ตำแหน่งที่อยู่และความจำเพาะต่อแอนติเจนของพลาสมาเซลล์ในรอยโรคปริทันต์อักเสบ

นางสาวสรัญญา ธาวนพงษ์

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คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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LOCALIZATION AND ANTIGEN-SPECIFICITY OF PLASMA CELLS IN PERIODONTITIS LESIONS

Miss Saranya Thawanaphong

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By	Miss Saranya Thawanaphong
Field of Study	Periodontics
Thesis Advisor	Professor Dr.Rangsini Mahanonda
Thesis Co-Advisor	Sathit Pichyangkul, Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Dentistry

(Assistant Professor Dr.Suchit Poolthong)

THESIS COMMITTEE

.....Chairman

(Dr.Chantrakorn Champaiboon)

.....Thesis Advisor

(Professor Dr.Rangsini Mahanonda)

.....Thesis Co-Advisor

(Sathit Pichyangkul, Ph.D.)

.....External Examiner

(Emeritus Professor Stitaya Sirisinha, Ph.D.)

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ในรอยโรคปริทันต์อักเสบจะพบบีเซลล์และพลาสมาเซลล์จำนวนมาก การศึกษานี้ใช้วิธีโฟลไซโทเมทรีในการตรวจระบุพลาสมาเซลล์ โดยเซลล์ที่ย้อมติดซีดี19^{+}ซีดี27^{+}ซีดี38^{+}เอชแอลเอ-ดีอาร์$^{ต่ำ}$ ถูกระบุเป็นพลาสมาเซลล์ในรอยโรคปริทันต์อักเสบ ซึ่งเซลล์เหล่านี้สามารถตรวจระบุด้วยซีดี138^{+} เช่นกัน การตรวจทางอิมมูโนพยาธิวิทยาโดยการย้อมด้วยซีดี138 ในรอยโรคปริทันต์อักเสบขั้นรุนแรงพบว่ามีพลาสมาเซลล์จำนวนมากกระจายเป็นกลุ่มเล็กๆ ในชั้นเนื้อเยื่อเกี่ยวพัน บริเวณที่พบพลาสมาเซลล์ที่ย้อมติดซีดี138 อยู่ร่วมกับทีเซลล์ที่ย้อมติดซีดี3 อย่างหนาแน่นมากที่สุดได้แก่ บริเวณที่เป็นส่วนฐานของร่องลึกปริทันต์ อย่างไรก็ตามไม่พบเซลล์ที่ย้อมติดซีดี138 หรือพบน้อยมากในเนื้อเยื่อที่ไม่เป็นโรค เมื่อทำการตรวจหาความจำเพาะต่อแอนติเจนของพลาสมาเซลล์ด้วยวิธีการอีไลสปอต พบว่าพลาสมาเซลล์ที่ผลิตอิมมูโนโกลบูลินมีความจำเพาะต่อแบคทีเรียก่อโรคปริทันต์อักเสบพอร์ไฟโรโมแนส จิงจิวาลิส และมีส่วนน้อยที่จำเพาะต่อเชื้อแอคกรีเกทิแบคเทอร์แอคทิโนมัยซิเทมโคมิแทนส์ และพบว่ามีเซลล์ที่ผลิตอิมมูโนโกลบูลินจีมากกว่าเซลล์ที่ผลิตอิมมูโนโกลบูลินเออย่างมีนัยสำคัญ แต่อย่างไรก็ตามไม่พบความจำเพาะของพลาสมาเซลล์ต่อแบคทีเรียประจำถิ่นสเตรปโตคอกคัส กอร์โดไนหรือคอลลาเจนตนเอง ทั้งนี้ต้องมีการศึกษาเพิ่มเติมต่อไปเพื่อให้เข้าใจบทบาทของพลาสมาเซลล์ในโรคปริทันต์อักเสบว่ามีบทบาทในการป้องกันหรือการก่อให้เกิดโรค

ภาควิชา	ปริทันตวิทยา	ลายมือชื่อนิสิต .....
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ปีการศึกษา	2557	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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SARANYA THAWANAPHONG: LOCALIZATION AND ANTIGEN-SPECIFICITY OF PLASMA CELLS IN PERIODONTITIS LESIONS. ADVISOR: PROF. DR.RANGSINI MAHANONDA, CO-ADVISOR: SATHIT PICHYANGKUL, Ph.D., 43 pp.

Periodontitis is characterized by large infiltration of B cells and plasma cells. In this study, $CD19^{+}CD27^{+}CD38^{+}HLA\text{-}DR^{low}$ as periodontitis tissue–plasma cells were identified by flow cytometry. And these cells were also $CD138^{+}$ (a plasma cell marker). In severe periodontitis lesions, large number of $CD138^{+}$ plasma cells which form small clusters disseminated in connective tissue were consistently demonstrated by immunostaining. The densest area of co-localization of $CD138^{+}$ plasma cells and $CD3^{+}$ T cells were at the base of pocket epithelium. But none or very few $CD138^{+}$ plasma cells was/were observed in clinically healthy gingiva. From ELISPOT assay, we detected Ig-producing plasma cells specific to a key periodontal pathogen, *Porphyromonas gingivalis* and to a lesser extent against *Aggregatibacter actinomycetemcomitans*. Total IgG spot forming cells (SFC) were significant higher than total IgA SFC. On the other hand, Ig specific for commensal plaque bacteria-*Streptococcus gordonii* or self-tissue collagen could not be detected. More studies are required to gain insight into the role of periodontal tissue-plasma cells in protection or pathogenesis of the disease.

Department:	Periodontology	Student's Signature	.....
Field of Study:	Periodontics	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
APC	Allophycocyanin
ASC	Antibody secreting cells
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BM	Bone marrow
cADPR	Cyclic adenosine diphosphate ribose
CD	Cluster of differentiation
Cy	Cyanine
DAB	3,3'-diaminobenzidine tetrahydrochloride
DPBS	Dulbecco phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FITC	Fluorescein isothiocyanate BD
GC	Germinal center
GCF	Gingival crevicular fluid
GMC	Gingival mononuclear cells
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
Ig	Immunoglobulin
IQR	Interquartile range
KPL	*Keyhole limpet haemocyanin*
mAbs	Monoclonal antibodies

MALT	Mucosa associated lymphoid tissue
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NBT	Nitroblue tetrazolium
P. gingivalis	*Porphyromonas gingivalis*
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.05% Tween 20
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein complex
RANKL	Receptor activator of nuclear factor kappa-B ligand
RPMI	Roswell Park Memorial Institute
S. gordonii	*Streptococcus gordonii*
SFC	Spot forming cell
Streptavidin-AP	Streptavidin-Alkaline Phosphatase
T. forsythia	*Tannerella forsythia*
TNF	Tumor necrosis factor

CHAPTER I

INTRODUCTION

1.1 Background of the present study

One of the most common chronic inflammatory diseases in human is periodontal disease. The etiology of periodontal disease is microbial plaque biofilm and the imbalance in host immune response that play key roles in the pathogenesis and progression of disease (Mahanonda, 2012). Moreover genetic and environment have an effect on the inflammation process result in periodontium breakdown (Kornman, 2008). Periodontal disease is divided by the severity of tooth supporting tissues breakdown. Gingivitis is the form which confined inflammation in the gingiva and no periodontium breakdown. The other one is periodontitis which the inflammatory process involves tooth supporting tissues including alveolar bone (Mahanonda, 2012). The one difference between healthy and periodontal disease revealed in many histologic study. Inflammatory lymphocyte infiltrates such as B cells and T cells are numerous in lamina propria of periodontal lesion (Brandtzaeg and Kraus, 1965, Page and Schroeder, 1976, Seymour and Greenspan, 1979, Lappin et al., 1999, Orima et al., 1999, Amunulla et al., 2008). When the severity of disease is increase, it bring about the transition from gingivitis to periodontitis, the lymphocyte infiltration shift from T cells to B cells and plasma cell (Seymour et al., 1979). At present the role of B cells and plasma cells in the pathogenesis of periodontitis remain unclear.

