC=0.63; 95% CI: 0.60 – 0.66), demonstrating a weak discriminatory ability (Figure 1B).

3.3 Anti-Rgp IgG levels associate with periodontal inflammation and alveolar bone loss

Next, we analyzed anti-Rgp IgG levels in relation to different clinical periodontal parameters; periodontal inflammation, measured as bleeding on probing, (BoP), periodontal probing pocket depth (PPD), and alveolar bone loss. We detected significantly higher anti-Rgp IgG levels in cases with high periodontal inflammation (Figure 2A). The average increase in anti-Rgp IgG levels in cases with high versus low periodontal inflammation was 50%, p<0.001 (Table 3). We also found higher anti-Rgp IgG levels in cases with high level of alveolar bone loss (Figure 2B). For cases with high versus low level of alveolar bone loss, the average increase of anti-Rgp IgG levels was 65%, p<0.002 (Table 3), and here we also observed a significant increase (40%) when comparing cases with high versus moderate level of alveolar bone loss, p=0.042. We found no association between anti-Rgp IgG levels and level of PPD (Figure 2C, Table 3). These analyses were adjusted for the confounding effects of age, age^2, sex and smoking.

3.4 RA-specific autoimmunity does not associate with periodontitis or anti-Rgp IgG

Next, we analyzed the presence of ACPA IgG in PerioGene North. When applying the manufacturer's suggested cutoff value of ≥25 U/mL, 21 individuals were considered ACPA-positive. Notably, 15 of these had periodontitis, giving a frequency of 3.1% ACPA-positive cases and 1.2% ACPA-positive controls, p=0.003 (Table 1). Among the ACPA-positive participants, 1 case and 2 controls had been diagnosed with RA. Yet, there was no significant association between periodontitis and ACPA after adjusting for age and smoking (data not shown). Furthermore, anti-Rgp IgG levels did not differ significantly between ACPA-positive and ACPA-negative individuals (data not shown).

4 Discussion

In this study, we demonstrate that serum anti-Rgp IgG levels cannot clearly discriminate individuals with periodontitis from periodontally healthy but define a subgroup of periodontitis patients with high periodontal inflammation and advanced alveolar bone loss. Moreover, we find no strong support for a link between periodontitis or anti-Rgp IgG and RA-specific autoimmunity, e.g. ACPA.

Antibodies against periodontal pathogens have been investigated for many years (24). In particular, antibodies against *P. gingivalis* have been shown to associate with periodontitis (25), and among 29 different *P. gingivalis* antigens, Hirai and co-authors recently showed that the gingipains elicited the most sensitive and specific IgG responses in patients with periodontitis (14), supporting the use of Rgp in our study.

The data presented herein clearly show that individuals with periodontitis present with higher anti-Rgp IgG levels than periodontally healthy controls, which is in accordance with our previous findings (17). It should be noted that high anti-Rgp IgG levels were also detected among some of the controls. Studies have suggested that periodontally healthy individuals are able to produce highly functional antibodies, protecting against *P. gingivalis* colonization, whereas periodontitis patients produce less functional antibodies with lower avidity (26). Hence, we speculate that the

TABLE 1 Study participant characteristics in PerioGene North.

Baseline characteristics	Control (n=509)	Case (n=478)	Total (n=987)	*P*-value
Age, median (IQR)	44.0 (39.0-50.0)	59.0 (49.0-66.0)	50.0 (41.0-60.0)	< 0.001
Sex (%)				
Male	218 (42.8)	198 (41.4)	416 (42.1)	0.699
Female	291 (57.2)	280 (58.6)	571 (57.9)	
Smokers[a], *n* (%)				
No	404 (79.4)	120 (25.1)	524 (53.1)	< 0.001
Yes	105 (20.6)	358 (74.9)	463 (46.9)	
Periodontal parameters				
Number of teeth	28 (27-28)	24 (21-27)	27 (24-28)	< 0.001
BoP %, median (IQR)	5 (1-12)	25 (13-44)	12 (4-31)	< 0.001
BoP level[b] (%)				
Low (< 20%)	N/A	185 (38.7)	N/A	
High (≥ 20%)	N/A	291 (60.9)	N/A	
PPD level[b] (%)				
None	N/A	7 (1.5)	N/A	
Low	N/A	75 (15.7)	N/A	
Moderate	N/A	193 (40.4)	N/A	
High	N/A	203 (42.5)	N/A	
Alveolar bone loss level[b] (%)				
Low	N/A	117 (24.5)	N/A	
Moderate	N/A	246 (51.5)	N/A	
High	N/A	115 (24.1)	N/A	
RA status and antibody levels				
Rheumatoid arthritis (%) *(ICD-10 code M05-06)*	2 (0.4)	3 (0.6)	5 (0.5)	0.604
anti-Rgp IgG, median AU (IQR)	154.2 (79.4-300.8)	237.2 (125.3-611.9)	191.8 (98.5-402.4)	< 0.001
ACPA+, *n* (%)	6 (1.2)	15 (3.1)	21 (2.1)	0.033

