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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1758024> since 2020-10-08T14:52:14Z

Published version:

DOI:10.1111/jre.12709

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UNIVERSITÀ DEGLI STUDI DI TORINO

*This is an author version of the contribution published on:
Questa è la versione dell'autore dell'opera:*

Deli F, Romano F, Gualini G, Mariani GM, Sala I, Veneziano F, Bertero L,
Cassoni P, Aimetti M.

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Journal of Periodontal Research 2020;55(2):324-30 doi:10.1111/jre.12709.

*The definitive version is available at:
La versione definitiva è disponibile alla URL:*

<http://onlinelibrary.wiley.com/doi/10.1111/jre.12709/epdf>

Resident memory T cells: possible players in periodontal disease recurrence.

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Running title: Tissue-resident memory cells in gingiva

Keywords: cellular immunity; gingiva; periodontitis; T cells.

ABSTRACT

Background and Objective: Tissue-resident memory T cells (Trm) represent a new subset of long-lived memory T cells that remain in barrier tissues after previous bacterial or viral infection to support early/immediate defense mechanisms, providing site-specific protection from pathogen challenge. As data on Trm cells in human gingiva are just emerging, the aim of the present study was to explore their presence and distribution in epithelial and connective periodontal tissues in relation to microbial exposure and periodontal damage.

Methods: Periodontitis tissue specimens were collected from 20 generalized chronic periodontitis patients at the time of osseous resective surgery. As a control, 18 healthy tissue specimens were harvested each from both the primary flap and the palatal graft in 18 periodontally healthy patients during mucogingival surgeries. As CD69 and CD103 are phenotypic markers associated with tissue residence, intraepithelial and stromal CD103+ and CD69+ cells per high power field were counted in areas with highest expression. Double immunohistochemistry for CD3 and CD69 was performed to identify T cells.

Results: CD69+ as well as CD103+ cells showed a lymphocytic morphology and double CD69 and CD3 staining confirmed the T cell phenotype of these cells. CD103 and CD69 expression was significantly enhanced in epithelial and connective tissues from patients with periodontitis compared with healthy controls ($p < 0.001$).

Significant positive correlation between PD and both CD103 and CD69 epithelial expression was observed in tissue specimens from periodontitis patients ($p < 0.001$).

Conclusion: Within the limits of the present study, these results indicate that Trm cells are higher in periodontitis lesions. They could orchestrate the host response to microbial challenge, leading to a faster reactivation of periodontal disease.

INTRODUCTION

Periodontitis is a biofilm-induced chronic inflammatory disease involving a complex interplay between immune/inflammatory mediator cascade and dysbiotic microbial communities, leading to periodontal tissue breakdown. The susceptibility to periodontitis appears to be determined by the host response, specifically, by the magnitude of the inflammatory response and the differential activation of immune pathways.¹ The first line defense strategies rely on the innate immunity system, involving neutrophil granulocytes and macrophages, followed by an adaptive phase, characterized by B lymphocytes, T lymphocytes and plasma cells.² During this second phase, also called acquired immunity, pathogen-activated cells belonging to the line of T lymphocytes differentiate into memory cells.² This T cell lineage retaining memory of previous bacterial or viral infection enables quicker and more effective protection against secondary infections by facilitating the recruitment of circulating effector cells.^{3,4} A subset of memory T cells has the capacity to enter peripheral tissues (effector memory T cells, Tem), while another to localize into lymph nodes (central memory T cells, Tcm).⁵ Until recently it was thought that Tem cells, which may remain in the body for lifetime, remain circulating in the bloodstream and patrol the various districts.^{6,7} Recent studies have shown, however, that the majority of these cells differentiate into a population of tissue-resident memory T cells (Trm) that persist locally and are unable to re-enter the circulation.^{8,9} In particular, they have been isolated in the skin and in the barrier mucosal tissue within the reproductive tract, lungs, and intestine.^{10,11}

Expression of CD69 and α E integrin CD103 is usually used to define memory T cells as tissue resident.¹² CD69 downregulates the expression of the receptor for sphingosine 1 phosphate (S1P) on the Trm cells surface, blocking their capacity to sense S1P gradients in the blood and supporting their stationary nature.^{13,14} Although

its role is incompletely understood, it does appear that CD103 expression is a marker of differentiation of Trm cells, rather than a functional requirement for tissue residence.^{12,15} In some tissues, CD103+CD69+ cells make up the majority of the Trm population and CD103– T cells are only transiently present and quickly re-enter the circulation.¹⁶ Some tissues, including the skin or the intestine, contain subsets of T cells that lack CD103 expression but are nonetheless capable of maintaining tissue residence.^{17,18} This phenotypic heterogeneity among Trm populations often depends on the tissue of residence.

