Multidisciplinary Approach in Dentistry 2016
การประชุมวิชาการเฉลิมพระเกียรติ 60 พรรษา สมเด็จพระเทพรัตนราชสุดาฯ สยามบรมราชกุมารี
โดย ราชวิทยาลัยทันตแพทย์แห่งประเทศไทย และทันตแพทยสมาคมแห่งประเทศไทยในพระบรมราชูปถัมภ์

CD103$^+$ Resident Memory T Cells in Periodontitis Lesions

ArsarnYongyuth,[1] Noppadol Sa-Ard-Iam,[2] Chantrakorn Champaiboon,[1] Rangsinee Mahanonda[1]

[1]*Department of Periodontology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand*
[2]*Research unit for Immunopathological/clinical Research in Periodontal Disease, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand*

Abstract

A large number of immune infiltrated cells such as T and B cells are found in periodontal tissues. Recent studies demonstrated that resident memory T cells (CD103$^+$ T cells), located in various tissues and possibly involved in pathogenesis of diseases. However, resident memory T cells in periodontal tissues have not been fully investigated.

Objectives: To investigate the presence of CD103$^+$ memory T cells in periodontal tissues.

Materials and Methods: Human periodontal tissues were obtained from individuals with severe chronic periodontitis and clinically healthy groups. Excised tissues were embedded in paraffin sections, stained with mouse anti-human CD3, CD4, CD8 and CD103 monoclonal antibodies, respectively and investigated under light microscope. For flow cytometric analysis, single cell suspensions were obtained from periodontal tissues and stained with mouse anti-human CD3, CD4, CD8 and CD103 monoclonal antibodies. CD103$^+$ T cells were analyzed by four-color flow cytometry. Mann-Whitney rank-sum test was used to determine the percentage of CD103-expressing T cells.

Results: CD3$^+$, CD4$^+$, CD8$^+$ and CD103$^+$ T cells were found in periodontitis tissues. CD103$^+$ T cells were dispersed throughout both epithelium and connective tissues. The percentage of T cells in periodontitis tissues (24.98?3.07%) was lesser than those in healthy tissues (34.78±2.58%) ($p>0.05$). The percentages of CD3$^+$CD103$^+$ T cells were 17.91±4.14% and 19.75±0.36% in healthy and periodontitis group, respectively. The expressions of CD4$^+$CD103$^+$ T cells in healthy and periodontitis tissues were comparable. While, CD8$^+$CD103$^+$ T cells were approximately 2 to 3-fold higher than CD4$^+$CD103$^+$ T cells both in healthy (34.28±5.51%) and periodontitis groups (41.92 2.30%).

Introduction

Periodontal disease is one of the most common chronic inflammatory diseases found in human[1]. The imbalance of host immune response to bacterial plaque biofilm is proposed to be the immunopathogenesis of the disease[2]. A large number of immune infiltrated cells such as T and B cells are found in periodontal lesions[3,4]. Stable gingivitis lesions are predominated by T cells whereas progressive periodontitis lesions are predominated by B cells and plasma cells[5]. The shift from a stable lesion to a progressive lesion was postulated to play a crucial role in the disease initiation and progression[3]. However, the role of T cells and subsets of T cellsinvolving in the pathogenesis of periodontal disease needs to be clarified.

Corresponding author: *Arsarn Yongyuth*, *Department of Periodontology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand*
E-mail: arsarnyongyuth@gmail.com

T cell population can be generally categorized into $CD4^+$ and $CD8^+$ T cell by cluster of differentiation (CD) on cell surfaces. In addition, T cells are also characterized by a phenotypic expression, the $CD45^+$ molecule. $CD45RA^+$ T cells represent naive T cells that have not yet encountered with their respective antigen. While, $CD45RO^+$ T cells represent a pool of memory T cells[6]. Moreover, memory T cells also classified by their migratory locations, they are then subdivided into central memory T (T_{CM}) and effector memory T (T_{EM}) cells. $CCR7^+$ TCM cells reside in lymphoid tissues and express lymphoid homing marker, CD62L. Whereas, $CCR7^+$ T_{EM} cells which migrate between blood circulation and peripheral tissues express tissue homing markers such as CCR1, CCR3 and CCR5[7].

