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FLOW CYTOMETRIC ANALYSIS OF B CELL PROFILE IN PERIODONTAL DISEASE

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ลักษณะเด่นที่พบในโรคปริทันต์อักเสบ คือการมีเซลล์ภูมิคุ้มกันอย่างหนาแน่นทั้งบีเซลล์และทีเซลล์ ซึ่งเซลล์เหล่านี้มีบทบาทสำคัญในพยาธิอิมมูนของการเกิดโรค การศึกษาถึงบีเซลล์ในโรคปริทันต์ส่วนใหญ่มุ่งเน้นศึกษาถึงบีเซลล์ในระยะกระตุ้น แต่ยังไม่มีการศึกษาใดที่วิเคราะห์กลุ่มย่อยของบีเซลล์ได้อย่างสมบูรณ์ทั้ง naïve B cells memory B cells และ antibody secreting cells (ASCs) ดังนั้นวัตถุประสงค์ของงานวิจัยในครั้งนี้เพื่อศึกษากลุ่มย่อยของบีเซลล์ในเนื้อเยื่อปริทันต์อักเสบที่ได้มาจากผู้ป่วยที่เป็นโรคปริทันต์อักเสบเรื้อรังระดับรุนแรง การศึกษาในครั้งนี้ได้ยืนยันผลของงานวิจัยที่ผ่านมาในอดีตที่พบว่ามีบีเซลล์เด่นในโรคปริทันต์อักเสบและพบทีเซลล์เด่นในเนื้อเยื่อปริทันต์ที่มีสุขภาพปกติ เมื่อวิเคราะห์กลุ่มย่อยของบีเซลล์ พบ ASCs (ซีดี19^{+}ซีดี27^{+}ซีดี38^{+}) (58.44 ± 3.79 เปอร์เซ็นต์) มากที่สุดในเนื้อเยื่อปริทันต์อักเสบเรื้อรังระดับรุนแรง (จำนวน 21 ตัวอย่าง) ขณะที่พบ memory B cells (ซีดี19^{+}ซีดี27^{+}ซีดี38^{-}) (86.59 ± 1.29 เปอร์เซ็นต์) มากที่สุดในเนื้อเยื่อปริทันต์ที่มีสุขภาพปกติ (จำนวน 29 ตัวอย่าง) โดยทั้งสองกลุ่มพบ naïve B cells เล็กน้อย (ซีดี19^{+}ซีดี27^{-}ซีดี38^{-}) (น้อยกว่า 7 เปอร์เซ็นต์) ปัจจุบันยังไม่ชัดเจนว่า ASCs ที่พบในเนื้อเยื่อปริทันต์อักเสบเป็นพลาสมาบลาสหรือพลาสมาเซลล์ การศึกษานี้ได้ใช้การแสดงออกของโมเลกุล human leukocyte antigen (HLA)-DR เป็นการบอกความแตกต่างของเซลล์ทั้งสองชนิดเป็นครั้งแรกในโรคปริทันต์อักเสบ ผลการศึกษาแสดงให้เห็นว่า ASCs ที่พบเป็นชนิดพลาสมาเซลล์ (การแสดงออกของ HLA-DR ในระดับต่ำ) ซึ่งไม่ใช่พลาสมาบลาส (การแสดงออกของ HLA-DR ในระดับสูง) นอกจากนี้ การศึกษานี้ถือเป็นการเปิดประเด็นเป็นครั้งแรกในการพบ memory B cells ในเนื้อเยื่อปริทันต์ที่มีสุขภาพปกติ ดังนั้นควรทำการศึกษาต่อไปถึงกลุ่มย่อยของบีเซลล์เพื่อให้เกิดความเข้าใจถึงบทบาทของเซลล์เหล่านี้มากยิ่งขึ้นถึงความเกี่ยวข้องกับการคงสภาวะความสมดุล หรือการป้องกันการเกิดโรคปริทันต์

ภาควิชา.....ปริทันตวิทยา..... ลายมือนิสิต.....

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KEYWORDS: B CELL PROFILE / NAIVE B CELLS / MEMORY B CELLS / ANTIBODY SECRETING CELLS / FLOW CYTOMETRY / PERIODONTAL DISEASE

WARATTAYA RATTANATHAMMATADA: FLOW CYTOMETRIC ANALYSIS OF B CELL PROFILE IN PERIODONTAL DISEASE. ADVISOR: ASSOC. PROF. RANGSINI MAHANONDA, Ph.D., CO-ADVISOR: SATHIT PICHYANGKUL, Ph.D., 60 pp.

Hallmark of periodontitis has been characterized by dense infiltration of immune cells including B cells and T cells, all of which play critical role in immunopathogenesis of the disease. Most studies of infiltrated B cells in periodontal disease focused on activation stage, and so far there has been no study with a complete analysis of all B cell subsets such as naïve B cells, memory B cells and antibody secreting cells (ASCs). Therefore, we investigated the B cell subsets in inflamed periodontal tissues from patients with severe chronic periodontitis. We confirmed previously described B cell-dominated lesion in periodontitis and T cell-dominated lesion in healthy periodontal tissue. Among three B cell subsets, we found that ASCs ($CD19^+CD27^+CD38^+$) (58.44 ± 3.79%) were the major cell type in severe chronic periodontitis tissues (*n* = 21), whereas memory B cells ($CD19^+CD27^+CD38^-$) (86.59 ± 1.29%) were the major cell type in healthy periodontal tissues (*n* = 29). Both clinical groups demonstrated low levels of infiltrated naïve B cells ($CD19^+CD27^-CD38^-$) (less than 7%). At present, it's not clear if ASCs in periodontitis tissues are plasmablasts or plasma cells. Human leukocyte antigen (HLA)-DR expression was first used to differentiate the two cell types in periodontitis. Our findings clearly showed that the observed infiltrated ASCs were plasma cells (low HLA-DR expression), not plasmablasts (high HLA-DR expression). We also first identified the presence of memory B cells in healthy periodontal tissues. Further study on these B cell subsets should provide a better insight into their role either in periodontal homeostasis or protection.

Department……Periodontology……. Student's Signature……..

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

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LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibactor actinomycetemcomitans*
APC	Allophycocyanine
ASC	Antibody secreting cell
cADPR	Cyclic adenosine diphosphate ribose
CD	Cluster of differentiation
CD40L	CD40 ligand
CXCL	CXC-chemokine ligand
DPBS	Dulbecco phosphate-buffered saline
FITC	Fluorescein isothiocyanate
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
Ig	Immunoglobulin
IL	Interleukin
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein complex
P. gingivalis	*Porphyromonas gingivalis*
RPMI	Roswell Park Memorial Institute
TNF	Tumor necrosis factor

CHAPTER I

INTRODUCTION

1.1 Background of the present study

Periodontal disease is one of the most common chronic inflammatory diseases in human. It involves tooth supporting structures including gingiva, periodontal ligament, cementum, and alveolar bone. The stable form of periodontal disease is so-called gingivitis which the inflammation is confined to the gingiva. The severe form of periodontal disease is periodontitis which the inflammatory process involves tooth supporting tissues including alveolar bone. In severe cases, periodontitis may lead to tooth loss. The etiology of periodontal disease is microbial plaque biofilm and host immune response to plaque bacteria leads to tissue pathology (Mahanonda, 2012). A large number of immune infiltrates such as B cells and T cells have been detected in periodontal lesions. Gingivitis lesions are predominated by T cells whereas periodontitis lesions are dominated by B cells and plasma cells (Seymour et al., 1979). At present, the role of B cells and plasma cells in the pathogenesis of periodontitis remains unclear.

B cells are generated in the bone marrow. They exit as precursor B cells (pre-B cells), which are immature and express immunoglobulin (Ig)M. These cells further mature into naïve B cells in secondary lymphoid tissues (lymph node and spleen). Naïve B cells aggregate and form primary follicles that become secondary follicles with germinal centers after antigen stimulation (primary immune response). During this process, naïve B cells

expressing surface Ig bind the antigen, receive signals from helper T cells, and proliferate. This proliferation produces short-live Ig-secreting plasmablasts and germinal center cells, many of which switch their Ig constant region from IgM to IgG, IgA, IgD or IgE and acquire somatic mutations in the variable region. Cells, that acquire Ig mutations, improve antigen binding. These cells gain a survival advantage and emerge as long-lived surface-switched Ig memory cells or surface Ig-plasma cells that maintain serum Ig levels. A small proportion of plasmablasts stay in the secondary lymphoid organ where they were generated. Most of the plasmablasts migrate either to inflamed tissue or to the bone marrow under the control of chemotactic factors and cytokines and differentiate into plasma cells (Abbas and Lichtman, 2005; Delves et al., 2011; Murphy et al., 2008). All these tissues (secondary lymphoid tissue, inflamed tissue, and bone marrow) have been considered as plasma cell survival niche (Radbruch et al., 2006). At present, there has been limited information regarding memory B cell trafficking. As memory B cells are generated, some stay in germinal centers and most of cells reside in the bone marrow, lymph nodes, and spleen (McHeyzer-Williams et al., 2011; Tarlinton, 2006).

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. Later studies using enzymes and surface antigen markers in indirect immunofluorescence confirmed the predominant B cell and plasma cell lesion of periodontitis (Daly et al., 1983; Page and Schroeder, 1976; Seymour and Greenspan, 1979). In the past, there were several investigations regarding activation stages of tissue B cells in periodontitis such as $CD25^{+}$ (Gemmell and eymour, 1991) or $CD69^{+}$

(Champaiboon et al., 2000), however, very little data were found in relation to B cell profiles including naïve B cells and memory B cells. This may be due to limited markers for B cell phenotype. Recently, more advanced approaches such as flow cytometry and availability of novel phenotype markers for naïve B cells ($CD19^{+}CD27^{-}CD38^{-}$) (Moir and Fauci, 2009), memory B cells ($CD19^{+}CD27^{+}CD38^{-}$) (Fink, 2012; Moir and Fauci, 2009), and antibody secreting cells (ASCs) ($CD19^{+}CD27^{+}CD38^{+}$) (Wrammert et al., 2008). Even two subsets of ASCs can be differentiated from each other. While plasmablast expresses Human leukocyte antigen (HLA)-DR^{high}, plasma cells express HLA-DR^{low} (Jacobi et al., 2010; Moir and Fauci, 2009; Odendahl et al., 2005).

