IL-37- and IL-35/IL-37-Producing Plasma Cells in Chronic Periodontitis

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Abstract

Periodontitis is one of the most prevalent chronic inflammatory diseases and is induced by the interaction between oral microorganisms and the host immune system. Plasma cells are of special interest in chronic periodontitis (CP), as they represent ~50% of infiltrated immune cells in periodontal lesions. Plasma cells constitute the only known cell type capable of antibody production; however, recent evidence supports an emerging role for distinct sets of plasma cells in cytokine production. However, the presence of cytokine-producing plasma cells in CP is unknown. In this study, we used immunohistochemistry to detect significantly elevated levels of IL-35 and IL-37 (2 recently identified anti-inflammatory cytokines) in CP gingival tissue as compared with healthy tissue. Remarkably, we demonstrate that CD138⁺ CD38⁺ plasma cells are the major immune cell type in CP gingival tissues and that these cells produce IL-35 and IL-37. We used immunofluorescence and confocal microscopy analysis to identify a subset of plasma cells with robust cytoplasmic expression of IL-37—we denote this subset as IL-37-producing plasma cells (CD138⁺ CD38⁺ P_{IL-37}). Another subset of plasma cells coproduces IL-35 and IL-37 and is denoted as IL-37/IL-35-coproducing plasma cells (CD138⁺ CD38⁺ P_{IL-35/IL-37}). We determined that these 2 plasma cell subsets are IgG⁺ plasma cells. Moreover, we show that human recombinant IL-35 and IL-37 exhibit a dose-dependent inhibition of osteoclast formation in vitro (~78.9% and 97.7% inhibition in 300 ng/mL of IL-35 and IL-37, respectively, $P < 0.05$). Overall, our findings suggest that P_{IL-37} and P_{IL-35/IL-37} exist as subsets of plasma cells in CP lesions and that these 2 new types of plasma cells may regulate periodontitis pathogenesis by inhibiting alveolar bone loss through directly blocking osteoclast formation. Importantly, these data suggest a novel role of plasma cells and offer potential new mechanistic and regulatory targets to be investigated in the context of periodontal health and disease.

Keywords: osteoclast, periodontal disease, inflammation, cytokines, pathogenesis, adaptive immunity

Introduction

Chronic periodontitis (CP) is a common disease of the oral cavity, consisting of chronic inflammation of periodontal tissues. Periodontitis affects an estimated 10% to 15% of the world's population (Gross et al. 2017), including 64.7 million US adults, with 8.9% of them having severe CP (Eke et al. 2012; Eke et al. 2016). It leads to the progressive destruction of the tooth-supporting tissues and is associated with certain systemic disorders (Lin et al. 2007; Pinho et al. 2013).

The initiation and progression of CP depend on the interaction between oral microorganisms and the host immune system (Darveau 2010; Hajishengallis and Lamont 2012). The host inflammatory response in periodontitis is complex, including innate (neutrophils and macrophages) and adaptive (T and B lineage cells) immune cells (Benakanakere and Kinane 2012; Gonzales 2015). Remarkably, clinical investigations demonstrated that among patients with CP, B lineage cells (including B and plasma cells) dominated the infiltrated immune cells in periodontal lesions. In fact, B lineage cells make up about 70% of the total immune infiltrates, while plasma cells represent approximately 50% of the total in periodontitis lesions (Afar et al. 1992; Berglundh et al. 2007). These findings suggest an important role of plasma cells in CP.

Plasma cells, differentiated from B lymphocytes, are the only cell type in the organism capable of producing antibodies. The traditional immunologic dogma has been that plasma cells are serving primarily as professional antibody factories. However, reports recently identified plasma cells as the major source of B lineage cells, which display the capacity to

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A supplemental appendix to this article is available online.

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produce cytokines (Fillatreau 2015). These studies challenge the traditional dogma and suggest the existence of distinct sets of plasma cells being involved in the production of different cytokines, including IL-10, IL-35, IL-17, GM-CSF, and iNOS (Bermejo et al. 2013; Shen et al. 2014; Wang et al. 2014). Cytokine-producing plasma cells may play the critical role of immune regulation during autoimmune and infectious diseases (Dang et al. 2014; Ries et al. 2014).

