เม็มโมรี่ทีเซลล์ที่แสดงออกซีดี103ในเนื้อเยื่อปริทันต์ปกติและโรคปริทันต์

นายอาสาฬห์ ยงยุทธ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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$CD103^+$ MEMORY T CELLS IN PERIODONTAL HEALTH AND DISEASE

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อาสาฬห์ ยงยุทธ : เม็มโมรี่ทีเซลล์ที่แสดงออกซีดี103ในเนื้อเยื่อปริทันต์ปกติและโรคปริทันต์ (CD103^{+} MEMORY T CELLS IN PERIODONTAL HEALTH AND DISEASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ทญ. ดร.จันทรกร แจ่มไพบูลย์, 64 หน้า.

โรคปริทันต์เป็นโรคที่มีการอักเสบเรื้อรังของอวัยวะรองรับฟัน ซึ่งประกอบด้วยโรคเหงือกอักเสบและโรคปริทันต์อักเสบ โรคเหงือกอักเสบจะมีทีเซลล์เป็นส่วนใหญ่ ขณะที่โรคปริทันต์อักเสบจะมีบีเซลล์มากในรอยโรค การเปลี่ยนแปลงจากรอยของโรคทีเซลล์ไปเป็นรอยโรคของบีเซลล์ถูกตั้งข้อสันนิษฐานว่าเป็นสาเหตุของพยาธิกำเนิดของโรคปริทันต์ เมื่อไม่นานมานี้ กลุ่มย่อยใหม่ของทีเซลล์และทิชชู-เรสซิเดินซ์ เม็มโมรี่ทีเซลล์ (ซีดี103^{+}ทีเซลล์) พบในโรคหลายชนิดและน่าจะเกี่ยวข้องกับพยาธิกำเนิดของการเกิดโรคนั้นๆ อย่างไรก็ตาม การศึกษาเกี่ยวกับประชากรของทีเซลล์เหล่านี้ในเนื้อเยื่อปริทันต์ยังมีอยู่จำกัด จุดประสงค์ของการศึกษานี้เพื่อตรวจสอบการมีอยู่ของกลุ่มย่อยใหม่ของทีเซลล์ในเนื้อเยื่อปริทันต์ เนื้อเยื่อปริทันต์ที่ได้จากผู้ป่วยโรคปริทันต์อักเสบเรื้อรังขั้นรุนแรงหรือผู้ที่มีเนื้อเยื่อปริทันต์ปกติจะถูกนำมาเตรียมในพาราฟินและวิเคราะห์หาซีดี3^{+} , ซีดี4^{+}, ซีดี8^{+}และซีดี103^{+}ทีเซลล์ด้วยวิธีอิมมูโนฮิสโตเคมี สารแขวนลอยเซลล์เดี่ยวนำมาใช้วิเคราะห์หาประชากรของทีเซลล์ด้วยการใช้วิธีโฟลไซโทเมทรีแบบ 6 สี จากการวิเคราะห์ทางอิมมูโนฮิสโตเคมี พบว่า ซีดี103^{+}ทีเซลล์อยู่ในชั้นเยื่อบุผิวและชั้นเนื้อเยื่อเกี่ยวพัน การวิเคราะห์ด้วยวิธีโฟลไซโทเมทรีแสดงให้เห็นว่าซีดี103^{+}เซลล์ส่วนใหญ่นั้นเป็นซีดี8^{+}ทีเซลล์ ซีดี8^{+}ซีดี103^{+}ทีเซลล์ในเนื้อเยื่อปริทันต์อักเสบมีจำนวนเป็น 2 เท่าของเนื้อเยื่อปริทันต์ปกติ (58.32±26.08% และ 32.70±10.90% ของจำนวนซีดี8^{+}ทีเซลล์ทั้งหมด, p<0.05, ตามลำดับ) การศึกษานี้ยังได้ตรวจสอบกลุ่มย่อยอื่นๆของทีเซลล์ในเนื้อเยื่อปริทันต์เป็นครั้งแรก ได้แก่ นาอีฝ (T_N), สเต็มเซลล์-ไลค์ เม็มโมรี่ (T_{SCM}), เซนทรัล เม็มโมรี่ (T_{CM}), อิเฟคเทอร์ เม็มโมรี่ (T_{EM}) และ เทอร์มินัลลี ดิฟเฟอเรนติเอเตค เม็มโมรี่ (T_{TE}) ทีเซลล์ ผลการศึกษาพบว่ากลุ่มประชากรทั้งห้ามีอยู่ในทั้งกลุ่มเหงือกปกติและกลุ่มปริทันต์อักเสบ อย่างไรก็ตาม โดยส่วนใหญ่ของกลุ่มย่อยของทีเซลล์เหล่านี้เป็นกลุ่มของเซนทรัล เม็มโมรี่ทีเซลล์ การศึกษาครั้งนี้เป็นครั้งแรกที่แสดงว่ามีกลุ่มย่อยของทีเซลล์เหล่านี้ในเนื้อเยื่อปริทันต์การศึกษาเพิ่มเติมก็มีความจำเป็นเพื่อให้เข้าใจบทบาทของทีเซลล์เหล่านี้ในด้านการป้องกันหรือการดำเนินของโรคปริทันต์

ภาควิชา ปริทันตวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา ปริทันตศาสตร์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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5675827032 : MAJOR PERIODONTICS

KEYWORDS: PERIODONTITIS / MEMORY T CELLS / RESIDENT T CELLS / CD103 / HUMAN TISSUE IMMUNOLOGY

ARSARN YONGYUTH: $CD103^+$ MEMORY T CELLS IN PERIODONTAL HEALTH AND DISEASE. ADVISOR: ASST. PROF. CHANTRAKORN CHAMPAIBOON, Ph.D., 64 pp.

Periodontal disease is a chronic inflammatory disease of tooth supporting structures, which comprises of gingivitis and periodontitis. Gingivitis is predominated by T cells, whereas periodontitis is a B cell dominated lesion. The shift of a T cell lesion to a B cell lesion was proposed to be pathogenesis of periodontal disease. Recently, novel subpopulations of T cells and tissue-resident memory T cells ($CD103^+$ T cells) were reported in several diseases and possibly involved in their pathogenesis. However, study of these populations in periodontal tissues is limited. The aim of this study is to investigate the presence of novel T cell subsets in periodontal tissues. Periodontal tissues obtained from either individuals with severe chronic periodontitis or clinically healthy subjects were prepared as paraffin sections and then analyzed for $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ T cells by immunohistochemistry. Single cell suspensions were used to analyze T cell populations using 6-color flow cytometry. From immunohistochemical analysis, $CD103^+$ T cells were detected in both epithelium and connective tissues. Flow cytometric analysis revealed that the majority of CD103 expressing cells were $CD8^+$ T cells. $CD8^+CD103^+$ T cells in periodontitis tissues were 2-fold higher than those of healthy periodontal tissues (58.32±26.08% and 32.70±10.90% of total $CD8^+$ T cells, $p<0.05$, respectively). The study also investigated other subsets of T cells in periodontal tissues i.e. naïve (T_N), stem cell-like memory (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory T (T_{TE}) cells. Our finding showed that these 5 novel populations were detected in both healthy and periodontitis groups. However, the majority of T cell populations were T_{CM} cells. This is the first time that showed the existence of these T cell subsets in periodontal tissues. Further studies are required to gain an insight into their role on periodontal homeostasis or pathogenesis.

Department: Periodontology Student's Signature .....

Field of Study: Periodontics Advisor's Signature .....

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LIST OF ABBREVIATIONS

APC	Allophycocyanin
APCs	Antigen presenting cells
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CD62L	CD62 ligand
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
HSV	Herpes simplex virus
IFN	Interferon
IEL	Intraepithelial lymphocyte
IL	Interleukin
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein complex
RANKL	Receptor activator of nuclear factor kappa-B ligand
RPMI	Roswell Park Memorial Institute
S1P1	Sphingosine-1-phosphate receptor
TCR	T cell receptor
TGF	Transforming growth factor

TNF	Tumor necrosis factor

CHAPTER I

INTRODUCTION

Background of the present study

Periodontal disease involves tooth-supporting structures including gingiva, periodontal ligament, cementum and alveolar bone. According to its severity, the disease can be simply categorized into gingivitis and periodontitis. The periodontitis, a severe form, is an inflammation of the periodontium leading to alveolar bone destruction then tooth loss (Seymour et al., 1979). The imbalance of host immune response to bacterial plaque biofilm is proposed to be the immunopathogenesis of the disease (Kornman, 2008). In immunological studies, a large number of immune infiltrated cells such as T and B cells are found in periodontal lesions (Seymour et al., 1979, Yamazaki et al., 1993, Page and Schroeder, 1976, Brandtzaeg and Kraus, 1965). Stable gingivitis lesions are predominated by T cells whereas progressive periodontitis lesions are predominated by B cells and plasma cells (Seymour and Greenspan, 1979). The shift from a stable lesion to a progressive lesion was postulated to play a crucial role in the disease initiation and progression (Seymour et al., 1979). However, the role of T and B cells in the pathogenesis of periodontal disease is not clarified.

T cell population is generally categorized into $CD4^+$ (cluster of differentiation) and $CD8^+$ T cells. While $CD4^+$ and CD 8^+ T cells are defined by cluster of differentiation on cell surfaces, T helper (T_h) cells are classified by their roles in immune response and their cytokine profiles. T_h1 cells predominantly mediate cell-mediated immune responses via interferon-gamma (IFN-γ) and interleukin 2 (IL-2). Whereas, T_h2 cells secrete IL-4, IL-5, IL-10 and IL-13 which play a vital role on the growth and differentiation of activated B cells (Mosmann and Coffman, 1989). The T_h1/T_h2 paradigm

was then proposed for explaining the pathogenesis of periodontal disease for last few decades (Seymour et al., 1993). However, the role of T_h1 and T_h2 cells in human periodontal disease remains inconclusive. Recently, T_h17 cells have been proposed (Cardoso et al., 2009). They secrete IL-17 which induces IL-6, IL-8 and prostaglandin-E2 production. Hence, T_h17 cells are thought to play a role in osteoclastic activity and thereby mediate bone resorption (Ohlrich et al., 2009). On the other hand, regulatory T cells (T_{reg}) which are critical in regulation of inflammatory responses by secreting transforming growth factor-beta (TGF-β) and IL-10 (Couper et al., 2008). Therefore, the counterbalance of T_h17 cells and T_{reg} cells is postulated to contribute to the pathogenesis of periodontal disease (Zhou and Littman, 2009).

Besides cytokine profiles, 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 (Michie et al., 1992). Several studies suggested that memory T cells showed superior migration to and accumulation at sites of chronic inflammation compared to naïve T cells (Damle and Doyle, 1990, Oppenheimer-Marks et al., 1990, Sanders et al., 1988). Even though a large number of memory $CD45RO^+$ T cells were detected in periodontitis tissues (Gemmell et al., 1992), the role of memory T cells in periodontal disease is not fully understood.

Sallusto et al. also identified the heterogeneity of T cells on the basis of the lymph node-homing C-C chemokine receptor-7 (CCR7) (Sallusto et al., 1999). Based on their migratory properties, memory T cell subsets are subdivided into central memory T (T_{CM}) and effector memory T (T_{EM}) cells (Sallusto et al., 1999). $CCR7^+$ T_{CM} cells reside in lymphoid tissues and express lymphoid homing marker, CD62L. Whereas, $CCR7^+$ T_{EM} cells migrate between blood circulation and peripheral tissues (Sallusto et al., 1999). Masopust et al. showed that in response to virus or bacterial infection, antigen-specific

memory $CD8^+$ T cells migrated to non-lymphoid tissues were present as long-lived memory cells (Masopust et al., 2001). Subsequently, Gebhardt et al. termed these cells as tissue-resident memory T (T_{RM}) cells (Gebhardt et al., 2009). Tissues-resident memory T cells represent the predominant memory T cell subsets in many peripheral tissue such as, skin (Clark et al., 2006), gut (Masopust et al., 2010) and lung (Teijaro et al., 2011). These T_{RM} cells provided a first line of defense against secondary infection at local sites. Collective evidences from mouse and human studies suggested that T_{RM} cells in peripheral tissues played a key role in mediating protective immunity against microbial pathogens (Jiang et al., 2012, Mackay et al., 2012, Shin and Iwasaki, 2012). Despite the protective effects of T_{RM} cells, several studies suggested their role in detrimental effects, such as fixed-drug eruption (Mizukawa et al., 2002) and psoriasis (Boyman et al., 2004). At present, the role of T_{RM} cells is not fully understood.

