การแสดงออกของสารสื่ออักเสบในซีดี103^{+} และซีดี103^{-} ทีเซลล์ที่สกัดจากเนื้อเยื่อปริทันต์อักเสบ

นางสาวเบญจรัตน์ อิสระพิทักษ์กุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2560

EXPRESSION OF INFLAMMATORY MEDIATORS IN CD103^{+} AND CD103^{-} T CELLS ISOLATED FROM PERIODONTITIS TISSUE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Periodontics
Department of Periodontology
Faculty of Dentistry
Chulalongkorn University
Academic Year 2017

Thesis Title	EXPRESSION OF INFLAMMATORY MEDIATORS IN $CD103^+$ AND $CD103^-$ T CELLS ISOLATED FROM PERIODONTITIS TISSUE
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Field of Study	Periodontics
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เบญจรัตน์ อิสระพิทักษ์กุล : การแสดงออกของสารสื่ออักเสบในซีดี 103^{+} และซีดี103^{-}ทีเซลล์ที่สกัดจากเนื้อเยื่อปริทันต์อักเสบ (EXPRESSION OF INFLAMMATORY MEDIATORS IN CD103^{+} AND CD103^{-} T CELLS ISOLATED FROM PERIODONTITIS TISSUE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ทญ. ดร. รังสินี มหานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.สาธิต พิชญางกูร, หน้า.

ถึงแม้ว่าทิชชู-เรสซิเด็นซ์ เม็มโมรี่ทีเซลล์ (T_{RM}) จะมีบทบาทสำคัญในภูมิคุ้มกันและพยาธิวิทยาของเนื้อเยื่อเมือก การศึกษาเกี่ยวกับ T_{RM} ในเนื้อเยื่อปริทันต์อักเสบยังมีอยู่จำกัด การศึกษาก่อนหน้าในห้องปฏิบัติการของเราตรวจพบ T_{RM} (ซีดี103^{+} ทีเซลล์)ในเนื้อเยื่อปริทันต์อักเสบ อย่างไรก็ตามยังไม่ทราบหน้าที่ของเซลล์เหล่านั้น จุดประสงค์ของการศึกษานี้เพื่อตรวจสอบการผลิตของสารสื่ออักเสบจากซีดี 103^{+} และซีดี103^{-} ทีเซลล์จากเนื้อเยื่อปริทันต์อักเสบ เนื้อเยื่อปริทันต์ได้จากผู้ป่วยโรคปริทันต์อักเสบเรื้อรังขั้นรุนแรง นำมาย้อมด้วยวิธี intracellular cytokine staining และวิเคราะห์การแสดงออกของซีดี103 อินเตอร์เฟอรอนแกมม่า (IFN-γ) อินเตอร์ลิวคิน-17 (IL-17) และแกรนไซม์บีโดยการใช้วิธีโฟลไซโทเมทรีแบบ 6 สี ทีเซลล์ที่แทรกซึมอยู่ในเนื้อเยื่อปริทันต์อักเสบส่วนใหญ่เป็นซีดี4^{+} ทีเซลล์ พบการแสดงออกของซีดี103 บนซีดี8^{+} ทีเซลล์เป็นหลักและพบเพียงเล็กน้อยบนซีดี4^{+} ทีเซลล์ ซีดี4^{+}ซีดี103^{+} และซีดี4^{+}ซีดี103^{-}ทีเซลล์ผลิต IFN-γ และ IL-17 ในขณะที่ซีดี8^{+}ซีดี103^{+} และซีดี8^{+}ซีดี103^{-} ทีเซลล์ผลิตเพียง IFN-γ ซีดี4^{+} และซีดี8^{+} ทีเซลล์ที่แสดงออกซีดี103 ผลิต IFN-γ ในสัดส่วนที่มากกว่าอย่างมีนัยสำคัญ พบการผลิตแกรนไซม์บีจากซีดี103^{+} และซีดี103^{-}ทีเซลล์ การศึกษานี้เป็นครั้งแรกที่พบการผลิตของสารสื่ออักเสบจากซีดี103^{+} และซีดี103^{-}ทีเซลล์ในเนื้อเยื่อปริทันต์อักเสบ ซึ่งการค้นพบนี้เสนอแนะบทบาทของเรสซิเด็นซ์ เม็มโมรี่ทีเซลล์ในกระบวนการอักเสบของอวัยวะปริทันต์และการละลายของกระดูก

ภาควิชา ปริทันตวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา ปริทันตศาสตร์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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5875819232 : MAJOR PERIODONTICS

KEYWORDS: CD103 / INFLAMMATORY MEDIATORS / PERIODONTITIS / T CELLS / TISSUE - RESIDENT MEMORY T CELLS

BENJARAT ISARAPHITHAKKUL: EXPRESSION OF INFLAMMATORY MEDIATORS IN $CD103^+$ AND $CD103^-$ T CELLS ISOLATED FROM PERIODONTITIS TISSUE. ADVISOR: PROF. RANGSINI MAHANONDA, Ph.D., CO-ADVISOR: SATHIT PICHYANGKUL, Ph.D., pp.

Despite the important role of tissue - resident memory T (T_{RM}) cells in immunity and pathology of mucosal tissues, the study of tissue-resident memory T cells in periodontitis tissues is limited. Previous investigation in our laboratory identified the localization of T_{RM} ($CD103^+$ T cells) in periodontitis tissue. However, their function is not known. The aim of this study is to investigate the production of inflammatory mediators by $CD103^+$ and $CD103^-$ T cells in periodontitis tissue. Human periodontal tissues were obtained from patients with severe chronic periodontitis. Intracellular cytokine staining was performed and expression of CD103, interferon gamma (IFN-γ), interleukin-17 (IL-17) and granzyme B was analyzed by 6-color flow cytometry. Majority of infiltrated T cells in periodontitis tissues were $CD4^+$ T cells. Expression of CD103 was mainly detected on $CD8^+$ T cells, but only minimally on $CD4^+$ T cells. $CD4^+CD103^+$ and $CD4^+CD103^-$ T cells produced IFN-γ and IL- 17 whereas $CD8^+CD103^+$ and $CD8^+CD103^-$ T cells produced only IFN-γ. $CD4^+$ and $CD8^+$ T cells that expressed CD103 phenotype significantly produced higher proportion of IFN-γ. Granzyme B production was detected from $CD103^+$ and $CD103^-$ T cells. We first identified the production of inflammatory mediators from $CD103^+$ and $CD103^-$ T cells in periodontitis tissue. This suggests the possible role of resident memory T cells in periodontal inflammation and bone resorption.

Department:	Periodontology	Student's Signature	.....
Field of Study:	Periodontics	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere admiration to my advisor, Professor Dr. Rangsini Mahanonda, for her continuous support, kindness, understanding, meticulous comments, valuable time and devotion in guiding me throughout my Master degree program. I am extremely grateful to my co-advisor, Dr. Sathit Pichyangkul, Department of Immunology and Medical Component, AFRIMS, for providing the laboratory facilities, his supervision and advice in correcting this thesis. Besides, my advisors, I also wish to thank my thesis committee members; Professor Dr. Stitaya Sirisinha and Assistant Professor Dr. Chantrakorn Champaiboon for their suggestions and insightful comments.

Sincere appreciation is expressed to Mr. Noppadol Sa-Ard-Iam for assistance in laboratory work, statistical advice and proofreading this manuscript. I also would like to thank Ms. Pimprapa Rerkyen for her kind advice and technical support in preparing this manuscript.

I would like to acknowledge research grant from the Chulalongkorn Academic Advancement into Its 2nd Century Project and Thailand Research Fund and Chulalongkorn university (BRG5880003) for the financial support of this study. I would also like to thank the staff of Periodontology Department and Assistant Professor Dr. Keskanya Subbalekha, Department of Oral Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University for the generosity in collecting tissue sample. Lastly, I am very grateful to my father, mother, sisters and friends for their love and encouragement during the hard times.

CONTENTS

Page

Page

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

CCR	C–C chemokine receptor
CD	Cluster of differentiation
CD62L	CD62 Ligand
CXC	Cysteine X Cysteine
CXCR2	CXC chemokine receptor 2
Del-1	Developmental Endothelial Locus-1
GCF	Gingival crevicular fluid
G-CSF	Granulocyte colony-stimulating factor
HSV	Herpes simplex virus
IFN	Interferon
IL	Interleukin
LCMV	Lymphocyte choriomeningitis virus
mAbs	monoclonal antibodies
NK	Natural killer cells
NKT	Natural killer T-cells
RANKL	Receptor activator of nuclear factor kappa-B ligand
RPMI	Roswell Park Memorial Institute
SEB	Staphylococcal enterotoxin B
S1P1	Sphingosine-1-1phosphate receptors
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1

CHAPTER I
INTRODUCTION

Background of the present study

Periodontitis is an oral disease of tooth supporting structure including gingiva, cementum, periodontal ligament and alveolar bone. According to severity, the disease could be classified into two forms, gingivitis and periodontitis. Destruction of gingivitis is limited to gingiva (Page 1986), while periodontitis involves connective tissue attachment, loss of bone and consequently leads to tooth loss (Ranney 1993). Pathogenesis of periodontitis is host immune response to bacterial challenge. T and B cells account for adaptive immunity. Seymour et al. (1979) described the transition from gingivitis, which was mentioned as stable gingival lesion to progressive periodontal lesion (periodontitis) as a result of change from predominantly T cell response to B cell and plasma cell. The change was hypothesized to occur from an imbalance in T cell control induced by specific pathogenic bacteria. However, the role of T cell is still not clear.

