ปฏิกริยาของเชื้อแอคติโนแบซิลลัสแอคติโนไมซีเตมโคมิแทนส์
ต่อแอนติเจนพรีเซนติ่งเซลล์ (เดนไดรติกเซลล์)

นาง ปิยะวดี โพธิรักษานนท์

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INTERACTION OF *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* WITH ANTIGEN-PRESENTING CELLS (DENDRITIC CLLS).

Mrs. Piyawadee Pothiraksanon

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Periodontics

Department of Periodontology

Faculty of Dentistry

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ปิยะวดี โพธิรักษานนท์ : ปฏิกริยาของเชื้อ*แอคติโนแบซิลลัสแอคติโนไมซีเตมโคมิแทนส์*ต่อแอนติเจนพรีเซนติ่งเซลล์ (เดนไดรติกเซลล์) (INTERACTION OF *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* WITH ANTIGEN-PRESENTING CELLS (DENDRITIC CELLS).). อ. ที่ปรึกษา : ผศ.ดร. รังสินี มหานนท์, อ. ที่ปรึกษาร่วม : ดร. สาธิต พิชญางกูร, 63 หน้า. ISBN 974-17-1602-8

เป็นที่ทราบแน่ชัดว่าปฏิกิริยาระหว่างเซลล์เดนไดรติก (ดีซีส์) กับเชื้อก่อโรคว่ามีบทบาทสำคัญในการกำหนดประสิทธิภาพในการตอบสนองของระบบภูมิคุ้มกันและผลลัพธ์ในการเกิดโรค โรคปริทันต์อักเสบเป็นโรคติดเชื้อที่ทำให้เกิดการสูญเสียเนื้อเยื่อปริทันต์และกระดูกเบ้าฟัน โดยมีพยาธิสภาพทางอิมมูโนของโรคเกี่ยวข้องกับปฏิกิริยาระหว่างกระบวนการตอบสนองของระบบภูมิคุ้มกันกับเชื้อแบคทีเรียแกรมลบและผลิตภัณฑ์ของเชื้อ เช่น ไลโปโพลีแซคคาไรด์ (แอลพีเอส) ในคราบจุลินทรีย์ การศึกษานี้มีวัตถุประสงค์เพื่อทำการศึกษาปฏิกิริยาระหว่างแอลพีเอสที่สกัดจากเชื้อ*แอคติโนแบซิลลัส แอคติโนไมซีเทมโคมิแทนส์*ซึ่งเป็นเชื้อหลักที่ก่อโรคปริทันต์อักเสบต่อดีซีส์ โดยเตรียมดีซีส์ที่เจริญไม่เต็มที่จากการเพาะเลี้ยงคลัสเตอร์ออฟดิฟเฟอเรนทิเอชั่น (ซีดี)14โมโนไซท์ในอาหารเพาะเลี้ยงที่มีไซโตไคน์ชนิดแกรนูโลไซท์แมคโครฟาจสทิมูเลติ่งแฟคเตอร์ (จีเอ็ม-ซีเอสเอฟ) ร่วมกับอินเตอร์ลิวคิน (ไอแอล)-4 ดีซีส์ที่เจริญไม่เต็มที่นี้เมื่อนำมากระตุ้นด้วย*แอคติโนแบซิลลัส แอคติโนไมซีเทมโคมิแทนส์*แอลพีเอสในหลอดทดลองเป็นเวลา 1 วัน จะกลายเป็นดีซีส์ที่เจริญเต็มที่และมีการเพิ่มขึ้นของการแสดงออกของโคสติมูแลทอรี่โมเลกุล (ซีดี 40 และซีดี 80) และเครื่องหมายการเจริญเต็มที่ (ซีดี 83) รวมทั้งฮิวแมนลิวโคไซท์แอนติเจน-ดีอาร์ *แอคติโนแบซิลลัส แอคติโนไมซีเทมโคมิแทนส์*แอลพีเอสที่ความเข้มข้น 1,000 นาโนกรัมต่อมิลลิลิตร สามารถกระตุ้นให้มีการหลั่งไซโตไคน์ในปริมาณเล็กน้อย เราตรวจพบปริมาณปานกลางของทูเมอร์เนคโครซิสแฟคเตอร์-แอลฟาในทุกราย แต่ไม่พบไอแอล-12 ส่วนในมิกส์ลิวโคไซท์รีแอคชั่นพบว่าดีซีส์ที่ถูกกระตุ้นด้วย*แอคติโนแบซิลลัส แอคติโนไมซีเทมโคมิแทนส์*แอลพีเอสส่งเสริมการตอบสนองของทีเฮลเปอร์-1 (ทีเอช1) โดยดูจากการตรวจพบไซโตไคน์ชนิดอินเตอร์เฟียรอน-แกมมาเป็นส่วนมาก แต่ไม่พบไอแอล-4 และไอแอล-10 ที่ผลิตจากอัลโลเจนีอิค ทีเซลล์ กล่าวโดยสรุปจากการศึกษานี้แสดงให้เห็นว่าสามารถเลี้ยงดีซีส์จากซีดี 14โมโนไซท์ที่ทำการคัดเลือกโดยวิธีโฟลไซโตมิทริ โดยเพาะเลี้ยงในจีเอ็ม-ซีเอสเอฟร่วมกับไอแอล-4 ดีซีส์ที่เจริญไม่เต็มที่จะกลายเป็นดีซีส์ที่เจริญเต็มที่ได้ เมื่อกระตุ้นด้วย*แอคติโนแบซิลลัส แอคติโนไมซีเทมโคมิแทนส์*แอลพีเอสจะส่งเสริมการตอบสนองของทีเอช1 อย่างไรก็ดี จะเป็นการด่วนสรุปเกินไปกับผลการทดลองของเชื้อ*แอคติโนแบซิลลัส แอคติโนไมซีเทมโคมิแทนส์*ที่ไปมีผลต่อการชักนำของดีซีส์เกี่ยวกับการตอบสนองของทีเฮลเปอร์ การพัฒนาของทีเฮลเปอร์จะมีความเกี่ยวข้องกับพยาธิสภาพของการเกิดโรคปริทันต์อักเสบได้อย่างไรนั้นต้องได้รับการศึกษาต่อไป

ภาควิชา ปริทันตวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา ปริทันตศาสตร์ ลายมือชื่ออาจารย์ที่ปรึกษา.....

