Memory B Cell as an Indicator of Peri-implantitis Status: A Pilot Study

Danying Chen, PhD\(^1\)/Xiayi Wu, PhD\(^1\)/Quan Liu, PhD\(^1\)/Huaxiong Cai, DDS\(^1\)/Baoxin Huang, PhD\(^1\)/Zhuofan Chen, PhD\(^1\)

**Purpose:** Gingiva-resident memory B cells found recently in healthy periodontal tissue may play important roles in maintaining homeostasis against bacterial plaque. Whether resident memory B cells exist in healthy peri-implant tissue and how they respond in peri-implantitis lesions are of interest. The aim of this study was to preliminarily investigate whether memory B cell activities are related to inflamed or healthy peri-implant status. **Materials and Methods:** Patients with peri-implantitis or healed implants were recruited. The gingiva samples were collected and divided into inflamed (n = 4), treated (n = 4), and healed (n = 3) groups, followed by a flow cytometry analysis staining with CD3, CD19, CD27, CD38, and RANKL. The biopsy samples were also cryo-embedded for immunofluorescent double staining of CD19 and CD27. **Results:** CD27+ CD38+ ASC comprised 83.3% ± 3.3% of the total B cells in the inflamed group, and this proportion in the treated group was reduced to 44.5% ± 13.4%. The proportion of CD27+ CD3+ T cells was found to be unchanged between the inflamed and treated groups. Immunofluorescent staining indicated that CD19+ CD27+ population infiltrated peri-implant connective tissue. RANKL was expressed by almost all B cells and a portion of T cells in the inflamed group, while the proportions of RANKL+ B and T cells were significantly reduced in the treated group. Barely any memory B cells were detected in the healed group. **Conclusion:** Memory B cells were markedly activated in peri-implantitis and responded to the suprastructure removal treatment. The lack of gingiva-resident memory B cells in the clinically healed implants serves as a hint for the weakness of peri-implant tissue against bacterial plaque. *Int J Oral Maxillofac Implants* 2021;36:86–93. doi: 10.11607/jomi.8641

**Keywords:** B lymphocyte, host response, peri-implantitis

Peri-implantitis is becoming the most concerning complication when dental implant restoration is to be considered. It has come to a consensus that this biologic complication refers to an inflammatory process due to bacterial infection, resulting in immune cell infiltration of soft tissue and bone loss.\(^1\) Peri-implantitis has many features in common with periodontitis, as it is also an infectious disease that occurs around natural teeth. In histologic observation, B cells (especially plasma cells) and other lymphocytes dominate peri-implantitis and periodontitis lesions.\(^2\) However, peri-implantitis is characterized by larger lesion size and larger proportions of polymorphonuclear leukocytes and macrophages than periodontitis, denoting that certain histopathologic differences exist between the two diseases.\(^2,3\)

The B cell response has been attracting increasing attention in recent years, although T cell response to periodontal pathogens is considered to play a key role in the regulation of periodontal diseases.\(^4,5\) Memory B cells, which produce antibodies for antigens met in the past, are generated from naive B cells in the germinal center and can be recruited rapidly to the site of inflammation. Memory B cells were reported to express receptor activator for nuclear factor-kB ligand (RANKL), leading to increased alveolar bone loss and periodontal osteoclastogenesis in periodontitis\(^6,7\) and rheumatoid arthritis.\(^8\) Moreover, a cluster of memory B cells was found residing in the connective tissue subjacent to the junctional epithelium in healthy periodontal gingiva,\(^9\) implying their importance in maintaining periodontal homeostasis. However, memory B cells in healthy peri-implant gingiva and peri-implantitis lesions are not well-documented to date.

Semaphorins are a class of cell surface proteins that mediate cell-to-cell communication. They are essentially involved in inflammatory processes\(^10\) and bone metabolism.\(^11\) Gene expressions of semaphorin members (SEMA3A, SEMA3B, SEMA4A, and SEMA4D) were reported...
as significantly changed in the inflamed peri-implant gingiva compared with gingiva in healthy conditions. A novel marker of human memory B cells induced under Th2 conditions, SEMA4C, is also a semaphorin member. A detailed profile of SEMA4C expression in the periodontal and peri-implant tissue remains hazy. The aim of the present study was to preliminarily investigate whether memory B cell activities are related to inflamed or healthy peri-implant status. The present data would provide a feasible research design and statistical references for further studies.