In general, the measurement of immune response in humans is conducted using peripheral blood mononuclear cells. Very recently, tissue-specific immune response has been in focus. Assessment of immune response at the site of infection would provide a better insight into protective immune response or pathogenesis compared to that of peripheral blood. Most studies so far have intensively investigated tissue-specific memory T cells in skin (Ariotti et al., 2012; Clark et al., 2006; Jiang et al., 2012), lung (Purwar et al., 2011; Teijaro et al., 2011), and gut (Casey et al., 2012; Masopust et al., 2010), and provide their critical role in protection especially against viral infection. However, there has been limit information about tissue-specific memory B cells in humans. One study in mouse has described the persistence of lung tissue-specific memory B cells against influenza virus (Onodera et al., 2012). This memory B cell subset harbored in infected lung may play critical role in protection against pulmonary virus reinfection. In this study, we propose to investigate different profiles of B cell located in periodontal tissues (chronic periodontitis

compared with healthy). We anticipate that our data will provide insight into periodontal tissue specific B cell response.

1.2 Objectives

To characterize B cell profiles (such as naïve B cells, memory B cells, and ASCs) in inflamed periodontal tissues from patients with severe chronic periodontitis and compared with healthy tissues from healthy periodontal subjects.

1.3 Hypothesis

Memory B cells and ASCs are present in periodontal tissues.

1.4 Field of research

Human immunology

1.5 Criteria inclusions

1.5.1 Inflamed periodontal tissues and peripheral blood samples 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 root length with hopeless periodontal prognosis).

1.5.2 Healthy tissues and peripheral blood samples 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.

1.5.4 Phenotypic markers of B cell profiles were determined by flow cytometry.

1.6 Limitation of research

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

1.7 Application and expectation of research

1.7.1 New scientific information of different profiles of B cells located in periodontal tissues (chronic periodontitis compared with healthy) to provide novel insight into periodontal tissue specific B cell response.

1.7.2 Publication in the national peered-reviewed journal.

1.8 Keywords

B cell profile, Naïve B cells, Memory B cells, Antibody secreting cells, Flow cytometry, Periodontal disease

CHAPTER II

LITERATURE REVIEW

2.1 Periodontal disease

Periodontal disease is one of the most common chronic inflammatory diseases in human. It involves tooth supporting structures including gingiva, periodontal ligament, cementum, and alveolar bone. These diseases can be grouped into two major categories, gingivitis and periodontitis. The stable form of periodontal disease is so-called gingivitis which the inflammation is limited to the gingiva and does not affect the other attachments of teeth. The clinical features are characterized by swelling, redness, and bleeding of the gingiva. The advanced form of periodontal disease is called periodontitis that destroys the tooth supporting structures including connective tissue attachment and alveolar bone, resulting in formation of periodontal pockets. In severe cases, periodontitis may lead to tooth mobility and finally loss of teeth. 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). In healthy and gingivitis lesions, the composition of 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, Gram negative anaerobes and the majority of these bacteria are so-called key periodontal pathogens as *Porphyromonas gingivalis*, *Aggregatibactor actinomycetemcomitans*, and *Tannerella*

forsythia (Socransky et al., 1998). Histology studies revealed large number of inflammatory infiltrates such as B cells and T cells in periodontal lesion (Brandtzaeg and Kraus, 1965; Lappin et al., 1999; Orima et al., 1999; Page and Schroeder, 1976; Seymour and Greenspan, 1979). When the disease transition from gingivitis to periodontitis, the lymphocyte infiltration shift from T cells predominated in gingivitis to B cells dominated in periodontitis lesions (Seymour et al., 1979).

2.2 B cell biology

B cells are recognized for their role in the humoral mediated immunity with the primary function of antibody production. In addition, B cells are recognized as one of the professional antigen presenting cells for T cell activation (Abbas and Lichtman, 2005; Delves et al., 2011; Murphy et al., 2008). B cells are generated from hematopoietic stem cells (HSC) in the bone marrow and give rise to immature B cells. These immature B cells express surface-IgM and migrate to secondary lymphoid organs, such as spleen or lymph node, which reside as naïve B cells (Figure 1) (Abbas and Lichtman, 2005; Delves et al., 2011; Murphy et al., 2008). Upon antigen encounter, naïve B cells uptake and present membrane-bounded antigen in association with major histocompatibility complex (MHC) class II molecules as well as co-stimulatory B7 signals to helper T cells. In turn, the T cell up-regulates CD40 ligand (CD40L) that can provide critical co-stimulation to the B cell as well as produces cytokines (interleukin (IL)-2 and IL-4). These factors enable naïve B cells to become fully activated and undergo clonal expansion, somatic mutation and class switching, resulting in the development of effector B cells, either ASCs or memory B cells with IgG, IgA, IgD, IgE, or IgM (Figure 1 and Figure 2) (Delves et al., 2011).

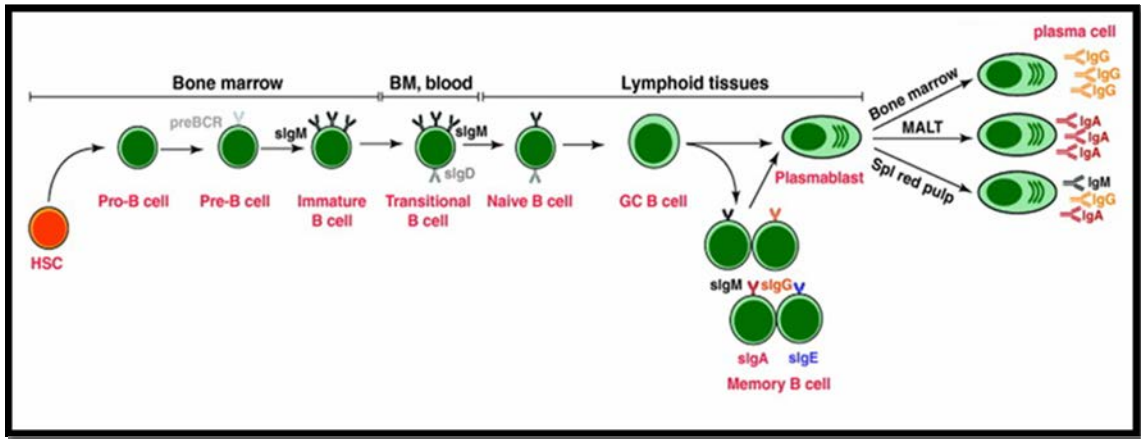


Figure 1. B cell development and differentiation: from HSC to plasma cells (Tangye, 2011). HSC, hematopoietic stem cell; BM, bone marrow; GC B cell, germinal center B cell; MALT, mucosal associated lymphoid tissue; Spl red pulp, splenic red pulp.

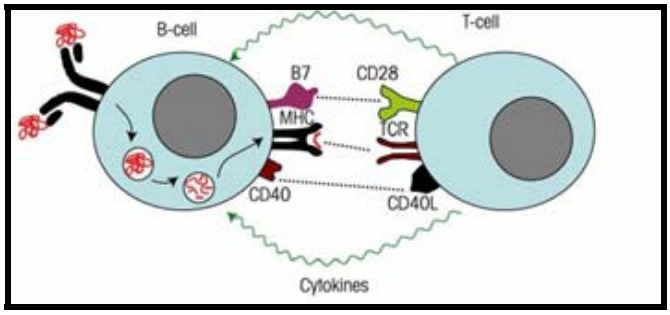


Figure 2. Helper T cell mediated activation of B cells (Delves et al., 2011).

ASC subsets consist of plasmablasts and plasma cells (Fairfax et al., 2008; Murphy et al., 2008; Odendahl et al., 2005). Plasmablasts are cells that have begun to secrete antibody, yet are still dividing and still express many of the characteristics of activated B cells (Murphy et al., 2008). Most of plasmablasts reside in secondary lymphoid tissues,

where they are generated and differentiate into plasma cells (Delves et al., 2011; Murphy et al., 2008). Apart from lymphoid tissues, recent studies in the area of vaccination demonstrated transient plasmablasts in human peripheral blood that peak at day seven after immunization with Influenza vaccine, tetanus vaccine, or yellow fever vaccine (He et al., 2011; Odendahl et al., 2005; Querec et al., 2009; Wrammert et al., 2008). Plasmablasts were also found presence in lamina propria of inflamed human gut tissue (Benckert et al., 2011). Migration of plasmablasts to bone marrow or inflamed tissues is thought to be under the control of chemokines such as CXCL12 (Delves et al., 2011; Radbruch et al., 2006). These cells then stop dividing and differentiate into plasma cells. It is interesting to note that resolution of inflammation in inflamed tissue eliminates the resident plasma cells (Radbruch et al., 2006).

Memory B cells, long-lived lymphocytes, are important in the secondary immune response. They provide a more rapid response to re-encountered antigen and a more efficient antibody production with high affinity Ig than the primary naïve B cell response. These cells are well recognized for their role in immune surveillance in circulating blood and lymphoid organs (Delves et al., 2011; Murphy et al., 2008). However, there is a lack of information regarding tissue resident specific memory B cells at different sites of the body.