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4276113132 : MAJOR PERIODONTICS

KEYWORD : DENDRITIC CELL / *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* / LIPOPOLYSACCHARIDE / PERIODONTITIS

PIYAWADEE POTHIRAKSANON : INTERACTION OF *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* WITH ANTIGEN-PRESENTING CELLS (DENDRITIC CELLS). THESIS ADVISOR : ASSISTANT PROFESSOR RANGSINI MAHANONDA, Ph.D. THESIS CO-ADVISOR : SATHIT PICHYANGKUL, Ph.D. 63 pp. ISBN 974-17-1602-8

It becomes clear that the interaction between dendritic cells (DCs) and pathogens is a significant factor in determining the effectiveness of the immune response and the outcome of disease. Periodontitis is a chronic bacterial infection which is characterized by a destructive inflammatory process affecting periodontal tissues and bone. The immunopathogenesis of the disease involves the interaction between host defense mechanisms and Gram negative bacteria and their products such as lipopolysaccharide (LPS) in dental plaque. We therefore attempted to investigate the interaction of LPS derived from *Actinobacillus actinomycetemcomitans,* one of the key periodontal pathogens, with dendritic cells. Immature dendritic cells were generated from $CD14^+$ monocytes culturing in medium supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4. These immature DCs when stimulated with *A. actinomycetemcomitans* LPS *in vitro* for 24 h. led to cell maturation associated with the enhanced expression of co-stimulatory molecules (CD40 and CD80), a maturation marker (CD83) and human leukocyte antigen (HLA)-DR. *A. actinomycetemcomitans* LPS at 1,000 ng/ml induced minimally production of cytokines. Moderate amount of tumor necrosis factor-α was consistently observed, however, we could not detect any IL-12 production. In mixed leukocyte reaction, *A. actinomycetemcomitans* LPS treated DCs promoted T-helper 1 (Th1) response as indicated by predominant production of interferon-γ but not IL-4 and IL-10 by allogeneic T cells. In conclusion, our study demonstrated that we were able to generate DCs from flow cytometrically sorted $CD14^+$ monocytes in culture with GM-CSF and IL-4. The immature DCs became mature when stimulated with *A. actinomycetemcomitans* LPS and capable to drive Th1 response. However, it is too early to draw any conclusion concerning the effect of *A. actinomycetemcomitans* on DC induced Th response. How this Th development associated with the pathogenesis of periodontitis would require further investigation.

Department Periodontology Student's Signature……

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

Academic Year 2002 Co-advisor's Signature……. ……..

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

page

LIST OF TABLES

Table page

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

ABBREVIATIONS

A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
Ab	antibody
APCs	antigen-presenting cells
CD	cluster of differentiation
DCs	dendritic cells
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
FACs	fluorescence-activated cell sorter
GM-CSF	granulocyte macrophage-colony stimulating factor
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysacharide
mAbs	monoclonal antibodies
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
NaCl	sodium chloride
NK cell	natural killer cell
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffer saline

ABBREVIATIONS (Continued)

P. gingivalis	*Porphyromonas gingivalis*
PMA	phorbol myristic acetate
TCR	T cell receptor
Th	T helper
TLRs	toll-like receptors
TNF	tumor necrosis factor
Tris-HCl	Tris-hydrochloride

CHAPTER 1

INTRODUCTION

1.1 Background of the present study

For decades, innate immunity was discussed as an evolutionary throwback before giving way to the more exquisite adaptive immune response. But now all that is changing. Over the past few years, researchers have begun to realize that the innate system is a powerful screening tool. In an early hour of infection, it can distinguish between different classes of pathogenic bacteria, viruses and fungi. And it is becoming clear that the innate immune response is also crucial for initiation and instruction of the slower-acting adaptive immune system (Banchereau and Steinman, 1998).

Recent advances are starting to reveal the important role of dendritic cells (DCs) , one of the cell types involved in the innate immunity, as their functions are initiation and modulation of the immune response. DCs are the most potent professional antigen-presenting cells (APCs). They capture, process and present antigens efficiently to both naïve and memory T cells. In addition they instruct the type of T helper (Th) cell differentiation (Pulendran et al., 2001). Generating the right type of response is necessary for protection of the disease. Since T cells alone are not capable of making the complex decision how to respond to antigens with high specificity, these decisions are made jointly by the nature of the microbe and by DCs or in other words the interaction between the microbes and DCs. However, there are other factors such as cytokines in that microenvironment that could also influence the type of Th response (Jahkovic et al., 2001).

Periodontitis, a chronic inflammatory disease, affects tooth supporting structure. It involves gingival inflammation and alveolar bone resorption and in a severe case, tooth exfoliation may occur. The disease is initiated and perpetuated by Gram negative bacteria such as *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* and their products such as lipopolysacharide (LPS). Although the bacterial pathogens are essential, they are insufficient for the disease to occur. Periodontitis results from the interaction between host defense mechanisms and bacterial plaque pathogens. Numerous infiltrated T and B cells as well as high levels of inflammatory mediators and cytokines in periodontal lesions are the characteristics of periodontitis. So far, many studies have investigated the types of cytokines (drive Th1 and Th2 subset) that associated with the pathogenesis of periodontitis (Taubman et al., 1994; Yamazaki et al., 1994; Page et al., 1997). This may be a key question for periodontal pathogenesis since Th1 and Th2 paradigm could be a major determinant of the progression from gingivitis to periodontitis, or the transition from a stable non-progressive periodontitis lesions to a burst of destructive activity. The studies of Th1 and Th2 cells based on their cytokine profiles have produced somewhat conflicting results. However, accumulated data seem to suggest mixed Th1 and Th2 type of responses in periodontitis. (Fujihashi et al., 1993; Fujihashi et al., 1996; Yamazaki et al., 1997; Salvi et al., 1998). So far this issue has not yet been clarified, therefore further evaluation is needed. T cells and DCs must interact to initiate immune responses against periodontopathic bacteria. Several previous studies have focused on the role of T cells in pathogenesis of the disease, however, less effort has been made to elucidate the interaction of periodontopathic bacteria with antigen-presenting cells, especially DCs. Therefore in the present study, the interaction of *A. actonomycetemcomitans* with DCs was investigated.

1.2 Objectives

1.2.1 To establish the immature monocyte derived DCs from monocyte

1.2.2 To examine whether *A. actinomycetemcomitans* LPS could induce DC maturation as monitored by up-regulation of co-stimulatory molecules cluster of differentiation (CD)40, CD80 and cytokine production.

1.2.3 To investigate the type of Th subset development induced by *A. actinomycetemcomitans* LPS-stimulated DCs in mixed leukocyte reaction(MLR).

1.3 Hypothesis

The interaction of *A. actinomycetemcomitans* LPS with DCs results in dendritic cell maturation (up-regulation of co-stimulatory molecules and cytokine production). These *A. actinomycetemcomitans* LPS-activated DCs could induce allogeneic T cell response in MLR and Th1 cytokine production.