Plasma cell is the one of terminal stage of B cells which can produce and secrete antibody. Origin of B cells is the bone marrow. They mature in lymph node and spleen

(secondary lymphoid tissues) into naïve B cells. When antigen stimulate them, primary immune response occur. Naïve B cells which expressing surface immunoglobulin (Ig) bind the antigen, aggregate and form primary follicles that become secondary follicles with germinal centers. In T-cell dependent activation, helper T cells signal is important for naïve B cells maturation to plasma cell. This signal lead to immunoglobulin isotype switching that change IgM to IgG, IgA or IgE and somatic mutation to improve antigen binding. These cells gain a survival advantage and emerge as long-lived surface Ig- plasma cells that maintain serum Ig levels or surface-switched Ig memory cells (Honjo et al., 2004, Abbas and Lichtman, 2005, Murphy et al., 2008, Delves et al., 2011).

In 1965, Brandzaeg and Kraus described the presence of large number of plasma cell (like morphology) in severe periodontitis tissue under light microscope, therefore suggesting the involvement of immune cells in disease pathogenesis. Later studies using enzymes and surface antigen markers in indirect immunofluorescence and immunohistochemistry confirmed the predominant B cell lesion of periodontitis (Page and Schroeder, 1976, Seymour et al., 1979, Daly et al., 1983, Yamazaki et al., 1993). And these B cells in periodontitis consist mainly of antibody secreting cells (ASC), especially plasma cells. Local ASC in periodontal tissues were identified by intracellular staining immunofluorescence IgG, IgM and IgA (Mackler et al., 1977, Seymour et al., 1979, Okada et al., 1983, Takahashi et al., 1996). Recent studies used CD138 as a marker of plasma cell revealed that B cells in periodontitis consist mainly plasma cells (Amunulla et al., 2008, Kim et al., 2010, Mizutani et al., 2014, Thorbert-Mros et al., 2014)

Antigen-specificity of ASC in periodontitis tissues was investigated by the technique of enzyme-linked immunosorbent spot (ELISPOT assay). Major isotype of spot forming cells (SFC) was IgG followed by IgA (Ogawa et al., 1989b). One study reported antigen specificity was fimbriae and lipopolysaccharide of *Porphyromonas gingivalis (P.*

gingivalis) (Ogawa et al., 1989a). However, this bacterium is not the only key pathogen in periodontal disease. ASC specific to other pathogens including *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* and *Tannerella forsythia* (*T. forsythia*) need further investigation. Besides bacterial etiology, autoimmune reaction has been thought to play role in periodontal tissue pathology. For example, antibody against collagen was reported in GCF of periodontitis patients (Sugawara et al., 1992). At present, there is little knowledge about the antigen specificity of antibody that secreted by plasma cells in periodontitis lesion, therefore, requires further study.

Our laboratory recently revisited the role of B cells in periodontitis. We confirmed that the predominant B cells in periodontitis tissue were antibody secreting cells with plasma cell phenotype ($CD19^{+}CD27^{+}CD38^{+}HLA\text{-}DR^{low}$), but not plasmablasts ($CD19^{+}CD27^{+}CD38^{+}HLA\text{-}DR^{high}$). In the present study, the localization and antigen-specificity of plasma cells in periodontitis tissues (chronic periodontitis compared with healthy) were investigated. We anticipate that our data will provide further insight into periodontal tissue specific B cell response.

1.2 Objectives

1.2.1 To investigate the localization of plasma cells in periodontal tissues from patients with severe chronic periodontitis.

1.2.2 To investigate the antigen-specificity of plasma cells in periodontal tissues from patients with severe chronic periodontitis.

1.3 Hypothesis

Local plasma cells are scattered in connective tissue of periodontitis lesion. These cells produce antibodies specific to *P. gingivalis* and *A. actinomycetem-comitans.*

1.4 Field of research

Human immunology

1.5 Criteria Inclusions

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

1.5.2 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).

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

1.6 Limitation of research

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

1.7 Application and expectation of research

8.1 New scientific information of plasma cell located in periodontal tissues (chronic periodontitis compared with healthy) and their antigen-specificity to provide novel insight into periodontal tissue specific B cell response.

8.2 Publication in the national peered-review journal.

1.8 Keywords

Periodontitis tissue, Plasma cells, Antigen-specificity

CHAPTER II

LITERATURE REVIEW

2.1 Periodontal disease

One of the most common chronic inflammatory diseases in human is periodontal disease. These diseases can be grouped by the severity of tooth supporting tissues breakdown into two major categories, gingivitis and periodontitis. Gingivitis is the form which confined inflammation in the gingiva and not affect the other attachments of teeth. The clinical features are characterized by increased redness, swelling, and bleeding of the gingiva during tooth brushing or when probing. The other one is periodontitis which inflammation of the periodontium extends beyond the gingiva, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both. The clinical features include gingival inflammation and clinically detectable attachment loss and periodontal probing depth is equal or more than 4 mm. When periodontitis is advanced, the teeth became mobile and finally tooth loss may occur (Mahanonda, 2012).

The etiology of periodontal disease is microbial plaque biofilm and the imbalance in host immune response that play key roles in the pathogenesis and progression of disease (Mahanonda, 2012). Moreover genetic and environment have an effect on the inflammation process result in periodontium breakdown (Kornman, 2008). In healthy and gingivitis lesions, the composition of subgingival microbial plaque is majority of gram positive facultative bacteria, such as *Streptococci* and *Actinomyces* species. On the contrary, periodontitis lesion appears a specific group of bacteria in subgingival plaque

biofilm (Socransky et al., 1998). Gram negative anaerobes and the majority of these bacteria are so-called key periodontal pathogens as *P. gingivalis*, *A. actinomycetem-comitans*, and *T. forsythia. P. gingivalis* is a keystone pathogen of periodontitis. It is a black-pigmented, non-motile, obligate anaerobic, gram-negative coccobacilli, normally residing in the human oral cavity and abnormally colonizing the lesion of periodontitis (Holt et al., 1999). *P. gingivalis* and *A. actinomycetemcomitans* can invade tissues and have the capacity to survive and spread to neighboring cells within the host epithelial cells (Meyer et al., 1996, Yilmaz et al., 2006). During chronic periodontitis both cellular and humoral immune responses are activated for infection control (Haffajee and Socransky, 2005).

2.2 B cell biology

Lymphocytes come in two major varieties namely T cells and B cells. B cells are able to produce antibody to generate humoral immune response. Moreover, B cells are recognized as one of the professional antigen presenting cells for T cell activation. (Abbas and Lichtman, 2005, Delves et al., 2011). Origin of B cells is the hematopoietic stem cells (HSC) in the bone marrow. They give rise to immature B cells which express surface-IgM. Then they become transitional B cells which just left the bone marrow but still unable to respond to antigen. These cells migrate to lymph node and spleen (secondary lymphoid tissues) where mature into naïve B cells (Figure 1) (Abbas and Lichtman, 2005, Delves et al., 2011, Bemark et al., 2012).