2.3 B cell in periodontal disease

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. The classic studies of Page and Schroeder (1976) as well as Seymour and Greenspan (1979) have indicated the detection of B cells and plasma cells in periodontitis lesion according to cell morphology and the presence of surface Ig. They used enzymes and surface antigen markers in indirect immunofluorescence for cell type identification. For fifty years, B cells have been recognized for their role in the pathogenesis of the periodontal diseases. The conversion of a stable gingivitis to a progressive periodontitis shift from a predominantly T cell lesion to B cell and plasma cell lesion (Seymour et al., 1979). Immunohistological studies had shown a predominance of B cells and plasma cells in advanced lesion of chronic periodontitis lesion (Mackler et al., 1977; Reinhardt and Bolton, 1988). Several investigations focused on activation stages of tissue B cells in periodontitis. These B cell activation markers included $FMC7^{+}$ (Seymour et al., 1985), $CD25^{+}$ (Gemmell and Seymour, 1991), and $CD69^{+}$ (Champaiboon et al., 2000). The frequency of activated B cells has been reported to be much higher in periodontitis than gingivitis (Yamazaki et al., 1993). IgG and IgA antibodies appear to be the major Ig class that secreted by gingival cells, indicating antigen exposure of memory B cells in periodontitis lesions (Daly et al., 1983; Ogawa et al., 1989b). It was proposed by Okada et al. (1987) and Amunulla et al. (2008) that B cell polyclonal activation with T cell help drives local B cell differentiation into ASCs, which needs to be investigated. To date, there has been very little data of infiltrated B cell profiles including naïve B cells, memory B cells, and ASCs, and their role in protection or pathogenesis of periodontal disease. This may be due to limited markers for B cell phenotype.

2.4 The novel surface markers to identify the different B cell profiles

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. Human Cell Differentiation Molecules council finds out current CD up to 363 molecules in 2011 of the International Workshop and Conference on Human Leukocyte Differentiation Antigens. The monoclonal antibodies (mAbs) against a variety of cell surface molecules, together with multicolor flow cytometric analysis, have facilitated the characterization of several B cell subsets. Single marker to identify the populations is a hazard when use alone. Combining of these markers via a multicolor flow cytometry allows to further identify B cell subsets (Llinas et al., 2011).

Selective mAbs have been used to identify B cell subsets including mAbs against CD19, CD27, and CD38. CD19 is expressed on all stage of B cell maturation including fully mature plasma cells (Odendahl et al., 2005). It is a cell surface molecule that forms B cell co-receptor complex. This complex enables to interactions with the B cell receptor, thus enhances B cell activation (Delves et al., 2011; Zikherman and Weiss, 2009). CD27 or tumor necrosis factor (TNF) receptor family is commonly used marker for human memory B cells (Agematsu et al., 2000). It is a receptor that promotes differentiation and survival of B cells (Borst et al., 2005; Darce et al., 2007; Lens et al., 1995). CD38 is expressed on germinal centers and ASCs. It is an enzyme that hydrolysis of cyclic adenosine diphosphate ribose (cADPR) for regulation of calcium mobilization and promote signal transduction (Deaglio et al., 2001; Malavasi et al., 2006; Morabito et al., 2006). A few markers have been used to

identify ASC subsets. These include mAbs against CD138, Ki-67, and HLA-DR. Previous studies used CD138 as a plasma cell marker (Jego et al., 2001; MacLennan et al., 2003). But later on, it was demonstrated that CD138 is expressed not only plasma cells but also plasmablasts (Qian et al., 2010). Recently, intracellular signaling molecule Ki-67 was used to differentiate plasma cell from plasmablast since Ki-67 is expressed in a proliferating plasmablast, not a plasma cell (Qian et al., 2010; Wrammert et al., 2008). Another marker, HLA-DR is also commonly used to identify ASC subsets (Jacobi et al., 2010; Odendahl et al., 2005). HLA-DR is known as one of three different types of MHC class II, which play important role in antigen presentation to T cells (Delves et al., 2011; Murphy et al., 2008). It is expressed on naïve B cells, memory B cells, and plasmablasts but not plasma cells (Murphy et al., 2008). (Table 1)

Table 1. Phenotypic markers of B cell profiles (Fink, 2012; Jacobi et al., 2010; Odendahl et al., 2005; Wrammert et al., 2008)

Markers	Function	Naïve B cells	Memory B cells	ASCs	
				Plasmablasts	Plasma cells
CD3	Surface molecule; part of T cell co-receptor complex	-	-	-	-
CD19	Surface molecule; part of B cell co-receptor complex	+	+	Low	Low
CD27	TNF receptor family	-	+	++	++
CD38	Signaling molecule and enzyme; formation of cADPR	-	-	++	++
HLA-DR	MHC class II	High	High	High	Low

Recent advances in flow cytometry and availability of new mAbs, cell surface markers of B cell subsets have been established. Naïve B cells are classified as $CD19^{+}CD27^{-}CD38^{-}$ cells (Moir and Fauci, 2009), memory B cells are classified as $CD19^{+}CD27^{+}CD38^{-}$ cells (Fink, 2012; Moir and Fauci, 2009), ASCs are classified as $CD19^{+}CD27^{+}CD38^{+}$ cells (Wrammert et al., 2008). Even two subsets of ASCs can be differentiated from each other. While plasmablasts express HLA-DR^{high}, plasma cells express HLA-DR^{low} (Jacobi et al., 2010; Moir and Fauci, 2009; Odendahl et al., 2005).

CHAPTER III

MATERIALS AND METHODS

3.1 Reagents

Roswell Park Memorial Institute (RPMI)-1640, collagenase type I, and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Gibco (Grand Island, NY, USA). Fetal calf serum was obtained from HyClone UK Ltd (Northumberland, UK).

3.2 Monoclonal Antibodies

Fluorescence-conjugated mouse anti-human CD3, 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).

3.3 Subject selection and ethical considerations

Periodontal tissues and heparinized peripheral blood samples were obtained from patient with untreated severe chronic periodontitis, patient with treated chronic periodontitis, and subjects with clinically healthy periodontal tissues. Since the project involved human tissues and blood samples, an ethical approval would be required. This ethical approval was obtained from the Ethics committee of the Faculty of Dentistry, Chulalongkorn

University (HREC-DCU 2013-016). Informed consent of each subject was obtained before the operation. All data of subjects were kept securely confidential.

3.4 Periodontal tissue and peripheral blood collection

Periodontal tissues and heparinized peripheral blood samples 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. 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 root length or more). 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. (Figure 3)

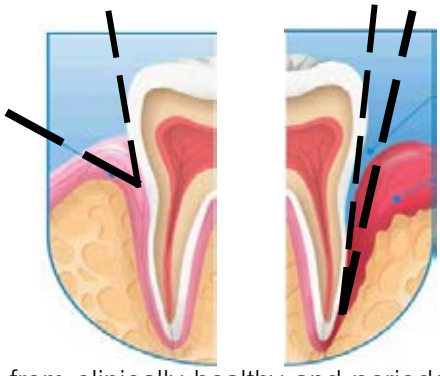
Healthy periodontal tissues  Periodontitis tissues

Figure 3. Periodontal tissue from clinically healthy and periodontitis tissues were prepared by internal bevel incision and intrasulcular incision according to treatment plan. (Adaptation from http://kevinconnellydmd.com/services/periodontal-treatment-and-care.)

We also collected tissue specimens from two patients after initial periodontal therapy (scaling and root planning and oral hygiene instruction). One treated patient with moderate periodontitis responded well to the treatment and returned for the procedure of apically flap surgery. The other treated patient with severe periodontitis (questionable prognosis) did not respond well to the treatment and returned for extraction.

The excised tissues were immediately placed in sterile tubes that contain RPMI-1640 medium. Three milliliters of peripheral blood were collected by nurse at the examination room on 4th floor, Somdejya 93th building. The samples were transferred to the laboratory within a few hours for phenotypic study.

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). 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 analysed for viability by trypan blue exclusion method.

3.6 Flow cytometric analysis

In this experiment, we studied the profiles of different B cells (naïve B cells, memory B cells, and ASCs) in periodontal tissues and heparinized peripheral blood. Isolated gingival cells were stained with anti-human CD3 (PerCP), anti-human CD19 (FITC), anti-human CD27 (PE), and anti-human CD38 (APC) mAbs at 4°C for 30 minutes while whole blood was stained at room temperature for 30 minutes. The stained cells were washed with stain buffer (BD Biosciences), treated with red blood cell lysing solution (FACs Lysing Solution, BD Biosciences) for 10 minutes at room temperature in the dark, washed, and then fixed with 1% paraformaldehyde. Analysis of flow cytometry samples were performed by four-color flow cytometry, FACSCalibur (BD Biosciences). $CD19^+CD3^-$ cells were gated and then analyzed for the expressions of CD27 and CD38 (Figure 4).

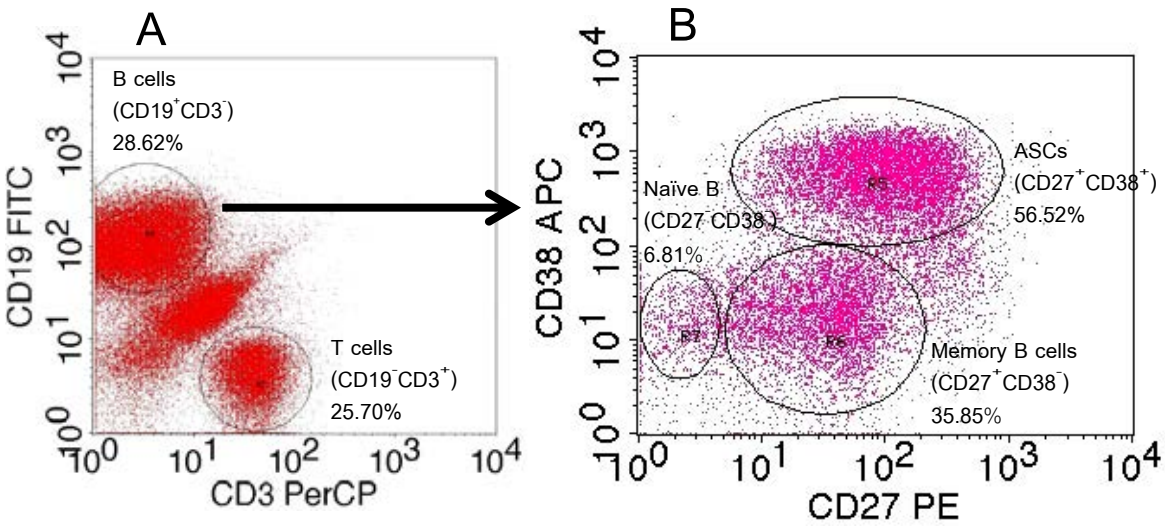


Figure 4. Flow cytometric analysis of cells from periodontitis tissue. Naïve B cells were classified as $CD19^{+}CD27^{-}CD38^{-}$, memory B cells were classified as $CD19^{+}CD27^{+}CD38^{-}$, and ASCs were classified as $CD19^{+}CD27^{+}CD38^{+}$ (B). PerCP, Peridinin Chlorophyll Protein complex; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; APC, Allophycocyanine.