1.4 Field of research

To investigate the effect of *A. actinomycetemcomitans* LPS on DCs maturation which may influence the specific type of Th response.

1.5 Criteria inclusion

1.5.1 Peripheral blood samples were collected from healthy adult volunteers.

1.5.2 Subjects who had clinically healthy periodontium with probing depth less than 4 mm. were included.

1.5.3 Subjects had not taken any antibiotics or anti-inflammatory drugs within the past 3 months prior to blood donation.

1.5.4 LPS of *A. actinomycetemcomitans* were used.

1.5.5 Mononuclear cells were obtained from peripheral blood samples using gradient centrifugation.

1.5.6 DCs were derived from human $CD14^{+}$ monocytes by culturing in medium supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF) plus interleukin (IL)-4

1.5.7 Cell surface marker analysis was determined by flow cytometry.

1.5.8 Cytokine production was analyzed by enzyme-linked immunosorbent assay (ELISA).

1.5.9 Type of Th response induced by *A. actinomycetemcomitans* LPS-stimulated DCs in MLR was determined by cytokine production using intracellular cytokine staining.

1.6 Limitation of research

The experiments used an *in vitro* monocyte-derived DCs as analogue to Langerhans cells.

1.7 Application and expectation of research

In this study, monocyte-derived DCs could be generated *in vitro* which enable us to investigate the interaction between APCs and LPS derived from plaque bacteria. This would provide the mechanism how APCs respond to

periodontopathic bacteria. And subsequently how it controls the type of T cell response (Th1 / Th2). We expect that the establishment of monocyte-derived DCs would enhance further evaluation of oral plaque bacteria and their pathogen-associated molecular patterns (PAMPs) on DCs.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Periodontal disease is a chronic bacterial infection which causes gingival tissue inflammation and alveolar bone resorption. According to the severity, the disease can be categorized into 2 major groups; gingivitis (the stable form) and periodontitis (the more advanced and severe form). Gingivitis involves gingival inflammation with no alveolar bone destruction whereas periodontitis involves gingival inflammation and loss of alveolar bone which may eventually lead to exfoliation of the teeth. Both forms of the disease appear to be global problems regarding oral health and particularly periodontitis which is considered to be one of the major causes of tooth loss in adults (Panos, 1996).

2.2 Plaque bacteria

It is now well recognized for the role of plaque bacteria and their products such as LPS as the etiology in periodontal disease. Current concepts of the periodontal diseases emphasize a specific bacterial etiology of periodontitis rather than a nonspecific indictment of the plaque mass (Loesche and Syed, 1978). Studies of the microbial progression from periodontal health through gingivitis to periodontitis have indicated a substantial increase in spirochetes and a change from a predominantly Gram-positive to a Gram-negative microbiota (Tanner et al., 1979; Slots, 1986). In addition, the ecology appears to become increasingly anaerobic as the severity of the disease increases. In fact, the recent

World Workshop on clinical periodontics (concensus report, 1996) has identified *A. actinomycetemcomitans, Porphyromonas gingivalis and Bacteroides forsythus* as causative agents of human periodontitis.

2.2.1 ***Actinobacillus actinomycetemcomitans*** *A. actinomycetemcomitans* is a small, nonmotile, Gram-negative, saccharolytic, capnophilic, round-ended rod. This species was first recognized as a possible periodontal pathogen by its increased frequency of detection and higher numbers in lesions of localized juvenile periodontitis (Newman et al., 1976; Slots, 1976; Newman and Socransky, 1977; Mandell and Socransky, 1981; Chung et al., 1989) and rapidly progressive periodontitis (Ohta et al., 1986) compared with numbers in plaque samples from other clinical conditions including gingivitis and health. According to the 1999 international workshop for a classification of periodontal diseases and conditions (AAP, 1999), juvenile periodontitis and some of rapidly progressive periodontitis are replaced with the term “aggressive periodontitis”. This group is a specific type of periodontitis with clearly identifiable clinical and laboratory findings. The common features are rapid attachment loss, bone destruction, and elevated proportion of *A. actinomycetemcomitans*. Subgingival colonization of *A. actinomycetemcomitans* has been demonstrated to increase the relative risk for periodontal disease progression three to four folds (Haffajee and Socransky, 1994).

The majority of subjects with localized juvenile periodontitis have an enormously elevated serum antibody response to this species (Ebersole et al., 1980; Genco et al., 1980; Lisgarten et al., 1981; Tsai et al., 1981; Altman et al., 1982; Ebersole et al., 1982; Ebersole et al., 1983) and the local antibodies specific to *A. actinomycetemcomitans* can also be detected (Schonfeld and

Kagan, 1982; Ebersole et al., 1985; Smith et al., 1985; Tew et al., 1985). When localized juvenile periodontitis patients were treated successfully, the species was eliminated or lowered in numbers. On the contrary, treatment failures were associated with failure to lower the numbers of the species in treated site (Slots and Rosling, 1983; Haffajee et al., 1984; Christersson et al., 1985; Kornman and Robertson, 1985; Mandell et al., 1986; Preus, 1988).

A. actinomycetemcomitans express a variety of potential virulence factors such as collagenases, LPS, leukotoxin, polyclonal B cell activators, alkaline and acid phosphatase (Wilson et al., 1996). *A. actinomycetemcomitans* leukotoxin binds to neutrophils, monocytes and a subset of lymphocytes and form pores in the membranes of these target cells thus leading to cell death (Taichman et al., 1987; Iwase et al., 1990). *A. actinomycetemcomitans* is invasive. Many investigators demonstrated its ability to invade human gingival epithelial cells *in vitro* (Mayer et al., 1991; Blix et al., 1992; Sreenivasan et al., 1993).

***A. actinomycetemcomitans* lipopolysaccharide.** It is known that the Gram negative bacterial plaque live in a highly organized biofilm community that subgingivally attach to the root surfaces and becomes recalcitrant to host defense removal (Darveau et al., 1997). These bacteria do not normally invade the lesional tissues in large numbers but they provide a platform for constitutively release their products and components. These released molecules subsequently penetrate periodontal tissue. It is believed that the interaction between the released bacterial products with the host cells or host immune system is the basis for the inflammatory response in periodontitis (Gemmell et al., 1997). LPS, the cell wall of Gram negative bacteria, has been identified as an important factor in the pathogenesis of periodontitis. It is found in periodontally involved cementum,

gingival crevicular fluid and gingival tissues of patients with periodontal disease (Daly et al, 1980; Mayrand and Holt, 1988). LPS is a complex glycolipid composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A that is responsible for most of the LPS-induced biological effects.