IQR, interquartile range; BoP, bleeding on probing; PPD, pocket probing depth; AU, arbitrary units; N/A, not applicable as only cases were subcategorized according to BoP, PPD and alveolar bone loss.

a) Smokers include current and former smokers.

b) Subcategorization of periodontal parameters within the case group. Group categorization was based on a PPD/alveolar bone loss score. None = ≤ 1.00, low = 1.01-1.49, moderate = 1.50 – 1.99, high = ≥ 2.00. The score was calculated by assigning each tooth a score of 1-3 depending on the degree of PPD/alveolar bone loss. The total score for the entire dentition was summed and then divided by the number of teeth.

presence of anti-Rgp antibodies in periodontally healthy controls in our study reflects a healthy immune response, while high levels of anti-Rgp IgG that we see in a subset of periodontitis patients indicate high *P. gingivalis* counts and high pathogenic gingipain load. In support of this scenario, several studies showed decreased anti-*P. gingivalis* antibody levels in response to successful periodontal treatment, following lower bacterial counts (27–29).

Our previous data (17), based on 41 cases and 39 controls from PerioGene North, indicated a very good capacity for anti-Rgp antibodies to discriminate cases from controls (AUC=0.79). Thus, we wanted to further validate our data in the entire cohort. However, in the present study, anti-Rgp IgG had poor discriminatory ability to distinguish cases from controls (AUC=0.63). The discrepant data between the two studies suggests that the sub-cohort is unrepresentative of the entire PerioGene North cohort. Moreover, the results in the present study are in agreement with our previous findings in PAROKRANK, a case-control study designed to investigate periodontal health in relation to myocardial infarction, where anti-Rgp IgG also could not separate periodontitis patients from controls efficiently, but was still significantly elevated in individuals with severe forms of periodontitis compared to controls (17).

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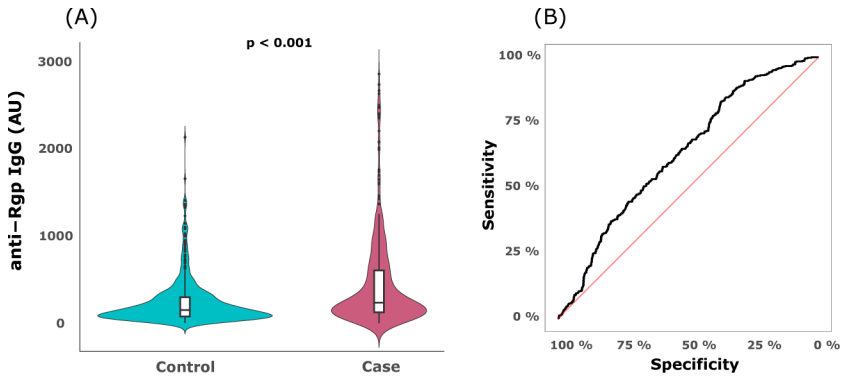

FIGURE 1
Serum anti-Rgp IgG levels are elevated in periodontitis, but do not discriminate cases from controls. **(A)** The violin plot shows distribution of serum anti-Rgp IgG levels in PerioGene North cases (n=478) and controls (n=509). Integrated box plots show median AU values and 25th and 75th percentiles as horizontal lines; whiskers indicate the 10th to 90th percentile; outliers plotted as dots. **(B)** Receiver operating characteristic (ROC) curve for prediction of cases and controls based on serum levels of anti-Rgp IgG. Rgp = arginine gingipain; AU = arbitrary units.

In here, we present significant associations between anti-Rgp IgG and specific clinical periodontal parameters, periodontal inflammation and alveolar bone loss. Thus, collectively, these data indicate that anti-Rgp antibodies are not likely to perform well as predictive biomarkers for periodontitis *per se*, while they may still be valuable indicators of risk for severe periodontal damage.