3.7. HLA-DR staining

We investigated ASC subsets, plasmablasts and plasma cells in some tissue specimens. It is known that plasmablast is a dividing ASC and express MHC class II (HLA-DR) while plasma cell is non-dividing terminally differentiated ASC with no MHC expression (Murphy et al., 2008; Oracki et al., 2010; Tarlinton et al., 2008). For HLA-DR staining, gingival cells were stained with anti-human CD19 (FITC), anti-human CD27 (PE), anti-human CD38 (APC), and anti-human HLA-DR (PerCP) mAbs. Mouse isotype IgG1 mAb used as control. Cells were stained at 4°C for 30 minutes, treated with red blood cell lysing solution, washed, fixed, and analyzed by flow cytometry.

Table 2. Monoclonal antibodies used for flow cytometric analysis

Monoclonal antibodies	Populations
$CD3^{+}CD19^{-}$	T cells
$CD3^{-}CD19^{+}$	B cells
$CD19^{+}CD27^{-}CD38^{-}$	Naïve B cells
$CD19^{+}CD27^{+}CD38^{-}$	Memory B cells
$CD19^{+}CD27^{+}CD38^{++}HLA\text{-}DR^{high}$	Plasmablasts
$CD19^{+}$ $CD27^{+}CD38^{++}HLA\text{-}DR^{low}$	Plasma cells

3.8 Statistical analysis

The data were analyzed using the computer program SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Results were presented as means ± S.E. The nonparametric Mann-Whitney's U-test was used to determine the differences of percentages of B cells and T cells, and percentages of B cell subsets between periodontitis and healthy groups. The Wilcoxon Ranks Sum test was used to determine the differences between percentages of B cells and T cells, and between percentages of B cell subsets in each groups. A critical level of 0.05 was employed. Thus, p-values less than 0.05 were considered as statistically significant.

CHAPTER IV

RESULTS

In the present study, each severe periodontitis patient had a loss of clinical attachment of 5 mm or more with loss of alveolar bone of 50% or more of root length. Each healthy periodontal subject had a probing depth less than 4 mm without loss of clinical attachment level and alveolar bone.

4.1 Phenotypic characterization of B cells and T cells in periodontal tissues

Gingival cells were extracted from periodontal specimens of severe periodontitis patients and healthy periodontal subjects. Infiltrated B cells and T cells were identified by anti-CD19 and anti-CD3 mAbs, respectively and analyzed by flow cytometry. Mean percentages of B cells and T cells in periodontitis and healthy periodontal tissue are presented in Figure 5A. In periodontitis tissue, the mean percentage of B cells (34.41 ± 4.38%) was significantly higher than T cells (24.98 ± 3.07%) ($p < 0.05$) with a B cell to T cell ratio of 1.5 : 1. On the other hand, the mean percentage of B cells (6.12 ± 1.17%) in healthy periodontal tissue was significantly lower than T cells (34.78 ± 2.57%) ($p < 0.05$) with a B cell to T cell ratio of 1 : 6. Comparison of infiltrated B cells and T cells between the two clinical groups reveals a significant higher mean percentage of infiltrated B cells and a significant lower mean percentage of infiltrated T cells in severe periodontitis group ($p < 0.05$, Figure 5A). In addition, peripheral blood samples were obtained from severe

periodontitis patients and clinically healthy periodontal subjects and analyzed for B cells and T cells. We found no difference in mean percentages of circulating B cells (14.14 ± 1.12%) and T cells (50.90 ± 3.55%) in periodontitis patients as compared to those from healthy subjects (mean B cells was 8.84 ± 1.31%; mean T cells was 53.01 ± 4.48%).

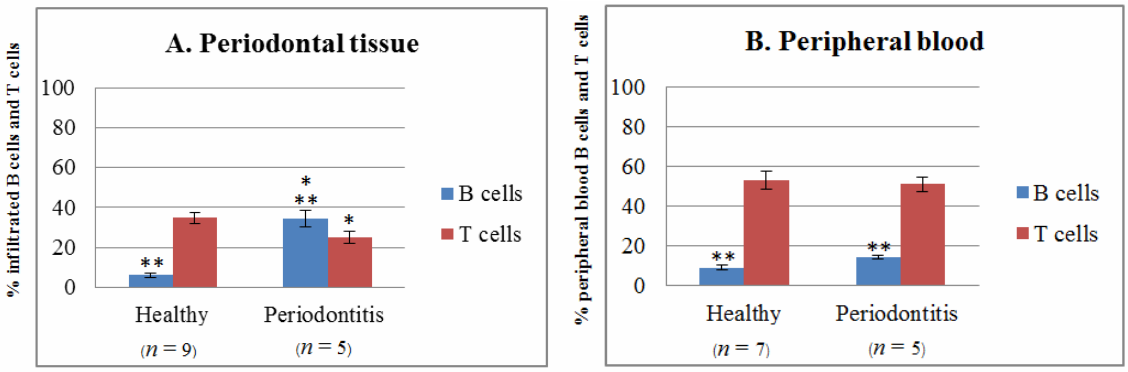


Figure 5. Mean percentages of infiltrated B cells and T cells in periodontal tissue and in peripheral blood. Cells extracted from periodontal tissue (A) and peripheral blood (B) in healthy and periodontitis were stained with mAbs specific to B cells and T cells, and then analyzed by flow cytometry. Data were presented as mean ± S.E.

*, $p < 0.05$, periodontitis group compared with healthy group

**, $p < 0.05$, B cells compared with T cells in each group

We also assessed infiltrated B cells and T cells from gingival tissues obtained from two treated patients who had received full-mouth scaling and root planing. One treated patient with moderate periodontitis responded well to the treatment and returned for the procedure of apically positioned flap surgery. The other treated patient with severe periodontitis did not responded well to the treatment and returned for extraction. After

plaque bacteria removal, the profiles of B cells and T cells in tissue were similar to those in healthy tissue with the B : T cell ratio of 1 : 9 and 1 : 3, respectively (Figure 6).

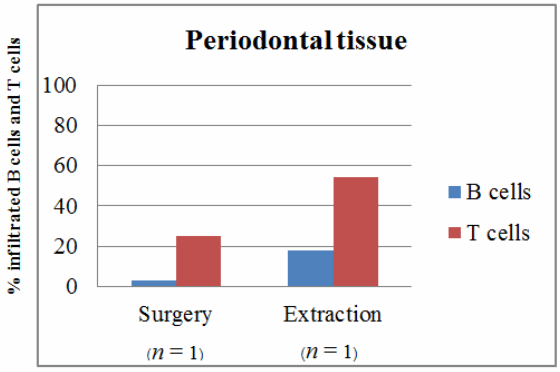


Figure 6. Percentages of infiltrated B cells and T cells in treated periodontal patients. Extracted gingival cells from flap surgical site and extraction site were stained with mAbs specific to B cells and T cells, and then analyzed by flow cytometry.

4.2 Phenotypic characterization of B cell subsets in periodontal tissues

Infiltrated B cells from each periodontal tissue specimens were categorized into three subsets: 1) ASCs ($CD19^{+}CD27^{+}CD38^{+}$), 2) memory B cells ($CD19^{+}CD27^{+}CD38^{-}$), and 3) naïve B cells ($CD19^{+}CD27^{-}CD38^{-}$). In all periodontitis tissues, infiltrated ASCs were the major cell type (58.44 ± 3.79%). The mean percentage of ASCs was significantly higher than memory B cells ($p < 0.05$) with the ratio of 1.5 : 1. In all healthy tissues, memory B cells were the majority (86.59 ± 1.29%). The mean percentage of memory B cells was significantly higher than ASCs ($p < 0.05$) with ratio of 13 : 1. Comparison of each B cell subset between the two clinical groups reveals that a significantly higher mean percentage of ASCs was observed in a periodontitis group ($p < 0.05$), whereas a significant higher

memory B cells was observed in a healthy group ($p < 0.05$). Very few naïve B cells (less than 7%) were detected in both groups (Figure 7A).

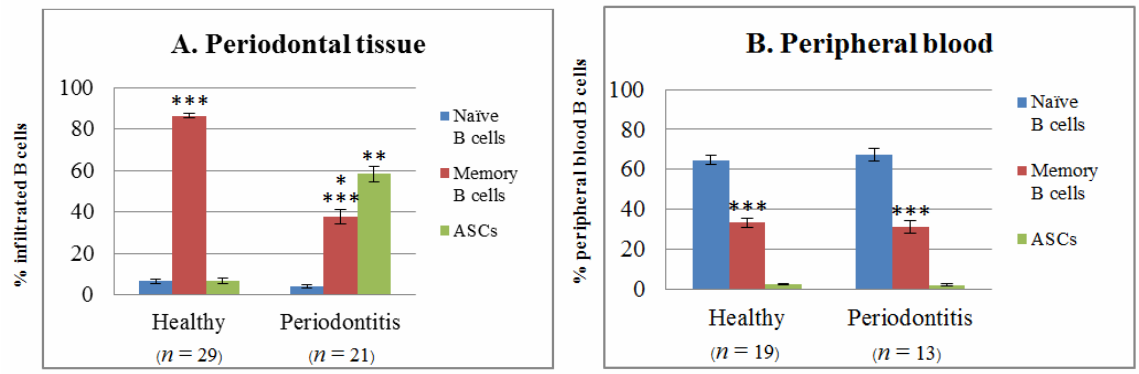


Figure 7. Mean percentage of B cell profiles in periodontal tissue and peripheral blood. Cells extracted from periodontal tissues (A) and peripheral blood (B) in healthy and periodontitis were stained with mAbs specific to naïve B cells, memory B cells, and ASCs, and then analyzed by flow cytometry. Data were presented using mean ± S.E.