Recently, other novel subpopulations of memory T cells have been observed eg. stem cell-like memory T (T_{SCM}) and terminally differentiated effector memory T (T_{TE}) cells (Farber et al., 2014, Gattinoni et al., 2011, Mahnke et al., 2013). So far, memory T cell subpopulations and resident memory T cells in periodontal tissues have not been fully investigated. This study analyzed the localization of periodontal tissue-resident memory T cells and a profile of memory T cells in periodontitis tissues compared to healthy periodontal tissues which may in turn, provide further information of periodontal tissue-specific memory T cells. Therefore, it may contribute to a better understanding in the pathogenesis of periodontal disease.

Objectives

1. To study anatomical localization of periodontal tissue-resident memory T cells that express CD103 by immunohistochemistry.

2. To investigate T cell subsets in periodontitis tissues as compared to healthy periodontal tissues by flow cytometric analysis: eg.
   - Naïve T (T_N) cells
   - Stem cell-like memory T (T_{SCM}) cells
   - Central memory T (T_{CM}) cells
   - Effector memory T (T_{EM}) cells
   - Terminally differentiated effector memory T (T_{TE}) cells
   - Periodontal tissue-resident memory T (T_{RM}) cells

Hypothesis

1. Periodontal tissue-resident memory T cells are detected in periodontal lesions.
2. Increased percentage of memory T cells in each subpopulation could be found in periodontitis tissues as compared to healthy tissues.

Field of research

Human immunology

Criteria Inclusions

1. Inflamed periodontal tissues were obtained from subjects with severe chronic periodontitis (gingival inflammation, clinical attachment loss 5 mm or more, severe bone loss approximately 50% of the root length or more, hopeless periodontal prognosis).
2. Healthy tissues were obtained from healthy periodontal subjects (no bleeding on probing, probing depth less than 4 mm, no clinical attachment loss and bone loss).

3. All subjects were in good general health, and none of them has taken antimicrobial or anti-inflammatory drugs within the previous 3 months.

Limitation of research

This study cannot investigate many periodontal tissue samples in each group due to limited time and expenses.

Application and expectation of research

1. New information of memory T cell profile and localization of $CD103^{+}$ periodontal tissue-specific memory T cells in periodontitis tissues comparing to healthy periodontal tissues.
2. Publication in the national peered-review journal.

Key words

Periodontitis, memory T cells, resident T cells, CD103, Human tissue immunology

CHAPTER II

LITERATURE REVIEW

Periodontal disease

Periodontal disease is a common chronic inflammatory disease and considered one of the most significant causes of tooth loss in adults (Genco et al., 2005). The disease involves tooth-supporting structures including gingiva, periodontal ligament, cementum and alveolar bone. According to its severity, the disease can be categorized into gingivitis and periodontitis. Gingivitis is an inflammation confined to the gingiva (Page, 1986), termed as a stable lesion (Seymour et al., 1979). The clinical features of gingivitis are characterized by increased redness, swelling and bleeding gum during brushing or probing (Mahanonda, 2012). The other form is periodontitis which the inflammation involves periodontium, leading to connective tissue and alveolar bone destruction (Ranney, 1993), termed as a progressive lesion (Seymour et al., 1979). The clinical features of periodontitis are gingival inflammation, attachment loss and periodontal probing depth is equal or more than 4 mm. When periodontitis progresses, teeth became mobile and tooth loss may finally occur (Mahanonda, 2012).

The imbalance of host immune response to bacterial plaque biofilm is proposed to be the immunopathogenesis of the disease. Moreover, genetic, environmental and acquired risk factors are also considered to play a role in initiating and progression of the disease (Kornman, 2008). In healthy periodontium and gingivitis lesions, the microbial plaque composition is a majority of gram positive facultative bacteria, such as *Streptococci* and *Actinomyces* species. On the other hand, gram negative anaerobes particularly, *Porphyromonas gingivalis, Tannerela forsythia* and *Aggregatibactor actinomycetemcomitans* seem to be key pathogens of microbial plaques in periodontitis

lesions (1996). A large numbers of immune infiltrated cells such as T and B cells are found in periodontal lesions (Seymour et al., 1979, Yamazaki et al., 1993, Page and Schroeder, 1976, Brandtzaeg and Kraus, 1965). Moreover, recent studies suggested that plasma cells are predominant in periodontitis lesions (Amunulla et al., 2008, Thorbert-Mros et al., 2014). However, there has been limited study of T cells in periodontal disease.

T cell biology

Lymphocytes derived in two major populations as T and B cells. T cells differentiate from hematopoietic stem cells (HSCs) in bone marrow (Abbas and Lichtman, 2005, Delves et al., 2011). Immature T cells migrate from the bone marrow through the blood circulation to the thymus. These immature T cells undergo functional maturation and develop into mature naïve T cells (Delves et al., 2011, Murphy et al., 2008). Naïve T cells constantly migrate between peripheral lymphoid organs and blood circulation where they interact with antigens (Abbas and Lichtman, 2005).

T cell populations are generally categorized into $CD4^+$ and $CD8^+$ T cells by cluster of differentiation (CD) on cell surfaces. $CD4^+$ T cells concurrently differentiate into T helper (Th) cells. Th1 cells mediate predominantly cell-mediated immune response to eradicate intracellular pathogens by their cytokines such as IFN-γ, IL-2 and tumor necrosis factor (TNF). Whereas, T_h2 cells play a vital role on the growth and differentiation of activated B cells by secreting IL-4, IL-5, IL-10 and IL-13 (Mosmann et al., 1986, Mosmann and Coffman, 1989). An experiment conducted in a mouse model demonstrated that mice resistant to intracellular *Leishmania major* infection developed T_h1 cell response whereas those susceptible to the infection developed T_h2 response (Mosmann et al., 1986, Mosmann and Coffman, 1989). The T_h1/T_h2 paradigm was then proposed for explaining the pathogenesis of periodontal disease (Seymour et al., 1993).

It was hypothesized that T_h1 cells are associated with a stable gingivitis lesion whereas T_h2 cells are associated with a progressive periodontitis lesion (Gemmell et al., 2002, Gemmell et al., 2007). On the other hand, some studies showed a mixed T_h1 and T_h2 responses in periodontitis (Prabhu et al., 1996, Berglundh et al., 2002, Fujihashi et al., 1996). Therefore, T_h1/T_h2 paradigm as the pathogenesis of periodontal disease is still in question. Unlike $CD4^+$ T cells, $CD8^+$ cytotoxic T lymphocytes directly eliminate host cells that infected by virus or intracellular pathogens by IFN-γ, TNF, granzyme and perforin (Lieberman, 2003, Kelso et al., 2002).

A separate lineage of $CD4^+$ T cell subset, T_h17 cells, has been recently described (Cardoso et al., 2009, Park et al., 2005). T_h17 cells are implicated in autoimmune and inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis and inflammatory bowel disease (Kryczek et al., 2008, Sarkar et al., 2010, Weaver et al., 2006). These cells secrete IL-17 which induces IL-6, IL-8 and prostaglandin-E2 production. Hence, T_h17 cells are also supposed to play a role in osteoclastic activity and mediates bone resorption (Ohlrich et al., 2009). Conversely, T_{reg} cells, another immune regulator secrete TGF-β and IL-10 (Couper et al., 2008, Sakaguchi, 2005). Therefore, the counterbalance of T_h17 cells and T_{reg} cells is also postulated to contribute to the pathogenesis of periodontal disease (Zhou and Littman, 2009). However, the evidence showed a role of T_h17/T_{reg} is still limited.

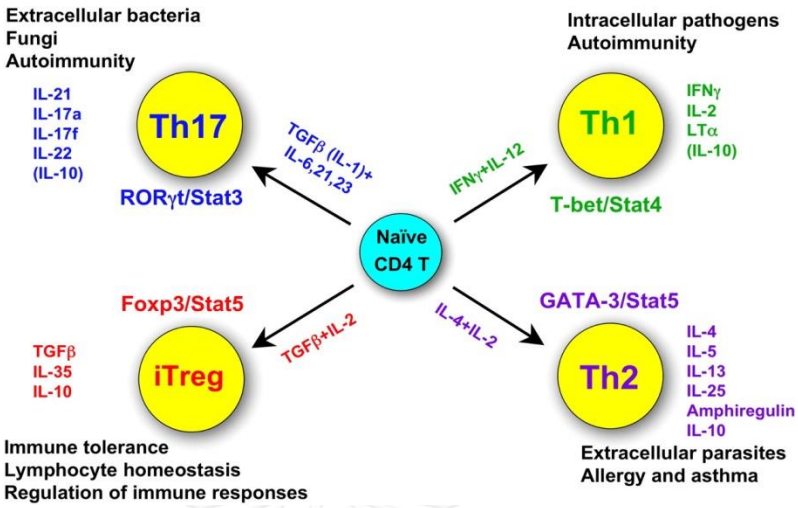


Figure 1. $CD4^+$ T helper cell fate: differentiation, their cytokine expression, characteristic transcription factors and cytokines critical for their fate determination (Zhu and Paul, 2008)

T cells recognized invading pathogens by means of cell-cell interaction. Antigens were carried by antigen presenting cells (APCs) and presented by major histocompatibility complex (MHC) molecule to T cell receptor (TCR). The interactions between T cells and APCs are described in figure 2. $CD4^+$ T cells recognize antigens that are presented with MHC class II, whereas $CD8^+$ T cells respond to antigens that are presented with MHC class I. The activation of T cells depends on the co-stimulation on APCs which include two related proteins called B7-1 (CD80) and B7-2 (CD86). These B-7 proteins are recognized by its ligands called CD28 which expressed on T cells. Signals from co-stimulators and binding of peptide-MHC molecules on the same APCs initiate T cells activation. Then, T cells expand by cell proliferation, differentiate into effector cells and secrete cytokines (Abbas and Lichtman, 2005, Delves et al., 2011). Once infection was cleared, majority of effector T cells die, but a small number of T cells developed into memory T cells (Mueller et al., 2013). These cells recognize their roles

as immunosurveillance throughout the body and provide lifelong protection against recurrent infection (Mueller et al., 2013, Farber et al., 2014). Memory T cells have self-renewal capability, highly protection and persist in non-lymphoid tissues for a long-term, especially at previously infected sites (Lanzavecchia and Sallusto, 2005).

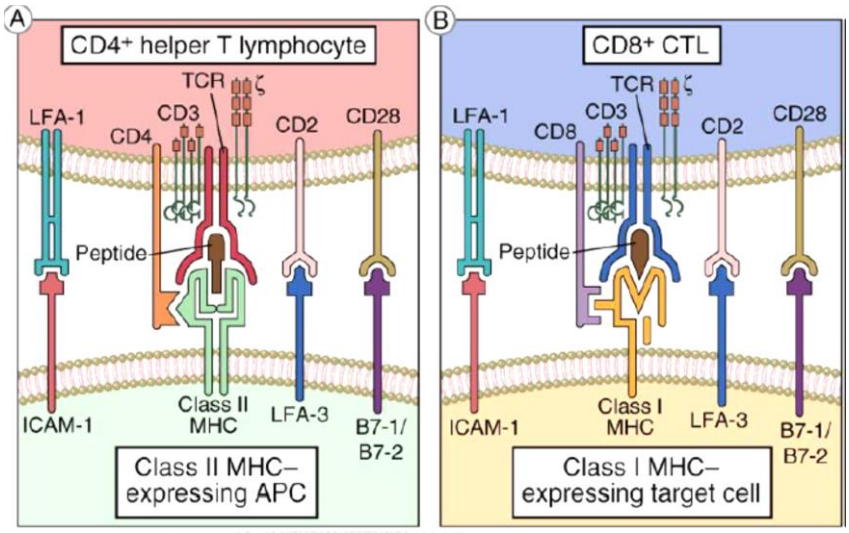


Figure 2. Interactions of accessory molecules between T cells and antigen presenting cells (Abbas and Lichtman, 2005).