Gingipains are known for their proteolytic capacity, including the degradation of components of the complement system and extracellular matrix structures (30). They can directly contribute to gingival bleeding and tissue breakdown by degrading fibrinogen (31). Interestingly, gingipains have also been shown to promote osteoclastogenesis *in vitro*, thus implicating a possible role in bone resorption (32). Noteworthy, both gingipain vaccines and gingipain-inhibitors have been used successfully to prevent *P. gingivalis*-induced periodontal damage in experimental models (33, 34). Moreover, we have previously shown elevated anti-Rgp antibody levels in RA versus controls (15), and given the results in the present study – demonstrating an association between high anti-Rgp antibody levels and advanced alveolar bone loss – anti-Rgp IgG levels should potentially also be examined in relation to articular bone loss in RA. Notably, a recent study links oral bacteremia to RA flares (35), and other studies have detected *P. gingivalis* DNA in RA synovial joints (36, 37). Thus, the mechanistic role of gingipains in inflammation-mediated bone resorption should be further explored.

TABLE 2 Association between serum anti-Rgp IgG and periodontitis.

	Anti-Rgp IgG level	*P-value*
	Exp(B) (95% CI)	
Category		
Control	Reference	
Case	1.71 (1.45-2.02)	< 0.001
Covariate		
Sex		
Men	Reference	
Women	0.92 (0.81-1.11)	0.226
Smoker[a]		
No	Reference	
Yes	0.92 (0.79-1.08)	0.302
Age	0.98 (0.93-1.03)	0.347
Age^2	1.00 (1.00-1.00)	0.375

Anti-Rgp IgG levels were analyzed in relation to periodontitis using a linear regression model. Age, age^2, sex and smoking were included in the model as covariates. B = regression coefficient; Exp(B) = exponential B (interpretable as multiplicative effect); CI = confidence interval. [a]Smokers includes current and former smokers.

Several studies have linked periodontitis and periodontal pathogens to ACPA-positive RA (15, 35, 38), including a recently published meta-analysis demonstrating that RA patients with periodontitis have significantly higher ACPA levels than RA patients without periodontitis (39). However, until now, it has not been clear if periodontitis alone is sufficient to elicit a systemic ACPA response, as previous studies are sparse and present conflicting results. The present study is, to the best our knowledge, the first to investigate ACPA status in a large and well-characterized periodontitis case-control study. Although we observed a higher frequency of ACPA-positivity among periodontitis patients compared to controls, in line with our previous report (17), this was not significant after adjustment for the confounding effects of smoking and age. Given that only 15 of 478 individuals with periodontitis were ACPA-positive, we were underpowered to analyze ACPA in relation to periodontal parameters.

In accordance with the data herein, a cross-sectional study by Lew and co-authors did not find differences in ACPA levels when comparing periodontitis patients without RA and healthy controls (40). In here, we did not find an association between ACPA status and anti-Rgp. Similarly, Svard et al. found no association between anti-Rgp antibodies in saliva and periodontitis or ACPA status (41).

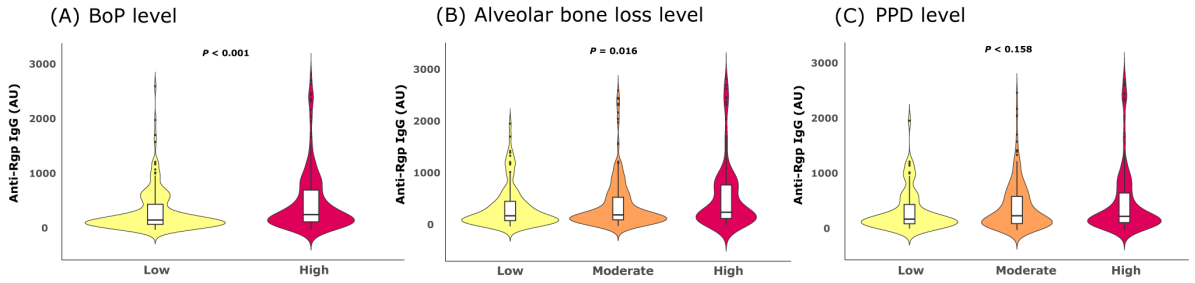

FIGURE 2
Anti-Rgp IgG levels are elevated in cases with periodontal inflammation and advanced alveolar bone loss. Violin plots show distribution of Rgp IgG levels in PerioGene North cases, based on: **(A)** degree of periodontal inflammation, measured as bleeding on probing (BoP); **(B)** level of alveolar bone loss and **(C)** periodontal pocket probing depth (PPD) level. Integrated box plots show median AU values and 25th and 75th percentiles as horizontal lines; whiskers indicate 10th to 90th percentile; outliers are plotted as dots. Rgp = arginine gingipain; AU = arbitrary units.