*, $p < 0.05$, memory B cells in periodontitis group compared with healthy group

**, $p < 0.05$, ASCs in periodontitis group compared with healthy group

***, $p < 0.05$, memory B cells compared with ASCs in each group

Profiles of circulating B cell subsets in periodontitis patients and healthy subjects were also evaluated. No differences were observed between the two groups with naïve B cells were the majority in both healthy (64.52 ± 2.35%) and in periodontitis group (67.24 ± 3.18%) (Figure 7B). The mean percentage of naïve B cells was significantly higher than memory B cells ($p < 0.05$) in healthy (33.24 ± 2.31%) and in periodontitis group (30.85 ± 3.16%). Very few ASCs (less than 3%) were detected in both groups (Figure 7B).

4.3 Phenotypic characterization of ASC subsets in periodontitis tissues

Both plasmablasts and plasma cells are capable of secreting antibodies (Delves et al., 2011; Murphy et al., 2008). Plasma cells express very low level of HLA-DR (Jacobi et al., 2010; Murphy et al., 2008; Odendahl et al., 2005). The levels of HLA-DR expression were then used to differentiate the two cell types in periodontitis tissues. It was found that ASCs in severe periodontitis tissues expressed significantly lower levels of HLA-DR (mean MFI (mean fluorescence intensity) 37.62 ± 7.54, $n = 10$) than naïve B cells (mean MFI 294.41 ± 27.28, $n = 10$, $p < 0.05$) and memory B cells (mean MFI 271.19 ± 33.06, $n = 10$, $p < 0.05$) suggesting that the infiltrated ASCs were plasma cells (Figures 8 and Table 3). On the other hand, circulating ASCs in peripheral blood showed high expression of HLA-DR (mean MFI was 229.47 ± 31.46, $n = 5$), a marker of plasmablasts (Figure 9 and Table 3).

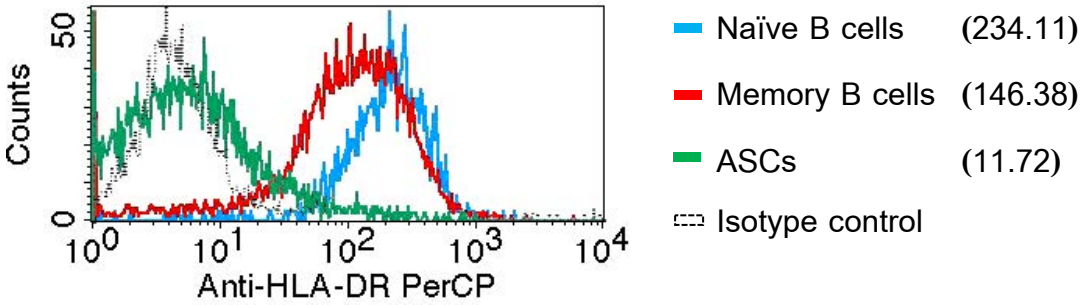


Figure 8. Flow cytometric analysis of HLA-DR expression on infiltrated B cell subsets. Cells extracted from periodontitis tissue were stained with mAbs specific to naïve B cells, memory B cells, and ASCs. Overlay histogram represents HLA-DR expression by ASCs (green line) compared with naïve B cells (blue line), memory B cells (red line), and Isotype control (gray dash line). Number in parentheses represents MFI of HLA-DR expression.

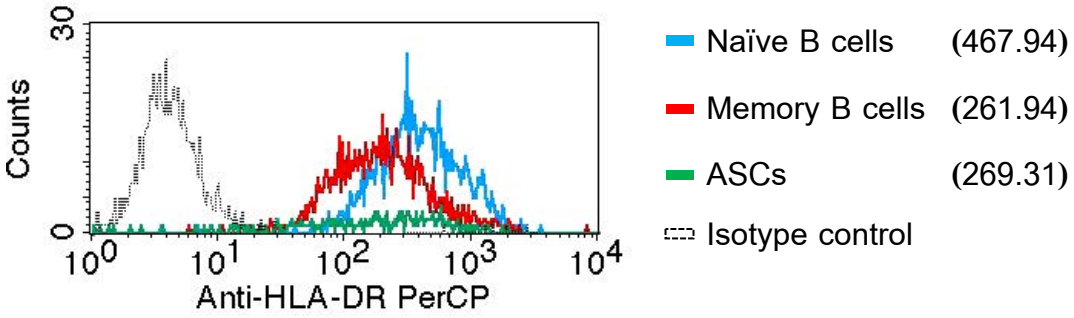


Figure 9. Flow cytometric analysis of HLA-DR expression on peripheral blood B cell subsets. Cells extracted from whole blood B cells of periodontitis patients were stained with mAbs specific to naïve B cells, memory B cells, and ASCs. Overlay histogram represents HLA-DR expression by ASCs (green line) compared with naïve B cells (blue line), memory B cells (red line), and Isotype control (gray dash line). Number in parentheses represents MFI of HLA-DR expression.

Table 3. Mean ± S.E. of MFI of HLA-DR expression on infiltrated B cell subsets from periodontitis tissues and peripheral blood of periodontitis patients.

B cell subsets	MFI of HLA-DR expression (Mean ± S.E.)	
	Periodontitis tissue (*n* = 10)	Peripheral blood (*n* = 5)
Naïve B cells	294.41 ± 27.28	288.71 ± 24.29
Memory B cells	271.19 ± 33.06	196.00 ± 16.54
ASCs	37.62 ± 7.54	229.47 ± 31.46

CHAPTER V

DISCUSSION AND CONCLUSION

Hallmark of periodontitis has been characterized by dense infiltration of immune cells including B cells and T cells, all of which play critical role in immunopathogenesis of the disease. The present study was carried out to investigate B cell profiles including naïve B cells, memory B cells, and ASCs in inflamed periodontal tissues from patients with severe chronic periodontitis. We confirmed the previously results describing B cell-dominated lesion in periodontitis and T cell-dominated lesion in healthy periodontal tissue (Seymour et al., 1979). Similar to previous studies, our results showed that the B cell to T cell ratio were 1.5 : 1 in periodontitis tissues (Amunulla et al., 2008; Lappin et al., 1999) and 1: 6 in healthy tissues (Lappin et al., 1999). In this study, peripheral blood B cells and T cells were used as control. Similar profiles of circulating B cells and T cells were observed in periodontitis patients and healthy periodontal subjects with the B cell to T cell ratio of 1 : 5. This ratio is within norm of the general population supported by other groups (Bisset et al., 2004; Blum and Pabst, 2007; Chng et al., 2004; Reichert et al., 1991). Hence, different profiles of infiltrated B cells and T cells evidenced in periodontitis tissue reflect active local immune response.

In this study, treated tissues were collected from two periodontitis patients. One patient had fair prognosis which required flap surgical procedure, while the other had questionable prognosis which required extraction. After initial periodontal therapy (removal of bacterial plaque biofilm), both showed reduced gingival inflammation. Unlike untreated

periodontitis tissue, treated tissues mainly consisted of infiltrated T cells. Our finding suggested that the initial treatment has reduced stimulatory effects from plaque bacteria, resulting in less inflammation and the ratio of B : T cell shifts towards healthy condition. However, it could not be concluded due to limitation in the number of treated periodontal tissues (n = 2) and more analysis should be performed.

In the present study, the identification techniques for B cell subsets have been refined. Double staining by anti-CD19 and anti-CD3 mAbs was selected to characterize B cells ($CD19^{+}CD3^{-}$). In the past, Gemmell and Seymour (1991), Lappin et al, (1999), and Amunulla et al. (2008) used anti-CD20 mAb for immunostaining of infiltrated B cells in periodontitis patients. CD20 is a surface molecule expressed in most B cells but not on ASCs (DiLillo et al., 2008; Edwards and Cambridge, 2006; Mason et al., 1990). In contrast, CD19 is expressed in all B cell subsets including ASCs, therefore it's a proper marker for B cell population. Previous studies of infiltrated B cells in periodontal disease focused mainly on activation stage, and so far there has been no study with a complete analysis of all B cell subsets such as naïve B cells, memory B cells, and ASCs. Among these three populations, we found that ASCs ($CD19^{+}CD27^{+}CD38^{+}$) were the major cell type in severe chronic periodontitis tissue whereas memory B cells ($CD19^{+}CD27^{+}CD38^{-}$) were the major cell type in healthy periodontal tissues. Both clinical groups demonstrated low levels of infiltrated naïve B cells ($CD19^{+}CD27^{-}CD38^{-}$). It has been previously shown that ASCs were present in periodontitis tissues however, it's not clear if these cells are plasmablasts or plasma cells. In this study, HLA-DR expression was first used to differentiate the two cell types in periodontitis tissues. Our findings clearly showed that the observed infiltrated ASCs were plasma cells with low HLA-DR expression compared to naïve B cells and memory B cells. It

is known that most plasma cells reside in bone marrow and in inflamed tissues (Delves et al., 2011; Murphy et al., 2008; Radbruch et al., 2006; Tarlinton et al., 2008). At present, it is not clear if periodontal tissue plasma cells are derived from antigen stimulation of naïve B cells or memory B cells in secondary lymphoid tissues such as lymph nodes or in resident periodontal tissue. It should be noted that the majority of infiltrated B cells in healthy periodontal tissues were memory B cells, thus suggesting that these observed plasma cells may be in part derived from local memory B cells. However, further research in this area is needed.