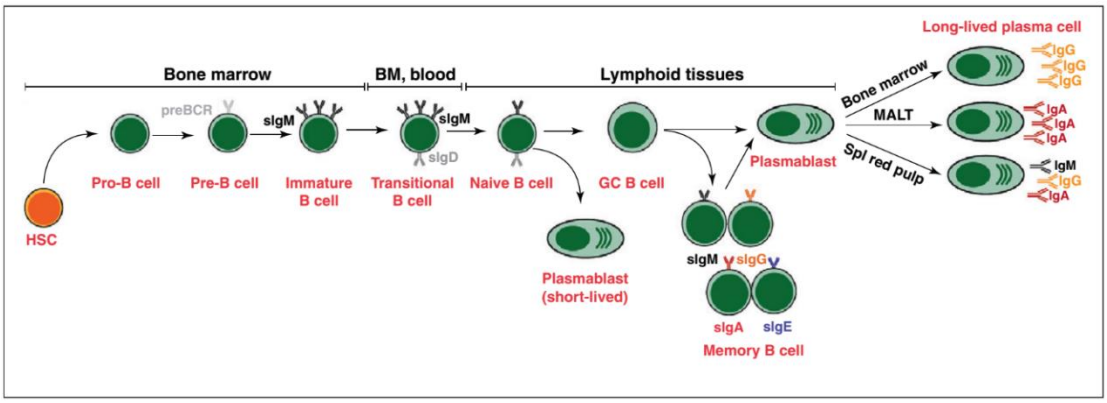

Figure 1. B cell development and differentiation: from HSC to plasma cells (Tangye, 2011) HSC, hematopoietic stem cell; BM, bone marrow; GC B cell, germinal center B cell; MALT, mucosal associated lymphoid tissue; Spl red pulp, splenic red pulp.

When antigen stimulate them, primary immune response occur. Naïve B cells uptake and present membrane-bounded antigen in association with major histocompatibility complex (MHC) class II molecules and co-stimulatory B7 signals to helper T cells. The critical co-stimulation CD40 ligand of the T cells as well as cytokines are required for naïve B cells to be fully activated (Figure 2). After activation, some naïve B cells differentiate to early IgM-producing plasma cells while others migrate to B cell follicles, where they form germinal centers. Then they are clonal expansion, immunoglobulin isotype switching that change IgM to IgG, IgA, or IgE and somatic hypermutation to selection of high affinity B cells which improve antigen binding. These cells gain a survival advantage and emerge as long-lived surface Ig-plasma cells that maintain serum Ig levels or surface-switched Ig memory cells (Honjo et al., 2004, Abbas and Lichtman, 2005, Murphy et al., 2008, Delves et al., 2011).

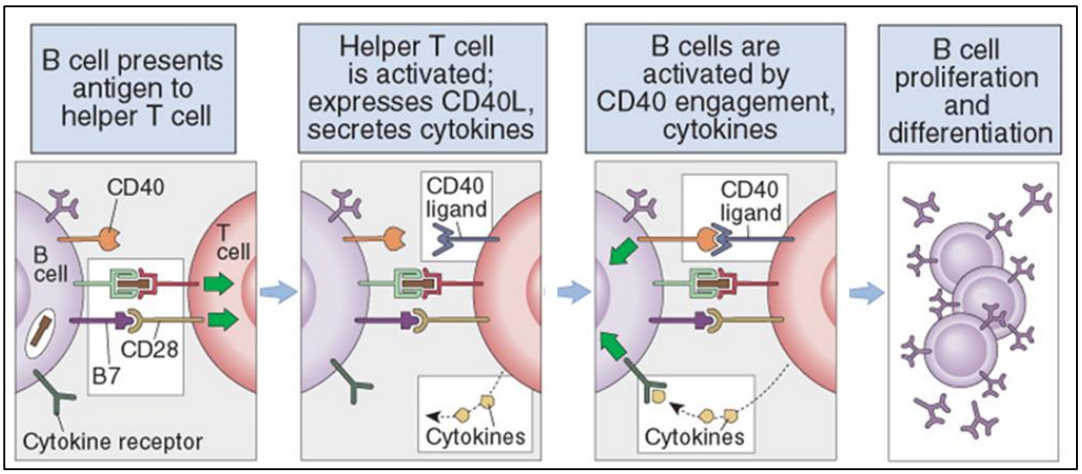

Figure 2. Helper T cell mediated activation of B cells (Abbas and Lichtman, 2005)

Plasma cell is the one of terminal stage of B cells which can produce and secrete large amounts of antibody. Antibody binds to the antigen by its specific recognition site and its constant structure regions activate complement through the classical pathway and phagocytes through their Fc receptors. Five different classes of Ig (IgG, IgM, IgA, IgD and IgE) fulfill different roles in immune protection. IgM produced early in the response switches to IgG, particularly with thymus-dependent antigens. The switch is largely under T cell control. IgG is the major antibody in serum and non-mucosal tissues whereas IgA is an antibody that plays a critical role in mucosal immunity which present as a dimer linked to a secretory component. (Bouvet and Fischetti, 1999, Abbas and Lichtman, 2005, Delves et al., 2011)

Memory B cells, long-lived surface-switched Ig lymphocytes, are important in the secondary immune response. These cells are well recognized for their role in immune surveillance in circulating blood and lymphoid organs. They provide a more rapid response to re-encountered antigen and a more efficient antibody production with high

affinity Ig than the primary immune response from naïve B cells (Delves et al., 2011; Murphy et al., 2008).

2.3 B cell in periodontal disease

The one difference in humoral immune response between healthy and periodontal disease revealed in many histologic study. Inflammatory lymphocyte infiltrates such as B cells and T cells are numerous in lamina propria of periodontal lesion (Brandtzaeg and Kraus, 1965, Page and Schroeder, 1976, Seymour and Greenspan, 1979, Lappin et al., 1999, Orima et al., 1999, Amunulla et al., 2008). When the severity of disease is increase, it brings about the transition from gingivitis to periodontitis, the lymphocyte infiltration shift from T cells to B cells and plasma cell (Seymour et al., 1979).

The study of B cells in periodontal disease has a long history. In 1965, Brandzaeg and Kraus described the presence of large number of plasma cells (like morphology) in severe periodontitis tissues under light microscope, therefore suggesting the involvement of immune cells in disease pathogenesis (Brandtzaeg and Kraus, 1965). Later studies using enzymes and surface antigen markers in direct or indirect immunofluorescence indicated the detection of B cells and plasma cells in periodontitis lesion according to cell morphology and the presence of surface Ig (Page and Schroeder, 1976, Mackler et al., 1977, Seymour and Greenspan, 1979, Okada et al., 1983, Jully et al., 1986, Gemmell and Seymour, 1991). Immunohistochemistry studies had shown the predominant B cells and plasma cell lesion of advanced chronic periodontitis (Reinhardt et al., 1988, Yamazaki et al., 1993, Liljenberg et al., 1994, Amunulla et al., 2008). Many studies focused on activated B cells or plasma cells in periodontitis tissue. The frequency of activated B cells has been reported to be much higher in periodontitis than gingivitis (Jully et al., 1986, Yamazaki et

al., 1993, Amunulla et al., 2008). Recent studies used CD138 as a marker of plasma cell revealed that B cells in periodontitis consist mainly plasma cells (Amunulla et al., 2008, Kim et al., 2010, Mizutani et al., 2014, Thorbert-Mros et al., 2014) Major isotypes of Ig that secreted by gingival cells are IgG and IgA indicating antigen exposure of memory B cells in periodontitis lesions (Daly et al., 1983, Ogawa et al., 1989b).

2.4 Novel surface markers to identify the plasma cells

CD or cluster of differentiation is a marker that is used for the investigation of cell surface molecules needed to identify cell types and stages of differentiation, and which is recognized by antibodies. CD molecules often act as receptors or ligands that play a role in cell signaling, and have other functions, such as cell adhesion. Classifying of B cell subsets can performed by multicolor flow cytometric analysis with combination of monoclonal antibodies (mAbs) against a variety of cell surface molecules (Llinas et al., 2011). CD19, CD27, CD38 and HLA-DR are selective mAbs which have been used to identify B cell subsets. CD19 (a cell surface molecule that forms B cell co-receptor complex) expressed on all stage of B cell maturation including fully mature plasma cells (Odendahl et al., 2005). CD27 (tumor necrosis factor receptor family) is a receptor that promotes differentiation and survival of B cells (Lens et al., 1995, Borst et al., 2005, Darce et al., 2007). It is expressed on human memory B cells (Agematsu et al., 2000) and antibody secreting cells (ASC) (Wrammert et al., 2008) which consist of plasmablast and plasma cells (Odendahl et al., 2005, Fairfax et al., 2008, Murphy et al., 2008). CD38 (an enzyme that hydrolysis of cyclic adenosine diphosphate ribose (cADPR) for regulation of calcium mobilization and promote signal transduction) is expressed on germinal centers and ASC (Deaglio et al., 2001, Malavasi et al., 2006, Morabito et al., 2006). HLA-DR is also commonly used to identify ASC subsets (Odendahl et al., 2005, Jacobi et al., 2010).