Since memory T cells in distant tissues is proposed to be crucial in prompt response when tissues exposed to the external stimuli or infection, several studies tried to demonstrate the presence of T cells in various tissues. The evidence showed that memory T cells persisted in multiple tissues such as lung, liver or kidney for long after virus or antigen was cleared[8]. CD103 was described as a marker fortissue resident memory (T_{RM}) cells[9-11]. CD103 involves in interaction with epithelial cells by binding to its ligand, E-cadherin[12]. Therefore, the $CD103^+$-E cadherin interaction may contribute to maintaining the resident status of T_{RM} cells in peripheral tissues[9].

Some evidences show that tissue resident memory T cells permanently reside in non-lymphoid tissues, could provide better protection compared to circulating memory T cells[10,11,13]. Eventhough, several studies showed a protective effect of resident $CD103^+$ T cells, some observations revealed the detrimental effects of T_{RM} cells in various peripheral organs[14,15]. For example, activation of $CD8^+CD103^+$ T cells in skin resulted in localized epidermal injury and rapid production of high levels of interferon-gamma[14]. Therefore, the role of memory T cells in tissues is still inconclusive.

B cells are known to be dominant in periodontal lesion. The shift from T cell lesion to B cell lesion was proposed to be related to the pathogenesis of periodontal disease. However, only few studies focus on the role of T cells in periodontal disease. The percentage of $CD3^+$ (T cells) and $CD45RO^+$ (memory T cells) cells were higher in periodontitis group compared to healthy group[16]. Tonetti and colleagues showed that a half of $CD3^+$ intraepithelial lymphocytes in periodontal tissues expressed $\alpha^{IEL}\beta^7$ integrin[17]. However, resident memory T cells and their roles in periodontal tissues have not been fully investigated.

Objectives

The aim of this study was to investigate the presence of $CD103^+$memory T cells in periodontal tissues compared to clinically healthy tissues.

Materials and methods

Samples

The Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University approved the study protocol (HREC-DCU2015-055) and all subjects signed informed consent before enrollment. Human periodontal tissues were obtained from patients with severe chronic periodontitis and subjects with clinically healthy periodontal tissues. All subjects are in good general health and none of them has taken antimicrobial or anti-inflammatory drugs within the previous 3 months.

Periodontitis tissues were collected from a site of extracted teeth with hopeless periodontal prognosis (gingival inflammation, clinical attachment loss 5 millimeters or more and bone loss 50% of the root length or more). Teeth with other dental diseases such as pulpal lesions were excluded. Each patient of periodontitis

Table 1 Mouse anti-human monoclonal antibodies used for immunohistochemical analysis.

Monoclonal antibodies	Populations
$CD3^+$	T cells
$CD4^+$	Helper T cells
$CD8^+$	Cytotoxic T cells
$CD103^+$	Resident memory cells

group had no history of periodontal treatment for the past 6 months. Healthy periodontal tissueswere collected from a site with clinically healthy gingiva (no bleeding on probing, probing depth less than 4 millimeters, no clinical attachment loss and bone loss) simultaneously to crown lengthening procedure. The subjects were recruited from Postgraduate Periodontology Clinic, Faculty of Dentistry, Chulalongkorn University. The excised tissues were immediately placed in a sterile tube that contain RPMI-1640 medium (Gibco, USA).

Immunohistochemistry

The excised periodontal tissues were washed thoroughly in DPBS. For paraffin embedded sections, they were fixed in 10% buffered formalin for a maximum of 24 hours and subsequently embedded in paraffin. Microtome serial 4-micron-thick sections were cut and mounted on glass slides. Sections were de-waxed. To inhibit endogenous peroxidase, slides were incubated with 0.3% hydrogen peroxide for 20 minutes. Slides were then placed into a 1mM EDTA pH 8.0 and heated at 95°C for 20 minutes for antigen retrieval.