In the literature only a study investigated the presence of Trm cells in gingival tissues in healthy and periodontitis patients.¹⁹ In healthy gingiva most of them were recirculating memory T cells and did not express CD69, while in periodontitis sites resident CD69+ memory T cells were prevalent. In both conditions only a minority of memory T cells expressed CD103.¹⁹ This finding is of particular interest because in treated periodontitis patients Trm cells could orchestrate an accelerated and enhanced host response to microbial challenge, leading to recurrence of periodontal disease. Because of the tendency of Trm to progressively accumulate in tissues with repeated antigen exposures, Trm-mediated inflammatory diseases tend to worsen over time leading to both faster onset and increased severity of inflammatory response with repeated exposure.^{3,4,20}

As data on Trm cells in human gingiva are just emerging, the aim of the present immunohistochemical study was devised to explore CD103 and CD69 expression in epithelial and connective periodontal tissue in relation to periodontal damage and microbial exposure.

MATERIAL AND METHODS

Patients

Participants were consecutively recruited among outpatients at the Section of

Periodontology, C.I.R. Dental School, University of Turin, Italy, between July 2017 and February 2018. In total, 20 patients (6 males and 14 females, mean age: 51.8 ± 7.5 years) with generalized chronic periodontitis (GCP) in accordance with the American Academy of Periodontology classification of 1999²¹ and 18 controls (8 males and 10 females, mean age: 47.6 ± 6.1 years) met all inclusion criteria. The study protocol was fully explained, and the participants signed an informed consent for the use of the excised tissue. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2002, and was approved by the local Ethic Committee (n° approval 0023162).

Each patient received a comprehensive periodontal examination and radiographic assessment. The clinical parameters assessed included presence/absence of bacterial plaque, presence/absence of bleeding on probing (BoP), probing depth (PD) and clinical attachment level.

For the GCP group, participants had completed the etiological periodontal treatment 3 to 4 months prior to enrolment and presented at least one posterior sextant requiring osseous resective surgery due to the presence of periodontal pockets associated with suprabony or intrabony component ≤ 3 mm as detected on radiographs. Controls had no history of periodontitis and presented mucogingival problems on the lower anterior teeth with indication to gingival augmentation procedures to increase the band of keratinized tissue. Exclusion criteria for both groups were: age younger than 18 years, systemic and immunologic diseases or usage of medications that could interfere with periodontal status or the healing process, current smoking, pregnancy, lactation, a full-mouth plaque score and a full-mouth bleeding score $> 20\%$ before the surgical procedure, previous periodontal surgery at the affected teeth. Subjects who had taken anti-inflammatory or antimicrobial drugs within the previous 3 months were also excluded.^{19,22} All enrolled

patients a week before surgery underwent a session of professional oral hygiene.

Gingival tissue biopsies

The GCP group received osseous resective surgery (ORS) with apical position of the flap.²³ All posterior sextants included in the analysis presented at least two teeth with residual periodontal pockets more than 4 mm deep and persisting BoP. In order to correct bone deformities and to achieve a physiologically scalloped gingival anatomy, the bone contour was reshaped and the flap margin was positioned at the level of the alveolar crest. The secondary flap containing the inflamed pocket wall tissue from diseased sites (periodontitis tissue) was collected for the immunohistochemical analysis.

Control group received the free gingival graft procedure as described by Sullivan and Atkins²⁴ or the laterally sliding flap procedure as described by Caffesse and Espinel²⁵ according to the clinical conditions. Two healthy tissue samples were harvested for each patient, one from the primary flap removed when exposing the recipient bed and one from the palatal graft so that the biopsy contained epithelium and connective tissue. Gingival sites exhibited no clinical sign of inflammation and PD \leq 3 mm. The palatal mucosa was taken as negative control. All grafts were removed from the area palatal to the first molar through the distal aspect of the canine. Each tissue specimen was approximately 0.2 to 0.4 cm x 0.2 cm in size.

The excised tissues were sutured on a sterile paper to identify the orientation of the tissue specimen, placed in a sterile tube containing 10% buffered formalin and transported on ice within a few hours to the Laboratory of the Anatomy Pathology Department, University of Turin, for the analysis.