It is expressed on plasmablasts but not on plasma cells (Murphy et al., 2008). CD138 (a cell membrane proteoglycan that functions as a matrix receptor) has been used as a plasma cell marker (Jego et al., 2001, MacLennan et al., 2003). But later on, it was revealed that CD138 is expressed on both plasma cells and plasmablasts (Qian et al., 2010).

In addition, monoclonal antibody with immunoperoxidase technique have facilitated to identify localization and distribution of immune cells in tissue. CD138 is expressed on the surface of mature epithelial cells, normal plasma cells and neoplastic plasma cells. Studies in humans have revealed that CD138 is expressed in the oral gingival epithelium, the junctional or pocket epithelium (Manakil et al., 2001, Kim et al., 2010, Kotsovilis et al., 2010, Mizutani et al., 2014, Thorbert-Mros et al., 2014). CD138 expression is highly sensitive and specific for plasmacytic differentiation and represents an excellent marker for evaluation of plasma cell disorders in routinely processed tissue samples (O'Connell et al., 2004). CD138 has been a marker for plasma cell in periodontitis lesions (Kim et al., 2010, Mizutani et al., 2014, Thorbert-Mros et al., 2014).

2.5 Antigen-specificity of plasma cells

Gingival tissue from periodontitis lesions is characterized by dense infiltration of plasma cells (Kim et al., 2010, Mizutani et al., 2014, Thorbert-Mros et al., 2014). However, antigens recognized by antibodies secreted from these immunocytes remain unclear. Using enzyme-linked immunosorbent assay (ELISA) technique, the serum from chronic periodontitis patients contains elevated levels of anti-*P. gingivalis* antibodies (Naito et al., 1984, Tew et al., 1985, Schenck et al., 1987, Pussinen et al., 2002, Lakio et al., 2009) and anti-*A. actinomycetemcomitans* antibodies (Tew et al., 1985, Ebersole et al., 2000,

Pussinen et al., 2002, Lakio et al., 2009). Further, antibodies against *P. gingivalis* were detected in gingival crevicular fluid (GCF) of chronic periodontitis patients (Naito et al., 1984, Tew et al., 1985, Condorelli et al., 1998). And antibodies against *A. actinomycetemcomitans* were also detected occur in GCF of chronic periodontitis patients (Tew et al., 1985, Ebersole et al., 2000). Antigen-specificity of plasma cells in periodontitis tissues was investigated by the technique of enzyme-linked immunosorbent spot (ELISPOT) assay. Major isotype of spot forming cells (SFC) was IgG followed by IgA and IgM respectively (Ogawa et al., 1989b, Ogawa et al., 1991). Two studies reported antigen specificity was fimbriae of *P. gingivalis* (Ogawa et al., 1989a, Ogawa et al., 1991). And one study reported antigen specificity was lipopolysaccharide of *P. gingivalis* (Ogawa et al., 1989a).

Besides bacterial etiology, autoantibodies and autoreactive B cells has been thought to play role in periodontal tissue pathology (Berglundh et al., 2007). Antibody against collagen was reported in serum (Hirsch et al., 1988, Anusaksathien et al., 1992, Sugawara et al., 1992) and GCF of periodontitis patients (Sugawara et al., 1992). In addition, gingival mononuclear cells (GMC) extracted from periodontitis tissue can secrete antibody against collagen (Hirsch et al., 1988, Jonsson et al., 1991). At present, there is little knowledge about the antigen specificity of antibody that secreted by plasma cells in periodontitis lesion, therefore, requires further study.

CHAPTER III

MATERIALS AND METHODS

3.1 Reagents

Roswell Park Memorial Institute (RPMI)-1640, Dulbecco phosphate-buffered saline (DPBS), L-glutamine, Penicillin Streptomycin, collagenase Type I and Fetal calf serum were obtained from Gibco (Grand Island, NY, USA). Phosphate-buffered saline (PBS), Streptavidin-AP, BCIP-NBT-blue system tablet and Tween 20 were obtained from Sigma (St. Louis, MO, USA).

3.2 Monoclonal Antibodies

Anti-human CD19, anti-human CD27, anti-human CD38, anti-human HLA-DR and mouse IgG1 mAbs were obtained from BD Biosciences (San Jose, CA, USA). Primary mouse-anti-human mAb against human CD3 were obtained from Dako (Denmark). Fluorescence-conjugated mouse anti-human CD138 and primary mouse-anti human-CD138 were obtained from BioLegend (USA). Unconjugated-goat anti-human IgG, goat anti-human IgG (H+L) biotin conjugated and goat anti-human IgA biotin conjugate were obtained from KPL (Gaithersburg, MD, USA). Goat $F(ab')^2$ anti-human IgA were obtained from Invivogen (San Diego, CA, USA).

3.3 Subject selection and ethical considerations

Human periodontal tissues were obtained from patient with untreated severe chronic periodontitis and subjects with clinically healthy periodontal tissues. Since the project involved human tissues, an ethical approval would be required. This ethical approval was obtained from the Ethics committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2014-009). Informed consent of each subject was obtained before the operation. All data of subjects were kept securely confidential.

3.4 Periodontal tissue collections

Human periodontal tissues were obtained from patients with severe chronic periodontitis and subjects with clinically healthy periodontal tissues after applied informed consents. These specimens were collected from patients at Periodontal Clinic and Oral Surgery clinic, Faculty of Dentistry, Chulalongkorn University. No other dental diseases such as pulpal disease were involved. All subjects were in good general health, and none of them had taken antimicrobial or anti-inflammatory drugs within the previous 3 months.

All subjects had no history of periodontal treatment for the past 6 months. Healthy periodontal tissue samples were collected from sites with clinically healthy gingiva (no bleeding on probing, probing depth less than 4 mm, no clinical attachment loss and bone loss) during crown lengthening procedure for prosthetic reasons. Severe chronic periodontitis tissues were collected from sites of extracted teeth with hopeless prognosis (gingival inflammation, clinical attachment loss 5 mm or more and severe bone loss 50% of the root length or more (Figure 3).

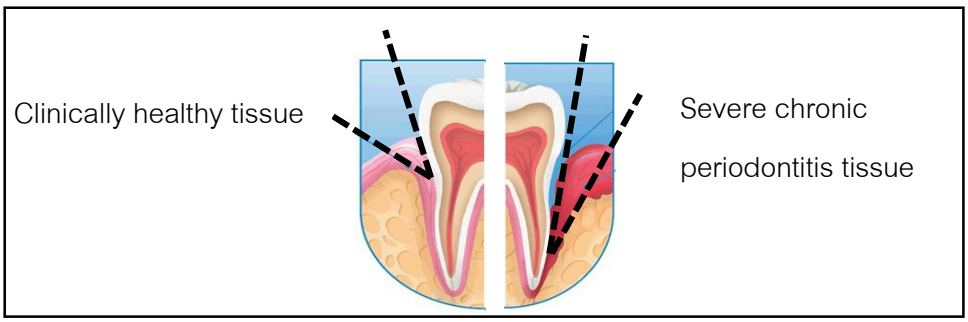

Figure 3. Periodontal tissue from severe chronic periodontitis and clinically healthy tissue were prepared by internal bevel incision and intrasulcular incision.

The excised tissues were immediately placed in sterile tubes that contain RPMI-1640 medium. The samples were transferred to the laboratory within a few hours.