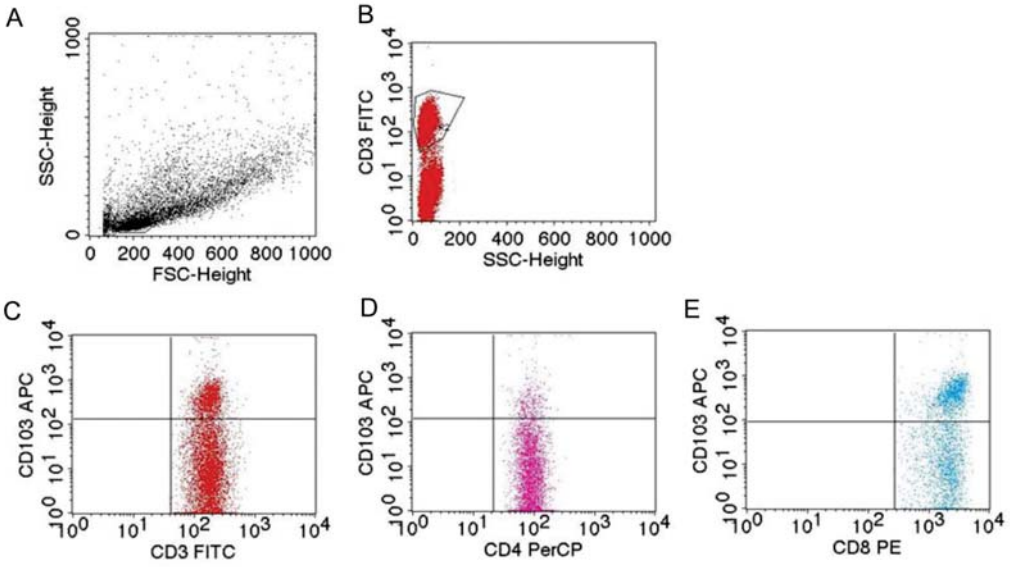

Figure 1 **Flow cytometric gating strategy to identify CD103-expressing cells in periodontal tissues.**

The analysis gates around lymphocyte **(A)**. Cells were analyzed on the basis of surface markers as T cell subpopulations; $CD3^+$ **(B)**, $CD3^+CD103^+$ **(C)**, $CD4^+$ $CD103^+$ **(D)** and $CD8^+CD103^+$ **(E)** T cells. **SSC**, side scatter; **FSC**, forward scatter.

For identifying localization of T cells in tissues, single immunohistochemical staining was performed via Polymer/HRP and DAB+chromogen system (DAKO EnVisionTM G/2 Double stain System) on sections. They were stained with primary mouse-anti-human CD3 (FITC), CD4 (PerCP), CD8 (APC-Cy7) and CD103 (APC) monoclonal antibodies (BD Biosciences) or isotype control (Table 1). Counterstaining was done with haematoxylin. They were investigated under light microscope.

Gingival cell preparation

Tissues were washed in RPMI-1640 medium and cut into small fragments (1-2 mm^3). These fragments were incubated at 37 oc in RPMI-1640 that contained 2 mg/ml of collagenase type I (Sigma Chemical Co.). The ratio of medium with collagenase to tissues was 1 ml per 100 mg of tissue. After 90 minutes of incubation at 37°C in 5% CO_2 atmosphere, residual tissue fragments were disaggregated by flushing several times with a pipette, until single cell suspensions were obtained. The single cell suspensions were then filtered through a filter of mesh size 70 µm (BD Biosciences).

Flow cytometric analysis of T cells

Extracted gingival cells from periodontal tissues were stained with mouse anti-human CD3 (FITC), CD4 (PerCP), CD8 (APC-Cy7) and CD103 (APC) monoclonal

antibodies (BD Biosciences) at 4°C for 30 minutes. The stained gingival cells were washed with PBS containing 0.1% albumin and 0.01% sodium azide and then fixed with 1% paraformaldehyde. Cells were then analyzed by four-color flow cytometry, FACSCailbur (BD Biosciences). $CD3^+$ cells were gated and then analyzed for the expression of CD4, CD8 and CD103 (Figure 1).

Statistical analysis

The data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Results were presented in mean±S.E. The non-parametric Mann-Whitney rank-sum test was used to determine the difference between the percentages of CD103-expressing T cells in both groups. A critical level of 0.05 was employed. Thus, p-value less than 0.05 were considered as statistically significant.