Previous study from our laboratory found that predominant B cells in periodontitis lesion were plasma cells and CD138 positive plasma cells when compared to CD3 positive T cells seem to be distributed in a similar location, scattering in connective tissue with dense area observed at the base of periodontal pocket, adjacent to pocket epithelium (Mahanonda et al. 2016). However, local function is still unknown. Apart from B cells, T cells also have an important role in gingivitis and healthy tissue. T helper 1 (Th1) cells mediate predominantly cell-mediated immune response to intracellular pathogens by producing cytokines such as Interferon gamma (IFN-γ), Interleukin-2 (IL-2) and tumor necrosis factor (TNF). Whereas, Th2 cells have a role in growth and

differentiation of activated B cells by secreting IL-4, IL-5, IL-10 and IL-13 (Mosmann and Sad 1996). The Th1/Th2 paradigm was proposed in explaining the pathogenesis of periodontal disease. Th1 cells are hypothesized to associate with stable gingivitis lesion, while Th2 cells are associated with progressive periodontitis lesion (Gemmell et al. 2002). However, it is still inconclusive on the role of Th1 and Th2 in periodontal disease . Another subset of T cell was introduced, Th17 provided potential alternative mechanism of periodontal disease. It is thought to play role in cell-mediated tissue damage, autoimmune and osteolytic process (Steinman 2007, Dong 2008). Th17 secretes IL-17, found in periodontal lesions and suggested to contribute to disease progression (Takahashi et al. 2005, Cardoso et al. 2009).

Periodontitis is a localized infection, specifically in the periodontal pocket area where periodontal tissue is very near to microbial plaque and continually exposed to this infection. In the past, there have been many studies of human T cells which conducted on a peripheral blood sample, not on a periodontal tissue sample where the immune response takes place. Hence, peripheral blood T cell information could mislead the understanding of immune response in a local periodontal tissue.

Recently, our laboratory conducted a study on CD 103^{+} memory T cells in periodontal tissue and identified 6 populations of T cell subsets including Resident memory T (T_{RM}), T_{N}, stem cell-like memory T (T_{SCM}), central memory T (T_{CM}), effector memory T (T_{EM}) and terminally differentiated effector memory T (T_{TE}) cells. The results showed that majority of T cells in periodontal tissues were T_{CM} cells both in health and disease. Significant proportions of $CD8^{+}CD103^{+}$ T cells were observed in periodontitis tissues compared to healthy tissues suggesting their possible role in tissue pathology (Yongyuth 2015). However, further investigations are needed in order to understand

their function of each T cell subpopulation. Therefore, this study was conducted to investigate cytokine production, such as interferon–gamma (IFN-γ), interleukin-17 (IL-17) or cytotoxic molecule, granzyme B to explain the role of memory T cells in periodontal pathogenesis.

Objectives

To determine cytokine expression in memory T cells isolated from periodontitis tissue.

Hypothesis

Memory T cells, $CD4^+$ and $CD8^+$ T cells from periodontitis tissue express IFN-γ, IL-17 and granzyme B.

Field of research

Human immunology

Criteria Inclusions

1. Inflamed periodontal tissues were obtained from patients with severe chronic periodontitis (gingival inflammation, clinical attachment loss of 5 mm or more, severe bone loss approximately 50% of the root length or more, hopeless periodontal prognosis).

2. All subjects were in good general health, no systemic disease or smoking and none of them had taken antimicrobial or anti-inflammatory drugs within the previous 3 months.

Limitation of research

This study could not investigate many periodontal tissue samples in each group due to limiting time and expenses.

Application and expectation of research

1. New information of cytokine expression from CD 103^{+} and CD 103^{-} T cells from periodontitis tissues and their possible roles in periodontal disease.

2. Publication in the national peered-review journal

Keywords

CD103, Inflammatory mediators, Periodontitis, T cells, Tissue-resident memory T cells

CHAPTER II
LITERATURE REVIEW

Periodontal disease

Periodontal disease is a common chronic inflammatory disease in humans, which affects tooth supporting structure including gingiva, cementum, periodontal ligament and alveolar bone. Two forms of periodontal disease severity could be classified as gingivitis and periodontitis. Gingivitis is a mild and stable form which inflammation is limited to soft tissue gingiva (Page 1986). An advanced and destructive form called periodontitis involves gingival inflammation and bone destruction, resulting in loss of connective tissue attachment and bone, and may consequently lead to tooth loss (Ranney 1993).

Dental plaque biofilms have been well recognized as etiologic agents. Dental plaque biofilm is a unique, complex and dynamic community consisting of multispecies that communicate, exchange genes and regulated via quorum-sensing (Socransky and Haffajee 2002, Hojo et al. 2009). Composition of microbial complexes in dental plaque varies and associated with periodontal health. In healthy and gingivitis sites, predominantly facultative gram-positive bacteria such as *Streptococci* and *Actinomyces* are found. While in periodontitis, a shift to gram negative anaerobes is observed. Key periodontal pathogens composed of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia* were documented (Socransky et al. 1998). Pathogenesis of periodontitis involves host immune response to plaque bacterial challenge. Large numbers of lymphocyte infiltrates including T and B cells are observed together with high levels of inflammatory mediators such as IL-8, IL-1β, tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), interferon-γ (IFN-γ), and receptor activator

of NF-κB ligand (RANKL) in tissues and gingival crevicular fluid of periodontitis patients (Page and Kornman 1997, Séguier et al. 1999, Belibasakis and Bostanci 2012). These mediators have been known to be involved in tissue and bone destruction (Graves et al. 2011).

B cells in periodontal disease

Since 1965, it has been known that immunoglobulin producing plasma cells predominated the periodontitis lesions (Brandtzaeg and Kraus 1965). B cells and plasma cells outnumbered T cells, when gingivitis progressed to periodontitis. Recent study from our laboratory confirmed the presence of predominant $CD138^{+}$ plasma cells in periodontitis tissues, scattering in connective tissue with dense area observed at the base of periodontal pocket, adjacent to pocket epithelium. These periodontitis plasma cells mainly secreted IgG specific to periodontal pathogens. Unlike periodontitis, majority of B cell subsets in healthy gingiva and gingivitis tissues were memory B cells. In healthy gingiva, these memory B cells resided in the connective tissue subjacent to the junctional epithelium, therefore suggesting a role of memory B cells in maintaining periodontal homeostasis (Mahanonda et al. 2016).

T cells in periodontal disease

Lymphocytes are categorized into T cells and B cells. T cells can be grouped into two major subdivisions : helper (Th, $CD4^{+}$) and cytotoxic (Tc or CTL, $CD8^{+}$) T cells. T helper cells function to stimulate B cells in antibody production or to activate function of macrophages or NK cells. Whereas cytotoxic T cells function to kill virally infected cells (Abbas et al. 2015). In 1979, Seymour, Powell, and Davies hypothesized that a

change from a T cell dominated-stable gingival lesion to a B cell and plasma cell dominated–periodontitis lesions may be due to an imbalance in T cell regulation induced by specific pathogenic bacteria. It is well recognized that T cells play important roles in immune responses and function by directly secreting soluble mediators or cytokines. Early T cell studies revealed the existence of different T cell subsets including Th1, Th2, Th17, and Treg (regulatory T cells) in periodontal lesions. However, the significance of these cell types in periodontal health and disease remain unclear and inconclusive. The concept of Th1 and Th2 was derived from mouse model. Th1 cells mediate predominantly cell-mediated immune response to intracellular pathogens by producing cytokines such as IFN-γ, IL-2 and TNF-α. Conversely, Th2 cells have a role in growth and differentiation of activated B cells by secreting IL-4, IL-5, IL-10 and IL-13. It was demonstrated in mouse model that resistant mice developed Th1 response, while susceptible mice developed Th2 cytokine production when subjecting to *Leishmania* infection (Mosmann et al. 1986, Mosmann and Sad 1996). The Th1/Th2 paradigm was then proposed in pathogenesis of periodontal disease. Th1 cells are hypothesized to associate with stable gingivitis lesion, while Th2 cells are associated with progressive periodontitis lesion (Gemmell et al. 2002). However, controversies arose as some studies showed predominantly Th1 response over Th2 in diseased periodontal tissue (Takeichi et al. 2000) and Th1 role in periodontal bone resorption (Taubman and Kawai 2001). Other studies also found comparable presence of both Th1 and Th2 cytokine response in advanced periodontitis (Fujihashi et al. 1996, Prabhu et al. 1996, Berglundh et al. 2002). The role of Th1 and Th2 in periodontal disease is still inconclusive.

A discovery of another subset of helper T cell, Th17 provided potential alternative mechanism of periodontal disease. Th17 is thought to play role in cell-mediated tissue damage, autoimmune and osteolytic process (Steinman 2007, Dong

2008). Th17 secretes IL-17, found in periodontal lesions and suggested to contribute to disease progression (Takahashi et al. 2005, Cardoso et al. 2009). Furthermore, Treg (regulatory T cells) have also been described. Treg secretes IL-10 and TGF-β and oppositely against Th17 cells by inhibiting inflammation and self-tolerance (Awasthi and Kuchroo 2009).

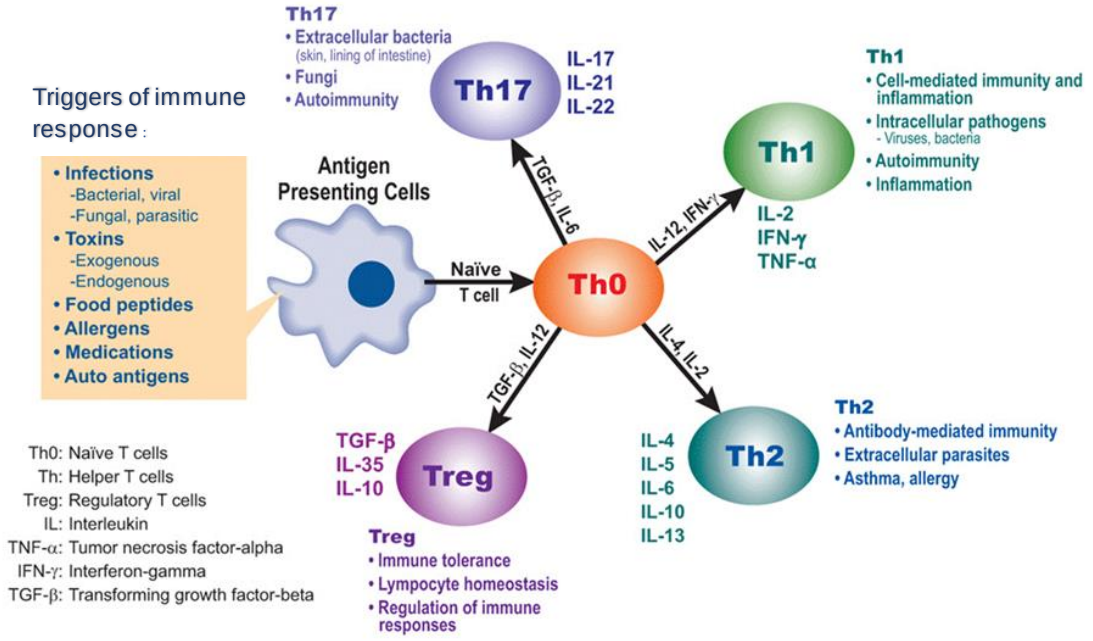


Figure 1. $CD4^+$ T helper cell fate: differentiation, their cytokine expression, characteristic transcription factors and cytokine production. Modified from (Li 2017).