Moreover, both $CD4^+$ and $CD8^+$ T cells can be separated into subsets based on their phenotypic expression of CD45 different isoforms. *In vitro* antigenic stimulation demonstrated that $CD45RA^+$ cells expressed CD45RO and lost CD45RA. This data suggested that $CD45RA^+$ cells are naïve while $CD45RO^+$ cells are memory T cells (Michie et al., 1992). Memory T cells display an increased expression of cell adhesion molecules (Sanders et al., 1988) and enhanced migration through endothelial cells (Oppenheimer-Marks et al., 1990). Moreover, increased endothelial permeability of $CD45RO^+$ memory T cells was suggested to be a dominant factor contributing to their preferential migration to and accumulation at inflammation sites (Damle and Doyle, 1990).

Conventionally, memory T cells have been classified into two subsets, central memory T (T_{CM}) and effector memory T (T_{EM}) cells. Earlier studies defined these subsets on their phenotypic markers, anatomical location and functions (Sallusto et al., 1999, Michie et al., 1992). T_{CM} cells express high levels of CD62L and CCR7 which home to secondary lymphoid organs and bone marrow. T_{CM} cells produce IL-2 which provides more proliferative potential and robust recall responses. On the other hand, T_{EM} cells are defined based on lack of CD62L and CCR7. They are commonly found in blood and non-lymphoid tissues. T_{EM} cells are less proliferative and exhibit effector cytokines such as IFN-γ and TNF-α (Sallusto et al., 1999). Memory T cell subsets, T_{CM} and T_{EM} cells, are described in figure 3.

Recent studies have revealed another subset of memory T cells, stem cell-like memory T (T_{SCM}) cells. T_{SCM} cells have self-renewal capacity and ability to generate other subsets of memory T cells *in vitro* (Gattinoni et al., 2011, Zhang et al., 2005). They express high levels of CD45RA, CD62L, CD28 and CD95 while T_{CM} lack CD45RA. Another subset is terminally differentiated effector (T_{TE}) cells which are derived from T_{EM} cells by IL-15. T_{TE} cells express CD45RA and CD95 whereas T_{EM} cells express CD45RO (Lugli et al., 2010). Although the lineage relationship of human T cell subsets is not fully determined. Data from Farber et al. suggested the hierarchical organization of memory T cell subsets. T_{SCM} cells are located at an intermediate position between T_N cells and T_{CM} cells, which are committed progenitor cells prone to T_{EM} cell differentiation (**Fig. 4**)

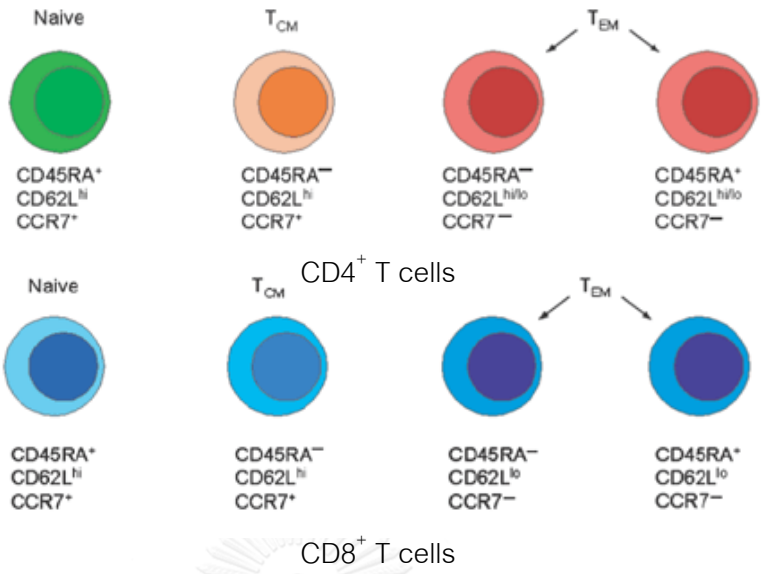


Figure 3. Subsets of memory T cells. $CD4^+$ and $CD8^+$ T cell subsets based on differential expressions of CD45, CD62L and CCR7 have been characterized from human peripheral blood mononuclear cells. (Modified from Sallusto et al., 1999)

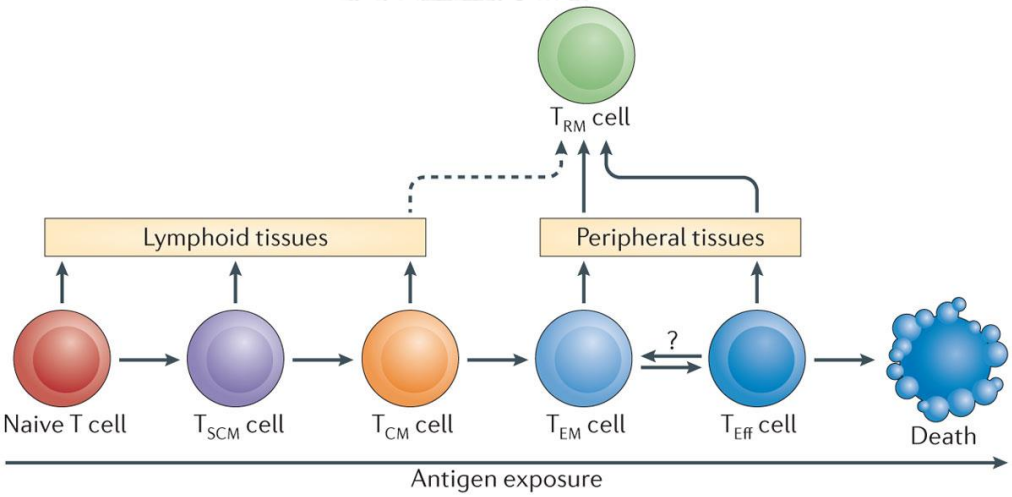


Figure 4. A model for the generation of human memory T cell subsets. A progressive differentiation pathway places naive, T_{SCM}, T_{CM} and T_{EM} cells in a differentiation hierarchy (Farber et al., 2014).

In 2001, emerging evidences from a mouse model showed that antigen-specific memory $CD8^+$ memory T cells persist long-term in multiple tissues such as lung, liver or

kidney after virus or antigens were cleared which confirmed the existence of T_{CM} and T_{EM} cells in mouse lymphoid and peripheral tissues (Masopust et al., 2001). Gebhardt et al. demonstrated that memory T cells also remained in mouse skin epithelia and latently infected sensory ganglia, called tissue-resident memory (T_{RM}) cells. These cells express CD103 and CD69, which are distinct from lymphoid memory T cells (Gebhardt et al., 2009). Collective evidences showed that tissue resident memory T cells permanently reside in non-lymphoid tissues, could provide better protection compared to circulating memory T cells (Teijaro et al., 2011, Gebhardt et al., 2009, Jiang et al., 2012). For examples, challenging of herpes simplex virus (HSV) or vaccinia virus subcutaneously generated population of $CD8^+$ T cells that concentrated at the sites long after resolution. They also provided superior viral clearance after skin re-infection compared to circulating memory T cells (Mackay et al., 2012, Jiang et al., 2012). Lung $CD4^+$ T_{RM} cells generated by influenza infection, did not recirculate and also enhanced viral clearance and survival to influenza virus re-challenge (Purwar et al., 2011). Moreover, T_{RM} cells established in a vaginal epithelial layer provided greater protection against a lethal herpes simplex virus type 2 (HSV-2) challenge than circulating HSV-2-specific memory T cells did (Shin and Iwasaki, 2012). Even though, several studies showed a protection effect of resident $CD103^+$ T cells, some evidences revealed the detrimental effect of T_{RM} cells in various peripheral organs (Smyth et al., 2007, Mizukawa et al., 2002). Activation of $CD8^+CD103^+$ T cells in skin resulted in localized epidermal injury and rapid production of high level of IFN-γ (Mizukawa et al., 2002). The treatment of psoriasis by anti E-selectin to inhibit T cell migration from blood was also ineffective which suggested a role of resident T cells in pathogenesis of psoriasis, but not circulating T cells (Bhushan et al., 2002).

The novel surface markers to identify the distinct T cell subpopulations

CD or cluster of differentiation is a numerical designation used for the investigation of cell surface proteins that define a particular cell types and stages of cell differentiation (Abbas and Lichtman, 2005). CD molecules often act as receptors or ligands that play a role in cell signaling and have other functions, such as cell adhesion or migration. The development of immunological methods, i.e. monoclonal antibodies and flow cytometry, has provided update information about antigen specific memory T cells. However, a single marker or combination of two markers does not allow the identification of T cell subsets. Combining of various markers via a multicolor flow cytometry allows to further identify T cell subsets (Mahnke et al., 2013).

Selective monoclonal antibodies have been used to identify T cell subsets including monoclonal antibodies against CD45RA, CD28, CD95 and CD103. CD45RA is a cell surface molecule expressed on T cells that characterizes for naïve T lymphocytes. Whereas CD45RO cells represent a memory T cell population (Michie et al., 1992). CD28 is a co-stimulatory receptor that expresses on T cells (Effros, 1997). It involves in T cell activation by binding its ligands, B7-1 (CD80) and B7-2 (CD86), which are expressed by APCs, activated B cells and monocytes. CD28 interaction with CD80 or CD86 mediate antigen-specific T cell responses, up-regulation cytokine expression and promoting T cell expansion and differentiation (Lenschow et al., 1996). CD95, also known as Fas or tumor necrosis TNF receptor, is a death receptor expressed on T and B cells (Li-Weber and Krammer, 2002, Huck et al., 1998). The binding of CD95 to CD95 ligand results in apoptotic cell death which is known to be a mechanism for control of ongoing immune response (Krammer, 2000). CD103 involves in interaction with epithelial cells via binding to its ligand, E-cadherin (Cepek et al., 1994). Therefore, the $CD103^{+}$-E cadherin interaction may contribute to maintaining the resident status of T_{RM} cells in peripheral tissues (Pauls et al., 2001). CD69 is a traditionally marker associated

with recent activation (Gebhardt et al., 2009) and functions by inhibiting sphingosine-1-phosphate receptor (S1P1) which results in retention of newly primed T cells in draining lymph nodes (Shiow et al., 2006). At present, CD103 and CD69 are known to be used to identify T_{RM} cells (Gebhardt and Mackay, 2012, Turner et al., 2014).

Recent advances in immunological methods have provided more information about antigen-specific memory T cells and their surface markers. Availability of multi-color flow cytometry and new monoclonal antibodies have established for the identification of memory T cell subsets based on expression of surface markers (shown in table 2). Naïve T (T_N) cells were classified as $CD28^+CD95^-CD45RA^+$ T cells. Stem cell-like memory T (T_{SCM}) cells were classified as $CD28^+CD95^+CD45RA^+$ T cells. Central memory T (T_{CM}) cells were classified as $CD28^+CD95^+CD45RA^-$ T cells. Effector memory T (T_{EM}) cells were classified as $CD28^-CD95^+CD45RA^-$ T cells. Terminally differentiated effector memory T (T_{TE}) cells were classified as $CD28^-CD95^+CD45RA^+$ T cells. Resident memory T (T_{RM}) cells were classified as $CD103^+$ T cells (Mahnke et al., 2013, Farber et al., 2014). Therefore, these technologies will provide a better tool to study T cell subpopulations and then may lead to identify a specific subpopulation involved in disease pathogenesis.