Results

The determination of CD3, CD4, CD8 and CD103 positive cells in periodontitis tissue were made by immunohisto-chemistry. Paraffin-embedded sections from a periodontitis patient are presented in Figure 2. Numerous $CD3^+$ cells resided at epithelium, connective tissues and epithelial-connective tissue junction (Figure 2B). A few CD4 positive cells were found in connective

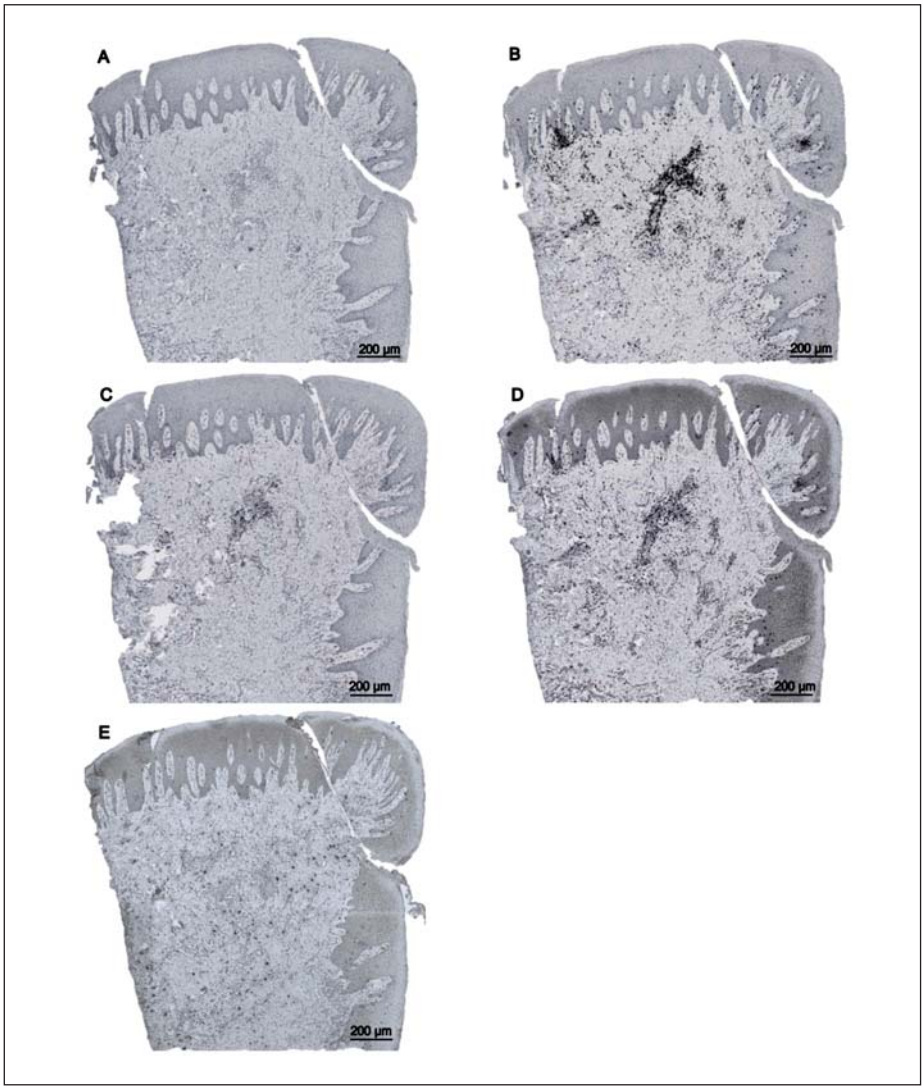

Figure 2 **Immunohistochemical analysis of T cells in a periodontal tissue.** Periodontal tissue biopsy obtained from a representative periodontitis patient illustrating; negative control **(A)**, CD3 staining **(B)**, CD4 staining **(C)**, CD8 staining **(D)** and CD103 staining **(E)**. Bar is 200 mm.

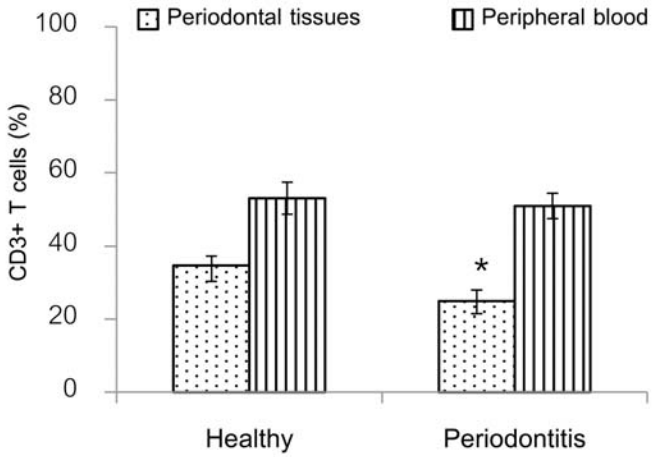

Figure 3 Mean percentage of $CD3^+$ T cells in periodontal tissues and peripheral blood.
Cells extracted from healthy and periodontitis individuals were stained with anti-human CD3 monoclonal antibodies, then analyzed by flow cytometry. Data were presented in mean±S.E. *, $p < 0.05$.