Memory T cells

Following positive and negative selection, T cells are released from the thymus as mature, naïve T cells harboring a given epitope specificity. In response to cognate antigen encountered, naïve T cells proliferate and differentiate into effector cells, the vast majority of which migrate to peripheral tissues and inflamed sites to facilitate destruction of infected targets reviewed in (Sallusto et al. 1999). Following antigen

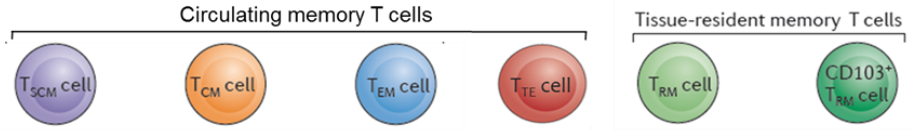
clearance, such as that in smallpox vaccination, >95% of the effector cells die while a small pool of T cells ultimately develops into long-lived memory T cells (Zhang et al. 2005). The memory T cells found in the blood can be divided into subsets based on the differential expression of markers of migration, CD62L and CCR7 (Sallusto et al. 1999). Those with high expression of CD62L and CCR7 are termed central memory T cells (T_{CM}) because they are enriched in the secondary lymphoid organs. By contrast, memory T cells lacking CD62L and CCR7 expression migrate between blood and non-lymphoid tissue, exhibiting rapid effector capabilities on stimulation and so are termed effector memory T cells (T_{EM}). These T_{EM} can be differentiated into terminal effector memory T cells (T_{TE}) by IL-15 (Lugli et al. 2010). Studies in both mice and humans have led to identification of another subset of memory T cells which retain stem cell-like properties similar to hematopoietic stem cells and generate multiple subsets of memory T cells *in vitro* (Zhang et al. 2005, Gattinoni et al. 2011). These cells are known as stem cell memory T cells (T_{SCM}). T_{CM} and T_{SCM} express CD28 and CD95, while T_{EM} and T_{TE} express CD28 but not CD95. In recent years, a new subset of memory T cells that permanently reside in non-lymphoid tissues has been identified; they are now widely referred to as tissue-resident memory T (T_{RM}) cells (Di Meglio et al. 2011, Ariotti et al. 2012, Sathaliyawala et al. 2013).

Tissue resident memory T cells (T_{RM})

T_{RM} in mice, nonhuman primates and humans express CD103 and CD69. The ligand for CD103, E-cadherin is expressed on epithelial cells suggesting that the interaction of CD103 and E-cadherin may contribute to maintaining the resident status of T_{RM} in peripheral tissue (Pauls et al. 2001). It should be pointed out that $CD103^-$ T_{RM} also can be detected and localize to the same clusters that $CD103^+$ T_{RM} do (Wakim et al.

2010). $CD103^+$ expression is more predominant in $CD8^+$ T_{RM} than $CD4^+$ T_{RM}, which is expressed by a proportion of skin memory $CD4^+$ T cells (Pauls et al. 2001, Gebhardt et al. 2011). $CD103^+$ T cells had been widely studied in protective barrier organ and lymphoid tissues including in skin, lung, intestine, vagina and brain of mouse model and human (Gebhardt et al. 2009, Wakim et al. 2010, Mackay et al. 2012, Sathaliyawala et al. 2013, Turner et al. 2014).

Another key cell surface marker of T_{RM} is CD69. In addition to its association with recent activation, CD69 inhibits the function of sphingosine-1-phosphate receptor 1 (S1P1), resulting in retention of newly primed T cells in draining lymph nodes (Shiow et al. 2006). Therefore, the CD69/S1P1 may play a role in inhibiting T_{RM} cell egress from tissue. $CD69^+$ memory $CD4^+$ T cells have been recognized in the skins, intestines and lungs of human and mice (Jiang et al. 2012, Turner and Farber 2014) while blood circulating memory T cells lack $CD69^+$ expression. In human, $CD69^+$ memory T cells (T_{CM} and T_{RM}) were also found in lymph nodes and spleens, but not expressing $CD103^+$ (Sathaliyawala et al. 2013). This CD103/$CD69^+$ phenotype is not expressed among pathogen-specific memory $CD8^+$ T cells in blood (Mueller et al. 2013). It should be pointed that T_{RM} can be further classified as T_{RM} $CD103^-$ and T_{RM} $CD103^+$ (Farber et al. 2014).



CD45RA	+	-	-	+	-	-
CCR7	+	+	-	-	-	-
CD28	+	+	-	-	-	-
CD95	+	+	+	+	+	+
CD69	-	-	-	-	+	+
CD103	-	-	-	-	-	+
IL-2	+++	+++	++	+/-	+/-	+/-
IFN	+	++	+++	+++	+++	+++
TNF	+	++	+++	+++	+++	+++

Figure 2. Schematic of human memory T cell heterogeneity in the blood and in tissues. Four circulating populations include stem cell memory T cells (T_{SCM}), central memory T cells (T_{CM}), effector memory cells (T_{EM}), and terminal effector memory T cells (T_{TE}). Two tissue populations include tissue-resident memory T (T_{RM}) cells with $CD103^+$ T_{RM} and $CD103^-$ T_{RM}. Modified from (Farber et al. 2014).

While circulating memory T cells provide efficient protection against systemic infections, their ability to deal with localized infections in the periphery is often limited. In a mouse model of viral infection, it was clearly demonstrated that T_{RM} provide superior protection against viral infection relative to the circulating memory T cell (Gebhardt et al. 2009, Gebhardt and Mackay 2012, Jiang et al. 2012). T_{RM} cells generated in skin and salivary glands after Vaccinia virus or LCMV infection, mediate potent protection from infection rechallenge even when T cell recirculation is pharmacologically inhibited (Liu et al. 2009, Hofmann and Pircher 2011, Teijaro et al. 2011). T_{RM} cells established in the vagina epithelial layer by exogenous chemokine treatment provide better protection

against a lethal vagina HSV-2-challenge compared to circulating HSV-2-specific memory T cells (Shin and Iwasaki 2012). Infection of mice with influenza virus leads to the generation of both resident and transient circulating memory T cells in the lungs; however, lung T_{RM} $CD4^+$ T cells and $CD8^+$ T cells show optimal protection against influenza challenge compared with circulating memory T cells (Teijaro et al. 2011, Turner et al. 2014, Wu et al. 2014). Collective evidence suggests that T_{RM} in peripheral tissues play a key role in mediating T cell–dependent protective immunity against microbial pathogens. Furthermore, these findings also suggest that peripheral blood immune response may differ from those at the tissue sites where they are needed.

Immune response is like double edge sword. Evidence also suggests that T_{RM} cells can cause immunopathology, for example psoriasis and fixed drug eruption. A large number of $CD103^+$ T_{RM} cells expressing high levels of inflammatory cytokines, IL-17 and IL-22 were identified in psoriasis skin lesions (Cheuk et al. 2014). At the resting stage of fixed drug eruption patient (no clinical active skin lesion), $CD103^+$ T_{RM} cells reside in the epithelial area at the junction between epidermis and dermis and minimally express IFN-γ. Upon re-exposure to the same drug, a significantly increase of T_{RM} with high expression of IFN-γ could be observed (Mizukawa et al. 2002).

Memory T cells in periodontitis

It was known that majority of T cells in periodontitis tissue express CD45RO, a memory cell phenotype (Gemmell et al. 1992, Yamazaki et al. 1993). So far there has been very limited data on memory T cell subsets in periodontal disease. Preliminary flow cytometric analysis from our laboratory revealed the presence of different memory T cell subsets including T_{SCM}, T_{CM}, T_{EM}, T_{TE}, and T_{RM} in periodontal tissue, both in health and

disease. Immunohistochemical analysis showed that $CD103^+$ T_{RM} cells were localized in both epithelial layer and connective tissue. The majority of CD103 expressing cells in periodontal tissue both in health and disease were $CD8^+$ T cells. $CD8^+$ T cells were mainly detected in epithelial layer, while $CD4^+$ T cells were mainly detected in connective tissue. Our laboratory finding agreed with early study by Tonetti et al. (1995) which showed intraepithelial lymphocytes express $\alpha^{IEL}\beta^7$ integrin, the surface molecule known as CD103 and found 49–54% of $CD3^+$ intraepithelial lymphocytes expressed $\alpha^{IEL}\beta^7$ integrin with majority locating in epithelium.

Inflammatory mediators in periodontal disease

Cytokines are polypeptides that function as messenger molecules and communicate signals from one cell to another. Cytokines also instruct cell to proliferate, differentiate and secrete additional cytokines. In periodontitis several proinflammatory cytokines were found to involve in the pathogenesis including IL-1, IL-6, IL-12, IL-17, IL-18, IL-21, TNF-α and IFN-γ. These cytokines could be detected in the gingival crevicular fluid (GCF), exudates collected at the gingival margin, and in gingival tissue (Yucel-Lindberg and Båge 2013). Among these cytokines, IL-1, IL-6 and TNF-α have the most prominent roles in periodontitis. In this context, focused will be on mediators expressed by T_{RM} cells including IFN-γ and IL-17. Granzyme B will also be reviewed.