MATERIALS AND METHODS

Study Population
Patients with at least one functioning implant-supported restoration, or with at least one osseointegrated implant not yet restored by a dental prosthesis, or with uncontrolled periodontitis, or with problems in the impacted third molar were selected. Informed consent of all participants was obtained prior to enrollment in this study. Participants who were older than 18 years of age, nonsmokers, and in general good health were eligible. Subjects who fulfilled one or more of the following criteria were excluded: antibiotic or anti-inflammatory medication history within the previous 3 months, chronic medical disease or condition, smoking, and people who declined to enroll in the study (enrollment process in Appendix Fig 1; see Appendix in online version of this article at quintpub.com).

Subjects with a restoration supported by one to two implants functioning for 4 to 15 years were enrolled during follow-up visits. Clinical and radiographic peri-implant examinations were taken. Clinically, the probing depth and associated bleeding or suppuration were recorded. The level of vertical marginal bone (distance from the implant shoulder to the first bone-to-implant contact [DIB]) was calculated by averaging the mesial and distal sites of the vertical distance from the implant-abutment junction to the first point of bone-to-implant contact in the radiographic image, with the known implant length as a calibration if applicable. Subjects who fulfilled the following criteria were diagnosed as having peri-implantitis: radiographic evidence of crestal bone loss > 3 mm, and probing depth > 5 mm, associated with bleeding on probing and/or suppuration. In this particular study, the peri-implantitis subjects were further divided into two groups. In the “inflamed” group, probing depth was > 5 mm, and the suprastructure was removed after diagnosis immediately followed by a surgical debridement and soft tissue biopsy. In the “treated” group, probing depth was > 5 mm, and the suprastructure was removed, followed by a surgical debridement and soft tissue biopsy 1 to 2 weeks later. The probing depth and bone loss measurement was done on the day of biopsy. Soft tissue biopsy specimens were obtained around the implants where inflammatory granulation formed (Appendix Fig 2).

Subjects with at least one nonsubmerged implant healing for at least 3 months were enrolled in the “healed” group when they returned for stage-two gingival plastic surgery. After radiographic and clinical examination, subjects who showed successful osseointegration and excessive keratinized soft tissue (thickness > 2 mm and width > 4 mm) were included. The soft tissue biopsy specimens were obtained from the buccal pocket by an inverse bevel incision extending to the bottom of the pocket followed by a crevicular incision around the implant healing abutment.

From September 2018 to February 2019, a total of 12 implants diagnosed as peri-implantitis from eight patients were included in the inflamed group and the treated group. The eight biopsy samples (at least 4 × 2 × 2 mm³ in size) diagnosed as peri-implantitis (four in the inflamed group and four in the treated group) and three biopsy samples (approximately 2 × 2 × 2 mm³ in size) from the healed group were further divided into two pieces for the flow cytometry assay (at least 1 × 2 × 2 mm³ in size) and the immunofluorescent staining assay (at least 1 × 2 × 2 mm³ in size). Detailed clinical assessment data of the two groups are shown in Table 1, Appendix Table 1, and Appendix Table 2. Notably, all 12 implants were restored with cement-retained restorations. Other samples diagnosed as peri-implantitis were used in the real-time quantitative polymerase chain reaction (RT-qPCR) assay.

Subjects with radiographic evidence of crestal bone loss > 3 mm, probing depth > 5 mm, associated with bleeding on probing and/or suppuration of at least one natural tooth, and no history of past periodontal treatment, were assigned to the “periodontitis” group. Biopsy samples were obtained from the inflammatory tissues around the pocket by an inverse bevel incision extending to the bottom of the pocket followed by a

<table>
<thead>
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<th>Table 1: Clinical Data of Evaluated Patients</th>
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<tr>
<td>Inflamed</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>No. of individuals</td>
</tr>
<tr>
<td>No. of implants</td>
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<tr>
<td>Age (y)</td>
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<td>Crestal bone loss (mm)</td>
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<td>Restoration time (y)</td>
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Data were presented as mean ± SD. *P < .05, Mann-Whitney test.
crevicular incision. Subjects who were periodontally healthy and had no bleeding on probing when they needed to undergo surgical extraction of third molars were assigned to the “healthy” group. Samples were used in the RT-qPCR assay.