In summary, the data we present are based on a large and well-characterized periodontitis cohort and suggest that there is not convincing evidence for that a systemic ACPA response is connected to periodontitis, or elevated anti-Rgp IgG levels in periodontitis patients. Still, this does not rule out that locally produced ACPA in inflamed gingival tissue are involved in onset and/or progression of RA in susceptible individuals. Notably, ACPA have been detected in gingival crevicular fluid from non-RA individuals (42).

The main strength with the present study is the significant power of PerioGene North, owing to its large number of study participants and the high resolution clinical periodontal data provided by trained specialists. Within this material, we have previously described serum levels of hs-CRP that are in line with results presented in a meta-analysis (43), implying that PerioGene North is well suited for investigating, and detecting, serum markers associated with periodontitis. Unlike the periodontitis cases in PAROKRANK, where more than half were affected by myocardial

TABLE 3 Average increase in anti-Rgp IgG levels between different clinical subgroups of periodontitis cases.

Cases	BoP (level)		PPD (level)		Alveolar bone loss (level)	
	Exp(B) (95%CI)	*P-value*	*Exp(B) (95%CI)*	*p-value*	*Exp(B) (95%CI)*	*p-value*
Category						
Low	Reference					
Moderate	N/A	N/A	1.18 (0.88-1.59)	0.262	1.22 (0.92-1.64)	0.228
High	1.50 (1.22-1.83)	< 0.001	1.32 (0.98-1.76)	0.064	1.65 (1.17-2.33)	0.002
Moderate vs High	N/A	N/A	1,12 (0.84-1.48)	0.749	1.4 (1.0-1.8)	0.0417
Covariate						
Sex						
Men	Reference					
Women	0.82 (0.67-1.00)	0.048	0.82 (0.67-1.00)	0.055	0.81 (0.67-0.99)	0.044
Smoker[a]						
No	Reference					
Yes	0.75 (0.60-0.94)	0.015	0.73 (0.58-0.92)	0.008	0.72 (0.57-0.91)	0.006
Age	0.99 (0.93-1.05)	0.745	0.98 (0.92-1.04)	0.479	2.43 (0.28-20.9)	0.418
Age^2	1.00 (1.00-1.00)	0.616	1.00 (1.00-1.00)	0.407	3.47 (0.40-29.7)	0.257

a) Smokers include current and former smokers.
Anti-Rgp IgG levels were analyzed in relation to periodontitis subcategories using a linear regression model. Age, age^2, sex and smoking were included in the model as covariates. Comparisons between BoP level is only applicable between low and high groups since it is a dichotomous variable. B = regression coefficient; Exp(B) = exponential B (interpretable as multiplicative effect); CI, confidence interval; BoP, bleeding on probing; PPD, pocket probing depth; N/A, not applicable.

infarction, the overall presence of general diseases in PerioGene North is low (19). Therefore, the elevated anti-Rgp IgG levels observed in PerioGene North are less likely to be affected by the presence of other systemic diseases.

A limitation with our study was that the case group, due to the original purpose of PerioGene North to study genetic polymorphisms, were not matched to controls on age. However, all controls were 34 years or older, which is a strength as many other studies have included young adults as periodontally healthy controls. Still, this resulted in an older case group and all analyses were subsequently adjusted for age as well as for smoking, both well-known risk factors for periodontitis (44, 45). In our previous report (19), *post-hoc* analysis within different age groups confirmed that the distinguishable protein profile was not a result of the case group being older.

In conclusion, our data show that periodontitis *per se* is not associated with the presence of a systemic ACPA response and that ACPA-positive individuals in this cohort do not have an increased antibody response to *Pg* virulence factor arginine gingipain. While anti-Rgp IgG showed poor ability to separate patients with periodontitis from periodontally healthy in an efficient manner, elevated levels associate significantly with the subset of patients that have active periodontal inflammation and advanced alveolar bone loss. Prospective studies could reveal whether high anti-Rgp IgG levels can serve as a biomarker to predict a more aggressive disease course. Future studies should also address the actions of gingipains in the context of bone resorption, to clarify molecular mechanisms, and further explore whether blocking gingipains could be a future treatment for periodontitis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Regional Ethical Review Board at Umeå University, the Regional Ethical Review Board at Uppsala University and the Swedish Ethical Review Authority. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in the study.

Author contributions

EK: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. Cd: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. MW: Formal analysis, Investigation, Methodology, Writing – review & editing. BP: Methodology, Resources, Writing – review & editing. JP: Funding acquisition, Methodology, Resources, Writing – review & editing. SL: Methodology, Project administration, Writing – review & editing. AE: Formal analysis, Methodology, Supervision, Writing – review & editing. KL: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. PL: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor TV declared a past co-authorship with the author JP.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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