Many studies have demonstrated the ability of LPS derived from *A. actinomycetemcomitans* to activate immune cells as well as residential cells. *A. actinomycetemcomitans* LPS are potent stimulators for monocytes/macrophages as measured by the release of mediators and cytokines. These include collagenases, IL-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α, all of which mediate a variety of important inflammatory effects on connective tissue and bones involving in chronically inflamed periodontal tissues (Wahl, 1974; Cury et al., 1988; Saglie et al., 1990; Zadeh et al., 1999) In addition, *A. actinomycetemcomitans* LPS were shown to induce *in vivo* and *in vitro* bone resorption (Iino et al., 1984; Ishihara et al., 1991; Nishihara et al., 1995; Ueda et al., 1998). Kikuchi et al. (2001) demonstrated enhanced expression of osteoclast differentiation factor on mouse osteoblast after stimulation with *A. actinomycetemcomitans* LPS, thus implying the important role of *A. actinomycetemcomitans* LPS in bone resorption.

2.3 Host response to plaque bacteria

It is now clearly understood that bacterial plaque pathogens are essential for the disease initiation, however they are not sufficient for periodontitis to occur. The disease results from the interaction between host defense and bacterial pathogens. Such interaction plays an important role in the pathogenesis of

periodontitis. Histologically periodontitis lesion is characterized by dense infiltration of T and B cells in the extravascular gingival connective tissue (Seymour et al., 1993). It is suggested that inappropiate production of cytokines particularly from T cells plays a significant role in the pathogenesis of the disease (Seymour et al., 1993). Study of cytokines in periodontitis during the past 10 years provides variable results with regard to the type of cytokines, whether Th1 or Th2, detected. For example, the expression of mRNA of IL-2, interferon (IFN)-γ and IL-5 was detected from patients' tissues by one group (Tekeichi et al., 2000), while the other group reported the detection of IFN-γ and IL-5 but little or no IL-2 or IL-4 mRNA (Fujihashi et al., 1993). As for the protein detection, IL-4 and IL-6 producing memory T cells detection by immunochemistry were observed in periodontal tissue (Aoyagi et al., 1995). The difference in findings among these observation may relate to the technique used to process tissues as well as to detect cytokine production. So far, the issue of Th1 and Th2-type response in periodontitis is still inconclusive. Why and how the specific type of Th response is so crucial in the disease process will be discussed.

2.4 T cells in periodontitis

Infiltrated T cells in periodontitis lesions have been identified as antigen-specific memory T cells and the majority of which are in activated stage as indicated by high expression of CD45RO and human leukocyte antigen (HLA)-DR (Gemmell et al., 1992; Yamazaki et al., 1993). Activated T cells are known to release cytokines that are potent regulators of the local immune response.

It is now known that $CD4^+$ T cells or Th cells can be devided into, at least 2 subsets i.e. Th1 and Th2 according to their cytokine production. The first Th cell types reported were mouse Th1 and Th2 cells. Mouse Th1 cells were found to

secrete IFN-γ, while Th2 cells secreted IL-4 (Mosmann et al., 1986). In human, Th1 cells were also identified that secrete IFN-γ and TNF-β while Th2 cells produce IL-4, IL-5, IL-6 and IL-13 (Seder and Paul, 1994; Romagnani, 1997). IL-10 is also classified as a Th2-type cytokine in the mouse, but in human, both Th1 and Th2 subsets secrete IL-10 (Ferrick et al., 1995; Katsikit et al., 1995). The ensuring Th1- and Th2-type immune responses both include potent humoral and cell-mediated components, but the effector cells and antibody (Ab) isotypes involved are quite distinct (Abbas et al., 1996). Th1 cells are responsible for the activation of macrophages to a microbicidal state, the induction of immuno globulin (Ig) G Abs that mediate opsonization and phagocytosis, and the support of $CD8^+$ antiviral effector T cells. By contrast, Th2 cells stimulate the growth and differentiation of mast cells and eosinophils, as well as the production of Ab isotypes, including IgE, which can mediate the activation of these cells. Th1 and Th2 cells are not precommitted phenotypes but rather, represent endpoints of a multistep differentiative process whereby a common precursor population acquires a distinct cytokine secretion profile (Romagnani, 1997). A key question is how this differentiation decision is made.

Two key cytokines that bridge the gap between innate and acquired immunity are IL-10 and IL-12. Both are produced by monocytes, macrophages and dendritic cells in response to microbes (D'Andrea et al., 1992; Hsieh et al., 1993; Giambartolomei et al., 1999), but they have largely opposite properties. IL-12 is a T-cell stimulatory cytokine which activates T cells and natural killer (NK) cells to secrete IFN-γ and to lyse target cells (Trinchieri, 1994). T cells that are influenced by IL-12 during antigen presentation will mature into IFN-γ-producing cells (Gerosa et al., 1996; Trinchieri, 1997). IL-10, in contrast, downregulates T-cell cytotoxicity, IL-12, IFN-γ production and decreases presentation of antigens for T

cells (de Waal Malefyt et al., 1991; Fiorentino et al., 1991; D'Andrea et al., 1993). Instead, IL-10 stimulates B-cell maturation and antibody production (Rousset et al., 1992).

Study of the numerous animal models, together with the few studies in humans, have revealed that the ability of a host to eradicate effectively an invading pathogen depend on the type of effector-specific immune response (Th1- or Th2-type response) that is mounted (Romagnani,1997). This has been suggested to be a major branch point in the immune response since it is an important determinant of whether the response to infectious pathogen will lead to protection of host or dissemination of the disease. In general, Th1 cells are more suitable for protection against intracellular parasites including bacteria, protozoa, fungi and viruses such as *Mycobacterium tuberculosis* (Brightbill et al., 1999), *Toxoplasma gondii* (Gazzinelli et al., 1994), whereas Th2 cells protect against extracellular parasites (most helminthic parasites) such as *Schistosoma mansoni* (Velupillai and Harn, 1994), and *Acanthoceilonema vitae* (Whelan et al., 2000). On the other hand, generating the inappropriate type of immune response can induce pathogenesis or even the matter of life and death such as a lethal Th2-type response found in *Leishmania major* infection in mouse model (Liew, 1989; Locksley et al., 1991).