Flow Cytometry
For the flow-cytometry assay, samples from the inflamed, treated, and healed groups were used. The biopsy specimens were washed using RPMI 1640 medium (Gibco) and cut into pieces, followed by incubation in medium containing 2 mg/mL collagenase type I (Gibco) for 90 minutes at 37°C. Then, the isolated cell suspension was obtained by filtering through a 70-μm mesh. The isolated cells were washed using a staining buffer (eBiosciences) and then stained with anti-human CD19-FITC (Clone HIB19, BD Biosciences, 1:5 dilution), CD27-PE (Clone M-T271, BD Biosciences, 1:5 dilution), CD3-PerCP (Clone SP34-2, BD Biosciences, 1:5 dilution), and CD38-APC (Clone HIT2, BD Biosciences, 1:5 dilution) mAbs on ice for 30 minutes. The stained cells were then treated with RBC lysing buffer (Beyotime Biotechnology) according to the manufacturer’s instruction. Briefly, cells were stained with anti-human CD19 (FITC), CD27 (PE), and CD38-APC (Clone HIT2, BD Biosciences, 1:5 dilution) mAbs (BD Biosciences) as mentioned earlier. Then, washed cells were fixed and permeabilized on ice for 20 minutes. The cells were further washed with the wash buffer and incubated with the anti-human RANKL-APC (BioLegend no. 347507, 1:20 dilution) or an isotype control mAbs (BioLegend no. 347507, 1:20 dilution) mAbs on ice for 30 minutes. The stained cells were then treated with RBC lysing buffer (Beyotime Biotechnology) for 15 minutes on ice and then washed. For intracellular staining of RANKL, the following procedure was conducted using a Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer’s instruction. Brieﬂy, cells were stained with anti-human CD19 (FITC), CD27 (PE), and CD3 (PerCP) mAbs (BD Biosciences) as mentioned earlier. Then, washed cells were fixed and permeabilized on ice for 20 minutes. The cells were further washed with the wash buffer and incubated with the anti-human RANKL-APC (BioLegend no. 347507, 1:20 dilution) or an isotype control mAbs (BioLegend no. 400122, 1:20 dilution) for another 30 minutes on ice. Finally, single-cell suspension was prepared with a strainer mesh.

Analysis was performed using four-color flow cytometry (CytoFLEX, Beckman Coulter). All of the stained cells were collected. The proportion of characterized cells was studied retrospectively.

Immunofluorescent Staining
For the immunofluorescent staining assay, samples from the inflamed (n = 4), treated (n = 4), and healed (n = 3) groups were used. The biopsy specimens were washed by saline, then fixed in 4% paraformaldehyde (PFA) for 24 hours, and 4-μm-thick cryo-embedded sections were made. For immunofluorescent double staining of CD19 and CD27, the sections were incubated at room temperature for 30 minutes, washed, then unmasked using the ethylenediaminetetraacetic acid (EDTA) antigen retrieval solution (Solarbio) for 10 minutes. Nonspecific binding was blocked by 5% bovine serum albumin (BSA) for 30 minutes at room temperature. Primary antibodies (anti-CD19 antibody 2E2B6B10 and anti-CD27 antibody EPR8569, Abcam, 1:100 dilution) were incubated at 4°C overnight, and secondary antibodies (mouse anti-rabbit IgG-FITC and anti-mouse IgGk BP-PE, Santa Cruz Biotechnology, 1:500 dilution) were incubated at room temperature for 1 hour. The slides were washed by phosphate-buffered saline (PBS) and then stained with DAPI (Solarbio). Finally, the slides were mounted and observed under an inverted fluorescence microscope.

RT-qPCR Assay
For the RT-qPCR assay, samples from the peri-implantitis, periodontitis, and healthy groups were used. The biopsy specimens were washed with saline and then dissolved in TRiZol (Invitrogen). Total RNA was extracted, and approximately 1 μg was reverse transcribed to cDNA using the PrimeScript RT Master Mix Kit (Takara) according to the manufacturer’s instructions. The RT-qPCR primer sequences were described as follows: SEMA4C (Forward – TTGTGCGCGTAAAGACAGTG, Reverse – CGGTCAGCGTCAGTGTCAG), GAPDH (Forward – TACGCAATGCCCTCCTGCAC, Reverse – TCTGCGTGCAGTGAC). The reaction was performed in a total volume of 20 μL, using SYBR green (Takara) as the nuclear dye, under an initial denaturation process of 30 seconds at 95°C followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds on the LightCycler real-time PCR instrument (Roche). The expression of SEMA4C was normalized to that of GAPDH and further compared with a healthy sample using the 2-△△CT method.