IL-35 and IL-37 are recently discovered immune-suppressing cytokines (Hu 2017). IL-35 belongs to the IL-12 family and plays an inhibitory role in arthritis, asthma, and inflammatory bowel disease (Behzadi et al. 2016). IL-37 is a new member of the IL-1 family and functions as a master regulator with broad anti-inflammatory activities (Palomo et al. 2015). Some evidence supports a role for IL-35 and IL-37 in the pathogenesis of periodontitis (Mitani et al. 2015; Giacoppo et al. 2017). Moreover, IL-37 was shown to be expressed in plasma cells associated with breast cancer tissue (Kumar et al. 2002). It was also demonstrated that activated B cells, particularly plasma cells, can robustly produce IL-35 for immune regulation in mice (Shen et al. 2014).

Thus far, it remains unknown whether the infiltrated plasma cells in CP lesions display immune regulatory function by producing anti- or proinflammatory cytokines. To address this knowledge gap, we sought to investigate the production of IL-35 and IL-37 by plasma cells infiltrated in human gingival tissue from patients with CP. We demonstrate significantly elevated IL-35 and IL-37 levels in CP gingival tissue as compared with healthy tissue. In addition, we report a novel finding of 2 subtypes of CD138⁺CD38⁺IgG⁺ plasma cells that can produce IL-35 and IL-37 in CP gingival tissues. We further demonstrate the inhibitory role of IL-35 and IL-37 on the formation of osteoclasts *in vitro*. Taken together, our data suggest a new role of infiltrated plasma cells to potentially regulate bone loss by producing IL-35 and IL-37 in periodontitis.

Materials and Methods

Tissue Sample (Biopsy) Collection

This study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (15-0335). All subjects enrolled into this study provided written informed consent. Two gingival biopsies were harvested from healthy tissue and chronic lesions ($n = 10$) from patients with periodontitis, per traditional periodontal disease classification from the American Academy of Periodontology/American Dental Association (American Academy of Periodontology Task Force 2015). Notably, biopsies were collected only from the papillae of posterior teeth (≤ 0.5 cm thick), capturing the base of the periodontal pocket or gingival sulcus. Each biopsy contained oral epithelium and gingival connective tissues. Detailed inclusion and exclusion criteria are included in the Appendix.

Immunohistochemistry and Quantification

The gingival biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemistry (IHC), immunofluorescence staining, and analysis. They were sectioned in the sagittal direction, including the epithelial and connective tissue in the same slide (5 μ m). The slides were stained with anti-human IL-35, IL-37, CD138, CD38, IgG, and IgM antibody (Appendix Table 1) according to the manufacturer's instructions and counterstained with hematoxylin. Photo images were captured with an Olympus BX61 microscope. The IHC stained sections were digitally imaged (20 $\times$ objective) in an Aperio ScanScope XT with line-scan camera technology (Leica Biosystems). The Genie algorithm was used to segment tissue for the epithelial and nonepithelial (connective tissue) regions of interest (ROIs). Epithelial ROIs were excluded from the analysis. The Aperio eSlide Manager Software (Aperio color deconvolution algorithm and the Genie pattern recognition software) was used to quantify brown stain of the nonepithelial ROIs in digital images.

Immunofluorescent Staining and Confocal Microscopy

Colocalization of IL-35, IL-37, CD138, CD38, and human IgG was determined with distinct fluorescence-conjugated secondary antibodies (Appendix Table 2). The combination of secondary antibodies is listed in Appendix Table 3. Nuclei were stained by DAPI staining according to the manufacturer's instructions (10236276001; Sigma). Photo images for fluorescence staining were captured with a Zeiss LSM 700 confocal microscope.

Recombinant Human IL-35 (rhIL-35) and IL-37 (rhIL-37b)

rhIL-35 was purchased (8608-IL-050; R&D Systems) and reconstituted into sterile phosphate-buffered saline for an *in vitro* osteoclast assay. The mature form of rhIL-37b (21-218 amino acid) was cloned into pET-30a (+) with 6 His tag at C-terminal. It was expressed in *Escherichia coli* BL21 (DE3) and purified through an Ni-NTA agarose gel column. Purified proteins were freeze-dried by lyophilization and kept in -20°C for phosphate-buffered saline reconstitution before the *in vitro* osteoclast assay.