T cell in periodontitis

In 1979, Seymour and Greenspan hypothesized that the conversion from gingivitis to periodontitis involved a shift from T cell-dominated lesions to B cell and plasma cell-dominated lesions (Seymour and Greenspan, 1979). The shift from a stable lesion to a progressive lesion was postulated to play a crucial role in the disease initiation and progression (Seymour et al., 1979). In 1988, Reinhardt et al. reported increased number of T cells in periodontal lesions (Reinhardt et al., 1988). Yamazaki et al. also reported that the number of T cells in periodontal lesions was much larger than

healthy gingiva. The majority of T cells was $CD4^+CD45RO^+$ memory T cells, indicating that activated T cells localized in periodontal tissues (Gemmell et al., 1992, Yamazaki et al., 1993, Yamazaki et al., 1995).

$CD4^+$ T cells are classified into distinct populations on the basis of their cytokine production. T_h1 cells preferentially produce IL-2 and IFN-γ , whereas T_h2 cells produce IL-4, IL-5, IL-6, and IL- 13 (Mosmann et al., 1986). It was hypothesized that T_h1 cells are associated with the stable gingivitis lesion, whereas T_h2 cells are associated with the progressive periodontitis lesion (Gemmell et al., 2002, Gemmell et al., 2007). However, some studies showed a mixed T_h1 and T_h2 responses in periodontitis (Prabhu et al., 1996, Berglundh et al., 2002, Fujihashi et al., 1996). The mixture of T_h1 and T_h2 cytokine profiles in periodontal disease may indicate diverse stages of periodontal disease progression (Taubman and Kawai, 2001).

In terms of T cells subsets, the $CD4^+$ to $CD8^+$ T cell ratio has been investigated. Decreased $CD4^+$ to $CD8^+$ T cell ratio in periodontitis tissues was observed when compared with healthy periodontal or gingivitis tissues (Stoufi et al., 1987, Cole et al., 1987, Yamazaki et al., 1993). The normal periodontal tissues exhibited $CD4^+$ T cell to $CD8^+$ T cell ratio almost comparable to those of peripheral blood (Cole et al., 1987, Stoufi et al., 1987). Whereas the disease tissues showed decreased $CD4^+$ T cell to $CD8^+$ T cell ratio when compared with those of peripheral blood (Stoufi et al., 1987). It is suggested that local T cell immunoregulatory function was distinct from peripheral blood.

T_h17 cells have also been described in human periodontal disease. These cells produce a pro-inflammatory cytokine, IL-17 (Takahashi et al., 2005). It was demonstrated that *Porphyromanas gingivalis* infection induced a significant increase of IL-17 production (Oda et al., 2003). They were suggested to be associated with gingival inflammation and alveolar bone destruction (Cheng et al., 2014). IL-17 showed to

induce osteoclastic bone resorption by receptor activator of nuclear factor kappa B ligand (RANKL) production (Van bezooijen et al., 1999). Another $CD4^+$ T cell subset, T_{reg} cells, have been identified. T_{reg} cells expressed TGF-β and IL-10 which may play a regulatory role in periodontal disease (Nakajima et al., 2005), probably in down-regulation of RANKL expression (Ernst et al., 2007). However, the role of T_h17/T_{reg} cells is yet to be investigated.

A few decades ago, Tonetti et al. revealed the presence of intraepithelial lymphocytes in human gingival tissues. These cells expressed $\alpha^{IEL}\beta^7$ (now called CD103) and were detected especially in gingival epithelia. They also suggested that T cell subsets defined by $\alpha^{IEL}\beta^7$ molecule may underline the potential significance in their immune homeostasis (Tonetti et al., 1995). However, the evidence of $CD103^+$ T cells and their roles in periodontal homeostasis is still limited.

While novel subpopulations of T cells in different tissues have been studied, there is little knowledge of these T cells subpopulations in periodontal tissues. Therefore, this study will provide new insight into novel T cell populations in human periodontal tissues.

CHAPTER III

MATERIALS AND METHODS

Reagents

Roswell Park Memorial Institute (RPMI)-1640 and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Gibco (Grand Island, NY, USA). Fetal calf serum, collagenase, phosphate-buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Monoclonal antibodies

Fluorescence-conjugated mouse anti-human CD4, CD8, CD28, CD45RA, CD95, CD103 monoclonal antibodies and mouse isotype control monoclonal antibodies were obtained from BD Biosciences (San Jose, CA, USA).

Subject selection and ethical consideration

Periodontal tissue samples were obtained from subjects with severe chronic periodontitis and clinically healthy periodontal tissues. The ethical approval was obtained from the Ethics committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2015-055). An informed consent was obtained from all participating subjects before the operation. All data of subjects were kept securely.

Periodontal tissue collection

Periodontal tissue samples were collected from periodontitis and periodontally healthy subjects at the Periodontal Clinic or Oral Surgery Clinic, Faculty of Dentistry, Chulalongkorn University. Gingival tissues surrounding teeth with other dental diseases such as pulpal diseases were excluded. All subjects were in good general health and

none of them had taken antimicrobial or anti-inflammatory drugs within the previous 3 months. Each subject of periodontitis group had no history of periodontal treatment in the past 6 months.

Healthy periodontal tissues were collected from a site of extracted teeth with clinically healthy gingiva (no bleeding on probing, probing depth less than 4 millimeters, no clinical attachment loss and no bone loss) simultaneously to crown lengthening procedure. While periodontitis tissues were obtained 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) (**Fig. 5**).

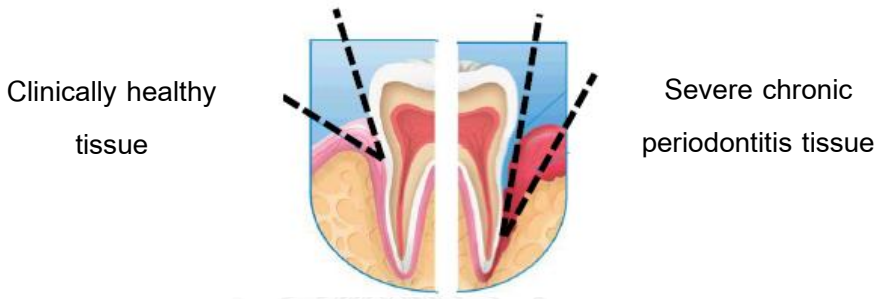


Figure 5. Periodontal tissue with severe chronic periodontitis and clinically healthy tissues were prepared by internal bevelled incision and intrasulcular incision.

The excised periodontal tissues were immediately placed in a sterile tube that contained RPMI-1640 medium and then transferred to the laboratory within a few hours for further investigation.

Immunohistochemistry

The excised periodontal tissues were washed thoroughly in normal saline solution, fixed in 10% buffered formalin for a maximum of 24 hours. Then, tissues were transferred to Department of Pathology, Faculty of Dentistry, Chulalongkorn University for embedding tissues in paraffin block. Microtome serial 4-micron-thick sections were

cut and mounted on glass slides. Sections were de-paraffinized in xylene and rehydrated through a graded ethanol series (100%, 95% and 80%) and distilled water. To inhibit endogenous peroxidase, slides were further incubated with 0.3% hydrogen peroxide solution for 20 minutes. Slides were then placed into a 1 mM EDTA pH 8.0 and heated at 95°C for 20 minutes for antigen retrieval.

For identifying localization of T cells in tissues, single immunohistochemical staining was performed via Polymer/HRP and DAB^+chromogen system (DAKO EnVisionTM G/2 Doublestain System) on tissue 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. Sections were then investigated T cell subsets under light microscope.

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

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

Gingival cell preparation (single 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 plus collagenase to tissues was 1 ml per 100 mg of tissue. After 90 minutes of incubation, residual tissue

fragments were disaggregated by flushing several times with a pipette, until single cell suspensions were obtained. The single cell suspensions were filtered through a filter of mesh size 70 μm (BD Biosciences).

Flow cytometric analysis

To determine, sets of surface markers shown in table 2 were used to determine T cell profiles (e.g. T_N, T_{CM}, T_{EM}, T_{SCM}, T_{TE} and T_{RM}) according to their phenotypic expressions. Isolated gingival cell suspensions from periodontal tissues were stained with anti-human CD4 (PerCP), CD8 (APC-Cy7), CD28 (PE), CD45RA (PE-Cy7), CD95 (FITC) and CD103 (APC) monoclonal antibodies at 4°C for 30 minutes. Gingival cells were washed with PBS containing 0.1% albumin and 0.01% sodium azide and then fixed with 1% paraformaldehyde. Analysis of flow cytometry samples were performed by six-color flow cytometry, FACSCalibur (BD Biosciences). First, $CD4^+$ and $CD8^+$ cells were gated (**Fig. 6B**). Then $CD28^+CD95^+$ cells were gated (**Fig. 6C**). Finally, these cells were analyzed for the expressions of CD45RA and CD103 (**Fig. 6D**).

Table 2. Mouse anti-human monoclonal antibodies used in flow cytometric analysis.

Monoclonal antibodies	T-cell populations
$CD28^+CD95^-CD45RA^+$	Naïve T (T_N) cell
$CD28^+CD95^+CD45RA^+$	Stem cell-like memory T (T_{SCM}) cell
$CD28^+CD95^+CD45RA^-$	Central memory T (T_{CM}) cell
$CD28^-CD95^+CD45RA^-$	Effector memory T (T_{EM}) cell
$CD28^-CD95^+CD45RA^+$	Terminally differentiated effector memory T (T_{TE}) cell
$CD103^+$	Resident memory T (T_{RM}) cell

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 T cell populations in healthy subjects and disease periodontal tissues. *P*-values less than 0.05 were considered as statistically significant.

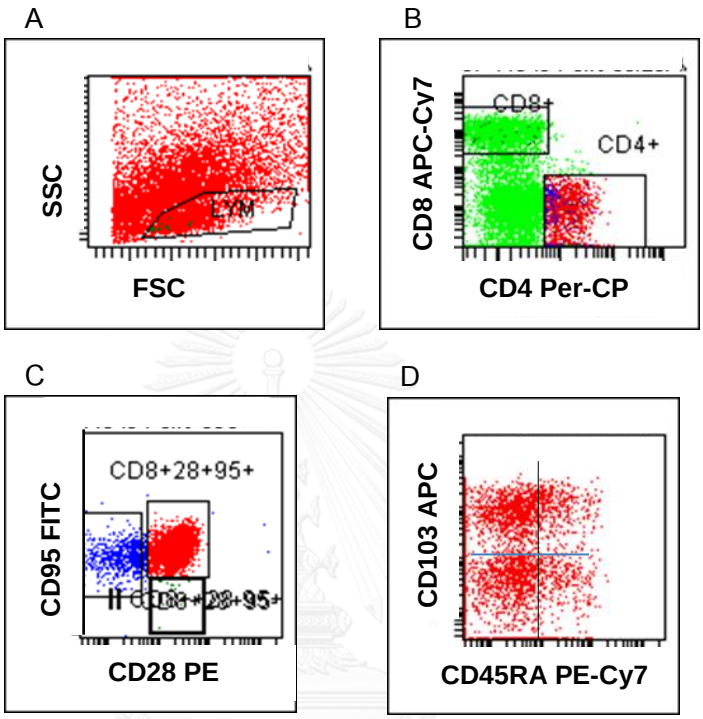


Figure 6. Flow cytometric gating strategy to identify T cell subsets in periodontal tissues. The analysis gates around lymphocyte **(A)**. Cells were analyzed on the basis of surface markers; $CD4^+$ and $CD8^+$ **(B)**, $CD28^+$ and $CD95^+$ **(C)** and $CD45RA^+$ and $CD103^+$ **(D)** T cells. SSC, side scatter; FSC, forward scatter.