Statistical Analysis
The sample capacity was estimated based on the parametric estimation method, and the examined hypothesis was H0: μ1 = μ2. Parameters from a previous study were used to calculate the total sample capacity by using the formula:

\[ n = \left( z_{1-\alpha/2} + z_{1-\beta} \right)^2 \cdot \sigma^2 \cdot (1+1/k) / \delta^2 \]

The result showed that approximately two samples were needed in each group for the flow cytometry assay, and approximately four samples were needed in each group for the RT-qPCR assay. Finally, two times of the estimated sample capacity was determined as the lowest sample size in this study. Inflammatory tissues obtained from the neighboring implants were collected and analyzed as one sample. Histologic and RT-qPCR experiments were performed in triplicate. Data analysis was performed with statistical software (GraphPad Prism 6.0) using a nonparametric statistical method (Mann-Whitney test). All demographic data were presented as median and 95% confidence intervals. The significance level was set at .05 (alpha = .05).
RESULTS

Participants
No significant differences were observed between the inflamed group and the treated group, except for the restoration time (inflamed group, 3.3 ± 1.0 years; treated group, 10.3 ± 4.6 years). As for the healed group, three implants from three patients were included. All included implants healed for 3 months. The average age of the patients was 54.7 ± 8.9 years in this group.

Quantification of the Memory B Cells
B cell subsets, including naive B cells, memory B cells, and antibody-secreting cells (ASCs), in the peri-implant gingiva under inflamed or treated conditions were characterized (Fig 1a). The ratio of CD19+ B cells vs CD3+ T cells was significantly higher in the inflamed group than in the treated group (Fig 1b, P < .05, Mann-Whitney test). CD27+ memory B cells comprised 83.3% ± 3.3% of the total B cells in the inflamed group (n = 4), whereas that amount was reduced to 31.2% ± 15.0% in the treated group (n = 4). CD27+ CD38+ memory B cells comprised 7.39% ± 4.4% of the total B cells in the inflamed group, which increased to 44.5% ± 13.4% in the treated group. The difference in the density of CD38+ and CD38- memory B cells in the inflamed compared with the treated group was statistically significant (Fig 1c, P < .05, Mann-Whitney test); moreover, the CD38 signal levels were substantially higher in the inflamed group than in the treated group. The density of the naive B cell subset was not significantly different between the inflamed group (3.0% ± 1.4%) and the treated group (13.7% ± 8.0%).

On the other hand, CD3+ CD27+ T cells, which were usually considered to be naive, and memory T cell populations, also comprised the majority of the CD3+ T cell population in the peri-implantitis gingiva. This was comparable in the inflamed group (80.8% ± 8.0%) and the treated group (83.7% ± 10.1%; Fig 1d, P > .05, Mann-Whitney test).

Fig 1  Flow cytometry data of B cell and T cell subsets in peri-implantitis gingiva. (a) Gating methods. (b) The ratio of CD19+ B cells vs CD3+ T cells. (c) The percentage of memory B cells and ASCs in B cell subsets. (d) The percentage of CD27+ memory T cells in T cell subsets. Data were presented as median and 95% confidence intervals. *P < .05, ns = not significant, Mann-Whitney test.
RANKL Expression in B and T cells
RANKL expression in the inflammatory site was involved in the progression of bone loss. In the inflamed group, almost all of the B cells (naive and memory B cells, 99.0% ± 0.8%) and a majority of T cells (94.0% ± 1.3%) were RANKL positive. In the treated group, the proportion of RANKL+ cells was significantly reduced in B cells and T cells (Fig 2, \( P < .05 \), Mann-Whitney test).

Location of the Memory B Cells
The anatomical location of the memory B cell population in histologic sections was investigated by immunofluorescent double-staining of CD19 and CD27. High-density CD19+ CD27+ population in the inflammatory cell infiltrate of the peri-implant connective tissue was found in the inflamed group (Figs 3a and 3b). In the treated group, the CD19+ CD27+ population scattered throughout the inflammatory cell infiltrate (Figs 3c and 3d).

It is of interest whether tissue-resident memory B cells exist in the healthy peri-implant tissue as in the natural teeth. The flow cytometry assay of the healed group (n = 3) showed that barely any B cells (0.07% ± 0.06%) or T cells existed (0.06% ± 0.03%; Fig 4a). Histologic staining also detected no cluster of CD19+ CD27+ cell population (Figs 4b and 4c).