rhIL-35 and rhIL-37 Inhibitory Assay in Osteoclast Formation

Four- to 6-wk-old mice were euthanized with CO₂, and bones (fibula and femurs) were excised under sterile conditions. Bone marrow was flushed out into α MED and cultured for 24 h

in a CO₂ incubator. Nonadherent cells were collected after 24-h incubation and plated with 30-ng/mL M-CSF (416-ML-050; R&D Systems). After 3 d, osteoclast precursors were detached in cold phosphate-buffered saline with a cell scraper and replated onto a 96-well plate at a density of 2×10^4 cells/well with 30-ng/mL CSF-1 and 10-ng/mL RANKL (462-TEC-010/CF; R&D Systems) for osteoclast formation. For the IL-35 and IL-37 inhibitory assay, the rhIL-35 or rhIL-37 was added into the culture medium with M-CSF and RANKL in gradient concentrations. Media were changed after 3-d differentiation with M-CSF and RANKL. Cells were fixed by 10% neutralized formalin for TRAP (tartrate-resistant acid phosphatase) staining on day 5. Images were captured by Leica WILD Macroscope M420. Osteoclast numbers per well were counted with ImageJ software.

Statistical Analysis

Two-tailed unpaired *t* tests were used to determine IL-35 and IL-37 expression differences between healthy and CP gingival biopsies, with a conventional statistical significance criterion of $P < 0.05$. The inhibitory activity of IL-35 and IL-37 to osteoclast formation in vitro was analyzed with 1-way analysis of variance, followed by Dunnett's post hoc test and a $P < 0.05$ criterion. Analyses were performed with GraphPad Prism 7.04.

Results

Expression of IL-35 and IL-37 in CP and Healthy Gingival Tissues

Previous studies detected IL-35 and IL-37 in human gingival crevicular fluid or saliva in periodontal disease (Koseoglu et al. 2015; Saglam et al. 2015); however, the cellular origin of IL-35 and IL-37 production is unclear. To determine the localization of IL-35 and IL-37 in human gingival tissue, we performed IHC staining of IL-35 and IL-37 with human gingival biopsies from CP and healthy tissue. We found positive staining of IL-35 and IL-37 (Fig. 1A, B).

We also determined the cell populations for IL-35 and IL-37 production in human gingival tissue by morphology. Our data indicated that IL-35 was mainly expressed by the infiltrated immune cells in the connective tissue (Fig. 1A, *a–c*). In contrast, IL-37 was predominantly expressed in epithelial cells, endothelial cells, and infiltrated immune cells of the connective tissue (Fig. 1B, *a–c*). Surprisingly, we observed that the morphology of the dominant inflammatory infiltrate cells that expressed IL-35 and IL-37 displayed a typical plasma cell morphology (eccentric nuclear and voluminous cytoplasm; Ribourtout and Zandecki 2015; Fig. 1A, *b*, 1B, *b*). These data suggest that the plasma cells in the gingival tissue might have the capacity to produce IL-35 and/or IL-37.

We next quantitated the expression of IL-35 and IL-37 levels by comparing the percentage of total positive signals in the connective tissues between CP gingival lesions and healthy tissue. Our data indicated that IL-35 (Fig. 1C) and IL-37 (Fig.

1D) levels in CP connective tissues were significantly increased as compared with healthy tissues. These data suggest that the infiltrated plasma cells might robustly produce IL-35 and/or IL-37 in CP gingival tissues.

Plasma Cells Are the Primary Source of IL-35 in Human CP Gingival Tissues

To validate our discovery that plasma cells serve as the primary inflammatory cell source of IL-35 in human gingival tissue (Fig. 1A), we examined the colocalization of IL-35 and plasma cells with plasma cell-specific markers: CD138 (syndecan 1; Nunez et al. 2016) and CD38 (Halliley et al. 2015; Ribatti 2017; coexpression of CD138 and CD38 on the surface of plasma cells have been well characterized). We stained serial gingival tissue slides with IL-35 and CD138 by IHC. The results suggest the colocalization between IL-35 and CD138 by serial slides (Fig. 2A, *a–d*). To evaluate this finding, we stained IL-35 with CD138 with immunofluorescence costaining and confocal microscopy. These data indicate that IL-35-producing cells in human gingival tissue exhibited CD138-positive staining on the surface of the cells (Fig. 2B, *a–f*).