CHAPTER IV

RESULTS

Localization of T cells in periodontal tissues by immunohistochemical analysis

Paraffin-embedded sections were prepared from 5 severe periodontitis tissues (**Fig. 7**) and 5 clinically healthy periodontal tissues (**Fig. 8**). The localization of T cells in periodontal tissues samples was observed by immunohistochemistry using monoclonal antibodies against CD3, CD4, CD8 and CD103. In severe periodontitis tissues, a large numbers of $CD3^+$ cells resided in epithelium, connective tissues and at epithelial-connective tissue junction. Some formed clusters of $CD3^+$ cells in connective tissues as well as at epithelial-connective tissue interface (**Fig. 7A**). A few numbers of $CD4^+$ cells were found in connective tissues and scarcely detected in the epithelia (**Fig. 7B**). Some clusters of $CD8^+$ cells were also observed at the interface and in underlying connective tissues (**Fig 7C**, shown in black arrow). $CD103^+$ cells were also dispersed throughout in both epithelium and connective tissues (**Fig. 7D**). In addition, clinically healthy periodontal tissues revealed the presence of CD3 CD4, CD8 and CD103 positive cells (**Fig. 8A**, **8B**, **8C** and **8D**, respectively). Moreover, $CD3^+$ and $CD8^+$ T cells were particular detected in connective tissues and at the interface (**Fig. 8A** and **8C**). $CD103^+$ cells were scattered throughout healthy periodontal tissues (**Fig. 8D**). However, a lesser extent of T cells in healthy tissues (Fig. 8) were observed when compared with periodontitis tissues (**Fig. 7** and **8**).

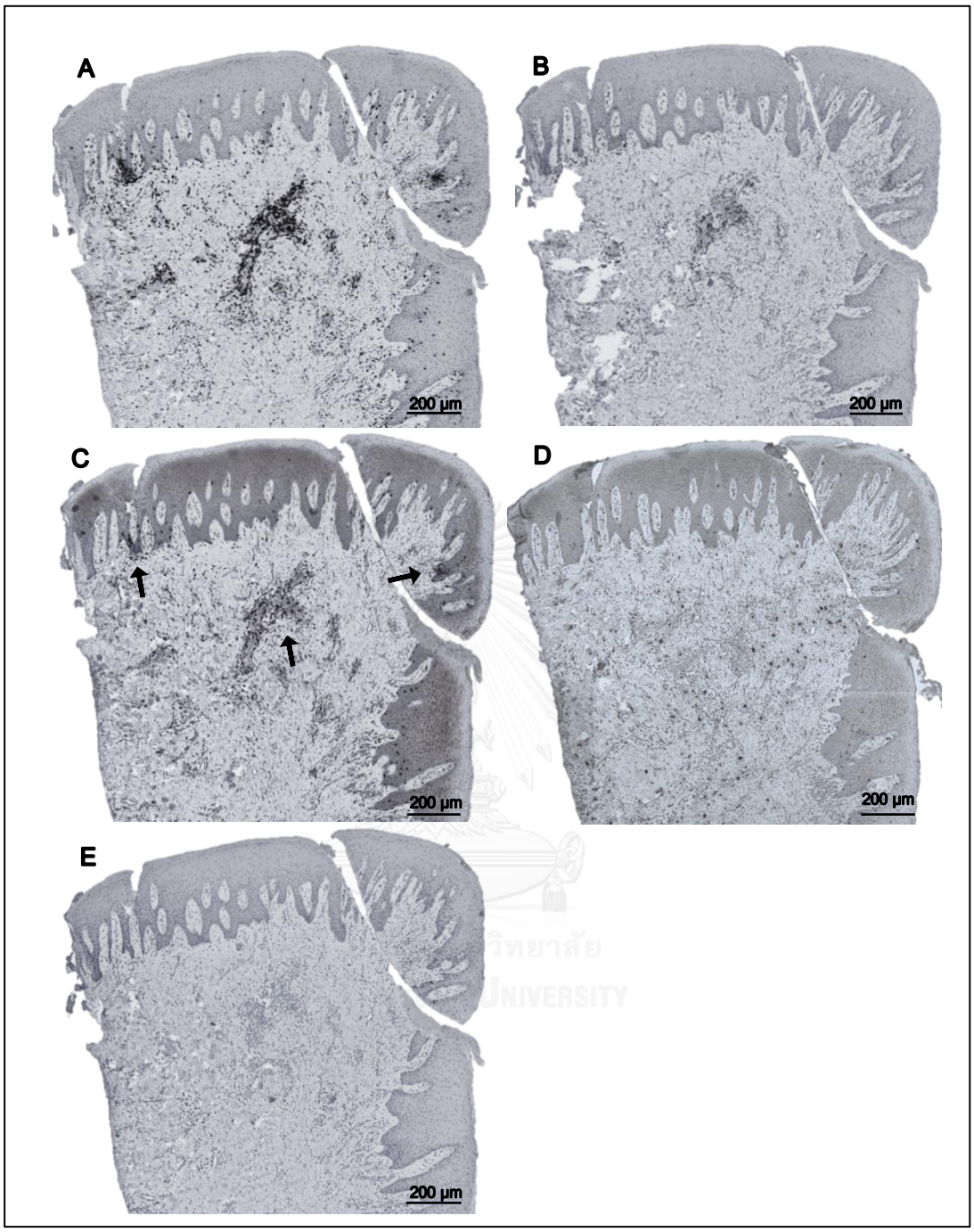


Figure 7. Immunohistochemical analysis of T cells in a periodontitis tissue. Periodontitis tissues were stained with anti-human monoclonal antibodies against; CD3 **(A)**, CD4 **(B)**, CD8 **(C)**, CD103 **(D)** and negative control **(E)**. Periodontal tissue dissections are representative of five individuals. Note black arrows show clusters of $CD8^+$ T cells. Bar is 200 µm.

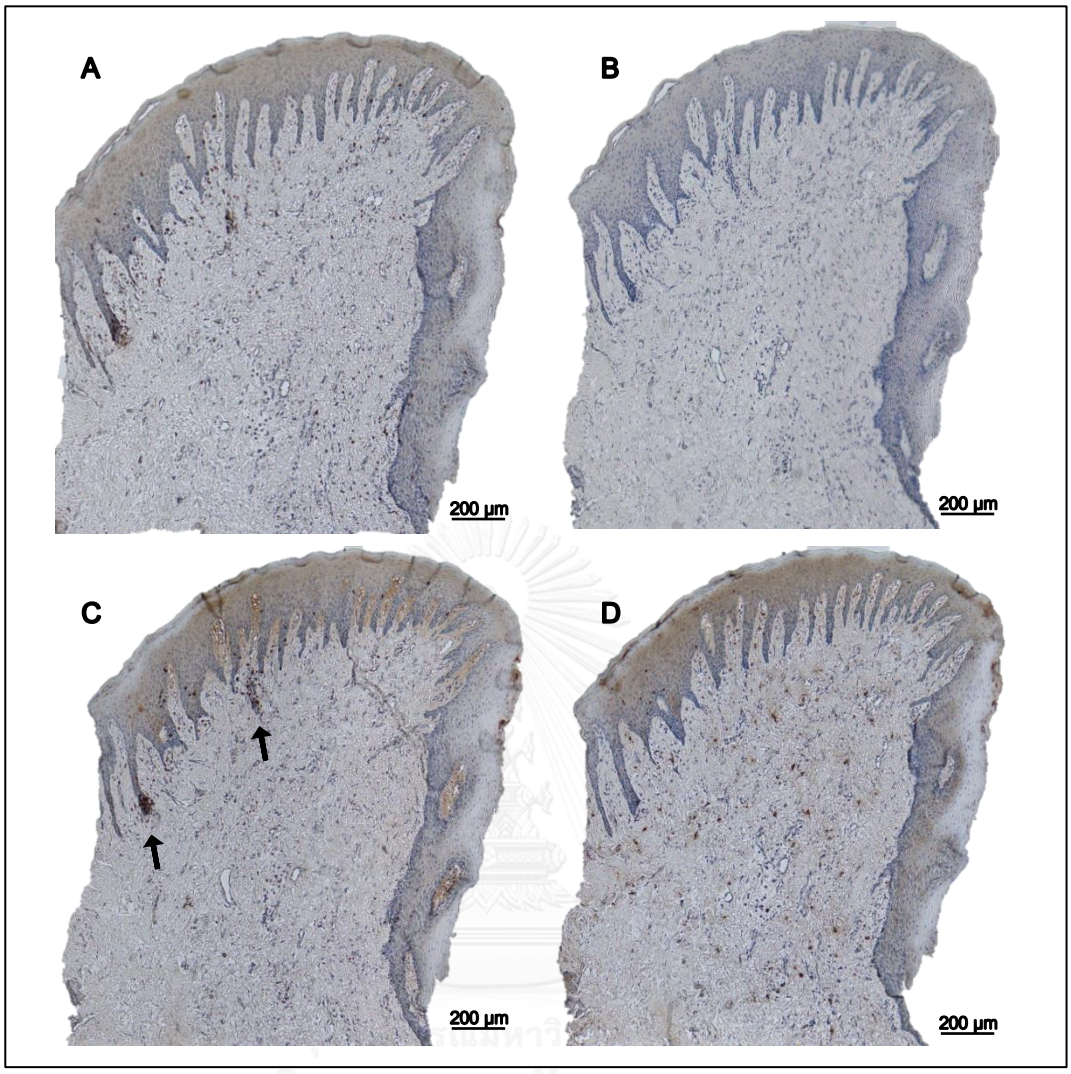


Figure 8. Immunohistochemical analysis of T cells in a healthy periodontal tissue. Periodontitis tissues were stained with anti-human monoclonal antibodies against; CD3 **(A)**, CD4 **(B)**, CD8 **(C)** and CD103 **(D)**. Periodontal tissue dissections are representative of five individuals. Note black arrows show clusters of $CD8^+$ T cells. Bar is 200 µm.

Flow cytometric analysis of T cell subsets in periodontal tissues

For flow cytometric analysis, gingival cells were extracted from 8 healthy periodontal subjects and 5 subjects with severe chronic periodontitis. Infiltrated T cells were determined by staining anti-CD4, CD8, CD28, CD95, CD45RA and CD103 mAbs and then analyzed by 6-color flow cytometry. Mean percentages of infiltrated cells in

periodontitis and healthy periodontal tissues were presented in figure 9. In periodontitis tissues, $CD4^+$ and $CD8^+$ T cells represented 29.07% and 17.71% of infiltrated T cells, respectively. Whereas, $CD4^+$ and $CD8^+$ T cells in healthy periodontal tissues consisted of 31.19% and 21.29% of total T cells, respectively. Even though, the numbers of $CD4^+$ and $CD8^+$ T cells in healthy tissues seemed to be higher than those of periodontitis tissues, the differences were insignificant. Within clinically healthy group, $CD4^+$ T cells were much larger than $CD8^+$ T cells (p = 0.028). However, the ratios of $CD4^+$ to $CD8^+$ T cells in the clinically healthy and periodontitis groups were comparable (1.37 and 1.64, respectively).

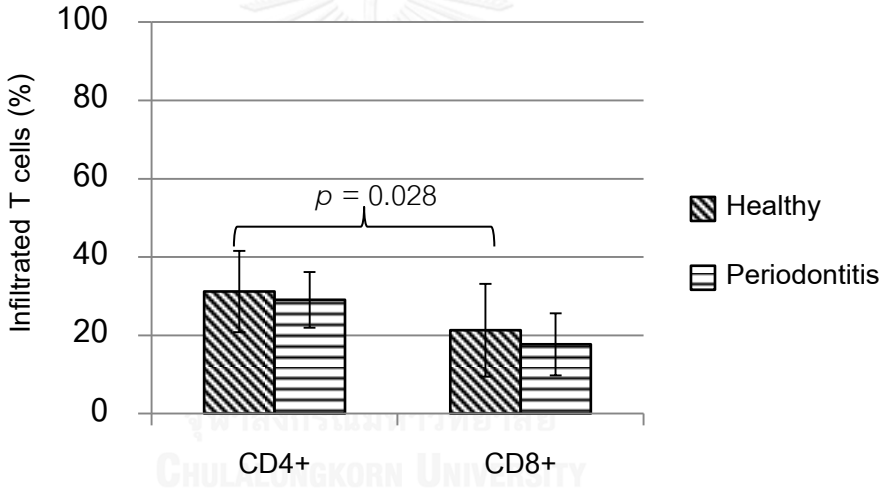


Figure 9. The mean percentages of infiltrated T cells in periodontal tissues. Cells extracted from healthy and periodontitis tissues were stained with either anti-human CD4 or anti-human CD8 monoclonal antibodies and then analyzed by flow cytometry.