3.5 Gingival cell preparation

The method for obtaining single cell suspensions from gingival tissues was modified from the method that was described by Mahanonda et al (2002) (Mahanonda et al., 2002). Briefly, the tissues were washed thoroughly in DPBS and then were cut into small fragments (1–2 mm^3). These fragments were incubated in RPMI-1640 medium that contained 2 mg/ml of collagenase type I. The ratio of medium plus collagenase to tissues was 1 ml per 100 mg of tissue. After 90 minutes of incubation at 37°C in 5% CO_2 atmosphere, residual tissue fragments were disaggregated by gentle flushing several times with a pipette, until single cell suspensions were obtained. The single cell suspensions were filtered through filter of mesh size 70 μm (BD Biosciences). The lymphocytes were counted in haemocytometer and analyzed for viability by trypan blue exclusion method.

3.6 Flow cytometric analysis

We studied the presence of plasma cells in severe periodontitis tissues which had phenotypic markers of $CD19^{+}CD27^{+}CD38^{+}CD138^{+}$. Extracted gingival cells from severe periodontitis patients were stained with a). anti-human CD19 (FITC), CD27 (PE), CD38 (APC), and HLA-DR (PerCP) monoclonal antibodies or b). anti-human CD19 (FITC), CD27 (PE), CD38 (APC) and CD138 (PerCP/Cy5.5) monoclonal antibodies at 4°C for 30 minutes. The stained gingival cells were washed with PBS containing 0.1% albumin and 0.01% sodium azide. Then they treated with red blood cell lysing solution (FACs Lysing Solution, BD Biosciences) in the dark at room temperature for 10 minutes, washed and fixed with 1% paraformaldehyde. Analysis of flow cytometry samples were performed by four-color flow cytometry, FACSCalibur (BD Biosciences). First, $CD19^{+}CD3^{-}$ cells were gated. Then $CD19^{+}CD27^{+}38^{+}$ cells were gated. Finally these cells were analyzed for the expressions of HLA-DR or CD138 (Figure 4).

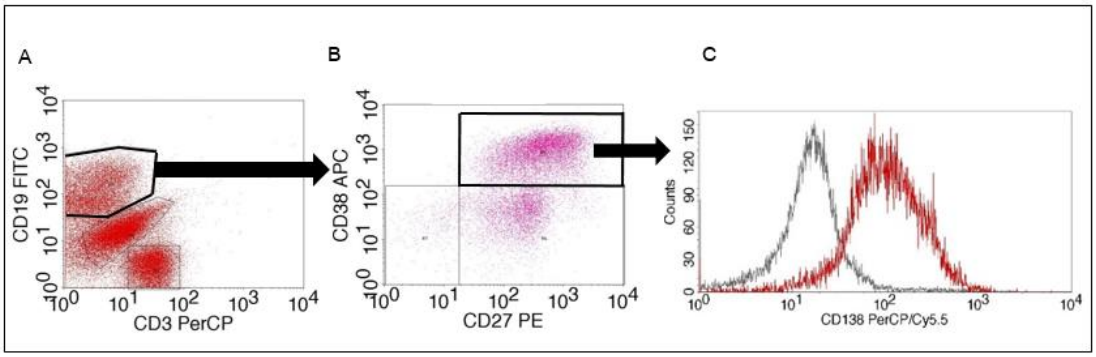

Figure 4. Flow cytometric analysis of plasma cells in periodontitis tissues. Plasma cells were classified as $CD19^{+}CD27^{+}CD38^{+}138^{+}$ (C). PerCP, Peridinin Chlorophyll Protein complex; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; APC, Allophycocyanine; Cy, Cyanine.

3.7 Immunohistochemistry

In our laboratory, we start using frozen sections to perform immunohistochemistry. Later on, we established immunohistochemistry with paraffin-embedded sections due to clear structure. The excised periodontal tissues were immediately washed in normal saline solution. They were fixed in 10% buffered formalin for a maximum of 24 hours and subsequently embedded in paraffin. Microtome serial 4-micron-thick sections were cut and mounted on glass slides. Sections were deparaffinized in xylene and rehydrated through a graded ethanol series (100%, 95%, 80%) and distilled water. To inhibit endogenous peroxidase, they were incubated with 0.3% hydrogen peroxide solution for 15 minutes. Placing the slides into a 1mM EDTA pH 8.0 and heating for 95°C for 20 minutes for antigen retrieval.

For identifying local plasma cells, single immunohistochemical staining was performed via Polymer/HRP and DAB^{+}chromagen system (DAKO EnVisionTM G/2 Doublestain System, Denmark) on the sections. They were stained with primary mouse-anti-human CD138 (plasma cells) (BioLegend, USA) or isotype control. For identifying local T cells, single immunohistochemical staining was performed via Polymer/HRP and DAB^{+}chromagen system (DAKO EnVisionTM G/2 Double stain System, Denmark) on the sections. They were stained with primary mouse-anti-human mAb against human CD3 (Dako, Denmark) or isotype control. Counterstaining was done with haematoxylin. They were investigated under light microscope.

3.8 Enzyme-linked immunosorbent spot (ELISPOT) assay

The frequency of antigen-specific antibody-secreting cells were measured by ELISPOT assay. Soluble antigens derived from key periodontal bacteria: *P. gingivalis*, *A.*

actinomycetemcomitans, and commensal bacteria: *S. gordonii* were provided by Professor Fuminobu Yoshimura, Department of Microbiology, School of Dentistry, Aichi Gakuin University, Nagoya, Japan. Collagen type I (Sigma) were included.

Multiscreen 96-well filtration plates (Millipore, Bedford, MA, USA) were coated with bacterial antigens, *P. gingivalis, A. actinomycetemcomitans, S. gordonii,* and collagen type I at predetermined concentration (10 µg/well). The plates were also coated with unconjugated-goat anti-human IgG or unconjugated-goat $F(ab')^2$ anti-human IgA at 5 µg/ml for positive control and keyhole limpet haemocyanin (KLH) at predetermined concentration (0.5 µg/well) for negative control. The coated plates were placed in a humidified chamber at 4°C overnight. The plate was washed twice with Dulbecco phosphate-bufferd saline (DPBS) and blocked with DPBS containing 10% FBS for 1 hour at 37°C. After washed twice with DPBS gingival mononuclear cells were added and incubated overnight at 37°C. After that, the plate was washed 3 times with PBS and another 3 times with PBS with 0.05% Tween 20 (PBST). Goat anti human IgG or IgA biotin conjugated (KPL, USA) was added into the plate. After 2 hours of incubation at 37°C, the plate was washed 4 times with PBST and streptavidin-alkaline phosphatase was added to the plate at a 1:1,000 dilution and incubated for 1 hour at 37°C. Then the plate was washed 3 times with PBST and another 3 times with PBS, after that a substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium [BCIP/NBT]; Sigma-Aldrich, St. Louis, MO) was added to allow spots to develop for 5-15 min, and then spots were counted by using an ELISPOT Reader (Cellular Technologies, Cleveland, OH, USA).

3.9 Statistical Analysis

Data from ELISPOT assay were analyzed using the computer program SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Results were presented as median and interquartile range. The nonparametric Mann Whitney's U-test was used to determine the differences of total IgG spot form cells (SFC)/10^6 gingival mononuclear cells (GMC) and total IgA SFC/10^6 GMC, and *P. gingivalis*-specific IgG SFC/10^6 GMC and *P. gingivalis*-specific IgA SFC/10^6 GMC. A critical level of 0.05 was employed. Thus, p-values less than 0.05 were considered as statistically significant.