Plasma cells are considered to produce and secrete large amounts of antibodies constitutively. There were previous reports of high levels of IgG and IgA in periodontal tissues as well as in gingival crevicular fluid of periodontitis patients (Kono et al., 1991; Ogawa et al., 1989b). The local antibodies showed specificity to P. gingivalis and A. actinomycetemcomitans (Ebersole et al., 2000; Engström et al., 1999; Ogawa et al., 1989a; Plombas et al., 2002). However, little is known about the function of the predominance periodontal plasma cells in periodontitis, particularly whether their role in disease pathogenesis or protection. In human gut, it was demonstrated that IgG^+ and IgA^+ ASCs generated antigen-specific antibodies against commensal and enteropathogenic microbes, play critical role in intestinal homeostasis (Benckert et al., 2011).

The presence of human memory T cells in healthy tissue has recently been demonstrated in different organs including skin, lung, and gut (Clark et al., 2006; Masopust et al., 2010; Purwar et al., 2011). These T cells are thought to play a crucial role in host protection especially against viral pathogens (Ariotti et al., 2012; Casey et al., 2012; Teijaro et al., 2011). However, the study of tissue memory B cells is limited. To the best of our

knowledge, the present study is the first to show memory B cells in healthy human periodontal tissues. At least one study in mice demonstrated the presence of lung memory B cells persisted for five months after influenza infection. These memory B cells were capable of secreting neutralizing antibodies against influenza virus (Onodera et al., 2012). It would be interesting to elucidate the functional role of memory B cells in periodontal tissues.

In conclusion, our study confirmed previous reports of large number of infiltrated B cells in periodontitis. We first used HLA-DR expression as a marker and identified these infiltrates to be plasma cells and not plasmablasts. We also first identified the presence of memory B cells in healthy periodontal tissues. Further study on these B cell subsets should provide a better insight into their role either in periodontal homeostasis or protection.

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APPENDIX

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

No.	Sex	Age (years)	Tooth No.	PD (mm)	BOP
1	Female	46	#48	2-3	-
2	Female	47	#23	2-3	-
3	Female	62	#25	2-3	-
4	Female	9	-	2-3	-
5	Female	56	#47	2-3	-
6	Female	57	#34	2-3	-
7	Female	42	#47	2-3	-
8	-	-	-	2-3	-
9	Female	59	#47	2-3	-
10	Female	23	#14-24	2-3	-
11	Female	24	#35-37	2-3	-
12	Female	60	#36	2-3	-
13	Male	68	#11	2-3	-
14	Female	31	#13-23	2-3	-
15	Female	66	#24-25	2-3	-
16	Female	37	#47	2-3	-
17	Female	52	#13-15	2-3	-
18	Female	30	#37	2-3	-
19	Male	21	#46	2-3	-
20	Female	51	#25	2-3	-
21	Female	33	#36	2-3	-
22	Female	17	#38	2-3	-
23	Male	62	#44-46	2-3	-
24	Female	31	#14-24	2-3	-
25	Male	65	#23	2-3	-
26	Male	62	#35-45	2-3	-
27	Male	13	#11	2-3	-
28	Female	48	#24	2-3	-
29	Male	55	#25	2-3	-

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	Male	60	#37	-	-	Severe bone loss (>50%)	-
2	Female	48	#27	8-9	11-13	Severe bone loss (>50%)	MO:1 FI:2
3	Male	47	#17	6-9	7-9	Severe bone loss (>50%)	MO:3 FI:1
4	Male	35	#37	-	-	Severe bone loss (>50%)	MO:3
5	Female	66	#16	8-10	9-14	Severe bone loss (>50%)	MO:2 FI:1-3
6	Female	41	#18	5-8	5-7	Severe bone loss (>50%)	FI:1
7	Female	46	#47	10-12	11-13	Severe bone loss (>50%)	MO:3 FI:2
8	Female	60	#17	5-7	8-11	Severe bone loss (>50%)	MO:2 FI:2
9	Male	-	-	-	-	Severe bone loss (>50%)	
10	-	-	-	-	-	Severe bone loss (>50%)	
11	Female	65	#47	5-12	5-13	Severe bone loss (>50%)	MO:3
12	Male	42	#26	5-7	7-10	Severe bone loss (>50%)	MO:2 FI:2
13	Male	50	#27	8-11	10-14	Severe bone loss (>50%)	MO:1 FI:1
14	Female	40	#27	5-10	-	Severe bone loss (>50%)	-

No.	Sex	Age (years)	Tooth No.	Clinical examination			
				PD (mm)	CAL (mm)	Bone loss	Others
15	Female	46	#27	5-10	8-15	Severe bone loss (>50%)	MO:1 FI:4
16	Male	66	#42	5-8	6-11	Severe bone loss (>50%)	MO:2
17	Female	42	#47	6-9	4-9	Severe bone loss (>50%)	MO:1 F:2
18	Female	61	#12, 22-23	-	-	Severe bone loss (>50%)	-
19	Female	45	#48	5-6	5-6	Severe bone loss (>50%)	MO:1 F:1
20	Male	65	#27	-	-	Severe bone loss (>50%)	-
21	Male	53	#46-47	6-12	10-13	Severe bone loss (>50%)	MO:2-3 F:3-4
				After hygienic phase (Periodontal tissue after treatment)			
1	Female	57	#28 Surgery	4-9	7-12	Moderate bone loss (25-50%)	MO:1
2	Female	52	#17 Extraction	8-10	8-10	Severe bone loss (>50%)	MO:3 F:2-3

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 : Phenotypic characterization of Infiltrated B cells and T cells in periodontal tissues

	No.	Tooth No.	Infiltrated B cells and T cells in periodontal tissue (%)	
			B cells	T cells
Healthy	1	#48	4.99	24.17
	2	#23	5.38	26.08
	3	#25	4.36	39.02
	4	-	5.06	38.92
	5	#47	9.40	31.43
	6	#34	6.77	29.46
	7	#47	3.68	34.81
	8	-	1.79	47.89
	9	#47	13.66	41.25
		Mean ± S.E.	6.12 ± 1.17	34.78 ± 2.57
Periodontitis	1	#37	37.53	34.24
	2	#17	26.01	21.20
	3	#37	28.62	25.70
	4	#16	50.14	16.02
	5	#47	29.73	27.76
		Mean ± S.E.	34.41 ± 4.38	24.98 ± 3.07
Treatment	1	#28 Surgery	2.70	24.99
	2	#17 Extraction	17.80	54.45

Descriptive statistics of percentages of B cells and T cells in periodontitis and healthy groups

	Healthy B cells	Healthy T cells	Periodontitis B cells	Periodontitis T cells
N	9	9	5	5
Mean	6.12	34.78	34.41	24.98
Std. Error of Mean	1.17	2.57	4.38	3.07
Std. Deviation	3.51	7.72	9.79	6.87
Minimum	1.79	24.17	26.01	16.02
Maximum	13.66	47.89	50.14	34.24

Mann-Whitney's U-test results of differences of percentages of B cells and T cells between periodontitis and healthy groups

	Groups	N	Mean Rank	Sum of Ranks
Percentage of B cells	Healthy	9	5.00	45.00
	Periodontitis	5	12.00	60.00
	Total	14		
Percentage of T cells	Healthy	9	9.22	83.00
	Periodontitis	5	4.40	22.00
	Total	14		

Test Statistics[b]

	Percentage of B cells	Percentage of T cells
Mann-Whitney U	.000	7.000
Wilcoxon W	45.000	22.000
Z	-3.000	-2.067
Asymp. Sig. (2-tailed)	.003	.039
Exact Sig. [2*(1-tailed Sig.)]	.001[a]	.042[a]
Exact Sig. (2-tailed)	.001	.042
Exact Sig. (1-tailed)	.000	.021
Point Probability	.000	.006

a. Not corrected for ties. b. Grouping Variable: Groups

Wilcoxon Ranks Sum test results of differences between percentages of B cells and T cells in periodontitis and healthy groups

		N	Mean Rank	Sum of Ranks
Healthy B cells – T cells	Negative Ranks	0[a]	.00	.00
	Positive Ranks	9[b]	5.00	45.00
	Ties	0[c]		
	Total	9		
Periodontitis B cells - T cells	Negative Ranks	5[d]	3.00	15.00
	Positive Ranks	0[e]	.00	.00
	Ties	0[f]		
	Total	5		

a. Healthy_B cells < T cells b. Healthy_B cells > T cells c. Healthy_B cells = T cells

d. Periodontitis_B cells < T cells e. Periodontitis_B cells > T cells f. Periodontitis_B cells = T cells

Test Statistics

	Healthy B cells - T cells	Periodontitis B cells - T cells
Z	-2.666[a]	-2.023[b]
Asymp. Sig. (2-tailed)	.008	.043
Exact Sig. (2-tailed)	.004	.063
Exact Sig. (1-tailed)	.002	.031
Point Probability	.002	.031

a. Based on negative ranks. b. Based on positive ranks.