Interferon gamma (IFN-γ)

IFN-γ is a proinflammatory mediator known to activate macrophages and macrophage-like cells such as endothelial cells, dendritic cells, Langerhans cells (Billiau

and Dijkmans 1990). IFN-γ contributes to Th1 polarization of $CD4^+$ T cell and selectively inhibits proliferation of Th2 cells (Mosmann and Sad 1996).

In periodontitis, the concentration of IFN-γ has been found to increase in GCF and reported to associate with progressive or more severe lesion (Dutzan et al. 2009). There were studies, which indicate that IFN-γ provokes bone resorption and also inhibits bone resorption. In mouse model, IFN-γ was found to produce inflammatory reaction and bone resorption to *A. actinomycetemcomitans* and *P. gingivalis*. Its proinflammatory effect leads to upregulation of TNF-α and IL-1β, mediators involved in bone loss mechanism. It also stimulates osteoclast formation via antigen-driven T cell activation and attraction of RANKL (Gao et al. 2007). However, IFN-γ has also been described to inhibit osteoclastogenesis (Takayanagi et al. 2005).

IFN-γ has been viewed not only through a destructive viewpoint, but also as a protective viewpoint by controlling infection. IFN-γ plays an important role in innate immunity. It is capable of acting on innate cells such as endothelial, fibroblast, macrophages and neutrophils. It provides leukocyte recruitment and activates inflammation, enhances phagocytosis and antigen uptake (Garlet 2010). In addition, IFN-γ has been involved in formation of extracellular traps of neutrophil (NETS) (Martinelli et al. 2004). IFN-γ mRNA has also been reported to express from γδ T cells (first line defense against infection) from chronic periodontitis (Lundqvist et al. 1994).

Interleukin-17 (IL-17)

IL-17 is a proinflammatory mediator produced from Th17, a subset of $CD4^+$ T cell. It has been implicated in many autoimmune and inflammatory conditions. Other than Th17, IL-17 can also be produced from mast cells, neutrophils, dendritic cells, γδ T

cells, macrophages, natural killer cells, and periodontal ligament (PDL) cells (Park et al. 2012).

Studies have indicated an increased level of IL-17 in periodontitis tissue (Ito et al. 2005, Takahashi et al. 2005, Lester et al. 2007, Ohyama et al. 2009). Emerging role of Th17 and IL-17 in pathogenesis of periodontitis have been widely described, (Cheng et al. 2014, Zenobia and Hajishengallis 2015). The potential mechanism involves its induction of several proinflammatory mediators such as IL-6, granulocyte colony-stimulating factor (G-CSF), IL-8/CXCL8, TNF-α, chemokines , matrix metalloproteinases (Dong 2006), RANKL expression by osteoblasts (Miossec and Kolls 2012) and periodontal ligament cells (Lin et al. 2015) resulting in connective tissue degradation and bone resorption.

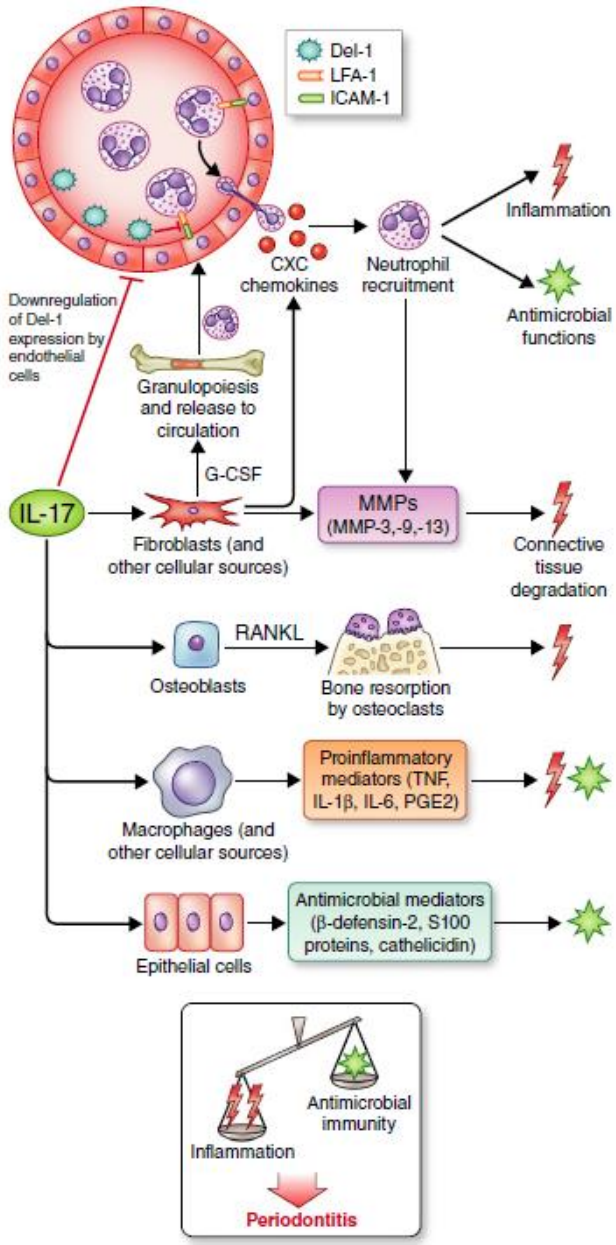


Figure 3. Biological functions of interleukin-17 and their role in periodontitis. (Zenobia and Hajishengallis 2015)

IL–17 has an important role in host innate immunity as G-CSF and CXC chemokines function by recruiting neutrophils. CXCR2 mediates neutrophil extravasation into gingival tissue (Zenobia et al. 2013). Mice lacking IL-17 have been shown to

produce lower level of neutrophil chemokines and more susceptible to infection (Ye et al. 2001). However, persistent recruitment of neutrophils could lead to chronic inflammatory conditions as IL-17 has been found to inhibit the expression of Del-1 (Eskan et al. 2012), an endothelial secreted glycoprotein involved in control of neutrophil transmigration and recruitment (Hajishengallis and Sahingur 2014).

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Granzyme B

Granzymes are serine proteases, consisted of His-Asp-Ser catalytic triad. There are five granzymes in humans (A, B, H, K and M). Granzyme A and B play particularly significant roles in cytotoxic process. Granzyme A activates proteolysis of nuclease inhibitor resulting in single-stranded DNA breaks within target cell. Granzyme B is the most extensively studied and may contribute to the pathogenesis of several chronic inflammatory diseases through both cytotoxic and extracellular mechanisms (Anthony et al. 2010).

Granzyme is generally only expressed, synthesized and stored in lymphoid cell line cells including T cells, NKT, and NK cells. Other type of cells require antigen stimulation to induce expression (Garcia-Sanz et al. 1990). Function of granzyme B in cytotoxicity is by inducing rapid DNA fragmentation of target cells. Granzyme B cleaves substrate and activates caspase-3, which cuts after aspartic residue. Caspase-3 activates a proteolytic cascade resulting in activation of caspase-activated deoxyribonuclease (CAD), the enzyme the degrades the DNA. Granzyme B can also activate caspase indirectly by cleaving Bid, a protein that promotes apoptosome (Ewen et al. 2012).

For extracellular mechanism, granzyme B can efficiently cleave different substrates such as extracellular matrix proteins and proteoglycans including vitronectin,

fibronectin and laminin leading to structural integrity of the skin and increase susceptibility to injury (Fig. 4) (Hiebert and Granville 2012). *In vitro* studies on granzyme B found that it is able to cause detachment of endothelial cell and chondrocytes inducing endothelial cell death (anoikis) (Buzza et al. 2005). This demonstrates the role of granzyme B in chronic wound pathogenesis.

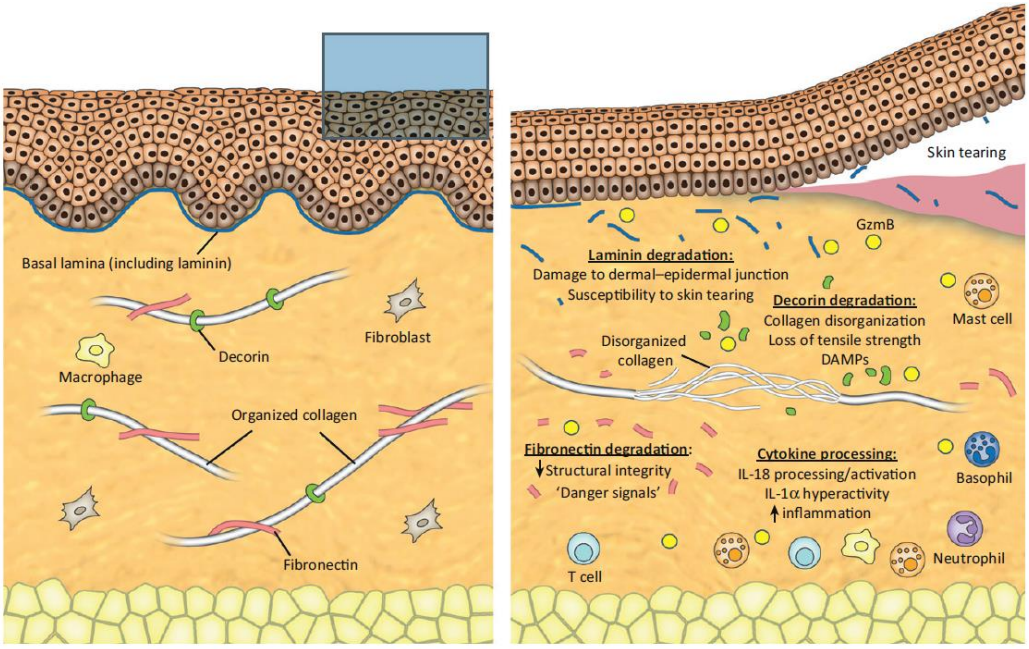

Figure 4. Proposed mechanisms of granzyme B-mediated injury and inflammation. (Hiebert and Granville 2012).

Granzyme B also promotes inflammation by influencing cytokine expression and processing. It is a potent IL-18 converting enzyme by cleaving pro-IL18 into mature IL-18, which was able to induce IFN-γ production leading to inflammation (Omoto et al. 2010). Subsequent study found that granzyme B triggers switching and proteolytic processing of IL-1α to mature forms resulting in the increase of cytokine activity several folds (Afonina et al. 2011). However, granzyme A and B have also been proposed in inflammation control by participating in regulatory T cells activity (Gondek et al. 2005).