CHAPTER IV

RESULTS

4.1 Flow cytometric analysis of plasma cells in periodontitis tissues

We previously used flow cytometry to demonstrate that the predominant B cells in periodontitis tissue were antibody secreting cells with plasma cell phenotype ($CD19^{+}CD27^{+}CD38^{+}HLA\text{-}DR^{low}$). However, it was not feasible to employ monoclonal antibodies against these 4 surface markers for gingival tissue immunostaining. Therefore we tested if anti-CD138 antibody could recognize gingival plasma cells. In this study, gingival cells from seven patients with severe chronic periodontitis were extracted. Our results in Figure 5 clearly showed that all $CD19^{+}CD27^{+}CD38^{+}HLA\text{-}DR^{low}$ plasma cells were positive for CD138.

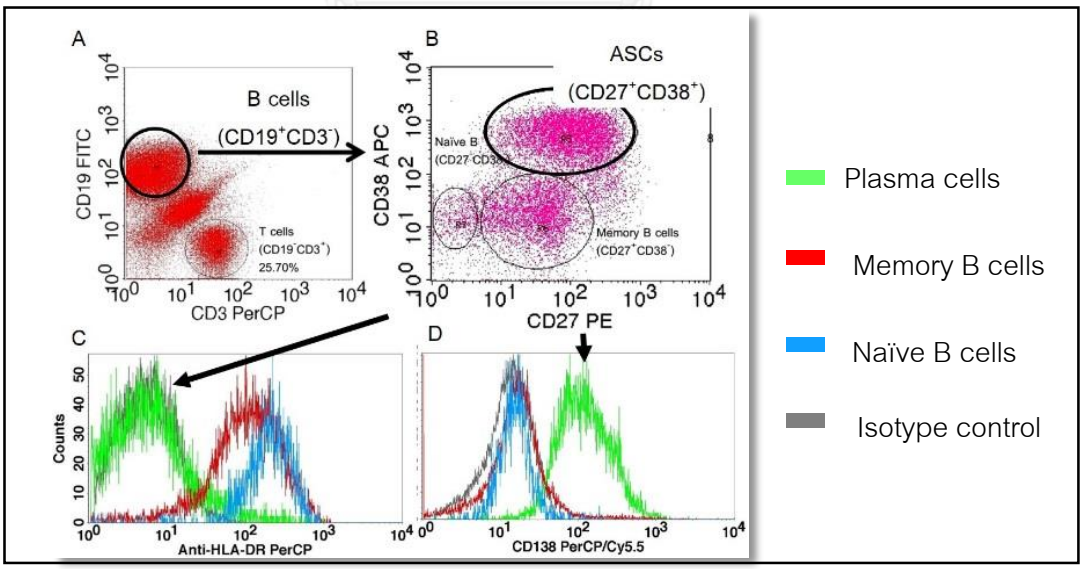

Figure 5. Flow cytometric analysis of plasma cells in periodontitis tissues. Cells extracted from periodontitis tissues were stained with monoclonal antibodies specific to antibody

secreting cells ($CD19^+CD27^+CD38^+$) (B). Plasma cells identified as $CD19^+CD27^+CD38^+$ HLA-DR^{low} cells (C) were also positive for CD138 (D). A representative from seven separate experiments.

4.2 Localization of plasma cells and co-localization of plasma cells and T cells in periodontal tissues

Paraffin-embedded sections were prepared from five severe periodontal tissues and five clinically healthy gingival tissues. The localization of plasma cells in gingival tissue samples was observed by immunohistochemistry using monoclonal antibody against CD138 to detect plasma cells. In all periodontal tissues, positive staining of CD138 was detected on plasma cells and epithelium. The small round cells with CD138 positive were consistently observed in large numbers, forming small clusters scattering in connective tissue, especially at the base of periodontal pocket, adjacent to pocket epithelium (a representative of five individuals, Figure 6A). On the other hand, none or very few (Figure 6F) CD138 positive cells was/were observed in clinically healthy gingiva.

We then investigated the location of these plasma cells in periodontitis in relation to T cells. We stained T cells using monoclonal antibody against CD3. Figure 6B and 6E showed that most of CD3 positive T cells scattered in connective tissue but to a lesser numbers when compared to CD138 positive plasma cells (Figure 6A). Some of CD3 positive T cells were also detected in epithelial layer (Figure 6B). More numbers of CD3 positive T cells were observed in periodontitis tissue when compared to those in healthy (Figure 6G). By single immunostaining in a consecutive tissue sections, CD138 positive plasma cells and CD3 positive T cells seem to be distributed in a similar location, scattering in connective tissue, suggesting co-localization of plasma cells and T cells.

Again the dense area of co-localization could be observed at the base of periodontal pocket, adjacent to pocket epithelium (magnified photographs in Figures 6D and 6E).

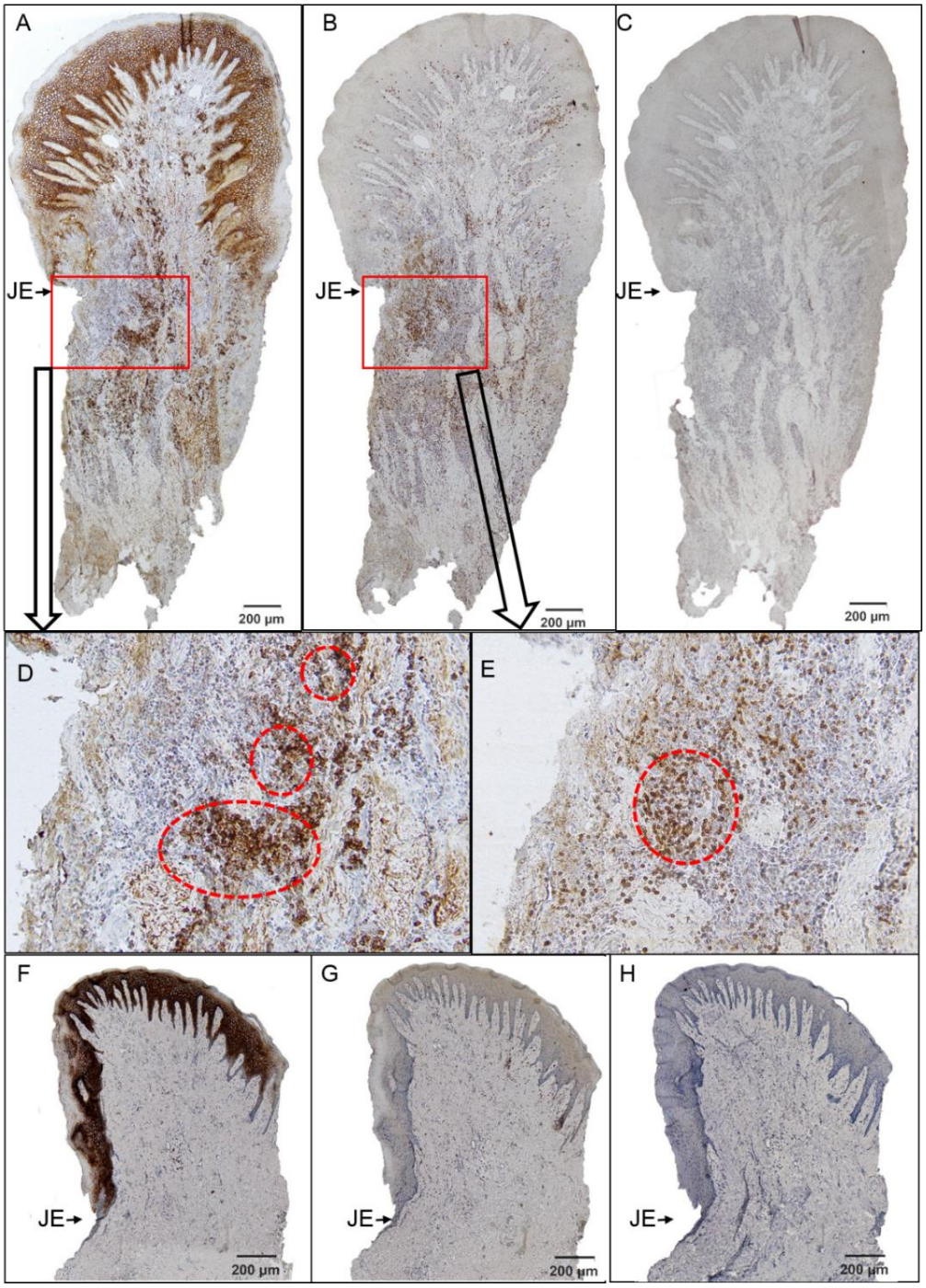

Figure 6. Localization of plasma cells and co-localization of plasma cells and T cells in periodontal tissues. Anti-CD138 monoclonal antibody was used to stain plasma cells in

periodontitis tissue (A) and clinically healthy gingiva (F). Anti-CD3 monoclonal antibody was used to stain T cells in periodontitis tissue (B) and clinically healthy gingiva (G). Magnified photographs of the area at the base of periodontal pocket suggests co-localization of $CD138^+$ plasma cells (D) and $CD3^+$ T cells (E). (C) and (H) showed negative control in severe periodontitis tissue and clinically healthy gingiva respectively. Data are representative of 5 different individuals in each clinical group. Magnifications 4x