We also stained plasma cell marker CD38 with IL-35 by immunofluorescence costaining, followed by confocal microscopy (Fig. 2C, *a–j*). The data confirmed the colocalization of IL-35 and CD38 in gingival tissue slides (Fig. 3B, *h–j*). These data also indicated that the plasma cells in gingival tissues are shown as CD138/CD38 double-positive cells (Fig. 2C, *f*, *g*), which is consistent with previous reports (Kim et al. 2012; Halliley et al. 2015). In sum, our results indicate that CD138⁺ CD38⁺ plasma cells represent a major inflammatory source of IL-35 in human gingival tissue. Given our findings, we currently refer to this type of CD138⁺ CD38⁺ plasma cells that can produce IL-35 as IL-35-producing plasma cells (P_{IL-35}).

Plasma Cells Are One of the Major Sources of IL-37 in Human CP Gingival Lesions

To confirm our observation that plasma cells infiltrated in connective tissue express IL-37 (Fig. 1B), we examined the colocalization of IL-37 with plasma cell-specific markers: CD138 (syndecan 1; Nunez et al. 2016) and CD38 (Halliley et al. 2015; Ribatti 2017). We stained serial gingival tissue slides with IL-37 and CD138 by IHC. The results demonstrate the possible colocalization in serial slides between IL-37 and CD138 (Fig. 3A, *a–d*). To further corroborate this finding, we stained IL-37 with CD138 via immunofluorescence costaining and confocal microscopy. The results indicate that the majority of the CD138-positive plasma cells in human gingival tissue robustly produce IL-37 (Fig. 3B, *a–e*). Intriguingly, IL-37-producing plasma cells are more ubiquitous than IL-35-producing plasma cells according to the observation from IL-37 staining results (Fig. 3B, *b*, *e*, *f*) as compared with IL-35 staining results (Fig. 2B, *b*, *e*, *f*). Notably and consistent with the previous finding, besides the plasma cells, the epithelial layer also exhibited

Figure 1. Expression level and localization of IL-35 and IL-37 in human gingival tissues. **(A)** The expression of IL-35 was characterized with anti-human IL-35-specific antibody by immunohistochemistry. Results are shown as representative 3,3'-Diaminobenzidine (DAB) staining of IL-35 in healthy (a) and CP (b) gingival tissues. No primary antibody incubation was used as a negative control staining (c). White arrowheads indicate IL-35-positive infiltrated immune cells. **(B)** The expression of IL-37 was characterized with anti-human IL-37-specific antibody by immunohistochemistry. Results are shown as representative DAB staining of IL-37 in healthy (a) and CP (b) gingival tissues. No primary antibody incubation was used as negative control staining (c). Red arrowheads indicate IL-37-positive epithelial cells. Black arrowheads indicate IL-37-positive endothelial cells. White arrowheads indicate IL-37-positive infiltrated immune cells. Scale bars are shown in the panels. The expression levels of **(C)** IL-35 and **(D)** IL-37 in healthy and CP gingival tissues were quantitated by Aperio color deconvolution algorithm ($n = 10/\text{group}$). Data are shown as mean $\pm$ SD percentage of total positive signals in the connective tissue per tissue section. Results were analyzed with a 2-tailed unpaired t test. **** $P < 0.0001$. CP, chronic periodontitis.

CD138-positive staining (Fig. 3A, a) because CD138 is known as a transmembrane protein present on the surface of plasma cells and epithelial cells (Kotsovilis et al. 2010).

We also stained plasma cell markers CD38 and IL-37 by IHC (immunofluorescence costaining could not be performed, as the primary antibody of CD38 and IL-37 is from the same

species). We found overlapping localization of IL-37 and IL-35 in serial gingival tissue slides (Fig. 3C, a–h). These results indicate that the plasma cells in human gingival tissue producing IL-37 are also CD38⁺. Given these findings, we define this type of CD138⁺ CD38⁺ plasma cells producing IL-37 as IL-37-producing plasma cells (P_{IL-37}).