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 CD3, anti-human CD4, anti-human CD8, anti-human CD103, anti-IFN-γ, anti-IL-17, anti-granzyme B human monoclonal antibodies and mouse isotype control monoclonal antibodies were obtained from BD biosciences (San Jose, CA, USA).

Subjects selection and ethical consideration

Periodontitis tissue specimens were collected from patients at Periodontal Clinic and Department of Oral Maxillofacial surgery, Faculty of Dentistry, Chulalongkorn University. The ethical approval by the Ethics Committee of Faculty of Dentistry, Chulalongkorn University and informed consent of all participating subjects were obtained before the operation (Ethical Approval number 119/2016).

Periodontal tissue collection

Periodontal tissue samples were collected from periodontitis 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 had no history of periodontal treatment in the past 6 months.

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).

The excised periodontal tissue specimens were immediately placed in a sterile tube that containing RPMI-1640 medium and then transferred to the laboratory within a few hours for further study.

Gingival cell preparation

Tissues were washed thoroughly and cut into small fragments (1-2 mm^3). They were then incubated in 2 mg/ml of collagenase (Sigma Chemical Co.) for 90 minutes at 37°C. Residual tissue fragments were disaggregated by flushing several times with pipette to obtain single cell suspensions and then were filtered through filter of mesh size 70 μm and 40 μm (Becton Dickinson).

Intracellular cytokine staining and flow cytometric analysis

To investigate the expression of IFN-γ, IL-17 and granzyme B, gingival cells were stimulated with Staphylococcal enterotoxin B (SEB) (4 μg/ml) and gingival cells cultured in medium served as negative controls. After 2 hours of stimulation, Golgiplug was added to inhibit cytokine secretion and the cell cultures were further incubated overnight. Gingival cells were first stained for anti-human CD3 (FITC), CD4 (PerCP), CD8 (APC-Cy7) and CD103 (APC) mAbs at 4°C for 30 minutes. The stained gingival cells

were washed with stain buffer and then fixed and permeabilized with BD Cytofix/Cytoperm kit (BD Pharmingen) on ice for 20 minutes and washed with BD Perm/Wash buffer (BD Pharmingen). The cells were then suspended in BD Perm/Wash buffer, and anti-human IFN-γ or anti-human IL-17 or anti-human granzyme B mAbs were added. After another 30 minutes of incubation on ice, cells were washed and then fixed with 1% paraformaldehyde. Analysis of flow cytometry samples was performed by 6-color flow cytometry (BD FACSCelesta™, Becton Dickinson). First, $CD3^+$ cells were gated. Then, $CD4^+$ and $CD8^+$ cells were gated. Finally, these cells were analyzed for the expressions of CD103, IFN-γ, IL-17 or granzyme B.

Statistical Analysis

The data were analyzed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Results were shown as mean±S.E. The Wilcoxon sign ranked test was used for analysis of dependent non-parametric data. Mann-Whitney U test was applied for analysis of independent non-parametric data. *P* values of 0.05 or less was considered significant.

CHAPTER IV
RESULTS

Determination of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ T cells in periodontitis tissues by flow cytometry

Flow cytometric analysis of infiltrated immune cells in periodontitis tissues (Fig. 5, Appendix B) demonstrates that 56.89%±3.00 of lymphocytes were $CD3^+$ T cells. Proportion of T cell subsets showed majority of T cells were $CD4^+$ T cells (57.28%±2.07), while percentage of $CD8^+$ T cells was 32.96%±2.82 (Appendix B).

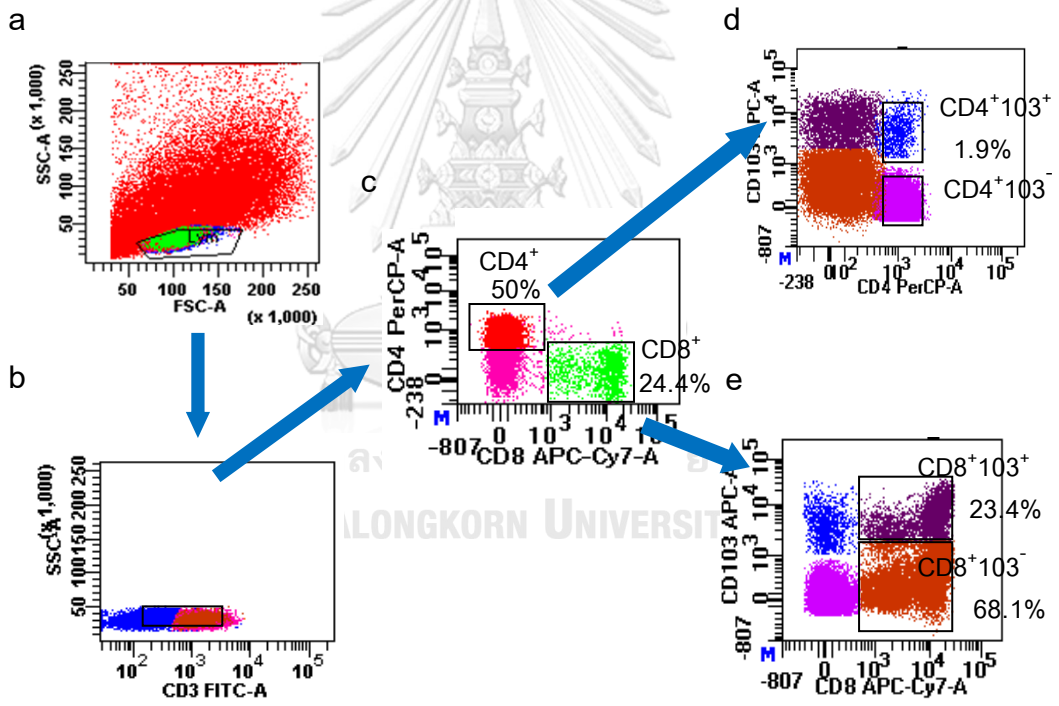

Figure 5 Flow cytometry gating strategy to identify CD103 expressing T cells isolated from periodontitis tissues; **(a)** Gating of lymphocytes; **(b)** Gating of $CD3^+$ T cells; **(c)** Gating of $CD4^+$ and $CD8^+$ T cells; **(d)** Gating of CD103 expression on $CD4^+$ T cells; **(e)** Gating of CD103 expression on $CD8^+$ T cells (representative of 7 samples).

In terms of CD103 expression, greater number of $CD103^-$ T cells were significantly present among both subsets of T cells (CD4; 94.14%±1.19 vs 5.86±1.19; p = 0.018 and CD8; 69.34%±4.10 vs 30.66%±4.10; p = 0.028) (Fig. 6, Appendix C). When comparing between $CD4^+$ and $CD8^+$ T cells, significantly greater percentage of tissue-resident marker, CD103 was found in $CD8^+$ T cells (5.86%±1.19 vs 30.66%±4.10; p = 0.02) (Fig. 6, Appendix C).

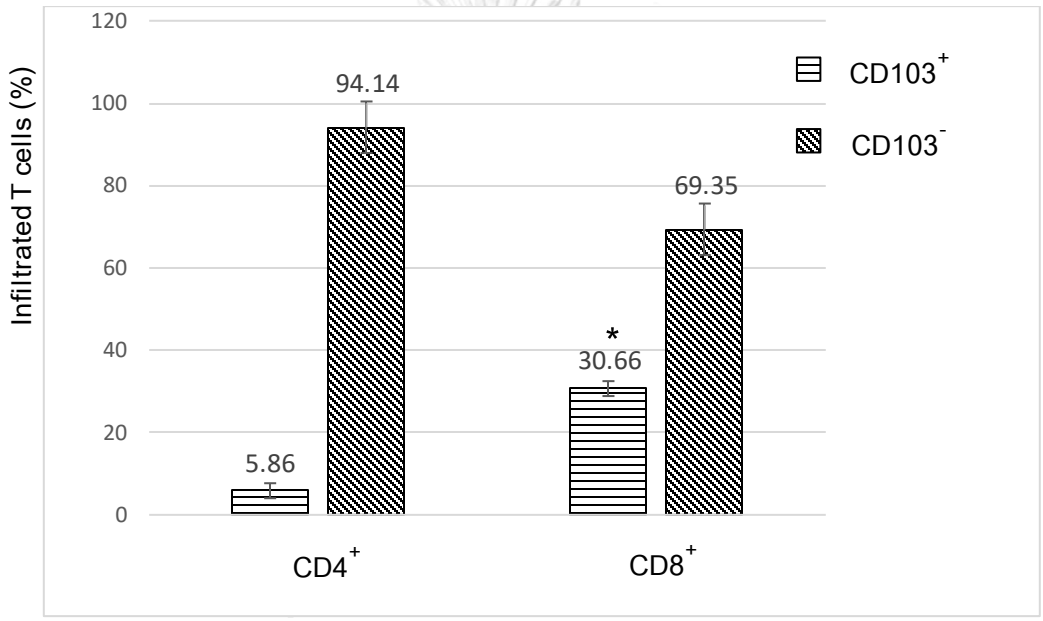

Figure 6. Mean percentage of $CD4^+CD103^+$, $CD4^+CD103^-$, $CD8^+CD103^+$ and $CD8^+CD103^-$ T cells isolated from periodontitis tissue. Mean±S.E. of n=7 are shown. *$p \leq 0.05$, ($CD4^+103^+$ vs $CD8^+103^+$ T cells), Wilcoxon signed-rank test.

Cytokine production of $CD103^+$ and $CD103^-$ T cells in periodontitis

To investigate the cytokine profiles of $CD103^+$ and $CD103^-$ T cells isolated from periodontitis, we assessed the expression of IFN-γ (n=7), IL-17 (n=4) and granzyme B (n=3) using intracellular cytokine staining following polyclonal stimulation with SEB (Fig. 7, Fig.9). The percentages of T cells expressing cytokines were obtained by deduction of control (no SEB stimulation) from SEB (with SEB stimulation).