4.3 Measurement of antigen-specific antibodies by ELISPOT assay

Gingival mononuclear cells (GMC) were isolated from five inflamed periodontitis tissues. Soluble bacteria antigens from key periodontal pathogens: *P. gingivalis, A. actinomycetemcomitans,* commensal bacteria: *S. gordonii*, and self-tissue antigen: type I collagen were used to assess specificity of antibodies secreted from gingival plasma cells. In all five patients, we detected gingival plasma cells producing IgG and IgA specific to *P. gingivalis*. Median of *P. gingivalis*-specific IgG spot forming cells (SFC) was $3683/10^6$ GMC (interquartile range [IQR] = 1094-8398.5). It seems to be higher than median of *P. gingivalis*-specific IgA SFC ($98/10^6$ GMC [IQR= 39.5-2018.5)]) but it is not statistically significant (p value= 0.056). On the other hands, median of total IgG SFC ($98679/10^6$ GMC [IQR= 80286.5-188998.5]) was significant higher than total IgA SFC ($8559/10^6$ GMC [IQR= 6804-26276]) (p value= 0.008). *A. actinomycetemcomitans*-specific IgG SFC were found in three patients (median= $14/10^6$GMC [IQR= 0-100.5]) but *A. actinomycetemcomitans*-specific IgA SFC was not found. We did not detected *S. gordonii*-specific IgG and IgA and type I collagen-specific IgG and IgA SFC in all studied periodontitis tissues (a representative from five separate experiments, Figure 7) (Table 1).

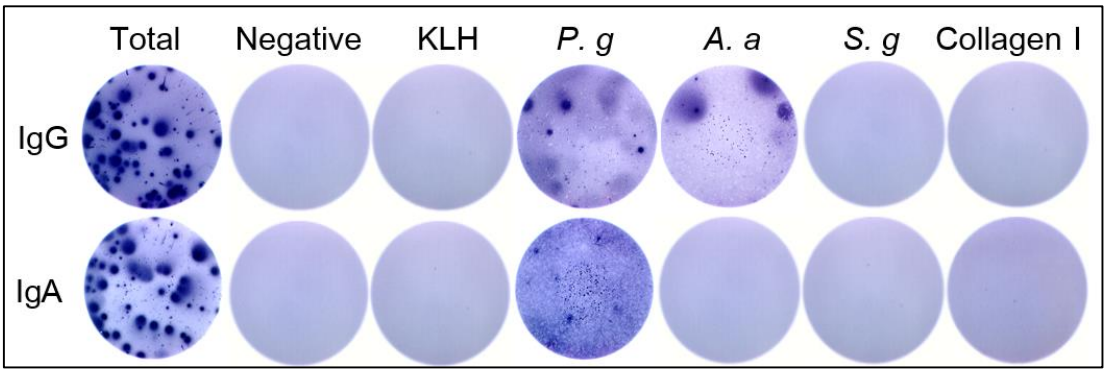

Figure 7. Antigen specificity of plasma cells by ELISPOT assay from one patient. KLH: keyhole limpet haemocyanin, *P. g: P. gingivalis, A. a: A. actinomycetem-comitans, S. g: S. gordonii,* GMC: gingival mononuclear cell. A representative from five separate experiments.

Table 1. Frequencies of immunoglobulin spot forming cells (SFC) and antigen-specific immunoglobulin SFC in 10^6 gingival mononuclear cells (GMC) (value are median SFC/10^6 GMC)

	Total Ig	*P. gingivalis*-specific		*A. actinomycetemcomitans*-specific		S. gordonii-specific		Collagen I-specific	
	SFC/10^6 GMC	n	SFC/10^6 GMC	n	SFC/10^6 GMC	n	SFC/10^6 GMC	n	SFC/10^6 GMC
IgG	98679*	5/5	3683**	3/5	14	0/5	0	0/5	0
IgA	8559*	5/5	98**	0/5	0	0/5	0	0/5	0

*p value= 0.008 (Mann Whitney's U-test)

**p value= 0.056 (Mann Whitney's U-test)

CHAPTER V

DISCUSSION AND CONCLUSION

Our laboratory recently revisited the role of B cells in periodontitis. We confirmed that the predominant B cells in periodontitis tissue were antibody secreting cells with plasma cell phenotype ($CD19^{+}CD27^{+}CD38^{+}$,$HLA\text{-}DR^{low}$) (Rattanathammatada, 2013). CD138 has been a well-known marker for plasma cell. In the present study, flow cytometric experiments suggest that extracted gingival cells from periodontitis tissue specimens (n=7) with phenotype of $CD19^{+}CD27^{+}CD38^{+}HLA\text{-}DR^{low}$ also expressed CD138 positive. We therefore performed immunohistochemistry staining using monoclonal antibody anti-human CD138 for identifying local plasma cells in periodontitis tissues as compared to those in clinically healthy gingiva.

Data from immunostaining with anti-CD138 monoclonal antibody revealed numerous plasma cells localization in periodontitis lesions that confirms the finding of previous studies (Page and Schroeder, 1976, Mackler et al., 1977, Seymour and Greenspan, 1979, Daly et al., 1983, Yamazaki et al., 1993, Kim et al., 2010, Mizutani et al., 2014, Thorbert-Mros et al., 2014). Gingival plasma cells appear to form small clusters within connective tissue. There are two possible scenarios to explain the formation of plasma cells in periodontitis tissues. First, these plasma cells migrate from the closest draining lymph node where B cells are activated and differentiated to plasma cells. The second scenario is based on the previous observations from our group demonstrating the presence of memory B cells ($CD19^{+}CD27^{+}CD38^{-}$) in clinically healthy gingival tissue (Rattanathammatada, 2013). These gingival memory B cells could rapidly activate upon

reencounter with oral bacteria and give rise to plasma cells. Further studies are required to understand the generation and maintenance of plasma cells in periodontitis.

In addition, our finding agree with previous studies (Yamazaki et al., 1993, Thorbert-Mros et al., 2014) that co-localization of B cells and T cells were observed in periodontitis lesions. The densest area of co-localization of CD138 positive plasma cells and CD3 positive T cells was in the connective tissue at the apical end of the pocket epithelium. The interesting question is whether these immune cells are able to form ectopic lymphoid structure. Ectopic lymphoid structure has been observed in several chronic inflammatory conditions such as in lung of rheumatoid arthritis patients with chronic pulmonary disease (Rangel-Moreno et al., 2006), and in stomach mucosa of chronic gastritis induced by *Helicobacter pylori* (Mazzucchelli et al., 1999). Such structure may contribute to a chronic stage of the diseases. In oral mucosal tissue, ectopic lymphoid structure was demonstrated in oral squamous cell carcinoma (Wirsing et al., 2014). In periodontitis, Nakajima et al. (2008) reported some periodontitis tissues displayed ectopic lymphoid structure consisting $CD19^{+}CD3^{+}$ cells together with follicular dendritic cells but some tissues did not (Nakajima et al., 2008). Therefore, there is a great need to further explore a clear component of ectopic lymphoid structure including high endothelial venules, follicular dendritic cells and germinal center B cells in periodontitis tissues.