2.5 Dendritic cells as potent antigen-presenting cells

Immunology has long been focused on antigens and lymphocytes, but the mere presence of these two parties does not always lead to immunity. A third party, the DC system of antigen-presenting cells is the initiator and modulator of the immune response. First visualized as Langerhans cells in the skin in 1868, the

characterization of DCs began only about 25 years ago. (Banchereau and Steinman, 1998) In oral cavity, Langerhans cells have been identified in human gingival epithelium within the stratum spinosum and stratum basale. These cells play a role in the defense mechanism of the oral mucosa (Saglie et al., 1987, Lindhe and Karring, 1997).

Among the 3 professional APCs, DCs are now recognized as the most potent APCs critical for T cell priming. DCs are optimized for antigen presentation in that they capture antigen with high efficiency, undergo recruitment and migration, express high levels of major histocompatibility complex (MHC) and co-stimulatory molecules (such as CD40 and B7 family, including CD80 and CD86) and produce T cell regulatory cytokines as well as chemokines. DCs represent a minor cell population in all tissues (0.5-2%) and are very difficult to isolate. A recent advance in tissue culture, it is now possible to generate large numbers of DCs *in vitro* (Caux et al., 1992). Currently human DCs can be generated *in vitro* from adherent monocytes or flowcytometrically sorted $CD14^+$ monocytes cultured with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). DCs reside in peripheral tissues in an immature stage to allow for optimal antigen uptake. Upon encountering a pathogen, DCs become activated, migrate to secondary lymphoid organ and differentiate into mature DCs, which mediate antigen presentation and stimulate naïve T cells. Not all pathogens can activate DCs. Measles virus and malaria parasite suppress DC activation and lead to inhibition of immune response (Grosjean et al. 1997, Urban et al., 1999). Other virus, such as HIV can infect DCs and vigorously replicate in DC-derived syncytia *in vitro* (Pope et al., 1997).

As mentioned earlier, the activation of the appropriate T cell subset is critical for providing protective immunity against a variety of pathogens. The current

hypothesis to explain the selectivity of T cell responses postulates that DCs influence T cell differentiation. The mechanism of how DCs induce Th1 or Th2 response has been the subject of intense investigation. Recent study demonstrates that the nature of DC activating signal controls the type of T cell differentiation (Kadowaki et al., 2000). The data imply that the interaction of DCs with pathogen could determine the type of immune response and the outcome of disease.

2.6 Dendritic cells in periodontal disease

Human periodontal tissues contain Langerhans cells and connective tissue or lamina propria DCs (Lombardi et al., 1993, Cutler et al., 1999). Langerhans cells were commonly reported to be presented in the suprabasal and spinous layers of keratinized and non-keratinized gingival epithelium which were identified by electron microscopy as having Birbeck granules (Schroeder and Theilade, 1966; Waterhouse and Squier, 1967) or by immunohistochemical analysis (CD1a, HLA-DR) (Hitzig et al., 1989). Their numbers were shown to be increased during human experimental gingivitis (Newcomb et al., 1982) and the increased numbers appeared to be significant when compared between clinically inflamed gingiva and healthy gingiva (Di Franco et al., 1985; Saglie et al., 1987). The presence of Langerhans cells were found to be in association with the presence of plaque bacteria or bacterial particles such as *A. actinomyce-temcomitans* (Saglie et al., 1987) and *P. gingivalis* (Saglie et al., 1987; Cutler et al., 1999). In periodontitis tissues the immature DCs or Langerhans cells were in pocket and oral epithelium (Cutler et al., 1999; Cirrincione et al., 2002) . These immature DCs showed a high expression of antigen capture molecules e.g. DEC 205, CD32 and CD11b, indicating antigen uptake capability (Cutler et al., 1999).

In lamina propria of adult periodontitis lesion, mature DCs were identified with surface expression of MHC II and co-stimulatory molecules such as CD40, CD80 and CD86 (Cutler et al., 1999). These lamina propria DCs were abundant and in close contact with $CD4^+$ and $CD8^+$ T cells as well as plasma cells (Cirrincione et al., 2002). Further works are needed to confirm these observations. To date the role of these immature and mature DC in the pathogenesis of periodontitis remains largely unknown. Further research are required to understand the interaction between DCs and other immune cells or residential cells in periodontal environment as well as the interaction between DCs and bacterial plaque pathogens.

CHAPTER 3

MATERIALS AND METHODS

3.1 Medium and monoclonal antibodies

RPMI1640 (Gibco Laboratory, Grand Island, NY) supplemented with 2 mM L-glutamine, 2 mg/ml of Sodium bicarbonate (Sigma Chemical Co., St. Louis, MO), 2 mg/ml of D-glucose (Sigma Chemical Co.), 5.94 mg/ml of HEPES (Sigma Chemical Co.), 80 μg/ml of gentamycin (SoloPak Laboratories Inc., Elk Grove Village, IL) and 10% heat inactivated autologous serum was used throughout the study.

Monoclonal antibodies (mAbs) used in this experiment were obtained from BD PharMingen (San Diego, CA). They are mAbs against CD1a, CD3, CD4, CD8, CD14, CD16, CD20, CD40, CD45RA, CD45RO, CD80, CD83, HLA-DR, IFN-γ, T cell receptor (TCR)-γδ, IL-4 and IL-10.

Monoclonal antibodies	specificity
Anti-CD1a	Dendritic cell, Langerhans cell
Anti-CD3	T cell
Anti-CD4	T cell subset
Anti-CD8	T cell subset
Anti-CD14	Monocyte
Anti-CD16	NK cell, granulocyte
Anti-CD20	B cell
Anti-CD40	Co-stimulatory molecule
Anti-CD45RA	Resting / naïve T cell
Anti-CD45RO	Activated / memory T cell
Anti-CD80	Co-stimulatory molecule
Anti-CD83	Mature dendritic cell
Anti-HLA-DR	Major histocompatibility complex Class
Anti-IFN-γ	II
Anti-TCR-γδ	IFN-γ
Anti-IL-4	T cell subset
Anti-IL-10	IL-4
	IL-10

Table 1 : Monoclonal antibodies used for flow cytometric analysis

3.2 Lipopolysacharide derived from *A. actinomycetemcomitans*

A. actinomycetemcomitans Y4 LPS was a kind gift from Associate Professor K. Yamazaki (Niigata University, Niigata, Japan). Briefly *A. actinomycetemcomitans* LPS was extracted from the lyophilized cells of *A. actinomycetemcomitans* Y4 by the hot phenol-water procedure (Westphal et al., 1965). The crude extract was treated with nuclease, and then washed extensively with pyrogen-free water by ultracentrifugation. This preparation was purified by chromatography on a Sephadex G-200 (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM Tris-hydrochloride (Tris-HCl) (pH 8.0) containing 0.2 M sodium chloride (NaCl), 0.25% (wt/vol) deoxycholate, 1mM EDTA and 0.002% sodium azide. The purified material was dialyzed against pyrogen-free water, and then lyophilized. The LPS preparation was reconstituted with pyrogen-free water and heated at 100°C, 30 minutes before use.