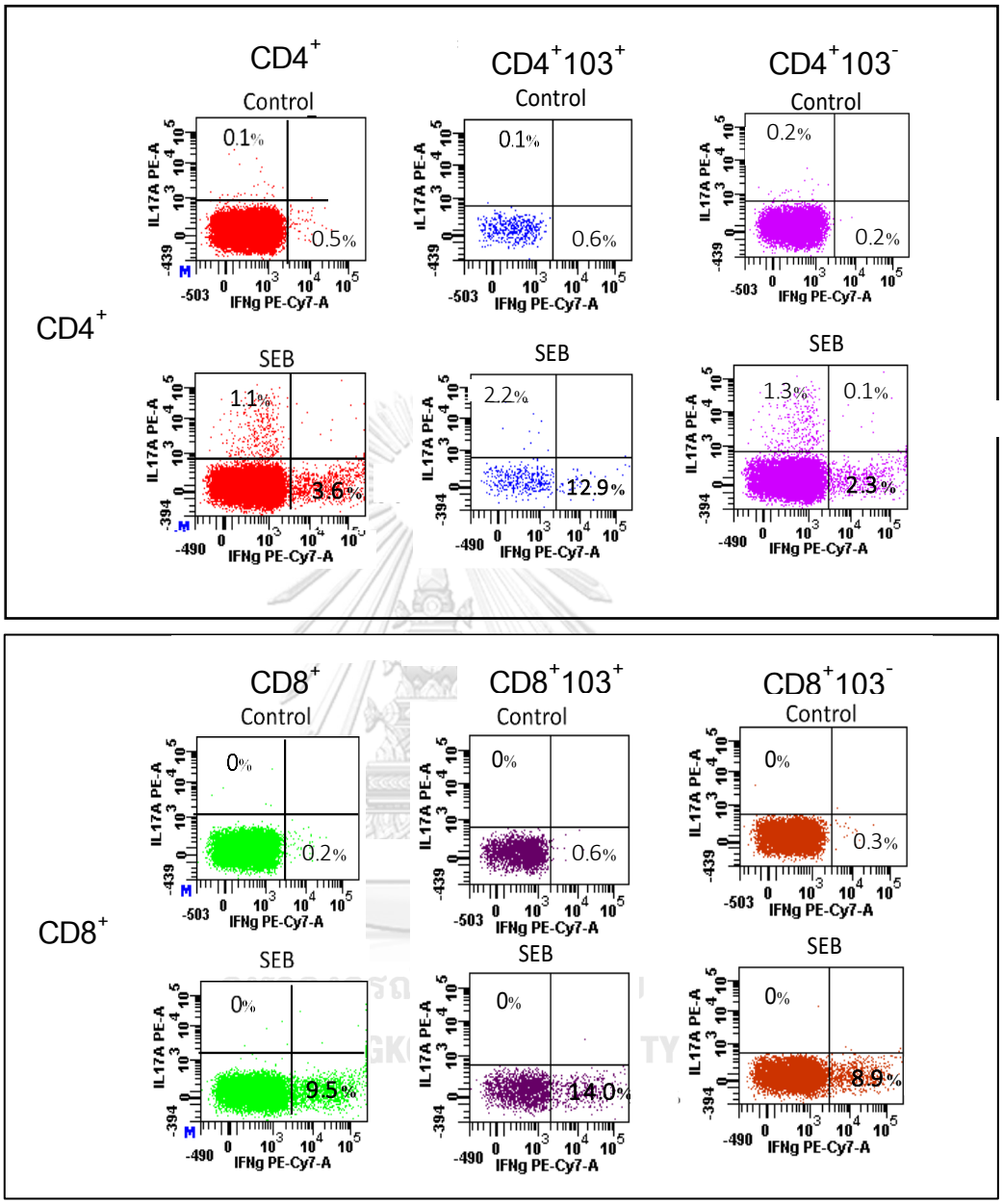

Figure 7. Flow cytometry analysis of IFN-γ and IL-17 produced by $CD4^{+}CD103^{+}$, $CD4^{+}CD103^{-}$, $CD8^{+}CD103^{+}$and $CD8^{+}CD103^{-}$ T cells (representative of 4 samples).

$CD3^+$ $CD103^+$ and $CD3^+$ $CD103^-$ T cells produced both IL-17 and IFN-γ. $CD4^+CD103^+$ and $CD4^+CD103^-$ T cells predominantly produced IL-17 alone (frequency = 1.33% ±0.47, 1.66% ±0.42 respectively) (Fig. 8, Appendix E) and IFN-γ alone (frequency = 18.48% ±4.20, 6.28% ±2.01 respectively) (Fig.8, Appendix D). The frequency of $CD4^+CD103^+$ and $CD4^+CD103^-$ T cells that produced IL-17 plus IFN-γ was negligible (Fig. 7). Unlike $CD4^+$ T cells, $CD8^+CD103^+$ and $CD8^+CD103^-$ T cells mostly produced IFN-γ alone (frequency = 14.88% ±1.36, 8.44±1.78 respectively) (Fig.8, Appendix D).

Measurement of IL-17 and IFN-γ production in $CD103^+$ T cells based on CD69 expression was not possible because *in vitro* stimulation with SEB leads to CD69 expression on most T cells. We found that the frequency of $CD4^+CD103^+$ memory T cells that produced IFN-γ was 2.9 folds higher than that of $CD4^+CD103^-$ memory T cells, the differences reached statistical significance (p = 0.02) (Fig. 8, Appendix D). The frequency of IL-17 producing cells between $CD4^+CD103^+$ and $CD4^+CD103^-$ memory T cells was similar (Fig. 8, Appendix E). A significant difference in the frequency of IFN-γ producing cells between $CD8^+$ $CD103^+$ and $CD8^+$ $CD103^-$ memory T cells was also observed (p = 0.03) (Fig.8, Appendix D).

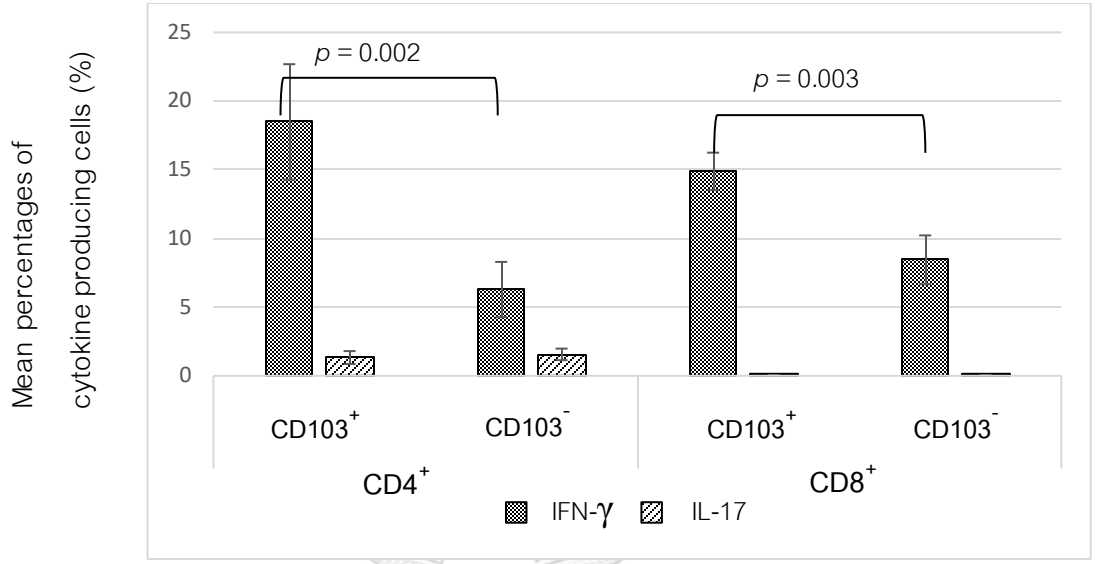

Figure 8. Production of IFN-γ and IL-17 by $CD4^+CD103^+$, $CD4^+CD103^-$, $CD8^+CD103^+$ and $CD8^+CD103^-$ T cells. Mean±S.E. of n=7 (IFN-γ) and n=4 (IL-17) are shown.

Granzyme B expression was detected in control unstimulated cultures and did not increase after SEB stimulation (Fig.9, Appendix F).

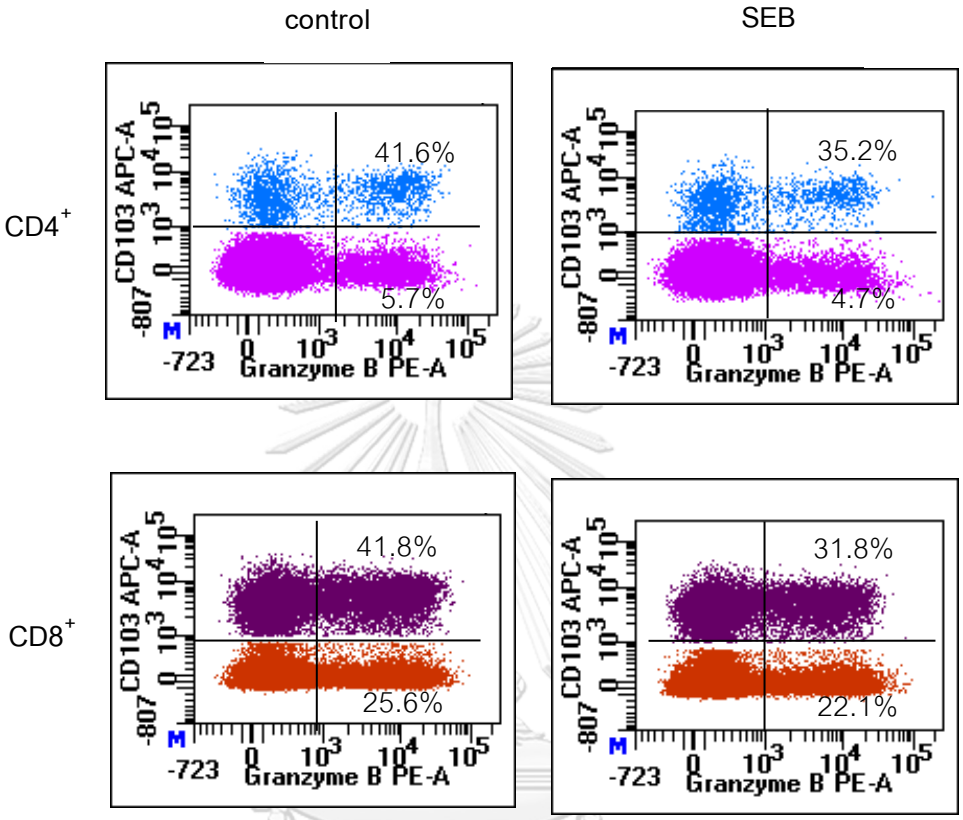

Figure 9. Flow cytometric analysis of granzyme B produced by $CD4^+CD103^+$, $CD4^+CD103^-$, $CD8^+CD103^+$ and $CD8^+CD103^-$ T cells (representative of 3 samples).