Samples processing and immunohistochemistry

Tissue samples from patients and controls were fixed in 4% buffered formaldehyde, routinely processed, and paraffin embedded. For each case, haematoxylin and eosin

slides were prepared and immunohistochemistry for CD103 (polyclonal, 1:200, Sigma-Aldrich, Saint Louis, MO, USA) and CD69 (polyclonal, 1:50, Sigma-Aldrich, Saint Louis, MO, USA) was performed on the Benchmark ULTRA platform (Ventana Medical Systems Inc., Tucson, AZ, USA) according to the manufacturer protocols. Double immunohistochemistry (IHC) staining for CD3 (clone 2GV6, Ventana Medical Systems Inc., Tucson, AZ, USA) and CD69 (clone 8B6, Invitrogen Corporation, Carlsbad, CA, USA) was performed using rabbit (CD3, AEC red staining) and mouse antibodies (CD69, DAB brown staining). CD3 reacts with ϵ -chain of CD3 part of the TCR/CD3 complex. This antibody is a pan T-cell reagent.

To quantify intraepithelial and stromal CD103 and CD69-positive cells, the whole samples were screened to select the areas with highest expression, and then 10 high power fields (HPF) were counted and mean value per HPF was determined. Positive and negative controls were used as appropriate in each staining run. Serial sections were routinely stained without primary antibodies or non-immune serum and served as negative controls. Reactive human tonsil tissue was used as the positive control.

Statistical analysis

Due to the explorer nature of this study a sample size calculation was not performed. A statistical software program (Stata for Mac, College Station, TX, USA) was used for data analysis. Sample distribution according to demographic data was assessed using chi-square test. Quantitative data were examined for normality by the Kolmogorov-Smirnov test. Because they did not achieve normality, analyses were performed using non-parametric methods and results were expressed with median and interquartile range (Q1-Q3). The Wilcoxon signed-rank test was employed to detect statistically significant difference in the expression of CD69 and CD103 in the epithelial and connective tissue within each experimental group. The comparison between periodontitis sites and healthy control specimens was performed using the

Kruskal-Wallis test, followed by post-hoc test. The statistical significance of correlations among variables was determined using the Spearman rank correlation coefficient. Two-tailed P values < 0.05 were considered statistically significant.

RESULTS

Demographic and clinical features of all participants are summarized in Supplementary Table 1, which shows no significant differences by age or sex. As expected, all clinical parameters were significantly increased in periodontitis (Supplementary Table 2). All harvested tissue specimens (n = 20 periodontitis tissue, n = 18 clinically healthy gingiva and n = 18 palatal mucosa) were immunohistochemically analysed. CD69+ as well as CD103+ cells showed a lymphocytic morphology (Supplementary Figure 1); to confirm the T cell phenotype of these cells, double IHC staining was performed (Supplementary Figure 2) suggesting a co-expression of both antigens, but evaluation was hampered by the intrinsic limitations of this approach (both CD3 and CD69 are membrane antigens, thus the two stains overlap).

Nonparametric Kruskal-Wallis yielded a p-value < 0.001, indicating significant differences among the three tissue specimens in median CD69+ T cells/HPF. Higher number of CD69+ T cells was found in both the epithelial (7.00 and 0.50-15.00, median and Q1-Q3) (Figure 1A) and the connective tissue (9.50 and 2.50-13.75, median and Q1-Q3) (Figure 1B) of periodontitis-involved sites compared with healthy gingiva (0.00 and 0.00-0.25 for both epithelium and connective tissue) and palatal mucosa (0.00 and 0.00-2.00 for epithelium, and 0.50 and 0.00-2.00 for connective tissue) in controls (p < 0.001) that showed similar expression levels. In the control group epithelial and stromal CD69 expression was absent in 11 gingival tissues and in 7 palatal mucosa specimens, respectively. As shown in Figure 2 comparable trend was observed for CD103 positivity with higher number of cells expressing this marker

in both epithelium (28 and 10.50-39.75, median and Q1-Q3) and stromal tissue (17 and 7.00-23.75, median and Q1-Q3) from periodontitis sites compared to healthy control sites ($p < 0.001$). Intra-group analysis demonstrated a greater CD103 positivity in the epithelium (Figure 2 A) compared with the stromal tissue (Figure 2B) in both the healthy gingiva (4.50 and 1.75-9.25 vs 2.00 and 0.00-5.00, median and Q1-Q3) and palatal mucosa (8.00 and 2.75-10.00 vs 2.50 and 0.75-6.00, median and Q1-Q3) in control participants ($p = 0.038$ and $p = 0.007$, respectively). Positivity for CD103 staining was absent in the epithelial layer of one gingival tissue and 3 palatal mucosa samples, while it was not detected in the connective tissue of 4 gingiva and 7 palatal mucosa specimens.