SEMA4C Expression in Gingival Tissue
To compare the memory B cell activation via Th2 response among healthy, periodontitis, and peri-implantitis gingiva, the expression level of SEMA4C was tested by the SYBR green RT-qPCR assay. No significant difference in the gene expression of SEMA4C was observed among the healthy (n = 8), periodontitis (n = 8), and peri-implantitis (n = 8) gingiva (Fig 5, \( P > .05 \), Mann-Whitney test).

DISCUSSION
A recent systematic review suggested that the peri-implantitis sulcus is populated by more complex and heterogenous microorganisms composed of predominantly noncultivable gram-negative species than in periodontitis, while the spectra of prevalent species are similar.\(^{15}\) On the other hand, the current evidence...
Fig 3  Memory B cell location in the gingiva tissue. White arrows indicate CD19⁺ CD27⁺ memory B cells. Scale bars: 400 μm. Green = CD27; red = CD19.

Fig 4  Lymphocytes in the healed peri-implant gingiva. (a) Flow cytometry assay barely identified CD3⁺ T cells or CD19⁺ B cells. (b, c) Histologic staining of CD19 and CD27 (n = 3). Scale bars: 400 μm. Green = CD27; red = CD19.

Fig 5  Relative quantification of SEMA4C mRNA expression level normalized to that of GAPDH. Tissue samples were harvested from healthy (n = 8), periodontitis (n = 8), and peri-implantitis (n = 8) subjects. Data were presented as median and 95% confidence intervals. No significant difference was detected between groups by Mann-Whitney test.
indicates that the host immune response to peri-implantitis should differ from periodontitis in intensity, but not particularity. B cells (CD20+ cells) and plasma cells (CD138+ cells) together accounted for the highest proportion (40% to 60%) of immune cells in peri-implantitis, as in periodontitis lesion.2,3

The major canonical human B cell subsets are recommended to be analyzed by a core marker set of seven markers; among those markers, it comes to a consensus that human memory cells can be defined by CD19+ and CD27+, besides the increasing CD38 level accompanied by their transformation to ASCs.16 The different cell surface markers and cardinal numbers used may have led to the comparable proportion of CD19+ B cells to CD3+ T cells (B/T ratio median 1.29) in the peri-implantitis lesions found in this particular study. In order to make a comparable preliminary study to the previous study in the periodontitis lesion,9 the flow cytometry assay in the present study was conducted by using the same markers to quantify memory B cell subsets in the peri-implantitis lesion. The present data displayed similar and seemingly more intense memory B cell activities (higher proportion of CD27+ CD38hi cells) in peri-implantitis gingiva than for periodontitis. Periodontal treatments such as scaling and root planing before the biopsy procedures of patients with periodontitis may affect the results; therefore, a strict control study is needed to compare memory B cell activity between the two diseases. Another two core markers, IgD and IgM, distinguish memory B cells into further heterogeneous subsets. Within the limitation of the sample size in this study, only IgD was tested, and it was observed that more than 98% of CD19+ CD27+ B cells were IgD in all tested samples, indicating that memory B cells in peri-implantitis lesion were pre-switched or switched B cells (data not shown). Interestingly, a cluster of CD19+ CD27- CD38+ B cells was observed in some samples (Fig 1a). Whether they represent the canonical "double negative" B cell subset (CD27- and IgD-) was not confirmed, for more markers like CD24 and CD21 were needed. Double negative B cell subset is one of the hotspot problems recently, which was found to be important in various diseases such as systemic lupus erythematosus, non-small cell lung cancer, and rheumatoid arthritis, whereas their phenotypic markers remain inconsistent.17 With their potential contribution to inflammation pathology, the presence of double negative B cells in peri-implantitis lesion deserves further validation.