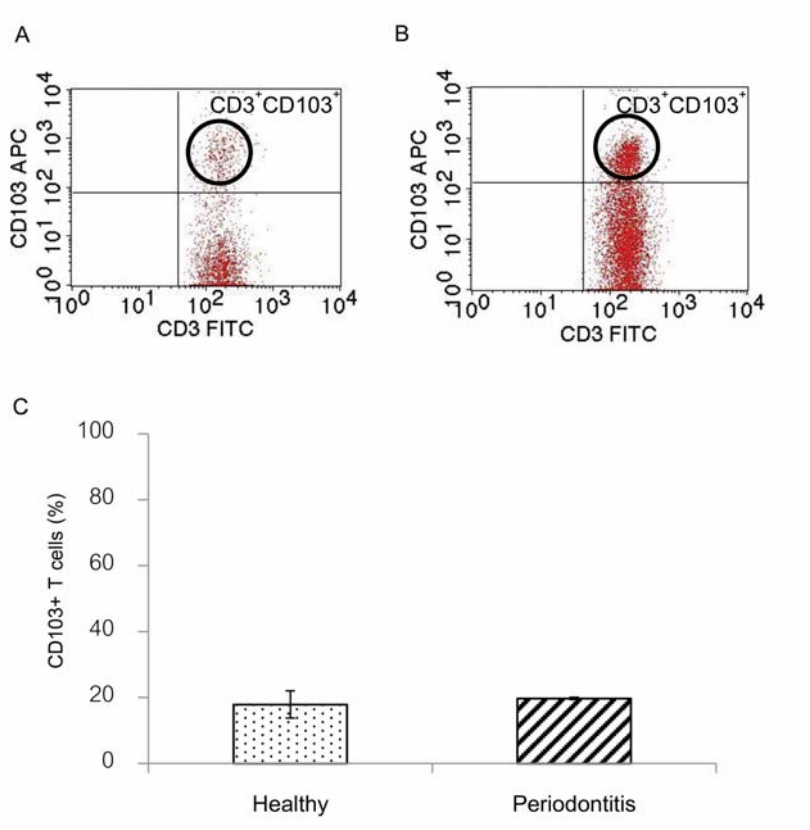

Figure 4 Mean percentage of CD103-expressing $CD3^+$ T cells in periodontal tissues.
Cells extracted from healthy and periodontitis tissues were stained with anti-human CD3 and co-stained with anti-human CD103 monoclonal antibodies, then analyzed by flow cytometry. The expression of CD103 marker on $CD3^+$ T cells isolated from healthy periodontal tissues **(A)** and periodontitis tissues **(B)**. Data were from a representative subject in each group. The mean percentages of $CD3^+CD103^+$ T cells from healthy periodontal and periodontitis tissues have shown in panel **(C)**.

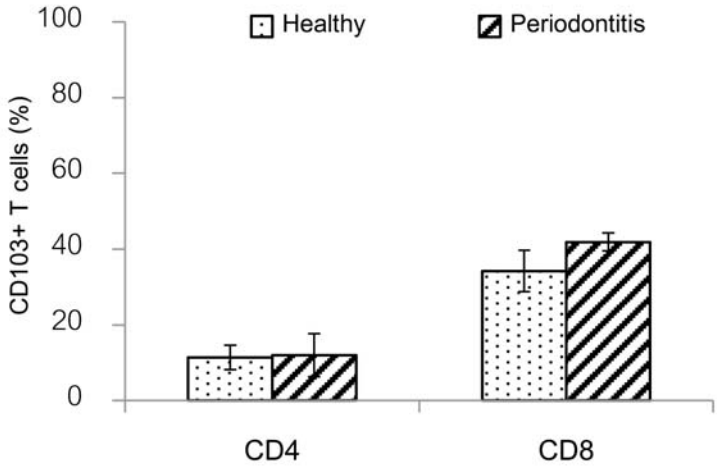

Figure 5 **Mean percentage of CD103-expression in $CD4^+$ and $CD8^+$ T cells in periodontal tissues.** Cells extracted from healthy and periodontitis tissues were stained with anti-human CD4, antihuman CD8 and co-stained with anti-human CD103 monoclonal antibodies and then analyzed by flow cytometry. Data were presented in mean±S.E.