Figure 2. Colocalization of IL-35 with plasma cells markers (CD138 and CD38) in CP lesions. **(A)** The localization of IL-35 and CD138 was determined in serial slides of CP gingival tissue with immunohistochemistry. (a–d) Results indicate representative DAB staining of IL-35 and CD138 with low (100 $\times$) and high (600 $\times$) magnification. **(B)** In addition, the colocalization of IL-35 and CD138 in human gingival tissue was performed with immunofluorescence and confocal microscopy. (a–d) Single layer of CD138, IL-35, DAPI, and bright field. (e, f) Merged layer of IL-35 + CD138 and IL-35 + CD138 + DAPI. Arrowheads indicate representative IL-35-positive plasma cells in the slides. **(C)** Moreover, the colocalization of IL-35 and 2 plasma cell markers (CD138 and CD38) in human gingival tissue was performed with immunofluorescence and confocal microscopy. (a–e) Single layer of CD138, CD38, IL-35, DAPI, and bright field. (f–j) Merged layers among CD138, CD38, IL-35, DAPI, and bright field. Arrowheads indicate representative IL-35-producing plasma cells in the slides. (h) IL-35 color switched from yellow to green because red and yellow could not merge. Scale bars are shown in the panel. CP, chronic periodontitis.

IL-35/IL-37-Coproducing Plasma Cells in Human CP Gingival Lesions

To elucidate the localization relationship between IL-35- and IL-37-producing plasma cells shown in Figures 2 and 3, we performed immunofluorescence costaining of IL-35 and IL-37 with CD138 in human gingival tissue, followed by confocal microscopy. Surprisingly, our results demonstrated that all of the IL-35-producing plasma cells also express IL-37 (Fig. 4, a–j). In contrast, not all of the IL-37-producing plasma cells are able to produce IL-35 (Fig. 3, g–i). Thus, these data indicate that IL-35-producing plasma cells in CP lesions can

simultaneously produce IL-37. Given this observation, we define this subset of plasma cells that can coproduce IL-35 and IL-37 as $P_{IL-35/IL-37}$; these cells may synergistically produce IL-35 and IL-37 during periodontitis development.

IL-37-Producing and IL-35/IL-37-Coproducing Plasma Cells in CP Lesions Are IgG Positive

Several distinct cytokine-producing plasma cells were IgM^+ (IL-10, IL-35, IL-17, and GM-CSF) or IgA^+ (NO and TNF- α ; Dang et al. 2014). We sought to determine whether the P_{IL-37} and $P_{IL-35/IL-37}$ that we identified can produce antibodies or not

Figure 3. Colocalization of IL-37 with plasma cells markers (CD138 and CD38) in CP gingival lesions. **(A)** The localization of IL-37 and CD138 was determined in serial slides of periodontitis gingival tissue with immunohistochemistry. (a–d) Results indicate representative DAB staining of IL-37 and CD138 in serial slides. **(B)** Furthermore, the colocalization of IL-37 and CD138 in human gingival tissue was performed with immunofluorescence and confocal microscopy. (a–d) Single layer of CD138, IL-37, DAPI, and bright field. (e, f) Merged layers of IL-37 + CD138 and IL-37 + CD138 + DAPI. Arrows indicate representative IL-37-positive plasma cells in the slides. **(C)** The localization of IL-37 and CD38 was determined with immunohistochemistry in serial slides of gingival tissue from patients with CP. Representative DAB staining of IL-37 (a) and CD38 (e) is shown with low (100×) magnification of serial slides. (b–d) Three representative regions of IL-37-positive staining with high (600×) magnification. (f–h) Three representative regions of CD38 staining with high (600×) magnification. Scale bars are shown in the panel. CP, chronic periodontitis.

and which type of antibody they are capable of producing. We profiled the plasma cells in gingival tissues with IHC and immunofluorescence costaining of IL-35 or IL-37 with anti-human immunoglobulin antibody. Surprisingly, our data indicated that P_{IL-37} and $P_{IL-35/IL-37}$ were IgG positive (Appendix Fig. 1A, a–h, B, a–h). We could not detect substantial amounts of IgM expression in the gingival tissues of CP lesions (Appendix Fig. 2). Collectively, these findings support that IL-37- and IL-35/IL-37-expressing plasma cells are CD138⁺ CD38⁺ IgG⁺ plasma cells.

Recombinant Human IL-35 (rhIL-35) and IL-37 (rhIL-37) Directly Inhibit Osteoclast Formation In Vitro

Besides their anti-inflammatory function, IL-35 and IL-37 were shown to be associated with bone erosion in a collagen-induced arthritis model and a lipopolysaccharide-induced bone resorption model, respectively (Li et al. 2016; Saeed et al. 2016). However, the mechanistic effects of these 2 cytokines on osteoclast differentiation are not well understood.