CHAPTER V
DISCUSSION AND CONCLUSION

Heavy infiltration of lymphocytes is commonly observed in periodontitis tissues. We detected more numbers of $CD4^+$ T cells (57%) compared to $CD8^+$ T cells (33%). These findings are consistent with results from previous reports (Yamazaki et al. 1995, Yongyuth 2015). The role of local tissue immunity has received more attention lately primarily due to the discovery of a new subset of memory T cells termed tissue-resident memory (T_{RM}) cells. These long-lived and non-recirculating T_{RM} cells permanently reside in non-lymphoid tissues including skin, brain, vagina and lung, and provide rapid, effective local protection against reinfection relative to circulating counterpart memory T cells (Hofmann and Pircher 2011, Teijaro et al. 2011, Jiang et al. 2012, Shin and Iwasaki 2012, Sakai et al. 2014). This novel memory T cell subset express CD103 and CD69 (C-type lectin), both of which are involved in cell adhesion and tissue retention (Shin and Iwasaki 2013). In our study, CD103 was selected over CD69 for T_{RM} identification. CD69 is also a well-known T cell receptor coupled activation marker. Periodontitis has a chronic inflammatory disease in nature, therefore T cells in periodontitis lesions are thought to be overwhelmingly activated.

There have been limited data regarding T cells expressing CD103 in periodontal disease. The presence of T cells expressed $\alpha^{IEL}\beta_7$ integrin (equivalent to CD103) in periodontal tissues was first described by immunohistochemical staining more than 20 years ago (Tonetti et al. 1995). They revealed that 49-54% of $CD3^+$ T cells expressed $\alpha^{IEL}\beta_7$ integrin and these cells were observed in underlying connective tissues as well as gingival epithelium in periodontitis patients (Tonetti et al. 1995). We confirmed the presence of $CD103^+$ T cells in periodontitis tissues and further investigated the

expression of CD103 on T cell subsets, $CD4^+$ and $CD8^+$ T cells. We demonstrated that there were greater numbers of $CD8^+CD103^+$ T cells than $CD4^+CD103^+$ T cells. Our findings agreed with the previous periodontitis study (Yongyuth 2015) and in line with observation in psoriatic skin (Pauls et al. 2001) with the preferential expression of CD103 on $CD8^+$ T cell population. However, the distribution of $CD103^+$ T cells in periodontitis tissue was different from those in skin. Previous immunohistochemical staining in our laboratory revealed that CD103 T cells in periodontitis tissues were scattering around connective tissue and epithelium (Yongyuth 2015) and not preferentially localized in epithelial layer as in psoriatic skin (50% of epidermal T cells vs 5% of dermal T cells expressed integrin αE(CD103)β7 (Pauls et al. 2001). They further emphasized the increase of $CD103^+$ T cells in epidermal layer of psoriasis as compared to healthy skin may associate with pathology of skin. In contrast, $CD103^+$ T cells were comparable between periodontitis tissue and healthy gingiva (Yongyuth 2015).

$CD4^+$ T cells isolated from periodontitis tissues produced either IL-17 or IFN-γ while $CD8^+$ T cells produced only IFN-γ. These findings agree with recent observations suggesting that the major source of IL-17 in periodontitis is $CD4^+$ T cells (Dutzan et al. 2016). In this study, we did not observe the differences in the magnitude of IL-17 responses between $CD4^+CD103^+$ and $CD4^+CD103^-$ T cells. However, $CD4^+CD103^+$ and $CD8^+CD103^+$ T cells showed a significant higher IFN-γ response compared with $CD4^+CD103^-$ and $CD8^+CD103^-$ memory T cells, respectively. It would have been more meaningful if we could have compared this cytokine production from periodontitis tissues with those obtained from healthy gingiva. Due to technical limitations, the number of T cells isolated from healthy gingiva was too low which limited cytokine investigation. In our current study, the results of granzyme B production could not be

interpreted. We observed that granzyme B was constitutively expressed in the control wells (no SEB stimulation) and no increase in the production was observed after SEB stimulation. Hence further investigation of granzyme B production from cells isolated from healthy gingiva is required.

The role of IL-17 and IFN-γ in pathogenesis of periodontitis has been well described. Expression of IFN-γ has been consistently reported in periodontitis tissues and may involve in tissue inflammation by recruitment of circulating memory T and B cells via VCAM-1 pathway (Schenkel et al. 2013). IL-17 has been detected in periodontitis and proposed as a major driving force of bone in periodontitis through the upregulation of RANKL and the activation of osteoclastogenesis (Zenobia and Hajishengallis 2015). It also induces the expression of matrix metalloproteinases in fibroblasts, endothelial cells and epithelial cells, leading to destruction of connective tissue (Miossec and Kolls 2012). Our study was the first study of cytokine production from $CD103^+$ T cells in periodontitis tissue. However, there has been only one recent investigation on T_{RM} marked by the surface marker CD69 in periodontitis tissue (Dutzan et al. 2016). We confirmed their finding that IFN-γ was expressed from $CD4^+$ and $CD8^+$ T cells, while IL-17 was only expressed from $CD4^+$ T cells (Dutzan et al. 2016).

In conclusion, we observed infiltration of $CD4^+$ and $CD8^+$ T cells in periodontitis tissues. CD103 expression was observed more on $CD8^+$ T cells than $CD4^+$ T cells. $CD103^+CD4^+$ and $CD103^-CD4^+$ T cells were able to produce IFN-γ, and IL-17, whereas $CD103^+CD8^+$ and $CD103^-CD8^+$ T cells produced only IFN-γ. Resident memory T Cells in the gingiva with the ability to produce such cytokines suggest their possible role in immunopathogenesis of periodontitis. Imbalance of subgingival bacteria community could damage gingival barrier allowing bacterial antigens to get access to the deeper

connective tissue where they activate resident memory T cells leading to deleterious inflammation; a hallmark of periodontitis.

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APPENDIX

Appendix A: Descriptive profile of gingival biopsy from periodontitis samples

NO.	Sex	Age (years)	Tooth No.	Clinical examination			
				PD (mm)	CAL (mm)	Bone loss (%)	Others
1	female	52	28	5-7	7-12	>75%	-
2	female	47	46	4-9	7-13	>50%	F2
3	male	39	47	5-10	7-12	-	MO1
4	male	30	16	6-8	7-11	>75	-
5	female	48	26	4-8	6-10	-	MO2
6	male	51	28	5-9	-	-	-
7	n/a	n/a	37	-	-	-	-

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 B: Phenotypic characterization of T cells in periodontal tissues.

No.	Tooth No.	Infiltrated T cells in periodontal tissues (%)		
		$CD3^+$ T cells	$CD4^+$ T cells	$CD8^+$ T cells
1	28	56.78	61.96	29.10
2	46	59.49	49.58	25.11
3	47	50.21	50.01	35.76
4	16	44.11	63.20	36.70
5	26	57.96	59.44	40.56
6	28	69.12	59.73	41.11
7	37	60.55	57.05	22.41
	Mean±S.E.	56.89 ±3.00	57.28 ±2.07	32.96 ±2.82

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

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
CD4	7	49.58	63.20	57.2814	5.47437
CD8	7	22.41	41.11	32.9643	7.45916
Valid N (listwise)	7				

Wilcoxon sign ranked test results of differences of percentages of $CD4^+$ and $CD8^+$ T cells in periodontitis tissue (data from control sample)

Ranks

		N	Mean Rank	Sum of Ranks
CD8 - CD4	Negative Ranks	7[a]	4.00	28.00
	Positive Ranks	0[b]	.00	.00
	Ties	0[c]		
	Total	7		

a. CD8 < CD4

b. CD8 > CD4

c. CD8 = CD4

Test Statistics[a]

	CD8 - CD4
Z	-2.366[b]
Asymp. Sig. (2-tailed)	.018

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

Appendix C: The expression of $CD103^+$ and $CD103^-$ T cells in periodontal tissues (data from control samples).

No.	Tooth No.	The expression of CD103 in periodontal tissues (%)			
		$CD4^+$ T cell		$CD8^+$ T cell	
		$CD103^+$	$CD103^-$	$CD103^+$	$CD103^-$
1	28	2.09	97.91	14.69	85.31
2	46	3.16	96.84	27.45	72.54
3	47	10.77	89.23	50.77	49.23
4	16	3.31	96.69	32.61	67.39
5	26	8.32	91.68	29.77	70.23
6	28	6.51	93.49	25.82	74.18
7	37	6.86	93.14	33.50	66.5
	Mean±S.E.	5.86 ±1.19	94.14 ±1.19	30.66 ±4.10	69.34 ±4.10

Descriptive statistics of the percentages of $CD103^+$ and $CD103^-$ T cells expressed from $CD4^+$ and $CD8^+$ T cells (data from control samples).

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
$CD4^+CD103^+$	7	2.09	10.77	5.8600	3.15139
$CD4^+CD103^-$	7	89.23	97.91	94.1400	3.15139
$CD8^+CD103^+$	7	14.69	50.77	30.6586	10.84622
$CD8^+CD103^-$	7	49.23	85.31	69.3400	10.84573
Valid N (listwise)	7				

Wilcoxon sign ranked test results of differences of percentages of $CD103^+$ and $CD103^-$ T cells expressed from $CD4^+$ and $CD8^+$ T cells.