tissues and very few in the epithelia (Figure 2C). Some clusters of CD3 and CD8 positive cells also detected in epithelial-connective tissue junction and underlying connective tissues (Figure 2B and 2D). CD103 positive cells were also dispersed throughout both epithelium and connective tissues (Figure 2E).

Analysis of T cell expression by flow cytometry

In peripheral blood, the percentages of CD3 expressing infiltrated cells in healthy subjects and periodontitis patientswere 53.01±4.48% and 50.90±3.55, respectively. No significant difference was found regarding to the percentage of T cells between these two patient groups. In healthy tissues, 34.78±2.58% of infiltrated cells expressed CD3, whereas 24.98±3.07% of infiltrated cells in periodontitis tissues were CD3 positive. There was a significant difference of the percentage of T cell between healthy group and periodontitis group (Figure 3).

CD103-expressing T cells in periodontal tissues

The percentages of $CD3^+$ T cells that expressed CD103 were 17.91±4.14% and 19.75±0.36% in healthy group and periodontitis group, respectively. There was no statistically significant difference between these 2 groups (Figure 4A, 4B and 4C).

The expression of CD103 in $CD4^+$ T cells in healthy and periodontitis tissues were quite similar, the percentages of $CD103^+CD4^+$ T cells were 11.36±3.23% and 11.99±5.71%, respectively. Surprisingly, $CD103^+$ $CD8^+$ T cells were approximately 2-fold higher than $CD103^+CD4^+$ T cells both in healthy (34.28±5.51%) and periodontitis groups (41.92±2.30%) (Figure 5).

Discussion

Our results showed that approximately 24-48% and 16-34% of immune infiltrated cells in healthy tissues and periodontitis tissues, respectively, were $CD3^+$ T cells which are comparable to the data reported by Berglundh and Donati[18]. They suggested that 13% of T cells expressed CD4 whereas 4% expressed CD8 in severe periodontitis tissues. However, no earlierstudy reports on the comparison between the mean percentages of T cells in healthy and periodontitis individuals. Our findings showed the significant difference between percentage of T cell in healthy and periodontitis tissues, but not in peripheral blood, which may indicate the distinct origin between T cells in peripheral blood and periodontal tissues.

Only one study reported that $\alpha^{IEL}\beta^7$-expressing $CD3^+$ T cells, resident T cells were detected in a severe stage of periodontal tissues and the number of resident cells detected is comparable to our data[17]. Our finding confirmed that intraepithelial lymphocytes located in both gingival epithelium and connective tissues. Furthermore, we extended the study to investigate whether T cell subsets in periodontal tissues express CD103 by flow

cytometry. Our observation revealed that more than 1/3 of $CD8^+$ T cells were $CD8^+CD103^+$ T cells, while only 11-15% of $CD4^+$ T cells expressed CD103. There was no statistically significant difference between periodontitis patient and healthy groups. However, the percentage of $CD8^+CD103^+$ T cells seemed to be higher than those of $CD8^+CD103^-$ T cells. Previous studies suggested that most $CD103^+$ tissue resident cells were a subset of $CD8^+$ T cells[19,20]. They have been described in various tissues, such as brain, intestine, skin and sensory ganglia[10,19-21]. CD103 also functions as a receptor for E-cadherin, an adhesion molecule expressed by epithelial cells[12] and thus maintaining the resident status in peripheral tissues. This may justify the presence of $CD8^+CD103^+$ T cells in the tissues. Even though, $CD4^+CD103^+$ T cells were also detected, the role and function of $CD4^+CD103^+$ T cells are not fully determined. The presence of CD103-expressing T cells in healthy and diseased periodontal tissues may be associated with localized periodontal infection since these populations may be able to give an early response to the infection. The further investigation is needed to clarify the function of these T cell populations including its role in periodontal homeostasis.