Unlike in healthy gingiva, there was no difference in median numbers of CD69+ and CD103+ T cells between epithelium and connective tissue harvested from periodontitis sites ($p = 0.07$ and $p = 0.586$). A significant positive correlation was found between both CD69 and CD103 epithelial expression and PD ($r = 0.793$ and $r = 0.668$, respectively, $p < 0.01$ Supplementary Figure 3). As expected, the percentage of bleeding sites into the ORS-treated area was not significantly correlated with CD69 ($r = 0.358$, $p = 0.121$) and CD103 ($r = 0.168$ and $p = 0.479$) epithelial expression.

Representative images of periodontal tissue sections with CD69 and CD103 immunostaining are presented in Figs. 3 and 4. These images showed that the intensity of CD69 and CD103 immunostaining was increased in patients with periodontal disease as compared to control patients, in both gingival and palatal tissue. The immunohistochemical analysis of periodontitis tissue failed to demonstrate a gradient of CD69 and CD103 staining from the gingival margin to the bottom of the periodontal pocket area in both the epithelium and the underlying connective tissue.

DISCUSSION

To the best of our knowledge this is the first immunohistochemical study regarding the CD69 and CD113 expression into the epithelial layer and lamina propria in clinically healthy and diseased periodontal tissues. We collected tissue samples from both gingiva and palatal area in periodontally healthy individuals to explore the presence and location of Trm cells in relation to microbial plaque exposure. Keratinized squamous epithelia, which are continuously triggered by commensal microbioma and dietary and airborne antigens, line the oral mucosa barrier in the hard palate.²⁶ Microbial communities colonizing shedding epithelia are different from those isolated from the tooth surface.²⁷ Therefore, biopsy tissues harvested from the palate were not challenged by bacterial species constituting tooth-adherent biofilm and were used as negative control in the present study.

Diseased and healthy gingival tissues demonstrated different numbers of CD69+ and CD103+ T cells with higher numbers in periodontitis sites. This could demonstrate that these differences might be the result of the host immune response to the bacterial exposure. This could be corroborated by the overall low number of CD69+/CD103+ T cells in healthy specimens as well as by the absence of statistically significant differences between the gingiva and the palate in the control group. Interestingly, epithelial and stromal CD69 expression was absent in 11 gingival tissues and 7 palatal mucosa specimens, respectively.

The precise surface profile denoting tissue residence of memory T cells is still contentious. Peripheral Trm cells typically express CD69, an early-activation marker that is thought to antagonize the S1P-mediated tissue egress.^{13,14} Furthermore, non-lymphoid Trm cells often express the α E-integrin CD103, which may enhance adhesion to epithelial cells and promote their prolonged tissue retention or may provide survival signals.^{12,15} Peripheral Trm cells may not require CD103 for sustained maintenance as demonstrated in the liver, intestine, and secondary

lymphoid organs.^{28,29}

Kumar et al. demonstrated in humans that CD69 is the key marker that distinguishes Trm cells in barrier tissues from their circulating counterpart, while CD103 expression is more variable and confined to a subset of CD8+ Trm cells, mainly in the gastro-enterical tract and the lung.³⁰ Notably, CD69+ tissue memory T cells are transcriptionally and phenotypically distinct from CD69- memory T cells in tissues and blood and exhibit an increased expression of adhesion, pro-inflammatory and regulatory molecules.³⁰

In the mouse model CD8 Trm cells with CD69 and CD103 phenotype are absent in the oral mucosa, but accumulate in the gut mucosa.³¹ In contrast CD69+ CD103+ CD4 Trm are highly enriched in the oral mucosa, but not in the spleen and lung.³¹ This may be influenced by tissue microenvironments. Importantly, data have been acquired from the entire oral mucosa and not from distinct locations within the oral cavity.

The extension of CD69 expression seems to be a function of bacteria and antigens exposure. In agreement with findings by Mahanonda et al. on periodontitis tissue specimens harvested from sites of extracted teeth, the present study also showed a higher number of CD69+ T cells in the epithelium and connective tissue collected during flap operation procedures.¹⁹ A positive correlation was also observed between number of CD69+ and CD103+ T cells and PD, but the immunohistochemical analysis failed to demonstrate a gradient of these cells from the gingival margin to the bottom of the periodontal pockets. It can be hypothesized that the longer exposure to the bacterial biofilm at the most damaged sites may account for these findings. Once memory T cells have switched to tissue-resident phenotype, they remain in the tissue lifetime and can spread to the neighbouring tissues. The length of time during which periodontal tissues had been exposed to the oral environment