Ranks

		N	Mean Rank	Sum of Ranks
$CD4^+CD103^-$ - $CD4^+CD103^+$	Negative Ranks	0[a]	.00	.00
	Positive Ranks	7[b]	4.00	28.00
	Ties	0[c]		
	Total	7		
$CD8^+CD103^-$ - $CD8^+CD103^+$	Negative Ranks	1[d]	1.00	1.00
	Positive Ranks	6[e]	4.50	27.00
	Ties	0[f]		
	Total	7		

a. $CD4^+CD103^- < CD4^+CD103^+$

b. $CD4^+CD103^- > CD4^+CD103^+$

c. $CD4^+CD103^- = CD4^+CD103^+$

d. $CD8^+CD103^- < CD8^+CD103^+$

e. $CD8^+CD103^- > CD8^+CD103^+$

f. $CD8^+CD103^- = CD8^+CD103^+$

Test Statistics[a]

	$CD4^+CD103^-$ - $CD4^+CD103^+$	$CD8^+CD103^-$ - $CD8^+CD103^+$
Z	-2.366[b]	-2.197[b]
Asymp. Sig. (2-tailed)	.018	.028

a. Wilcoxon Signed Ranks Test

b. Based on negative ranks.

Mann-Whitney's U-test results of differences of percentages of $CD103^+$ T cells expression comparing between $CD4^+$ and $CD8^+$ T cells.

Ranks

	T cell subset	N	Mean Rank	Sum of Ranks
$CD103^+$	$CD4^+$	7	4.00	28.00
	$CD8^+$	7	11.00	77.00
	Total	14		

Test Statistics[a]

	$CD103^{+}$
Mann-Whitney U	.000
Wilcoxon W	28.000
Z	-3.130
Asymp. Sig. (2-tailed)	.002
Exact Sig. [2*(1-tailed Sig.)]	.001[b]

a. Grouping Variable: T cell subset

b. Not corrected for ties.

Appendix D: The expression of IFN-γ produced by $CD103^+$ and $CD103^-$ $CD3^+$, $CD4^+$ and $CD8^+$ T cells (data from SEB - control sample).

No.	Expression of IFN-γ from $CD103^+$ and $CD103^-$ T cells (%)			
	$CD4^+$ T cell		$CD8^+$ T cell	
	$CD103^+$	$CD103^-$	$CD103^+$	$CD103^-$
1	6.46	2.77	12.89	6.21
2	12.31	2.18	13.77	8.61
3	37.8	10.26	17.23	10.89
4	13.19	2.27	19.83	5.36
5	29.34	9.51	11.81	5.36
6	12.11	15.14	10.28	4.75
7	18.14	1.81	18.37	17.88
Mean ±S.E.	18.48 ±4.20	6.28 ±2.01	14.88 ±1.36	8.44 ±1.78

Descriptive statistics of the percentages of $CD103^+$ and $CD103^-$ T cells producing IFN-γ expressed from $CD4^+$ and $CD8^+$ T cells (data from SEB – Control).

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
IFN-γ $CD4^+CD103^+$	7	6.46	37.80	18.4786	11.12376
IFN-γ $CD4^+CD103^-$	7	1.81	15.14	6.2771	5.32234
IFN-γ $CD8^+CD103^+$	7	10.28	19.83	14.8829	3.60514
IFN-γ $CD8^+CD103^-$	7	4.75	17.88	8.4371	4.70296
Valid N (listwise)	7				

Mann-Whitney's U-test results of differences of percentages of cells producing IFN-γ comparing between $CD103^+$ and $CD103^-$ T cells expressed from $CD4^+$ and $CD8^+$ T cells (data from SEB – Control).

Ranks

	CD 103 T cells	N	Mean Rank	Sum of Ranks
IFN-γ $CD4^+$	$CD103^+$	7	4.00	28.00
	$CD103^-$	7	11.00	77.00
	Total	14		
IFN-γ $CD8^+$	$CD103^+$	7	4.14	29.00
	$CD103^-$	7	10.86	76.00
	Total	14		

Test Statistics[a]

	$CD4^+$	$CD8^+$
Mann-Whitney U	.000	1.000
Wilcoxon W	28.000	29.000
Z	-3.130	-3.003
Asymp. Sig. (2-tailed)	.002	.003
Exact Sig. [2*(1-tailed Sig.)]	.001[b]	.001[b]

a. Grouping Variable: CD103 T cells

b. Not corrected for ties.

Mann-Whitney's U-test results differences of percentages of cells producing IFN-γ comparing between $CD4^+$ and $CD8^+$ T cells (data from SEB – Control).

Ranks

	T cells	N	Mean Rank	Sum of Ranks
IFN-γ $CD103^+$	$CD4^+$	7	7.71	54.00
	$CD8^+$	7	7.29	51.00
	Total	14		
IFN-γ $CD103^-$	$CD4^+$	7	6.29	44.00
	$CD8^+$	7	8.71	61.00
	Total	14		

Test statistics[a]

	IFN-γ CD103^{+}	IFN-γ CD103^{-}
Mann-Whitney U	23.000	16.000
Wilcoxon W	51.000	44.000
Z	-.192	-1.087
Asymp. Sig. (2-tailed)	.848	.277
Exact Sig. [2*(1-tailed Sig.)]	.902[b]	.318[b]

a. Grouping Variable: T cells

b. Not corrected for ties.

Appendix E: The expression of IL-17 produced by $CD103^+$ and $CD103^-$ $CD4^+$ and $CD8^+$ T cells (data from SEB - control samples).

No.	Expression of IL-17 from $CD103^+$ and $CD103^-$ T cells (%)			
	$CD4^+$ T cell		$CD8^+$ T cell	
	$CD103^+$	$CD103^-$	$CD103^+$	$CD103^-$
1	0.03	0.81	0	0
2	2.14	1.14	0	0
3	1.29	2.09	0.06	0.01
4	1.87	2.61	0	0.07
Mean ±S.E.	1.33 ±0.47	1.66 ±0.42	0.02 ±0.02	0.02 ±0.02

Descriptive statistics of percentages of $CD103^+$ and $CD103^-$ T cells producing IL-17 expressed from $CD4^+$ and $CD8^+$ T cells (data from SEB - control).

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
IL-17$CD4^+CD103^+$	4	.03	2.14	1.3325	.93795
IL-17$CD4^+CD103^-$	4	.79	2.61	1.6575	.83958
IL-17$CD8^+CD103^+$	4	.00	.06	.0150	.03000
IL-17$CD8^+CD103^-$	4	.00	.07	.0200	.03367
Valid N (listwise)	4				

Ranks

	CD103 T cells	N	Mean Rank	Sum of Ranks
IL17CD4^{+}	CD103^{+}	4	3.50	14.00
	CD103^{-}	4	5.50	22.00
	Total	8		
IL17CD8^{+}	CD103^{+}	4	4.00	16.00
	CD103^{-}	4	5.00	20.00
	Total	8		

Test Statistics[a]

	IL17CD4^{+}	IL17CD8^{+}
Mann-Whitney U	4.000	6.000
Wilcoxon W	14.000	16.000
Z	-1.155	-.661
Asymp. Sig. (2-tailed)	.248	.508
Exact Sig. [2*(1-tailed Sig.)]	.343[b]	.686[b]

a. Grouping Variable: CD103 T cells

b. Not corrected for ties.

Appendix F: The expression of granzyme B produced by $CD103^+$ and $CD103^-$ $CD4^+$ and $CD8^+$ T cells (data from control and SEB group).

No.	Expression of Granzyme B from $CD103^+$ and $CD103^-$ T cells (%)							
	$CD4^+$ T cell				$CD8^+$ T cell			
	$CD103^+$		$CD103^-$		$CD103^+$		$CD103^-$	
	Control	SEB	Control	SEB	Control	SEB	Control	SEB
1	42.00	40.28	5.73	5.26	41.92	40.57	25.91	24.81
2	50.00	47.52	19.18	20.98	42.64	38.86	33.61	32.12
3	30.58	40.92	10.77	11.88	43.93	46.17	7.12	7.24
Mean ±S.E.	40.86 ±9.76	42.91 ±4.01	11.89 ±6.80	12.71 ±7.89	42.83 ±1.02	41.87 ±3.82	22.21 ±13.63	21.39 ±12.79

Descriptive statistics of percentages of $CD103^+$ and $CD103^-$ T cells producing granzyme B expressed from $CD4^+$ and $CD8^+$ T cells.

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
$GZCD4^+103^+$	3	40.28	47.52	42.9067	4.00806
$GZCD4^+103^-$	3	5.26	20.98	12.7067	7.89254
$GZCD8^+103^+$	3	38.86	46.17	41.8667	3.82362
$GZCD8^+103^-$	3	7.24	32.12	21.3900	12.78772
Valid N (listwise)	3				

Mann-Whitney's U-test results of differences of percentages of cells producing granzyme B comparing between $CD103^+$ and $CD103^-$ T cells expressed from $CD4^+$ and $CD8^+$ T cells (data from SEB group).

Ranks

	CD103 T cells	N	Mean Rank	Sum of Ranks
$GZCD4^+$	$CD103^+$	3	5.00	15.00
	$CD103^-$	3	2.00	6.00
	Total	6		
$GZCD8^+$	$CD103^+$	3	5.00	15.00
	$CD103^-$	3	2.00	6.00
	Total	6		

Test Statistics[a]

	$GZCD4^+$	$GZCD8^+$
Mann-Whitney U	.000	.000
Wilcoxon W	6.000	6.000
Z	-1.964	-1.964
Asymp. Sig. (2-tailed)	.050	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[b]	.100[b]

a. Grouping Variable: CD103 T cells

b. Not corrected for ties.

VITA

Miss Benjarat Isaraphithakkul was born on 5th of April 1988 in Nakhon Si Thammarat. She graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Rangsit University in 2013, and became a general dentist at Nakhonpat hospital, a private hospital in Nakhon Si Thammarat. Presently, she studies in Master degree program in Periodontology at Graduate School, Chulalongkorn University.