3.3 Generation of monocyte-derived DCs

Heparinized peripheral blood was obtained from healthy adult volunteers. These subjects had clinically healthy periodontal conditions, with < 4 mm. probing depth and no bleeding upon probing. They had not taken antibiotics or anti-inflammatory drugs within the past 3 months. None of them had any symptoms of infection.

DCs were generated from peripheral blood mononuclear cells (PBMC) prepared from these volunteers. PBMC were separated over Ficoll-Hypaque (Sigma Chemical Co.), as previously described (Boyum 1968). T cells were depleted from PBMC by rosetting with neuraminidase-treated sheep red blood

cells. T cell depleted population was stained with mAb against CD14 and then they were positively sorted using fluorescence-activated cell sorter (FACs Vantage, Becton Dickinson, Mountain View, CA). Immature DCs were generated by culturing $1x10^5$ $CD14^+$ monocytes in 200 μl of RPMI1640 medium containing 10% heat-inactivated autologous serum, 50 ng/ml GM-CSF (Genzyme Co., Cambridge, MA) and 50 ng/ml IL-4 (Genzyme Co.) in 96-well flat microtiterplates (Costar, Cambridge, MA). The culture plates were incubated at 37°C in 5% CO_2 atmosphere for 5-7 days. Approximately half of the spent culture medium was removed and replaced with equal volume of fresh culture medium together with cytokines every other day. These cultured cells were characterized and identified as immature DCs, judging from expression of CD1a and negligible expression of CD83.

3.4 Stimulation of monocyte-derived DCs with *A. actinomycetemcomitans* LPS

In a dose response experiment, monocyte-derived DCs ($1x10^5$ cells/ 200 μl) were stimulated with varying concentrations of *A. actinomycetemcomitans* LPS (0, 100, 300, 1,000 ng/ml (predeterminant)) for 24 h. Then, cells were harvested for phenotypic analysis using flow cytometry. The culture supernatants were collected for evaluation of cytokine production by commercial ELISA kits.

3.5 Mixed leukocyte reaction and intracellular cytokine staining

Enriched T cell population were prepared from PBMC by rosetting with neuraminidase-treated sheep erythrocytes. Red blood cells were lysed with NH_4Cl-Tris. Allogeneic naïve $CD4^+$ T cells were obtained by negative sorting of enriched T cell population that had been stained with mAbs against CD8, CD20,

CD45RO, CD56 and TCR- γδ. This technique normally provided $CD4^+$, $CD45RA^+$ naïve T cells with purity >95%.

DCs were cultured in the presence of *A. actinomycetemcomitans* LPS (1,000 ng/ml) for 24 h. *A. actinomycetemcomitans* LPS treated DCs were co-cultured with allogeneic naïve T cells (DC:T ratio = 1:5). After 6 days, the cultures were restimulated with phorbol myristic acetate (PMA) (50 ng/ml) and ionomycin (50 μg/ml) for 5 h. and brefeldin (10 μg/ml) was added during the last 3 h. The stimulated cells were then stained with mAb against CD4, fixed and permeabilized and then stained with mAbs against IFN-γ, IL-4 and IL-10. $CD4^+$ T cells were gated and then analysed for intracellular cytokines by flow cytometry.

3.6 Flow cytometric analysis

To study the effect of *A. actinomycetemcomitans* LPS on DC activation, both control DCs and *A. actinomycetemcomitans* LPS-treated DCs were stained with mAbs anti-CD40 (APC), anti-CD80 (PE), anti-CD83 (FITC) and HLA-DR (PerCP). Mouse isotype mAbs conjugated with APC, PE, FITC and Per CP were used as control.

Monocytes-derived DCs were stained at 4°C for 30 min, washed in PBS and then reconstituted with 1% paraformaldehyde. Normally, 5,000-10,000 cells were analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA). The levels of surface molecule expression were presented by mean fluorescence intensity (MFI).

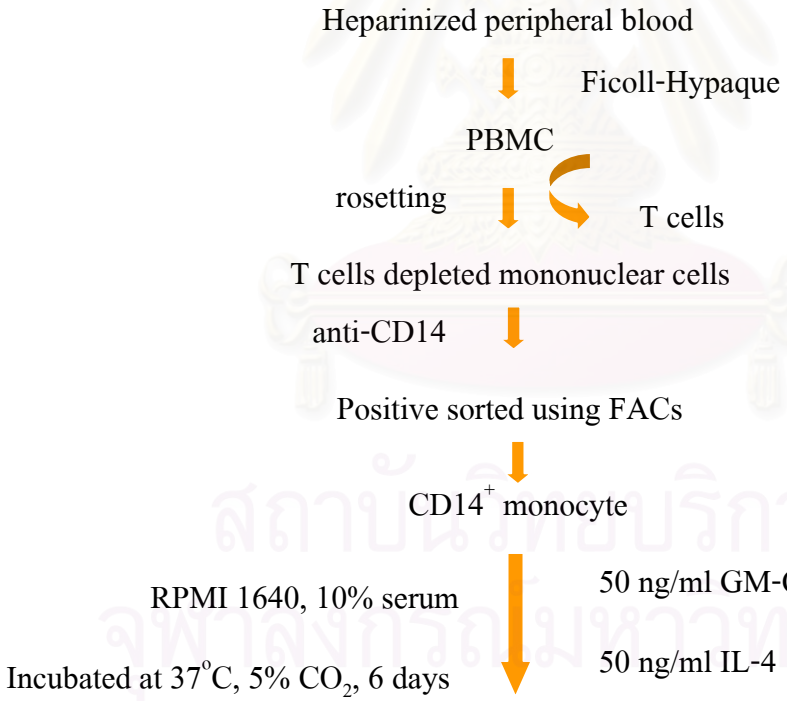
3.7 Cytokine determination

ELISA

Production of TNF-α, IL-10 and IL-12 in supernatants of *A. actinomycetemcomitans* LPS-stimulated DCs were measured by ELISA (R&D system, Minneapolis, MN). Detection limit of ELISA assay for TNF-α is 4.5 pg/ml, IL-10 is 3.9 pg/ml and IL-12 is 4 pg/ml.