could justify a more pronounced host response at sites with deeper pockets.³²

In the present study we did not discriminate between CD4 and CD8 T cells. Thus, we can only speculate that the persistence in periodontal tissue of CD69+ T cells, expressed by both CD4 and CD8 T subsets, could predispose to an increased tissue inflammatory response during secondary bacteria reinfection in periodontitis treated sites. As demonstrated by Zitzmann et al. in an experimental gingivitis model the larger size of the T-cell inflammatory infiltrate in gingival tissue from periodontitis patients may influence the tissue response to *de novo* plaque formation.³³ Regarding periods of activity and remission of periodontitis, previous investigations showed that the concentration of interferon (IFN)- γ in gingival tissue was higher in active versus inactive sites.^{34,35} It has been shown that T_{rm} cells produce IFN- γ and that high expression of IFN- γ is responsible of inflammatory bone loss.^{19,36} A higher frequency of CD4 memory T cells reactive to serotypes K1 and K2 of *Porphyromonas gingivalis* was detected in periodontitis tissue compared to healthy gingiva, suggesting a role of memory cells in the recurrence of periodontitis.³⁷

Many studies conducted in the other anatomical barrier districts demonstrated that therapies aimed at suppressing T_{rm} cells functions may lead to a transient clinical remission, but the disease will almost inevitably recur if these cells persist within the tissue.⁴ Therefore, the removal of T_{rm} cells during the surgical periodontal therapy, by the resection of the gingival tissue in which they are resident, could make the newly formed gingival unit less "reactive" to a subsequent plaque accumulation, contributing to a slower rate of disease recurrence or progression. This may be relevant in the clinical practice, mainly for periodontitis patients whose incomplete adherence to the supportive periodontal therapy increases the risk of recurrence of the disease over time. A previous study demonstrated that the dimension of inflammatory infiltrate was lower in areas treated by gingivectomy than in sextants

treated by open flap debridement.³² A recent study reported a milder inflammatory response in terms of gingival crevicular levels of interleukin- α in gingival recession sites after bilaminar root coverage procedures when compared to control sites treated by plaque control alone.³⁸

An important limitation of the present study is the difficulty in characterizing CD69+ and CD103+ cells to fully demonstrate their T cell phenotype. Based on morphological assessment (Supplementary Figure 1), these cells resemble mature lymphocytes and do not show features of other inflammatory cells, which can potentially express CD69 or CD103 (e.g. neutrophils, eosinophils, macrophages); considered that B cells do not usually localize within epithelial layers, the observed CD69+ and CD103+ cells were most likely T lymphocytes. Double IHC staining for CD3 and CD69 has been attempted (Supplementary Figure 2), but its evaluation is hampered by an intrinsic limitation: CD3 and CD69 (like CD4, CD8, CD103) are all membrane antigens, thus the two stains will inevitably overlap. Flow cytometry could be used to evaluate antigens co-expression, but requires tissue fragmentation, thus losing information regarding cell localization (e.g. intraepithelial or not). In this context the study by Mahanonda et al. demonstrated the presence of Trm cells in single cells suspension from periodontitis tissues of 4 patients but it did not distinguish between the epithelial or stromal localization.¹⁹ Novel approaches are now enabling precise spatial profiling of multiple antigens and could thus enable full characterization of these cells in future studies.³⁹

In conclusions, the present results would seem to indicate that Trm cells, based on CD69 and CD103 immunostaining, are higher in periodontitis lesions. They could orchestrate the host response to microbial challenge, leading to a faster recurrence of periodontal disease. Among clinical parameters assessed, PD was found the only significant predictor variable associated with CD69 and CD103 expression in

periodontitis patients. Future investigations on the inflammatory response before and after osseous resective surgery are needed to confirm the present findings on a larger population.

Acknowledgements

The authors declare that they have no conflict of interest to disclose. This study has no sources of funding. The authors are grateful to Dr. Valeria Manavella for her support in organizing and conducting the study.

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Figure legends

Figure 1. Box-and-whisker plots showing the number of intraepithelial (A) and stromal (B) CD69+ T cells per high power field (HPF) in periodontitis involved sites and healthy control sites (healthy gingiva and palatal mucosa). The box represents median, 25% and 75% percentiles; the whiskers represent data within 10% and 90% percentiles. ***P < 0.001 *versus* HS and *versus* healthy controls sites. ***P < 0.001 *versus* healthy controls sites.

Figure 2. Box-and-whisker plots showing the number of intraepithelial (A) and stromal (B) CD103+ T cells per high power field (HPF) in periodontitis involved sites and healthy control sites (healthy gingiva and palatal mucosa). The box represents median, 25% and 75% percentiles; the whiskers represent data within 10% and 90% percentiles. ***P < 0.001 *versus* healthy controls sites.