Conclusions

T cells were detected in periodontal tissues both in epithelial layers and connective tissues. Resident memory T cells were also found in both healthy and periodontitis tissues. The majority of resident memory T cells were $CD8^+$ T cells. The further investigation is needed to clarify the function of these T cell populations in the periodontal pathogenesis

Acknowledgements

We would like to thank Dr.Sathit Pichyangkul, a Director of Immunology and Medical Component, AFRIMS, Bangkok for providing laboratory facilities and setting experiments. We also would like to thank Faculty of Chulalongkorn University for research funding.

References

1. *Williams RC. Periodontal disease. N Engl J Med. 1990;322:373-82.*
2. *Kornman KS. Mapping the pathogenesis of periodontitis: a new look. J Periodontol. 2008;79:1560-8.*
3. *Seymour GJ, Powell RN, Davies WI. Conversion of a stable T-Cell lesion to a progressive B-cell lesion in the pathogenesis of chronic inflammatory periodontal disease: an hypothesis. J Clin Periodontol. 1979; 6:267-77.*
4. *Yamazaki K, Nakajima T, Aoyagi T, Hara K. Immunohistological analysis of memory T lymphocytes and activated B lymphocytes in tissues with periodontal disease. J Periodontal Res. 1993;28:324-34.*
5. *Seymour GJ, Greenspan JS. The phenotypic characterization of lymphocyte subpopulations in established human periodontal disease. J Periodontal Res. 1979;14:39-46.*
6. *Michie CA, McLean A, Alcock C, Beverley PC. Lifespan of human lymphocyte subsets defined by CD45 isoforms. Nature. 1992;360:264-5.*
7. *Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401:708-12.*
8. *Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. Science. 2001;291:2413-7.*
9. *Pauls K, Schon M, Kubitza RC, Homey B, Wiesenborn A, Lehmann P, et al. Role of integrin alphaE(CD103) beta7 for tissue-specific epidermal localization of $CD8^+$ T lymphocytes. J Invest Dermatol. 2001; 117:569-75.*
10. *Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, Carbone FR. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. Nat Immunol. 2009;10:524-30.*
11. *Wakim LM, Woodward-Davis A, Bevan MJ. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. Proc Natl Acad Sci U S A. 2010;107: 17872-9.*
12. *Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, et al. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature. 1994;372:190-3.*
13. *Jiang X, Clark RA, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS. Skin infection generates non-migratory memory $CD8^+$ T(RM) cells providing global skin immunity. Nature. 2012;483:227-31.*
14. *Mizukawa Y, Yamazaki Y, Teraki Y, Hayakawa J, Hayakawa K, Nuriya H, et al. Direct evidence for interferon-gamma production by effector-memory-type intraepidermal T cells residing at an effector site of immunopathology in fixed drug eruption. Am J Pathol. 2002;161:1337-47.*
15. *Smyth LJ, Kirby JA, Cunningham AC. Role of the mucosal integrin alpha(E)(CD103)beta(7) in tissue-restricted cytotoxicity. Clin Exp Immunol. 2007;149: 162-70.*

16. Amunulla A, Venkatesan R, Ramakrishnan H, Arun KV, Sudarshan S, Talwar A. Lymphocyte subpopulation in healthy and diseased gingival tissue. J Indian Soc Periodontol. 2008;12:45-50.
17. Tonetti MS, Straub AM, Lang NP. Expression of the cutaneous lymphocyte antigen and the alpha IEL beta 7 integrin by intraepithelial lymphocytes in healthy and diseased human gingiva. Arch Oral Biol. 1995;40:1125-32.
18. Berglundh T, Donati M. Aspects of adaptive host response in periodontitis. J Clin Periodontol. 2005; 32 Suppl 6:87-107.
19. Gebhardt T, Whitney PG, Zaid A, Mackay LK, Brooks AG, Heath WR, et al. Different patterns of peripheral migration by memory $CD4^+$ and $CD8^+$ T cells. Nature. 2011;477:216-9.
20. Wakim LM, Bevan MJ. Cross-dressed dendritic cells drive memory $CD8^+$ T-Cell activation after viral infection. Nature. 2011;471:629-32.
21. Masopust D, Choo D, Vezys V, Wherry EJ, Duraiswamy J, Akondy R, et al. Dynamic T cell migration program provides resident memory within intestinal epithelium. J Exp Med. 2010;207:553-64.