Infiltrated T cells from each periodontal tissue specimen were categorized into five subsets accordingly: 1) T_N ($CD28^+CD95^-CD45RA^+$), 2) T_{SCM} ($CD28^+CD95^+CD45RA^+$), 3) T_{CM} ($CD28^+CD95^-CD45RA^-$), 4) T_{EM} ($CD28^-CD95^+CD45RA^-$) and 5) T_{TE} cells ($CD28^+CD95^-CD45RA^-$) were then analyzed (**Fig. 10**). Figure 11 described the mean percentages of $CD4^+$ T cell subpopulations. In healthy tissues, T_N, T_{SCM}, T_{CM}, T_{EM} and T_{TE} cells represented 0.37%, 1.21%, 91.99%, 3.22% and 1.71% of $CD4^+$ T cells,

respectively (**Fig. 11A**). The mean percentages of $CD4^+$ T cell subpopulations in periodontitis tissues (0.34%, 1.66%, 90.71%, 5.02% and 0.78%, respectively) were comparable to those of healthy tissues (**Fig. 11B**). Most of $CD4^+$ T cell subsets were T_{CM} cells (shown in green), while T_N cells were barely detected in the tissues (shown in dark blue)

A

B

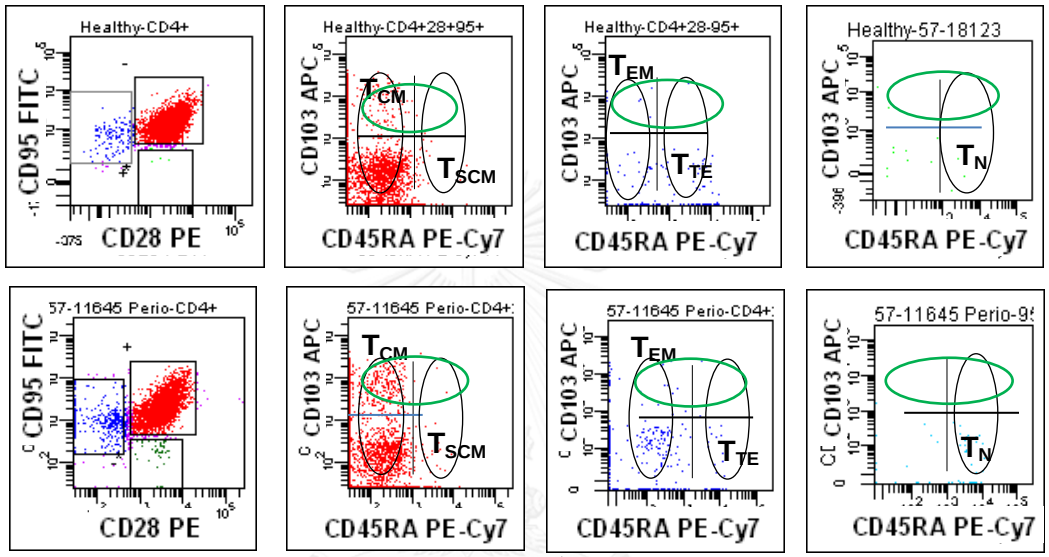
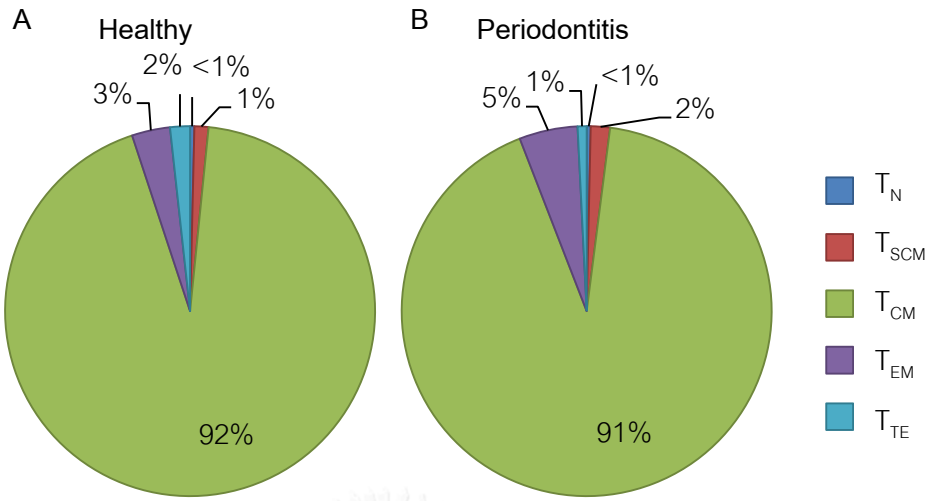


Figure 10. Flow cytometric analysis of $CD4^+$ T cells in clinical healthy periodontal tissues **(A)** and periodontitis tissues **(B)**. Note green ovals show T_{RM} cell population.

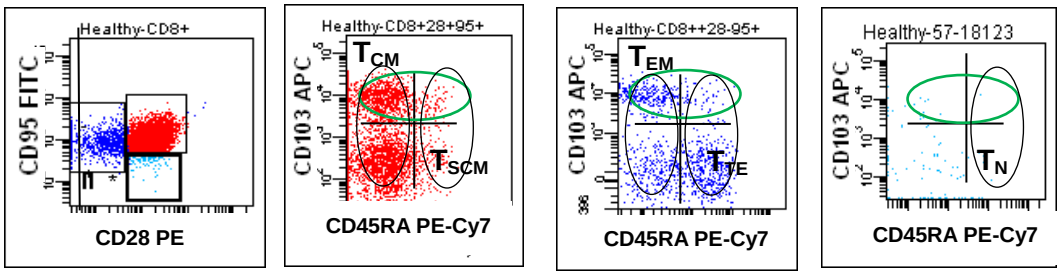


	T_N cells (%)	T_{SCM} cells (%)	T_{CM} cells (%)	T_{EM} cells (%)	T_{TE} cells (%)
Healthy	0.37±0.12	1.21±0.40	91.99±30.66	3.29±1.10	1.71±0.57
Periodontitis	0.34±0.20	1.66±0.74	90.71±40.57	5.02±2.25	0.78±0.35

Figure 11. The mean percentages of $CD4^+$ T cell subsets in clinically healthy **(A)** and periodontitis tissues **(B)** by flow cytometric analysis. The table showed the mean percentages of $CD4^+$ T cell subsets in each T cell subpopulation.

The percentages of $CD8^+$ T cell subsets were also investigated (**Fig. 12**). In healthy tissues, T_N, T_{SCM}, T_{CM}, T_{EM} and T_{TE} cells represented 0.62%, 7.04%, 67.02%, 12.50% and 9.10% of $CD8^+$ T cells, respectively (**Fig. 13A**). The percentages of $CD8^+$ T cell subsets obtained from periodontitis tissues were comparable to those of healthy tissues which are 0.15%, 6.17%, 75.71%, 9.81% and 6.82%, respectively (**Fig. 13B**). Similarly, the majority of $CD8^+$ T cells were T_{CM} cells (shown in green), while $CD8^+$ T_{EM} and T_{TE} cells were 2 to 9 fold higher than $CD4^+$ T_{EM} and T_{TE} cells, respectively (**Fig. 11** and **13**).

A



B

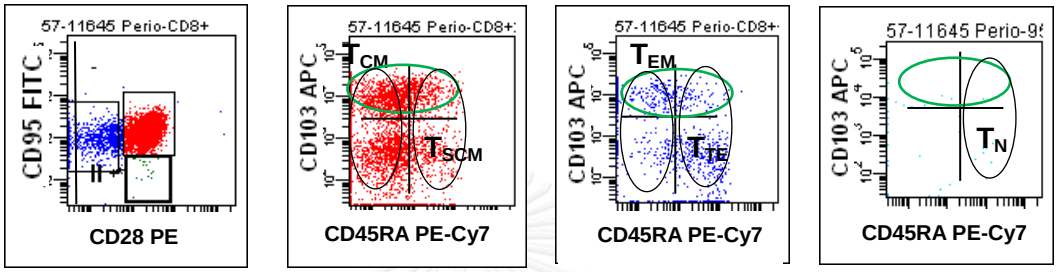
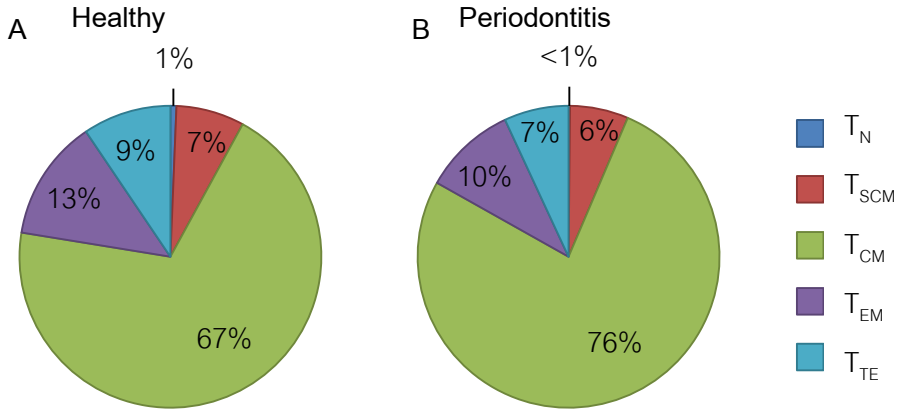


Figure 12. Flow cytometric analysis of $CD8^+$ T cells in clinically healthy periodontal tissues **(A)** and periodontitis tissues **(B)**. Note green ovals show T_{RM} cell population.

Overall, our finding showed the presence of T cell subpopulations in both $CD4^+$ and $CD8^+$ T cells in periodontal tissues. The majority of T cells subsets were T_{CM} cells. The mean percentages of $CD4^+$ and $CD8^+$ T cells subsets in healthy periodontal and periodontitis samples were similar which were depicted in figure 11 and 13, respectively. Note that the sizes of pie charts also illustrated the discrepancy on the overall number of $CD4^+$ and $CD8^+$ T cells between these two groups which indicated that total numbers of $CD4^+$ T cells were 2-fold larger than those of $CD8^+$ T cells (**Fig. 11** and **13**).



	T_N cells (%)	T_{SCM} cells (%)	T_{CM} cells (%)	T_{EM} cells (%)	T_{TE} cells (%)
Healthy	0.62±0.21%	7.04±2.35%	67.02±22.34	12.50±4.17%	9.10±3.03%
Periodontitis	0.15±0.09%	6.17±2.76%	75.71±33.86	9.81±4.39%	6.82±3.05%

Figure 13. The mean percentages of $CD8^+$ T cell subsets in clinically healthy **(A)** and periodontitis tissues **(B)** by flow cytometric analysis. The table showed the mean percentages of $CD8^+$ T cell subsets in each T cell subpopulation.

Since $CD103^+$ tissue-resident T cells were recently identified in several tissues and likely to be associated with the pathogenesis of several diseases, such as inflammatory bowel disease (Oshitani et al., 2003), multiple sclerosis (Sasaki et al., 2014) and fixed-drug eruption (Mizukawa et al., 2002). We further investigated whether CD103-expressing T cells are detected in either healthy or periodontal tissues (**Fig. 10** and **12**). Our results revealed that the expression of CD103 on $CD4^+$ T cells in healthy and periodontitis tissues were quite similar. The percentages of $CD4^+CD103^+$ T cells were 6.19±2.06% and 8.89±3.98% of total $CD4^+$ T cells in tissues, respectively. Unlike the numbers of $CD4^+CD103^+$ T cells, the numbers of $CD8^+CD103^+$ T cells in periodontitis tissues (58.32±26.08%) were higher than those of healthy periodontal tissues (32.70±10.90%, p=0.019, **Fig. 14**).