Figure 3. Periodontal tissue samples harvested from the secondary flap during osseous resective surgery. A,C,E: CD69 immunostaining. B,D,F: CD103 immunostaining. It is possible to recognize cells with residency phenotype in both the epithelium (black arrows) and connective tissue (white arrows). Magnification 100x (A,B) and 200X (C,D,E,F).

Figure 4. Tissue samples harvested from a periodontally healthy patient during a mucogingival surgery. A: Gingival tissue stained with CD69. C: Gingival tissue stained with CD103. B: Palatal tissue stained with CD69. D: Palatal tissue stained with CD103. Note the very low number of stained cells (black arrow). Magnification 400x.

Supplementary Tables

Supplementary Table 1. Demographic characteristics of study participants.

Supplementary Table 2. Clinical characteristics of tissue sample sites in periodontitis patients and healthy participants.

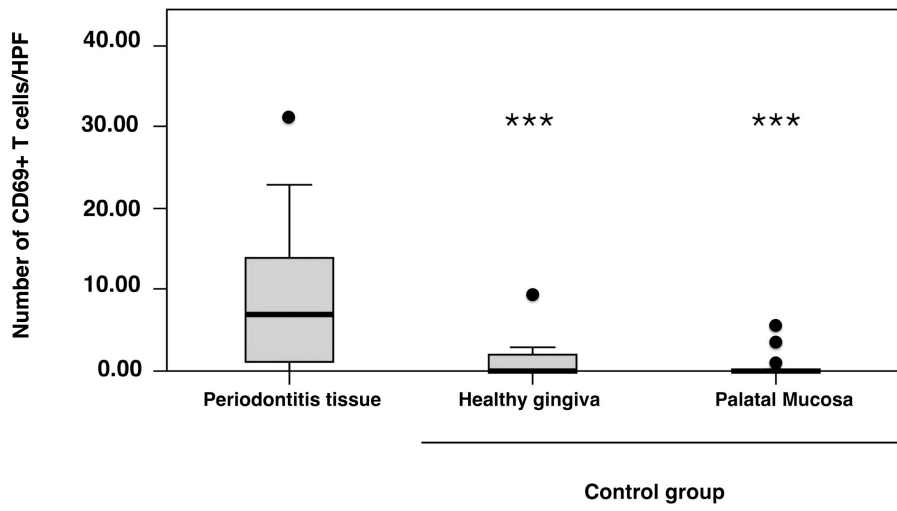
Supplementary Figures

Supplementary Figure 1. High-power HE (A) and CD103 immunohistochemistry (B) images showing a morphology consistent with intraepithelial lymphocytes. Original magnification 400X.

Supplementary Figure 2. High-power immunohistochemistry image with double staining for CD3 (red staining) and CD69 (DAB staining) suggesting a co-expression of these markers and thus the T lymphocytic phenotype of these cells. Original magnification 400X.

Supplementary Figure 3. Scatter diagrams of intraepithelial CD69+ (A) and CD103+ (B) T cells whose counts per high power field (HPF) were correlated significantly with the probing depths in subjects with periodontitis.