Appendix D : Phenotypic characterization of peripheral blood B cells and T cells

	No.	Tooth No.	Peripheral blood B cells and T cells (%)	
			B cells	T cells
Healthy	1	#48	11.16	44.09
	2	#23	6.09	42.99
	3	#25	4.28	35.32
	4	#47	8.04	65.21
	5	#34	9.73	60.38
	6	#47	7.73	62.45
	7	#35-37	14.84	60.60
		Mean ± S.E	8.84 ± 1.31	53.01 ± 4.48
Periodontitis	1	#37	11.37	60.69
	2	#16	17.37	40.04
	3	#18	13.58	47.75
	4	#47	16.02	49.83
	5	#17	12.36	56.18
		Mean ± S.E.	14.14 ± 1.12	50.90 ± 3.55

Descriptive statistics of percentages of B cells and T cells in periodontitis and healthy groups

	Healthy B cells	Healthy T cells	Periodontitis B cells	Periodontitis T cells
N	7	7	5	5
Mean	8.84	53.01	14.14	50.90
Std. Error of Mean	1.31	4.48	1.12	3.55
Std. Deviation	3.47	11.85	2.50	7.95
Minimum	4.28	35.32	11.37	40.04
Maximum	14.84	65.21	17.37	60.69

Mann-Whitney's U-test results of differences of percentages of B cells and T cells between periodontitis and healthy groups

	Groups	N	Mean Rank	Sum of Ranks
Percentage of B cells	Healthy	7	4.43	31.00
	Periodontitis	5	9.40	47.00
	Total	12		
Percentage of T cells	Healthy	7	6.86	48.00
	Periodontitis	5	6.00	30.00
	Total	12		

Test Statistics[b]

	Percentage of B cells	Percentage of T cells
Mann-Whitney U	3.000	15.000
Wilcoxon W	31.000	30.000
Z	-2.355	-.406
Asymp. Sig. (2-tailed)	.019	.685
Exact Sig. [2*(1-tailed Sig.)]	.018[a]	.755[a]
Exact Sig. (2-tailed)	.018	.755
Exact Sig. (1-tailed)	.009	.378
Point Probability	.004	.058

a. Not corrected for ties. b. Grouping Variable: Groups

Wilcoxon Ranks Sum test results of differences between percentages of B cells and T cells in periodontitis and healthy groups

		N	Mean Rank	Sum of Ranks
Healthy B cells – T cells	Negative Ranks	0[a]	.00	.00
	Positive Ranks	7[b]	4.00	28.00
	Ties	0[c]		
	Total	7		
Periodontitis B cells - T cells	Negative Ranks	0[d]	.00	.00
	Positive Ranks	5[e]	3.00	15.00
	Ties	0[f]		
	Total	5		

a. Healthy_B cells < T cells b. Healthy_B cells > T cells c. Healthy_B cells = T cells

d. Periodontitis_B cells < T cells e. Periodontitis_B cells > T cells f. Periodontitis_B cells = T cells

Test Statistics[b]

	Healthy B cells - T cells	Periodontitis B cells - T cells
Z	-2.366[a]	-2.023[a]
Asymp. Sig. (2-tailed)	.018	.043
Exact Sig. (2-tailed)	.016	.063
Exact Sig. (1-tailed)	.008	.031
Point Probability	.008	.031

a. Not corrected for ties. b. Grouping Variable: Groups

Appendix E : Phenotypic characterization of Infiltrated B cell subsets in periodontal tissues

	No.	Tooth No.	Infiltrated B cell subsets in periodontal tissues (%)		
			Naïve B cells	Memory B cells	ASCs
Healthy	1	#48	5.95	83.54	10.10
	2	#23	0.0	98.35	0.83
	3	#25	2.96	88.76	8.28
	4	-	12.26	84.63	2.92
	5	#47	3.81	94.25	1.94
	6	#34	7.25	84.10	8.65
	7	#47	6.86	85.39	6.86
	8	-	18.75	76.56	4.69
	9	#47	11.26	86.67	2.07
	10	#14-24	3.22	96.37	0.32
	11	#35-37	15.13	83.92	0.90
	12	#36	5.79	90.63	3.60
	13	#11	3.32	76.63	20.18
	14	#13-23	3.86	94.86	1.29
	15	#24-25	4.35	90.90	4.65
	16	#47	2.65	86.17	11.17
	17	#13-15	3.62	88.79	7.59
	18	#37	5.48	80.74	13.78
	19	#46	5.63	84.51	9.86
	20	#25	13.04	80.75	4.97
	21	#36	2.25	84.24	13.34
	22	#38	2.00	68.00	29.43
	23	#44-46	9.94	86.55	3.51
	24	#14-24	1.80	94.41	3.59
	25	#23	1.98	93.07	4.95
	26	#35-45	2.26	95.58	2.16
	27	#11	14.47	84.47	0.26

	No.	Tooth No.	Infiltrated B cell subsets in periodontal tissues (%)		
			Naïve B cells	Memory B cells	ASCs
	28	#24	4.18	90.92	4.90
	29	#25	16.97	77.37	5.84
		Mean ± S.E.	6.59 ± 0.95	86.59 ± 1.29	6.64 ± 1.19
Periodontitis	1	#37	4.74	32.08	63.34
	2	#27	3.92	33.29	62.36
	3	#17	3.03	32.99	63.94
	4	#37	6.81	35.85	56.52
	5	#16	13.83	42.43	43.49
	6	#18	7.18	39.94	52.16
	7	#47	6.56	51.60	41.53
	8	#17	14.50	55.70	29.76
	9	-	1.99	54.34	43.26
	10	-	1.65	19.11	79.04
	11	#47	0.58	3.54	95.36
	12	#26	0.83	43.49	55.52
	13	#27	1..23	44.43	54.38
	14	#27	1.47	40.97	57.46
	15	#27	2.61	62.31	35.10
	16	#11-21	0.64	24.25	74.51
	17	#47	0.11	10.45	89.25
	18	#12	5.63	55.23	38.82
	19	#28	0.82	27.48	71.77
	20	#27	1.72	27.42	70.76
	21	#46-47	1.25	54.20	48.99
		Mean ± S.E.	4.00 ± 0.88	37.67 ± 3.39	58.44 ± 3.79

Descriptive statistics of percentage of B cell subsets in periodontitis and healthy groups

	Healthy Naïve B cells	Healthy Memory B cells	Healthy ASCs	Periodontitis Naïve B cells	Periodontitis Memory B cells	Periodontitis ASCs
N	29	29	29	21	21	21
Mean	6.59	86.59	6.64	4.00	37.67	58.44
Std. Error of Mean	0.95	1.29	1.19	0.88	3.39	3.79
Std. Deviation	5.12	6.94	6.40	4.03	15.52	17.38
Minimum	0.00	68.00	0.26	0.11	3.54	29.76
Maximum	18.75	98.35	29.43	14.50	64.31	95.36

Mann-Whitney's U-test results of differences of percentage of B cell subsets between periodontitis and healthy groups

	Groups	N	Mean Rank	Sum of Ranks
Naïve B cells	Healthy	29	29.36	851.50
	Periodontitis	21	20.17	423.50
	Total	50		
Memory B cells	Healthy	29	36.00	1044.00
	Periodontitis	21	11.00	231.00
	Total	50		
ASCs	Healthy	29	15.00	435.00
	Periodontitis	21	40.00	840.00
	Total	50		

Test Statistics[a]

	Naïve B cells	Memory B cells	ASCs
Mann-Whitney U	192.500	.000	.000
Wilcoxon W	423.500	231.000	435.000
Z	-2.202	-5.985	-5.985
Asymp. Sig. (2-tailed)	.028	.000	.000
Exact Sig. (2-tailed)	.027	.000	.000
Exact Sig. (1-tailed)	.014	.000	.000
Point Probability	.000	.000	.000

a. Grouping Variable: Groups

Wilcoxon Ranks Sum test results of differences between percentage of B cell subsets in periodontitis and healthy groups

		N	Mean Rank	Sum of Ranks
Healthy Memory B cells – Naïve B cells	Negative Ranks	0[a]	.00	.00
	Positive Ranks	29[b]	15.00	435.00
	Ties	0[c]		
	Total	29		
Healthy ASCs – Naïve B cells	Negative Ranks	13[d]	15.92	207.00
	Positive Ranks	15[e]	13.27	199.00
	Ties	1[f]		
	Total	29		
Healthy ASCs – Memory B cells	Negative Ranks	29[g]	15.00	435.00
	Positive Ranks	0[h]	.00	.00
	Ties	0[i]		
	Total	29		
Periodontitis Memory B cells – Naïve B cells	Negative Ranks	0[j]	.00	.00
	Positive Ranks	21[k]	11.00	231.00
	Ties	0[l]		
	Total	21		
Periodontitis ASCs – Naïve B cells	Negative Ranks	0[m]	.00	.00
	Positive Ranks	21[n]	11.00	231.00
	Ties	0[o]		
	Total	21		
Periodontitis ASCs – Memory B cells	Negative Ranks	6[p]	7.00	42.00
	Positive Ranks	15[q]	12.60	189.00
	Ties	0[r]		
	Total	21		

a. Healthy_ Memory B cells < Naïve B cells b. Memory B cells > Naïve B cells c. Memory B cells = Naïve B cells
d. Healthy_ASCs < Naïve B cells e. ASCs > Naïve B cells f. ASCs = Naïve B cells
g. Healthy_ASCs < Memory B cells h. ASCs > Memory B cells i. ASCs = Memory B cells
j. Periodontitis_ Memory B cells < Naïve B cells k. Memory B cells > Naïve B cells l. Memory B cells = Naïve B cells
m. Periodontitis_ASCs < Naïve B cells n. ASCs > Naïve B cells o. ASCs = Naïve B cells
p. Periodontitis_ASCs < Memory B cells q. ASCs > Memory B cells r. ASCs = Memory B cells

Test Statistics

	Healthy Memory B cells - Naïve B cells	Healthy ASCs – Naïve B cells	Healthy ASCs - Memory B cells	Periodontitis Memory B cells - Naïve B cells	Periodontitis ASCs – Naïve B cells	Periodontitis ASCs - Memory B cells
Z	-4.703[a]	-.091[a]	-4.703[b]	-4.015[a]	-4.015[a]	-2.555[a]
Asymp. Sig. (2-tailed)	.000	.927	.000	.000	.000	.011
Exact Sig. (2-tailed)	.000	.937	.000	.000	.000	.009
Exact Sig. (1-tailed)	.000	.469	.000	.000	.000	.005
Point Probability	.000	.009	.000	.000	.000	.001

a. Based on negative ranks. b. Based on positive ranks.