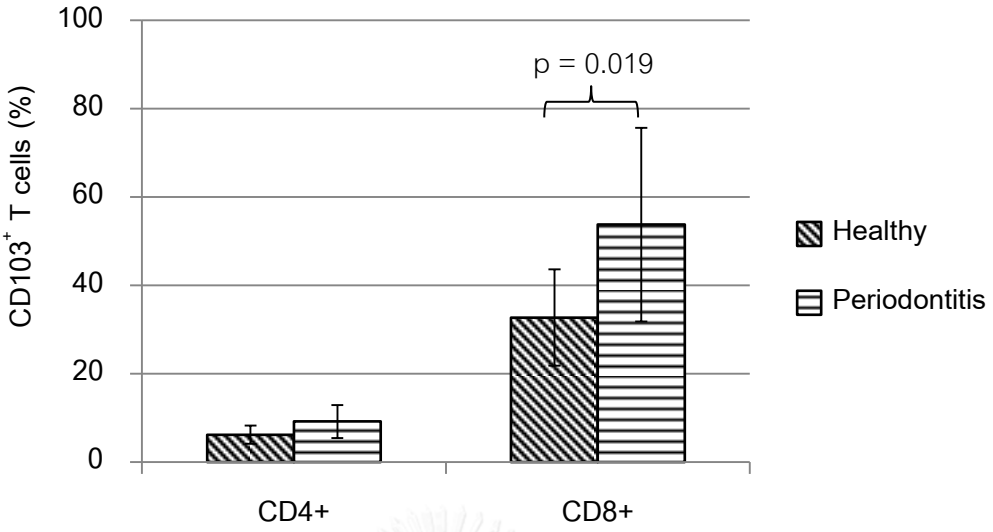


Figure 14. The mean percentages of CD103-expression in $CD4^+$ and $CD8^+$ T cells in periodontal tissues. Cells extracted from healthy and periodontitis tissues were stained with anti-human CD4, CD8 and co-stained with CD103 monoclonal antibodies and then analyzed by flow cytometric analysis.

CHAPTER V

DISCUSSION AND CONCLUSION

The interaction between periodontopathic bacteria and the host immune response is a crucial role in the pathogenesis of periodontal disease (Genco and Slots, 1984). Although periodontal bacteria including the key pathogens, including *Tannerella forsythia*, *Porphyromanas gingivalis* and *Aggregatibactor actinomycetemcomitans* were identified as causative agents (1996), host immune response to these bacteria were thought to be responsible for progression and the severity of periodontal disease (Seymour et al., 1993). Gingivitis or a stable lesion is T-cell dominated, while periodontitis, an progressive lesion is predominated by B cells and plasma cells (Seymour and Greenspan, 1979). The shift from a T-cell lesion to a B-cell lesion was suggested to be associated with periodontal homeostasis or pathogenesis (Seymour et al., 1979). However, the role of T cells in periodontal tissues is not fully clarified.

Periodontopathic bacteria-specific T cell clones derived from periodontal lesions and their cytokines were implicated in the pathogenesis of disease process (Seymour et al., 1993). In addition, T cell clones generated from inflamed gingival tissues with chronic periodontitis were specific to whole-cell antigens of *Porphyromanas gingivalis, Aggregatibactor actinomycetemcomitans, Prevotella intermedia* and collagen type I (Wassenaar et al., 1995). Other studies also confirmed that T cell clones derived from periodontitis tissues were specific to *Porphyromonas gingivalis* (Aroonrerk et al., 2003) *and Aggregatibactor actinomycetemcomitans* (Kawai et al., 1998). These antigen specific T cells were capable of IFN-γ production (Aroonrerk et al., 2003) and migration from the circulation to the gingival tissues (Kawai et al., 1998). The evidence suggested the role of T cells in periodontal disease.

The purpose of this study was to determine, by means of immunohistochemistry and flow cytometric analysis, subpopulations of T cells in clinically healthy periodontal and periodontitis tissues. A novel set of monoclonal antibodies was used to detect diverse T cell subsets, including anti-CD4, CD8, CD28, CD95, CD45RA and CD103.

Our immunohistological finding demonstrated the presence of T cells in both healthy and periodontitis tissues. The distributions of T cells in both healthy and periodontitis tissues were similar (Fig. 7 and 8). The majority of T cells were located in connective tissue and the interface between epithelium and connective tissues. However, periodontitis lesions contained larger numbers and higher densities of T cells than those of healthy periodontal tissues (Fig. 7 and 8). A large number of $CD103^+$ cells were also detected in lamina propria and the interface. Unlike in gut, $CD103^+$ T cells were mostly expressed in intraepithelial layers. Only 20-50% of T cells in lamina propria expressed CD103 (Kilshaw, 1999, Lefrancois et al., 1994). A previous study showed that $\alpha^{IEL}\beta^7$-expressing $CD3^+$ T cells, $CD103^+$ T cells, were detected in epithelial layers of severe periodontal tissues (Tonetti et al., 1995). Since $CD103^+$ T cells or T_{RM} cells were presumed to be the first line in response to external stimuli, the localization in the epithelium of the gingiva and gut was expected. Conversely, our finding is the first report of high density of $CD103^+$ cells detected in the lamina propria. Further studies are required to confirm their distribution and function. Note that the expression of CD103 was not only specific to T cells, but also lamina propria dendritic cells (Matteoli et al., 2010, Fujimoto et al., 2011), macrophages (Tiisala et al., 1995) and mast cells (Smith et al., 1994). Therefore, double staining of CD3 and CD103 should be performed to confirm the distribution of T_{RM} cells.

Since the limitation to perform double staining of CD3 and CD103 in immunohistological sections at this moment, periodontal tissues were extracted and stained with CD4, CD8 and CD103 and then analyzed by flow cytometry. Our

observation revealed that more than one-third of $CD8^+$ T cells were $CD103^+$ T cells, while only 3-16% of $CD4^+$ T cells expressed CD103. Focusing on T_{RM} cells, periodontitis lesions contained 2-fold higher $CD8^+$ T_{RM} cells than those of clinically healthy periodontal tissues. The presence of $CD8^+CD103^+$ T cells were proposed to be crucial to the development of these skin lesions, such as skin psoriasis and fixed-drug eruption (Mizukawa et al., 2002, Pauls et al., 2001, Cheuk et al., 2014). However, the role of T_{RM} in periodontal disease has not been well established. Since the retention of T_{RM} cells in tissues was suggested via binding to E-cadherin on epithelial cells (Cepek et al., 1994), the presence of CD103-expressing T_{RM} cells in periodontal tissues may be associated with prompt response of T cells in localized periodontal infection. Moreover, a mouse model demonstrated that $CD8^+$ T_{RM} cells in brain and kidney expressed up to 20-fold higher TCRs than splenic memory T cells did. The authors also suggested that increasing TCR affinity of T_{RM} cells would improve the ability of T_{RM} cells to detect infected cells (Frost et al., 2015). In addition, the function of $CD8^+$ T cells was suggested to be associated with development of cerebral malaria and cutaneous leishmaniasis via granzyme production (Haque et al., 2011, Santos Cda et al., 2013). Therefore, granzyme production by T_{RM} cells are needed to be further explored. Note that our report showed 2-fold increase of $CD8^+CD103^+$ T_{RM} cells in periodontitis tissues, it suggested that $CD8^+$ T_{RM} cells possibly contribute to periodontal pathogenesis. Even though, majority of T_{RM} cells in periodontal tissues were $CD8^+$ T cells which are similar to earlier reports in other tissues (Pauls et al., 2001, Zhou et al., 2008), $CD4^+CD103^+$ T cells were also observed. To date, the role of $CD4^+CD103^+$ T cells has not been fully clarified.

Besides the presence of T_{RM} cells in periodontal tissues, a novel subset of T cell populations, including T_N, T_{SCM}, T_{CM}, T_{EM} and T_{TE} cell has not yet been investigated in periodontal tissues. Our finding is the first study to identify these five unique populations

in periodontal tissues and the oral cavity. Interestingly, the majority of T cells in periodontal tissues are T_{CM} cells (>70% of total T cells in both periodontitis and healthy group). T_{CM} cells act as an antigen-specific T cells that expand upon re-challenge then become effector T cells (Sallusto et al., 1999). Zaph et al. showed that adoptive transfer of $CD4^+$ T_{CM} cell were capable of reducing parasite burden after re-challenging with *Leishmania major* in a mouse model (Zaph et al., 2004). Human T_{CM} cells were also found in secondary lymphoid organs and expressed lymph node-homing receptors, CCR7 and CD62L, which are markers for recirculating T cells through secondary lymphoid tissues (Sallusto et al., 1999). Tertiary lymphoid tissue in the terminal ileum also harbored with naïve, effector and central memory T cell subsets in a mouse model of Crohn-like ileitis (McNamee et al., 2013). In addition, forming of tertiary lymphoid structures was also detected during chronic inflammation (Carragher et al., 2008) and in oral squamous cell carcinoma (Wirsing et al., 2014). These findings suggested the possibility of retention of T cell subpopulations in the periphery or forming a tertiary-like lymphoid organ during periodontal disease progression.

$CD4^+$ T_{EM} and T_{TE} cells in healthy and periodontitis tissues represented about 5% and 6% of $CD4^+$ T cells, respectively. Whereas, those of $CD8^+$ T_{EM} and T_{TE} cells were 22% and 17% in healthy and periodontitis tissues, respectively. It confirmed the presence of these effector cells in periodontal tissues. However, their role is yet unknown. An *in vivo* study on peripheral blood mononuclear cells showed that $CD4^+$ T_{EM} cells expressed IFN-γ, IL-4 and IL-5, whereas, $CD8^+$ T_{EM} expressed IFN-γ (Sallusto et al., 1999). Further investigation on the role of these T cell subsets and cytokine profiles in periodontal tissues are required.

Very few T_N cells (less than 1.0%) and few T_{SCM} cells (less than 10%) were found in both two groups. Inversely, the other study reported that approximately 12-17% of $CD4^+$ T cells in periodontitis lesions were $CD45RA^+$ T_N cells (Yamazaki et al., 1993)

which are 20-fold higher than our result. This is possibly due to surface markers of T_N cells, CD45RA, used in their study are less specific to T_N cells compared to set of monoclonal antibodies used in our study. Similar to our result, T_{SCM} cells represented approximately 2 - 3% of circulating $CD4^+$ and $CD8^+$ T cells (Gattinoni et al., 2011). However, there are still limited data regarding to T_N and T_{SCM} cells in periodontal tissues.

In conclusion, our novel immunological methods first revealed that periodontal tissues both in health and disease contained various populations of T cells, including T_N, T_{SCM}, T_{CM}, T_{EM}, T_{TE} and T_{RM} cells. Interestingly, significant proportions of $CD8^+CD103^+$ T cells were observed in periodontitis tissues as compared to healthy tissues. This may suggest their possible role in tissue pathology. However, further investigations on the function of each T cell subpopulation, particularly cytokine production, such as IFN-γ, IL-17 or cytotoxic molecule, granzyme, may gain an insight into the role of periodontal tissue T cells in periodontal homeostasis and pathogenesis.