A



B

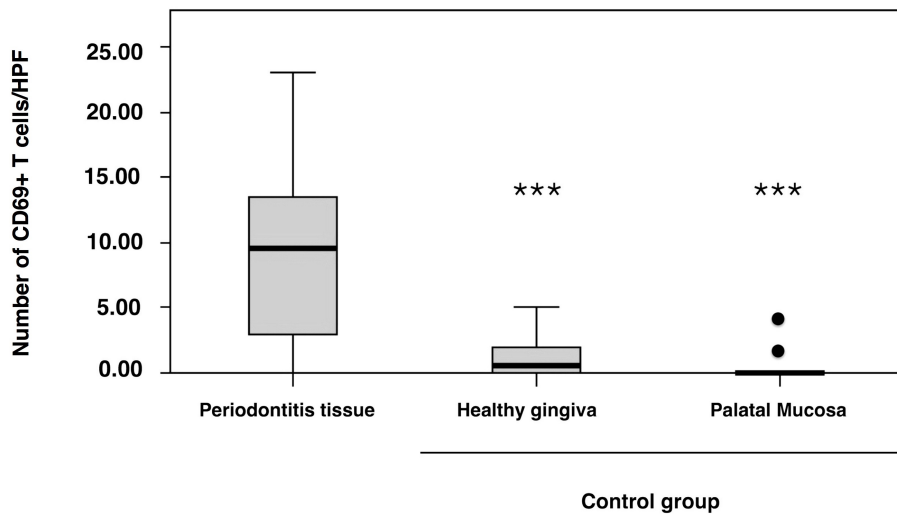


Figure 1

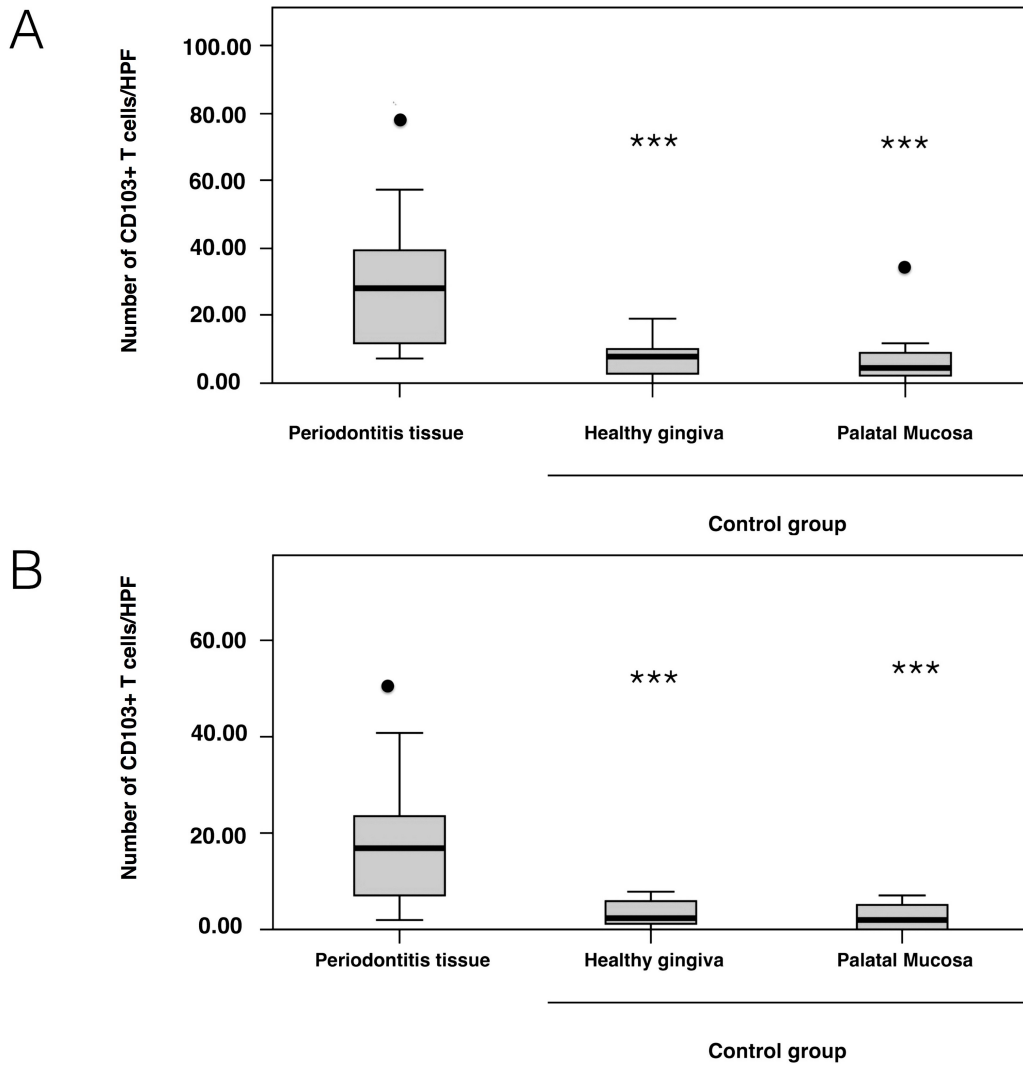