Various treatments for established peri-implantitis were intensively studied, including resective surgery, regenerative surgery, and nonsurgical therapies. A meta-analysis showed that single and combined nonsurgical therapies yielded greater probing depth reduction than surgical debridement only for treating peri-implantitis.18 Before those complex procedures, simple removal of the suprastructure was seemingly the basis of all treatment procedures for elimination of supragingival plaque and avoiding occlusal overload, yet few studies clearly define whether suprastructure removal is involved in the research baseline or whether suprastructure removal only affects peri-implant inflammation. In this study, removal of the suprastructure led to lower CD38 signal levels and significantly reduced the B/T ratio. On the other hand, the expression of RANKL in the memory B cells is emphasized and accounts for the alveolar bone loss in several periodontitis-related studies.6,7,19 In the present study, comparable RANKL signal levels were observed in almost all B cells and a majority of T cells in each peri-implantitis sample. The RANKL signal level of CD27+ cells was also consistent with that of CD27- cells in both B and T cells. The tremendous amount of RANKL+ cells accompanied by the wide-range infiltration together may explain the circumferential pattern of bone loss in the peri-implantitis lesions, whereas B and T cell infiltration was relatively restricted to the apical advancing front in the periodontitis lesions.3 Whether those RANKL+ CD27- cells are naive lymphocytes from blood contamination or truly reside in the gingiva remains unclear. The presented data suggested that suprastructure removal deserved attention and might be the first step in treating peri-implantitis. Moreover, whether the suprastructure is to be removed should be taken into consideration when setting up the research baseline.

Besides the memory B cell response in the inflamed peri-implant tissue, whether tissue-resident memory B cells guard in the healthy peri-implant tissue as in the natural teeth is also of interest. In this study, minute quantities of CD19+ B cells were detected using the flow cytometry assay at the 3-month healing time point, and barely any CD19+ CD27+ memory B cells were found histologically. These results raise even more interesting questions: First, do memory B cells truly reside in the peri-implant tissue as in healthy periodontal tissue? Second, if they do, how long does the residing process take? Also, do the gingiva-resident memory B cells contribute to the weakness of peri-implant tissue against bacterial plaque? Seeking those answers would require continually tracking for the tissue-resident memory B cells around healthy dental implants in animal models.

Tissue-resident memory T cells are commonly observed in human nonlymphoid tissues. Recently, CD69+ gingiva-resident memory T cells have been found to be an important part of immune surveillance to maintain periodontal homeostasis in the healthy condition,20,21 and a significant increase in the proportion of CD4+ CD69+ CD103- memory T cells was observed in the periodontitis tissue. Consistent with these findings, the present data also suggested that high proportions of CD27+ putative naive and memory T cells participated...
in the progression of peri-implantitis. CD4+ and CD8+ T cells activate B cells in Th1 and Th2 responses, respectively. Therefore, SEMA4C, whose expression is a feature of human tonsillar B cell activation specifically in Th2 responses, was also selected to explore the possible activation mechanisms of the memory B cells. The present study could not find significant differences, but only a slight increasing tendency of SEMA4C expression in the gingiva among healthy, periodontitis, and peri-implantitis conditions. The ignorance of the suprastructure removal treatment time point when sampling the peri-implantitis group may have led to the indifferent result between groups in the RT-qPCR assay. This again implied that suprastructure removal is of importance when setting up the research baseline. Since Sema4C is found to be critical for optimal regulatory cytokine production in plasma cells, further studies focusing on the cytokine regulation balance of peri-implantitis and peri-implant gingiva-resident memory T cells are essential to better understand the immunopathology of peri-implant diseases.

It should be pointed out that a small sample size was used in the present study, limiting the scientific plausibility to some degree.

CONCLUSIONS

Intensive memory B cell activation in the peri-implantitis gingiva was found in this preliminary study. The activation was significantly alleviated within 2 weeks after the suprastructure removal treatment. Thus, memory B cells could be regarded as an indicator of peri-implant disease status. In addition, a high level of RANKL expression may contribute to the circumsferential pattern of bone loss in the peri-implantitis lesions. Barely any gingiva-resident memory B cells were detected at the 3-month healing time point.

ACKNOWLEDGMENTS

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REFERENCES

Appendix Fig 1 (Left) The inclusion process of this study.

Appendix Fig 2 (Below) Images showing a typical biopsy surgery. (a) A scheme showing the biopsy method. Dash lines indicate the incisions. (b) Pretreatment. (c) Suprastructure removal. (d) Peri-implantitis lesion. (e) 9 months after the biopsy.

Appendix Table 1  Patient Information of Inflamed Group

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<th>ID</th>
<th>Sex</th>
<th>Age (y)</th>
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<th>Restoration time (y)</th>
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*aFDI tooth-numbering system.

Appendix Table 2  Patient Information of Treated Group

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*aFDI tooth-numbering system.