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APPENDIX

Appendix A: Descriptive profile of gingival biopsies from healthy periodontal samples

No.	Sex	Age (years)	Tooth No.	Clinical examination	
				PD (mm)	BOP
1	Male	42	44	2-3	-
2	Male	33	37	2-3	-
3	Female	17	15-25	2-3	-
4	n/a	n/a	43	2-3	-
5	Female	56	27	2-3	-
6	Female	60	17	2-3	-
7	Male	24	12-22	2-3	-
8	Female	66	33-43	2-3	-
9	n/a	n/a	15	2-3	-
10	Female	27	16-26	2-3	-
11	Female	35	26	2-4	-
12	Male	17	27	2-3	-
13	n/a	n/a	46	2-3	-

PD = Probing depth;

BOP = Bleeding on probing

Appendix B: Descriptive profile of gingival biopsies from severe chronic periodontitis subjects

No.	Sex	Age (years)	Tooth No.	Clinical examination			
				PD (mm)	CAL (mm)	Bone loss	Others
1	Female	71	28	7-10	8-12	> 50%	FI3, MO 1
2	Male	55	47	6-8	-	> 50%	-
3	Female	43	27	10	12	> 50%	FI1, MO1
4	Male	67	31, 42	5, 7	6, 8	> 50%	MO2
5	n/a	n/a	36	-	-	> 50%	-
6	Male	51	47	7-15	7-15	> 50%	MO3
7	Female	66	22-23	4-6	7-13	> 50%	MO3
8	Female	65	47	6-9	10-11	> 50%	MO2
9	Male	50	25	7-8	2-9	> 50%	MO2
10	n/a	n/a	17	-	-	> 50%	-

PD = Probing depth;

CAL = Clinical attachment level

MO = Tooth mobility (Miller's classification, 1950: Grade 0-3);

FI = Furcation involvement (Glickman's classification, 1958: Grade 1-4)

Appendix C: Phenotypic characterization of T cells in periodontal tissues

	No.	Tooth No.	Infiltrated T cells in periodontal tissues (%)	
			$CD4^+$ T cells	$CD8^+$ T cells
Healthy	1	12-22	36.33	26.68
	2	43	10.27	11.52
	3	17	26.66	12.84
	4	36-37	38.07	22.68
	5	27	34.94	41.42
	6	17	43.75	18.20
	7	26	26.59	16.95
	8	33-43	32.91	20.01
		mean±S.E.	31.19 ± 10.40	21.29 ± 7.10
Periodontitis	1	28	19.82	20.14
	2	47	30.37	21.85
	3	27	36.17	12.11
	4	31, 42	30.71	19.19
	5	25	28.27	15.25
		mean±S.E.	29.07 ± 11.87	17.71 ± 7.92

Descriptive statistics of the percentages of $CD4^+$ and $CD8^+$ T cells in healthy and periodontitis groups.

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
CD4	13	30.3738	8.57663	10.27	43.75
CD8	13	19.9108	7.82703	11.52	41.42
Condition	13	1.6154	.50637	1.00	2.00

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Healthy	16	26.2388	10.81102	10.27	43.75
Perio	10	23.3880	7.64680	12.11	36.17
CD	16	6.0000	2.06559	4.00	8.00

Mann-Whitney's U-test results of differences of percentages of $CD4^+$ and $CD8^+$ T cells in healthy and periodontitis groups.

Ranks

	Condition	N	Mean Rank	Sum of Ranks
CD4	Healthy	8	7.63	61.00
	Periodontitis	5	6.00	30.00
	Total	13		
CD8	Healthy	8	7.38	59.00
	Periodontitis	5	6.40	32.00
	Total	13		

Test Statistics[b]

	CD4	CD8
Mann-Whitney U	15.000	17.000
Wilcoxon W	30.000	32.000
Z	-.732	-.439
Asymp. Sig. (2-tailed)	.464	.661
Exact Sig. [2*(1-tailed Sig.)]	.524[a]	.724[a]

a. Not corrected for ties.

b. Grouping Variable: Condition

Ranks

	CD	N	Mean Rank	Sum of Ranks
Healthy	CD4	8	10.50	84.00
	CD8	8	6.50	52.00
	Total	16		
Periodontitis	CD4	5	7.60	38.00
	CD8	5	3.40	17.00
	Total	10		

Test Statistics[b]

	Healthy	Perio
Mann-Whitney U	16.000	2.000
Wilcoxon W	52.000	17.000
Z	-1.680	-2.193
Asymp. Sig. (2-tailed)	.093	.028
Exact Sig. [2*(1-tailed Sig.)]	.105[a]	.032[a]

a. Not corrected for ties.

b. Grouping Variable: CD

Appendix D: Phenotypic characterization of $CD4^+$ T cell subsets in periodontal tissues

	No.	Tooth No.	$CD4^+$ T cell subsets in periodontal tissues (%)				
			T_N	T_{SCM}	T_{CM}	T_{EM}	T_{TE}
Healthy	1	12-22	0.44	1.03	95.68	1.59	0.83
	2	43	-	0.59	87.30	10.35	1.17
	3	17	0.29	1.59	86.76	5.51	3.67
	4	36-37	0.92	1.80	92.51	1.61	0.74
	5	27	0.05	0.75	95.79	1.38	0.91
	6	17	0.38	2.17	96.24	0.56	0.16
	7	26	0.22	0.61	89.92	2.05	4.18
	8	33-43	0.29	1.14	91.68	3.29	2.01
		mean±S.E.	0.37 ± 0.12	1.21 ± 0.40	91.99 ± 30.66	3.29 ± 1.10	1.71 ± 0.57
Periodontitis	1	28	0.56	0.63	89.07	5.81	0.63
	2	47	-	2.85	96.80	0.04	0.00
	3	27	0.08	0.58	91.31	4.71	1.23
	4	31, 42	0.40	3.04	80.17	13.80	1.06
	5	25	-	1.19	96.19	0.75	0.96
		mean±S.E.	0.34 ± 0.20	1.66 ± 0.74%	90.71 ± 40.57	5.02 ± 2.25	0.78 ± 0.35

Descriptive statistics of the percentages of $CD4^+$ T cell subsets in healthy and periodontitis groups.

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
T_N	10	.3270	.26994	.04	.92
T_{SCM}	13	1.3823	.85604	.58	3.04
T_{CM}	13	91.4938	4.88532	80.17	96.80
T_{EM}	13	3.9577	4.11469	.04	13.80
T_{TE}	13	1.3500	1.24843	.00	4.18

Mann-Whitney's U-test results of differences of the percentages of $CD4^+$ T cell subsets in healthy and periodontitis groups.

Ranks

	Condition	N	Mean Rank	Sum of Ranks
T_N	Healthy	7	6.00	42.00
	Periodontitis	3	4.33	13.00
	Total	10		
T_{SCM}	Healthy	8	6.63	53.00
	Periodontitis	5	7.60	38.00
	Total	13		
T_{CM}	Healthy	8	7.00	56.00
	Periodontitis	5	7.00	35.00
	Total	13		
T_{EM}	Healthy	8	6.75	54.00
	Periodontitis	5	7.40	37.00
	Total	13		
T_{TE}	Healthy	8	7.75	62.00

	Periodontitis	5	5.80	29.00
	Total	13		

Test Statistics[b]

	T_N	T_{SCM}	T_{CM}	T_{EM}	T_{TE}
Mann-Whitney U	7.000	17.000	20.000	18.000	14.000
Wilcoxon W	13.000	53.000	56.000	54.000	29.000
Z	-.800	-.439	.000	-.293	-.878
Asymp. Sig. (2-tailed)	.424	.661	1.000	.770	.380
Exact Sig. [2*(1-tailed Sig.)]	.517[a]	.724[a]	1.000[a]	.833[a]	.435[a]

a. Not corrected for ties.

b. Grouping Variable: Condition

Appendix E: Phenotypic characterization of $CD8^+$ T cell subsets in periodontal tissues

	No.	Tooth No.	$CD8^+$ T cell subsets in periodontal tissues (%)				
			T_N	T_{SCM}	T_{CM}	T_{EM}	T_{TE}
Healthy	1	12-22	0.05	8.26	72.05	9.07	10.85
	2	43	-	9.41	74.39	11.85	2.79
	3	17	0.60	6.72	47.34	13.84	28.28
	4	36-37	1.47	15.17	62.20	7.88	9.44
	5	27	0.32	6.35	76.80	8.80%	5.47
	6	17	0.60	5.24	69.30	15.88	5.89
	7	26	0.94	1.71	72.24	14.06	6.74
	8	33-43	0.33	3.44	61.86	18.64	3.30
		mean±S.E.	0.62 ± 0.21	7.04 ± 2.35	67.02 ± 22.34	12.50 ± 4.17	9.10 ± 3.03
Periodontitis	1	28	0.20	6.45	75.40	9.33	6.20
	2	47	-	6.41	92.83	0.41	0.14
	3	27	0.26	8.55	66.20	15.77	7.55
	4	31, 42	0.00	5.76	70.52	9.72	13.00
	5	25	-	3.70	73.61	13.82	7.21%
		mean±S.E.	0.15 ± 0.09	6.17 ± 2.76	75.71 ± 33.86	9.81 ± 4.39	6.82 ± 3.05

Descriptive statistics of the percentages of $CD8^+$ T cell subsets in healthy and periodontitis groups.

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
T_N	10	.4770	.44853	.00	1.47
T_{SCM}	13	6.7054	3.32607	1.71	15.17
T_{CM}	13	70.3646	10.37698	47.34	92.83
T_{EM}	13	11.4669	4.68225	.41	18.64
T_{TE}	13	8.2200	6.91235	.14	28.28

Mann-Whitney's U-test results of differences of the percentages of $CD8^+$ T cell subsets in healthy and periodontitis groups.

Ranks

	Condition	N	Mean Rank	Sum of Ranks
T_N	Healthy	7	6.71	47.00
	Periodontitis	3	2.67	8.00
	Total	10		
T_{SCM}	Healthy	8	7.13	57.00
	Periodontitis	5	6.80	34.00
	Total	13		
T_{CM}	Healthy	8	6.00	48.00
	Periodontitis	5	8.60	43.00
	Total	13		
T_{EM}	Healthy	8	7.50	60.00
	Periodontitis	5	6.20	31.00
	Total	13		
T_{TE}	Healthy	8	6.88	55.00

	Periodontitis	5	7.20	36.00
	Total	13		

Test Statistics[b]

	T_N	T_{SCM}	T_{CM}	T_{EM}	T_{TE}
Mann-Whitney U	2.000	19.000	12.000	16.000	19.000
Wilcoxon W	8.000	34.000	48.000	31.000	55.000
Z	-1.943	-.146	-1.171	-.586	-.146
Asymp. Sig. (2-tailed)	.052	.884	.242	.558	.884
Exact Sig. [2*(1-tailed Sig.)]	.067[a]	.943[a]	.284[a]	.622[a]	.943[a]

a. Not corrected for ties.

b. Grouping Variable: Condition

Appendix F: The expression of $CD103^+$ T cells in periodontal tissues

	No.	Tooth No.	The expression of CD103 in periodontal tissues (%)			
			$CD4^+$ T cell		$CD8^+$ T cell	
			$CD103^+$	$CD103^-$	$CD103^+$	$CD103^-$
Healthy	1	12-22	9.20	90.53	29.90	69.89
	2	43	12.70	86.72	44.60	52.09
	3	17	4.06	94.88	23.27	73.92
	4	36-37	6.75	91.55	21.02	77.47
	5	27	5.03	94.01	34.11	64.68
	6	17	3.32	96.28	48.71	50.08
	7	26	3.44	94.30	26.26	71.70
	8	33-43	5.07	93.68	33.74	64.09
		mean±S.E.	6.19 ± 2.06	92.74 ± 30.91	32.70 ± 10.90	65.49 ± 21.83
Periodontitis	1	28	14.06	82.98	47.26	47.44
	2	47	3.82	95.87	99.40	0.39
	3	27	4.37	94.36	34.92	64.03
	4	31, 42	16.37	82.18	64.13	35.02
	5	25	5.84	93.26	45.88	52.46
		mean±S.E.	8.89 ± 3.98	89.73 ± 40.13	58.32 ± 26.08	39.87 ± 17.83

Descriptive statistics of the percentages of CD103-expressing T cells in healthy and periodontitis groups.

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
$CD4^+CD103^+$	13	7.2331	4.42688	3.32	16.37
$CD8^+CD103^+$	13	42.5538	20.89807	21.02	99.40
Condition	13	1.6154	.50637	1.00	2.00

Mann-Whitney's U-test results of differences of the percentages of CD103-expressing T cells in healthy and periodontitis.

Ranks

	Condition	N	Mean Rank	Sum of Ranks
$CD4^+CD103^+$	Periodontitis	5	8.20	41.00
	Healthy	8	6.25	50.00
	Total	13		
$CD8^+103^+$	Periodontitis	5	10.20	51.00
	Healthy	8	5.00	40.00
	Total	13		

Test Statistics[b]

	$CD4^+CD103^+$	$CD8^+CD103^+$
Mann-Whitney U	14.000	4.000
Wilcoxon W	50.000	40.000
Z	-.878	-2.342
Asymp. Sig. (2-tailed)	.380	.019
Exact Sig. [2*(1-tailed Sig.)]	.435[a]	.019[a]

a. Not corrected for ties.

b. Grouping Variable: Condition

VITA

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