Figure 4. Colocalization of plasma cell marker (CD138), IL-35, and IL-37 in chronic periodontitis gingival lesions. The colocalization of CD138, IL-35, and IL-37 in human gingival tissue was determined with immunofluorescence and confocal microscopy. (a–e) Single layer of CD138, IL-37, IL-35, DAPI, and bright field. (f–j) Merged layers among CD138, IL-37, IL-35, DAPI, and bright field. Arrows indicate representative IL-35/IL-37 double-positive plasma cells in the slides. Scale bars are shown in the panel.

Considering that alveolar bone loss is the severe and irreversible hallmark of periodontitis, we assessed the activity of IL-35 and IL-37 on the inhibition of osteoclast formation with an in vitro osteoclast differentiation assay with bone marrow-derived monocyte precursors. Our data showed that rhIL-35 and rhIL-37 inhibited osteoclast formation in vitro in a dose-dependent manner (Fig. 5A, B). We found that 30-ng/mL rhIL-35 could inhibit ~50% osteoclast formation and that 3-ng/mL rhIL-37 showed the ~50% inhibitory osteoclast formation. In addition, 300 ng/mL of IL-35 demonstrated ~78.9% inhibition, and 300 ng/mL of IL-37 exhibited ~97.7% inhibition in the assay (Fig. 5C, D). These results suggest that the P_{IL-37} and $P_{IL-35/IL-37}$ may have regulatory roles in the inhibition of alveolar bone loss by secreting IL-37 and IL-35/IL-37 into the gingival tissues of patients with CP.

Discussion

In this report, we present novel findings regarding the presence of anti-inflammatory cytokine-producing plasma cells in human gingival tissues. Recent studies suggested the existence of distinct sets of CD138^{hi} plasma cells being involved in the production of different cytokines, including IL-10, IL-35, IL-17, GM-CSF, iNOS, and TNF- α (Fillatreau et al. 2002; Neves et al. 2010; Rauch et al. 2012). Thus, besides the 2 subtypes of plasma cells (P_{IL-37} and $P_{IL-35/IL-37}$) identified in our current study, we postulate that there are more subtypes of plasma cells infiltrated into human gingival tissues. Identifying new types of cytokine-producing plasma cells and their proportion as well as function in human gingival tissues should clarify the biological importance and relevance of plasma cells in periodontitis.

Our study found that the predominant inflammatory plasma cells in gingival lesions could robustly produce IL-37 but that a subset could coproduce IL-35 and IL-37. We also observed plasma cells that did not produce IL-35 or IL-37 in the tissues—these could be traditional plasma cells, only able to produce antibodies. The difference/relationship between P_{IL-37} and $P_{IL-35/IL-37}$ and how different are they from traditional plasma cells (producing only antibody, not cytokines) remains to be explored. In parallel, a previous study reported that the IL-35-producing B cells, mainly plasma cells, could also coexpress IL-10 in mice (Wang et al. 2014). Therefore, these findings suggest that coproduction of cytokines by plasma cells for immune regulation may be a common biological phenomenon.

Previous studies showed that distinct subsets of cytokine-producing plasma cells were IgM⁺ or IgA⁺ plasma cells in animal models that play a regulatory role in vivo during autoimmune and infectious diseases (Dang et al. 2014). We found that P_{IL-37} and $P_{IL-35/IL-37}$ coexpress IgG in human gingival tissues. Considering that 1) the in vivo model used in previous studies to identify IgM⁺ plasma cells may mimic acute inflammation instead of chronic inflammation and 2) IgG⁺ plasma cells in our study were characterized from human gingival tissue of CP, we propose here that the potential explanation is the immunoglobulin isotype switching from isotype IgM to IgG during the development of the disease from acute to chronic. Actually, our current finding is consistent with previous studies demonstrating that the major isotype of immunoglobulin in human gingival tissues is IgG (Takahashi et al. 1997). Further studies are needed to investigate the mechanisms of developing B cells into CD138⁺ CD38⁺ IgG⁺ P_{IL-37} and $P_{IL-35/IL-37}$ in the process of CP.