Plasma cells are terminally differentiated B cells that can secrete antibody. Antigen-specificity of plasma cells in periodontal lesions was investigated by ELISPOT assay. Our finding agree with the previous studies that major isotype of spot forming cells was IgG followed by IgA (Ogawa et al., 1989b, Ogawa et al., 1991). Two studies reported antigen specificity was fimbriae of *P. gingivalis* (Ogawa et al., 1989a, Ogawa et al., 1991). And one study reported antigen specificity was lipopolysaccharide of *P. gingivalis*

(Ogawa et al., 1989a). Our findings confirm previous studies that plasma cells from periodontitis tissues produced antibody against *P. gingivalis*. We detected higher frequency of *P. gingivalis*-specific IgG plasma cells than *P. gingivalis*-specific IgA plasma cells but not statistically significance.

Our study extended to investigate the specificity of antibodies to other bacteria antigens and type I collagen, gingival plasma cells from three out of five patients had *A. actinomycetemcomitans*-specific IgG plasma cells. The frequency was lower when compared to *P. gingivalis*-specific IgG plasma cells. In all studied tissues, we could not measure the presence of plasma cells specific to commensal bacteria: *S. gordonii.* One of our limiting factors in ELISPOT assay is that we did not use different varieties of periodontal pathogenic bacteria such as *Tannerella forsythia* and *Treponema denticola*. Furthermore, the observed positive specificity against *P. gingivalis* may possibly cross-react with antigens from other plaque bacteria. Besides bacterial etiology, autoimmune reaction has been thought to play role in periodontal tissue pathology. No detection of collagen-specific plasma cells in our study was different from other groups. They reported the presence of collagen-specific plasma cells in majority in periodontitis tissue samples (Hirsch et al., 1988, Jonsson et al., 1991). Future studies using large sample sites and more sensitive assay are required.

Besides antibody production, activated B cells can be the cellular source of receptor activator of nuclear factor kappa-B ligand (RANKL) (Kawai et al., 2006, Yeo et al., 2011). RANKL is a TNF-related cytokine that involved in physiological osteoclastogenesis and pathological bone resorption. In periodontitis, more than ninety percent of B cells in periodontal tissue express RANKL (Kawai et al., 2006). In rheumatoid arthritis, high expression of mRNA for RANKL in B cells from synovial fluid has been reported (Yeo et al., 2011). Moreover, plasma cells in intestinal mucosa of patients with

inflammatory bowel disease express granzyme B that have cytotoxic function (Cupi et al., 2014). Further studies are required to investigate RANKL and granzyme B in periodontal tissues to better understand role of these plasma cells in pathogenesis of the disease.

In conclusion, we found that periodontitis tissue contained large numbers of plasma cells which co-localization with T-cells in connective tissue, especially at the base of periodontal pocket. Most of these gingival plasma cells produced antibodies against *P. gingivalis* and to a lesser extent to *A. actinomycetemcomitans*. Further studies are needed to gain insight into the role of periodontal tissue-plasma cells in protection or pathogenesis of the disease.

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APPENDICES

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

No.	Sex	Age (years)	Tooth No.	PD (mm)	BOP
1	Male	34	14	2-3	-
2	Female	52	25	2-3	-
3	Male	17	27	2-3	-
4	Female	49	14	2-3	-
5	Male	67	46	2-3	-

PD = Probing depth;

BOP = bleeding when examination with periodontal probe

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

No.	Sex	Age (years)	Tooth No.	Clinical examination			
				PD (mm)	CAL (mm)	Bone loss	Others
1	Female	61	17	9	9	Severe bone loss (>50%)	
2	Male	53	27	10-12	10-12	Severe bone loss (>50%)	MO:3
3	Male	77	27, 28	6-10	9-14	Severe bone loss (>50%)	FI:3
4	Male	65	26, 27	9-11	9-11	Severe bone loss (>50%)	FI:2 MO:1
5	Female	53	17	10-12	12-15	Severe bone loss (>50%)	FI:2 MO:2
6	Male	52	47	15	13	Severe bone loss (>50%)	FI:1 MO:3
7	Female	55	28	10	10	Severe bone loss (>50%)	MO:2
8	Male	45	15	7	8-9	Severe bone loss (>50%)	MO:2
9	Male	48	17, 18	5-10	7-11	Severe bone loss (>50%)	MO:1 FI:2
10	Male	49	15	10	13	Severe bone loss (>50%)	MO:2

PD = Probing depth;

CAL = Clinical attachment loss;

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

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

Appendix C: Frequencies of immunoglobulin spot forming cells (SFC) in 10^6 gingival mononuclear cells (GMC) from Enzyme-linked immunosorbent spot (ELISPOT) assay

No.	Total IgG SFC/10^6 GMC cells	Antigen used in ELISPOT (specific SFC/10^6 GMC cells)			
		P. gingivalis	*A. actinomycetemcomitans*	*S. godonii*	Type I collagen
1	64364.88	671.79	0	0	0
2	98678.51	1515.53	16.67	0	0
3	96207.84	11414.49	0	0	0
4	154948.98	3682.05	203.26	0	0
5	223047.28	5381.12	13.13	0	0

No.	Total IgA SFC/10^6 GMC cells	Antigen used in ELISPOT (specific SFC/10^6 GMC cells)			
		P. gingivalis	*A. actinomycetemcomitans*	*S. godonii*	Type I collagen
1	8558.50	97.14	0	0	0
2	13440.44	181.89	0	0	0
3	39110.69	3854.24	0	0	0
4	7988.50	27.50	0	0	0
5	5618.15	50.19	0	0	0

SFC= Spot Forming Cells

GMC= Gingival mononuclear cells

P. gingivalis = Porphyromonas gingivalis

A. actinomycetemcomitans = Aggregatibacter actinomycetemcomitans

S. godonii = Streptococcus gordonii

Descriptive statistics of frequencies of immunoglobulin spot forming cells (SFC) in 10^6 gingival mononuclear cells (GMC)

		Total IgG SFC/10^6 GMC cells	Total IgA SFC/10^6 GMC cells	P. gingivalis-specific IgG SFC/10^6 GMC cells	P. gingivalis-specific IgA SFC/10^6 GMC cells	A.actinomycetem comitans-specific IgG SFC/10^6 GMC cells
N	Valid	5	5	5	5	5
	Missing	5	5	5	5	5
Median		98679.00	8559.00	3683.00	98.00	14.00
Minimum		64365	5619	672	28	0
Maximum		223048	39111	11415	3855	204
Percentiles	25	80286.50	6804.00	1094.00	39.50	.00
	50	98679.00	8559.00	3683.00	98.00	14.00
	75	188998.50	26276.00	8398.50	2018.50	110.50

Mann-Whitney's U-test results of differences of frequencies of immunoglobulin spot forming cells in 10^6 gingival mononuclear cells between IgG isotype and IgA isotype

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Total Ig SFC/10^6 GMC cells is the same across categories of Group.	Independent-Samples Mann-Whitney U Test	.008[1]	Reject the null hypothesis.
2	The distribution of P. gingivalis-specific Ig SFC/10^6 GMC cells is the same across categories of Group.	Independent-Samples Mann-Whitney U Test	.056[1]	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

[1]Exact significance is displayed for this test.

VITA

Miss Saranya Thawanaphong was born on 1st of June 1984 in Bangkok. She graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Chiang Mai University in 2009, and became a faculty staff at the Faculty of Dentistry, Chiang Mai University in 2010-2013. She studied in Master degree program in Periodontology at Graduate School, Chulalongkorn University in 2012.