3.8 Study flow chart

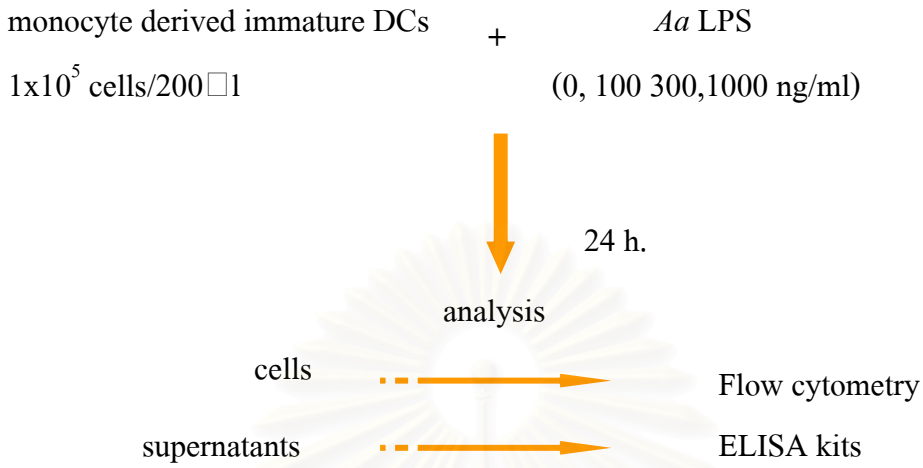
Generation of monocyte-derived DCs



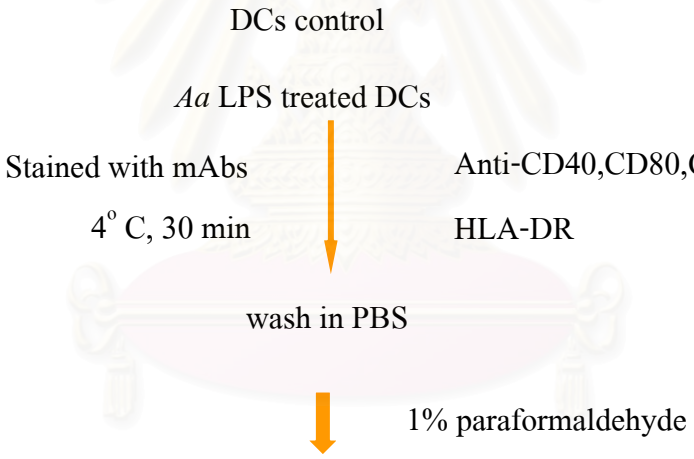
Monocyte-derived DCs

(Immature DCs were confirm by flow cytometry)

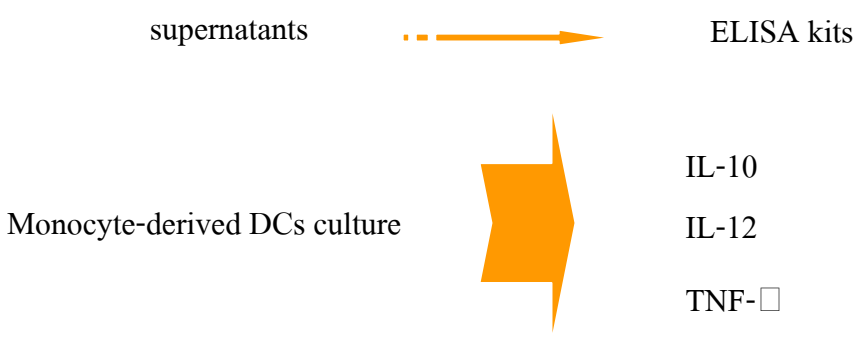
Stimulation of monocyte-derived immature DCs with *A. actinomycetemcomitans* LPS



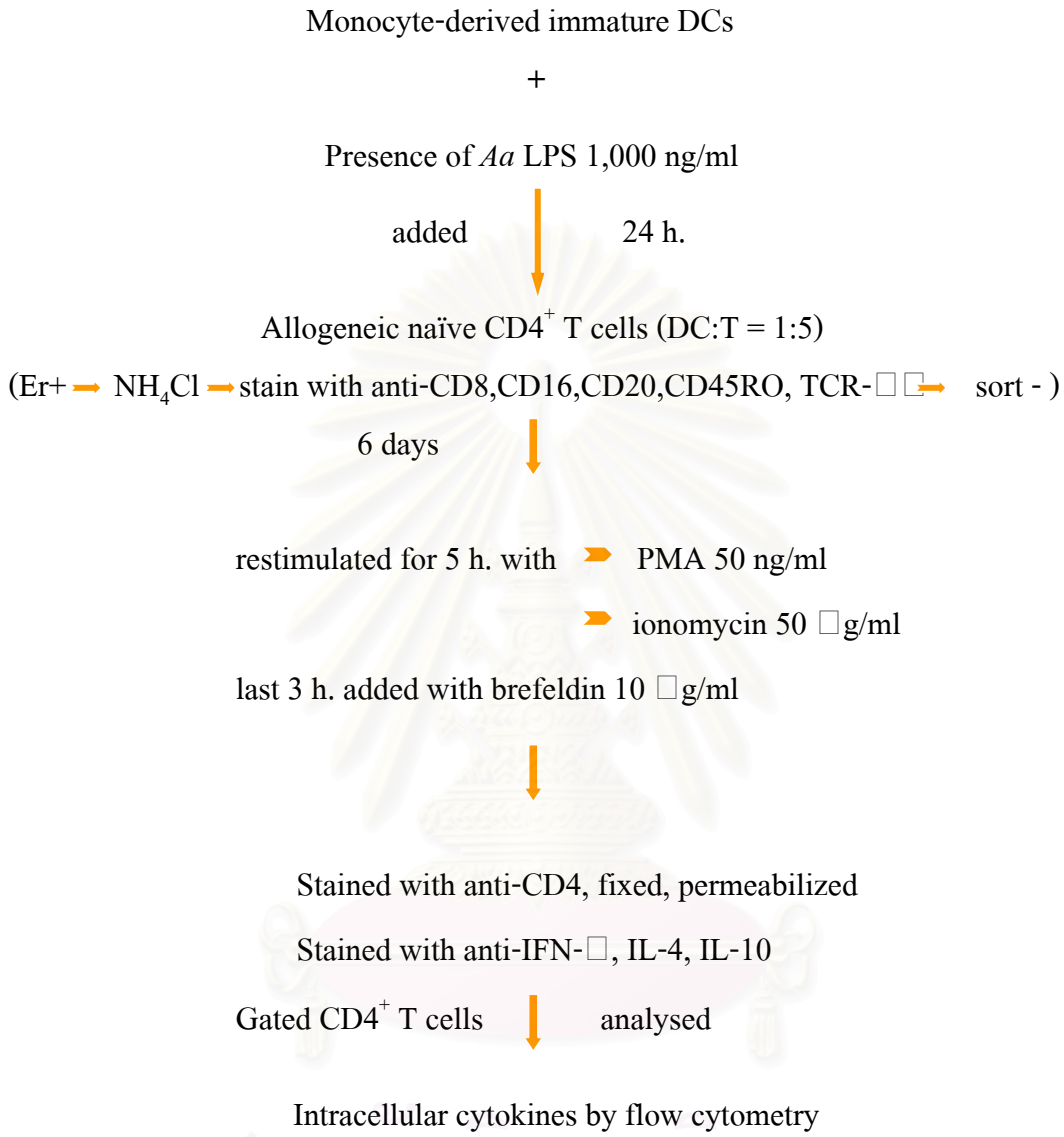
Flow cytometric analysis



Cytokine determination



Mixed leukocyte reactions



3.9 Statistic analysis

The data were analyzed using descriptive analysis.