Figure 2

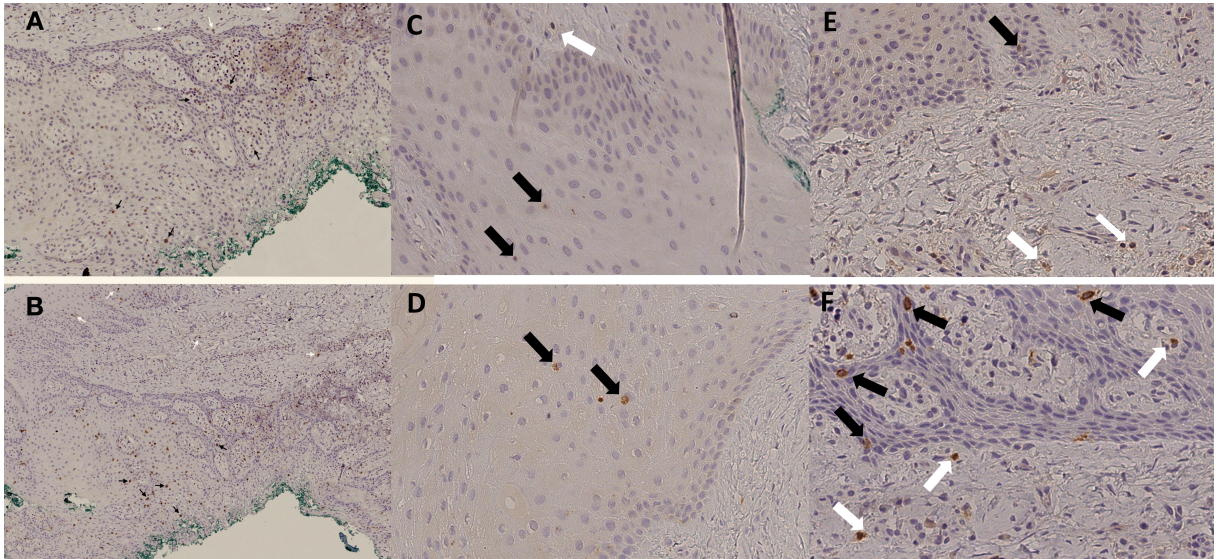


Figure 3

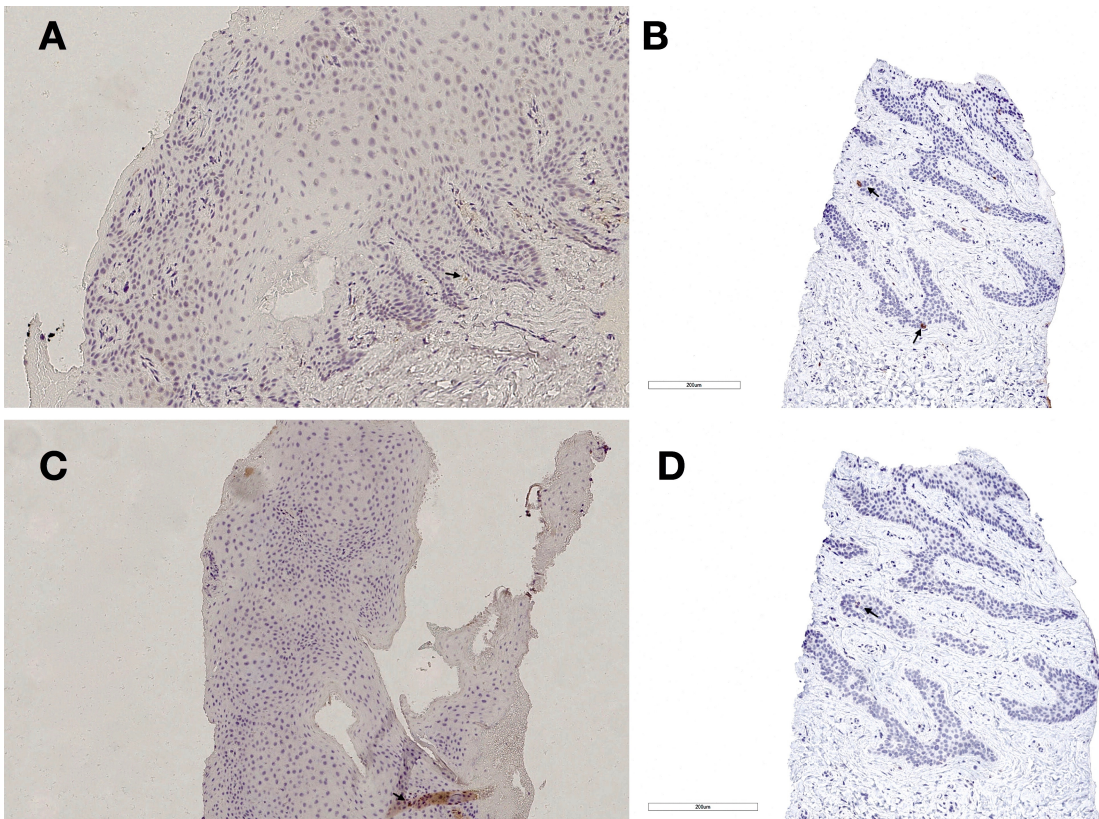


Figure 4