Appendix F : Phenotypic characterization of peripheral blood B cell subsets

	No.	Tooth No.	Peripheral blood B cell subsets (%)		
			Naïve B cells	Memory B cells	ASCs
Healthy	1	#48	41.51	56.98	1.20
	2	#23	61.82	33.58	3.36
	3	#25	71.50	28.25	0.17
	4	#47	46.48	50.68	2.73
	5	#34	71.97	26.96	0.88
	6	#47	63.09	34.87	1.91
	7	#35-37	75.07	22.03	2.75
	8	#36	70.51	28.51	0.98
	9	#11	75.84	20.57	3.48
	10	#13-23	72.57	25.22	2.04
	11	#11	61.66	37.22	1.06
	12	#24-25	79.44	20.06	0.49
	13	#47	62.03	33.79	4.07
	14	#13-15	62.40	34.62	2.97
	15	#37	56.27	41.42	2.14
	16	#46	60.27	35.50	4.11
	17	#25	79.91	18.31	1.54
	18	#44-46	54.75	43.28	1.76
	19	#14-24	59.18	36.86	3.77
	20	#23	51.27	47.46	1.28
	21	#25	77.32	21.82	0.79
		Mean ± S.E.	64.52 ± 2.35	33.24 ± 2.31	2.07 ± 0.27
Periodontitis	1	#37	71.53	27.61	0.65
	2	#16	73.64	20.13	5.98
	3	#18	56.82	42.30	0.83
	4	#47	59.48	39.14	1.40
	5	#17	76.44	21.69	1.84

	No.	Tooth No.	Peripheral blood B cell subsets (%)		
			Naïve B cells	Memory B cells	ASCs
Periodontitis	6	#47	69.49	29.27	1.12
	7	#26	76.09	23.29	0.63
	8	#27	63.13	35.16	1.59
	9	#27	60.33	39.98	0.63
	10	#42	51.70	41.59	6.59
	11	#47	83.44	15.20	1.21
	12	#48	83.44	15.20	1.21
	13	#27	48.62	50.51	1.10
		Mean ± S.E.	67.24 ± 3.18	30.85 ± 3.16	1.91 ± 0.55

Descriptive statistics of percentage of B cell subsets in periodontitis and healthy groups

	Healthy Naïve B cells	Healthy Memory B cells	Healthy ASCs	Periodontitis Naïve B cells	Periodontitis Memory B cells	Periodontitis ASCs
N	21	21	21	13	13	13
Mean	64.52	33.24	2.07	67.24	30.85	1.91
Std. Error of Mean	2.35	2.31	0.27	3.18	3.16	0.55
Std. Deviation	10.81	10.59	1.22	11.45	11.41	1.98
Minimum	41.51	18.31	0.17	48.62	15.20	0.63
Maximum	79.91	56.98	4.11	83.44	50.51	6.59

Mann-Whitney's U-test results of differences of percentage of B cell subsets between periodontitis and healthy groups

	Groups	N	Mean Rank	Sum of Ranks
Naïve B cells	Healthy	21	16.62	349.00
	Periodontitis	13	18.92	246.00
	Total	34		
Memory B cells	Healthy	21	18.10	380.00
	Periodontitis	13	16.54	215.00
	Total	34		
ASCs	Healthy	21	19.05	400.00
	Periodontitis	13	15.00	195.00
	Total	34		

Test Statistics[b]

	Naïve B cells	Memory B cells	ASCs
Mann-Whitney U	118.000	124.000	104.000
Wilcoxon W	349.000	215.000	195.000
Z	-.656	-.443	-1.152
Asymp. Sig. (2-tailed)	.512	.658	.249
Exact Sig. [2*(1-tailed Sig.)]	.529[a]	.675[a]	.261[a]
Exact Sig. (2-tailed)	.523	.668	.257
Exact Sig. (1-tailed)	.261	.334	.129
Point Probability	.005	.006	.004

a. Not corrected for ties. b. Grouping Variable: Groups

Wilcoxon Ranks Sum test results of differences between percentage of B cell subsets in periodontitis and healthy groups

		N	Mean Rank	Sum of Ranks
Healthy Memory B cells – Naïve B cells	Negative Ranks	19[a]	11.79	224.00
	Positive Ranks	2[b]	3.50	7.00
	Ties	0[c]		
	Total	21		
Healthy ASCs – Naïve B cells	Negative Ranks	21[d]	11.00	231.00
	Positive Ranks	0[e]	.00	.00
	Ties	0[f]		
	Total	21		
Healthy ASCs - Memory B cells	Negative Ranks	21[g]	11.00	231.00
	Positive Ranks	0[h]	.00	.00
	Ties	0[i]		
	Total	21		
Periodontitis Memory B cells – Naïve B cells	Negative Ranks	12[j]	7.50	90.00
	Positive Ranks	1[k]	1.00	1.00
	Ties	0[l]		
	Total	13		
Periodontitis ASCs – Naïve B cells	Negative Ranks	13[m]	7.00	91.00
	Positive Ranks	0[n]	.00	.00
	Ties	0[o]		
	Total	13		
Periodontitis ASCs - Memory B cells	Negative Ranks	13[p]	7.00	91.00
	Positive Ranks	0[q]	.00	.00
	Ties	0[r]		
	Total	13		

a. Healthy_ Memory B cells < Naïve B cells b. Memory B cells > Naïve B cells c. Memory B cells = Naïve B cells
d. Healthy_ASCs < Naïve B cells e. ASCs > Naïve B cells f. ASCs = Naïve B cells
g. Healthy_ASCs < Memory B cells h. ASCs > Memory B cells i. ASCs = Memory B cells
j. Periodontitis_ Memory B cells < Naïve B cells k. Memory B cells > Naïve B cells l. Memory B cells = Naïve B cells
m. Periodontitis_ASCs < Naïve B cells n. ASCs > Naïve B cells o. ASCs = Naïve B cells
p. Periodontitis_ASCs < Memory B cells q. ASCs > Memory B cells r. ASCs = Memory B cells

Test Statistics[b]

	Healthy Memory B cells – Naïve B cells	Healthy ASCs – Naïve B cells	Healthy ASC - Memory B cells	Periodontitis Memory B cells – Naïve B cells	Periodontitis ASCs – Naïve B cells	Periodontitis ASCs - Memory B cells
Z	-3.771[a]	-4.015[a]	-4.015[a]	-3.111[a]	-3.181[a]	-3.181[a]
Asymp. Sig. (2-tailed)	.000	.000	.000	.002	.001	.001
Exact Sig. (2-tailed)	.000	.000	.000	.000	.000	.000
Exact Sig. (1-tailed)	.000	.000	.000	.000	.000	.000
Point Probability	.000	.000	.000	.000	.000	.000

a. Based on positive ranks. b. Wilcoxon Signed Ranks Test

Appendix G : MFI of HLA-DR expression on B cell subsets from periodontitis tissues and peripheral blood of periodontitis patients.

No.	Tooth No.	MFI of HLA-DR expression (periodontitis tissues)		
		Naïve B cells	Memory B cells	ASCs
1	#17	234.11	146.38	11.72
2	#25	314.53	444.84	34.56
3	#47	216.63	178.35	15.24
4	#27	264.68	234.35	37.4
5	#27	349.38	405.12	44.95
6	#27	255.23	271.42	45.6
7	#12	508.03	389.76	95.65
8	#28	268.30	234.61	40.45
9	#27	225.02	205.58	17.32
10	#46-47	308.18	201.46	33.32
Mean ± S.E.		294.41 ± 27.25	271.19 ± 33.03	37.62 ± 7.53
No.	Tooth No.	MFI of HLA-DR expression (peripheral blood)		
1	#47	270.43	180.69	228.22
2	#26	324.17	153.86	133.31
3	#27	222.11	176.43	208.26
4	#28	265.53	226.47	249.22
5	#27	361.33	242.56	328.34
Mean ± S.E.		288.71 ± 24.33	196.00 ± 16.57	229.47 ± 31.52

Descriptive statistics of MFI of HLA-DR expression of B cell subsets in periodontitis group

	Periodontitis tissues			Peripheral blood		
	Naïve B cells	Memory B cells	ASCs	Naïve B cells	Memory B cells	ASCs
N	10	10	10	5	5	5
Mean	294.41	271.19	37.62	288.71	196.00	229.47
Std. Error of Mean	27.25	33.03	7.53	24.33	16.57	31.52
Std. Deviation	86.19	104.46	23.82	54.40	37.05	70.47
Minimum	216.63	146.38	11.72	222.11	153.86	133.31
Maximum	508.03	444.84	95.65	361.33	242.56	328.34

Wilcoxon Ranks Sum test results of differences between MFI of HLA-DR expressions of B cell subsets in periodontitis groups

		Periodontitis tissues			Peripheral blood		
		N	Mean Rank	Sum of Ranks	N	Mean Rank	Sum of Ranks
ASCs – Naïve B cells	Negative Ranks	0[a]	.00	.00	0[a]	.00	.00
	Positive Ranks	10[b]	5.50	55.00	5[b]	3.00	15.00
	Ties	0[c]			0[c]		
	Total	10			5		
ASCs – Memory B cells	Negative Ranks	0[d]	.00	.00	4[d]	3.50	14.00
	Positive Ranks	10[e]	5.50	55.00	1[e]	1.00	1.00
	Ties	0[f]			0[f]		
	Total	10			5		

a. Naïve B cells < ASCs b. Naïve B cells > ASCs c. Naïve B cells = ASCs

d. Memory B cells < ASCs e. Memory B cells > ASCs f. Memory B cells = ASCs

Test Statistics[b]

	Periodontitis tissues		Peripheral blood	
	ASCs – Naïve B cells	ASCs - Memory B cells	ASCs – Naïve B cells	ASCs - Memory B cells
Z	-2.803[a]	-2.803[a]	-2.023a	-1.753b
Asymp. Sig. (2-tailed)	.005	.005	.053	.080

a. Based on negative ranks. b. Wilcoxon Signed Ranks Test

BIOGRAPHY

Miss Warattaya Rattanathammatada was born on 13th of April 1985 in Phitsanulok. She graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Naresuan University in 2009, and became a governmental officer working as a dentist at Chaibadan Hospital, Lopburi. She studied in Master degree program in Periodontology at Graduate School, Chulalongkorn University in 2011.