Previous studies reported that IL-35 and IL-37 could alleviate bone erosion in a mouse model (Li et al. 2016; Saeed et al.

Figure 5. Recombinant human IL-37 and IL-35 suppress the differentiation/maturation of osteoclasts in vitro. Osteoclasts were differentiated from mouse bone marrow derived-macrophage with GM-CSF and RANKL in 96-well plates. Gradient concentrations of (A) IL-35 or (B) IL-37 were added into culture media during osteoclast differentiation. The cells were stained by TRAP (tartrate-resistant acid phosphatase) to detect the osteoclasts. Results are representative photos of TRAP staining from 3 independent experiments. The osteoclast numbers in the 96-well plates after (C) IL-35 or (D) IL-37 were counted by ImageJ and data shown as osteoclast numbers per well from representatives of 3 independent experiments. Data are shown as mean $\pm$ SD. Results were analyzed with 1-way analysis of variance, followed by Dunnett's post hoc test. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

2016). A previous study reported that IL-37 did not inhibit osteoclast formation on osteoclast precursors in vitro (Saeed et al. 2016), in contrast to our in vitro data, which demonstrated direct suppressive function of IL-35 and IL-37 in osteoclast formation. The potential explanation for the discrepancy in the findings might be the concentration of RANKL. Previous studies used a high concentration of RANKL (100 ng/mL) for osteoclast formation, in contrast to our low concentration of RANKL (10 ng/mL). A high concentration of RANKL may mask the inhibitory role of IL-37 in osteoclast formation in vitro. Interestingly, recent studies also indicated the suppressive function of IL-35 and IL-37 for osteoclast differentiation with the in vitro osteoclastogenesis assay (Tang et al. 2018; Yago et al. 2018). Clearly, further in vivo studies should be performed to clarify the potential direct role of IL-35 and IL-37 for osteoclast formation. In addition, the potential toxicity of a high concentration of IL-35 or IL-37 will need to be investigated in future preclinical applications. In sum, the data highlight the need to study and understand the inhibitory mechanistic role that P_{IL-37} and $P_{IL-35/IL-37}$ have in alveolar bone loss.

Although our data indicated that the majority of IL-35- and IL-37-producing cells in human gingival tissues of CP are plasma cells instead of B cells, there is no doubt that these plasma cells are differentiated from precursor B cells. As previous studies extensively reported that B cells also process the capacity to produce cytokines for immune regulation (Rosser

and Mauri 2015), it is plausible to hypothesize that the cytokine-producing plasma cells may be differentiated from cytokine-producing B cells. Fully understanding these biological mechanisms is likely to provide new insights into cytokine-producing B cells and plasma cells.

This study observed the existence of anti-inflammatory cytokine-producing plasma cells in human periodontitis tissues; however, future studies are definitely needed to fulfill the limitations of our work. First, our study does not quantitate the percentage of each anti-inflammatory cytokine-producing plasma cells over total plasma cells in human gingival tissues. Quantification data should provide additional clues to understanding the proportion of these anti-inflammatory cytokine-producing plasma cells in periodontitis. Thus, future quantification assays such as flow cytometry may be performed to answer this essential question. Second, our study did not investigate the function of anti-inflammatory cytokine-producing plasma cells. Although IL-35 and IL-37 have been extensively accepted as anti-inflammatory cytokines, which suggests that IL-35- and IL-37-producing plasma cells may occupy these

anti-inflammatory functions, direct evidence about the function of anti-inflammatory cytokine-producing plasma cells is unknown. Future ex vivo and in vivo investigation will be necessary to elucidate the function of these anti-inflammatory cytokine-producing plasma cells in periodontal disease.

Conclusion

In conclusion, this study identified 2 novel types of anti-inflammatory cytokine-producing plasma cells in human gingival tissues from subjects with CP. This observation extends our traditional insights of robustly infiltrated plasma cells in human gingival tissue and highlights a new, unrecognized function of plasma cells in CP.

Author Contributions

L. Jing, contributed to design and data acquisition, drafted the manuscript; S. Kim, contributed to data analysis, drafted and critically revised the manuscript; L. Sun, L. Wang, E. Mildner, contributed to data acquisition, critically revised the manuscript; K. Divaris, contributed to design and data acquisition, critically revised the manuscript; Y. Jiao, S. Offenbacher, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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