CHAPTER 4

RESULTS

4.1 Morphology and phenotypes of monocyte-derived DCs

DCs were generated from sorted $CD14^+$ monocytes (Fig.1A). To evaluate the efficiency of the generation of monocyte-derived DCs, we compared monocytes that were cultured in the absence (Fig.1B) and the presence (Fig.2A) of GM-CSF and IL-4 (50 ng/ml each). After 6-7 days in cytokine containing medium, the small round $CD14^+$ monocytes differentiated into monocyte-derived DCs with a morphology of irregular shape cell. These cells possess long thin cytoplasmic projection and very often formed small clumps (Fig. 2A and 2B). Phenotypic analysis by flow cytometry indicated that monocyte-derived DCs up-regulated several co-stimulatory molecules including HLA-DR, CD40 and CD80. Dendritic cell marker (CD1a) was found to be expressed on monocyte-derived DCs but the monocyte marker (CD14) and CD83 were negligibly expressed suggesting that monocytes cultured with GM-CSF and IL-4 had acquired typical characteristics of immature DCs (Fig. 3).

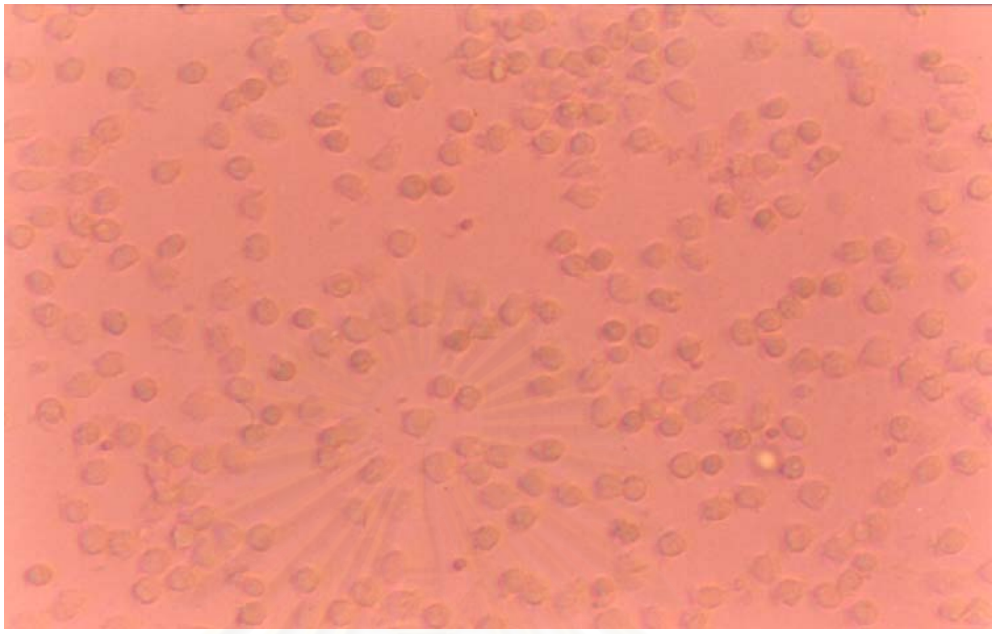


Figure 1A. Morphology of freshly isolated $CD14^+$ monocytes. (200x)

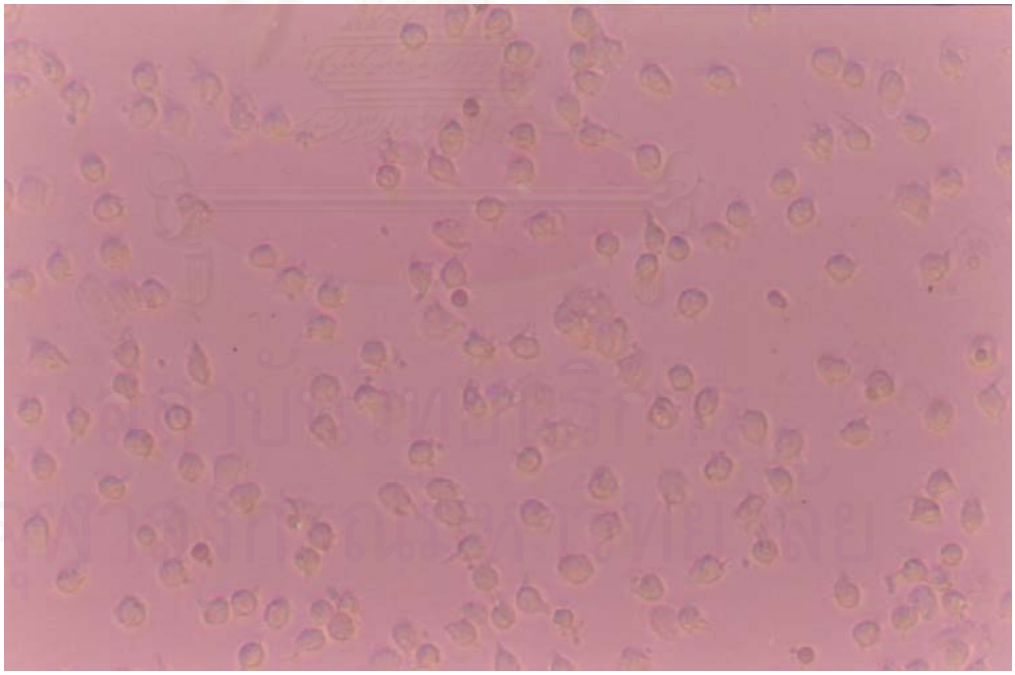


Figure 1B. Morphology of $CD14^+$ monocytes that had been cultured without cytokines, GM-CSF and IL-4 for 6 days. (200x)

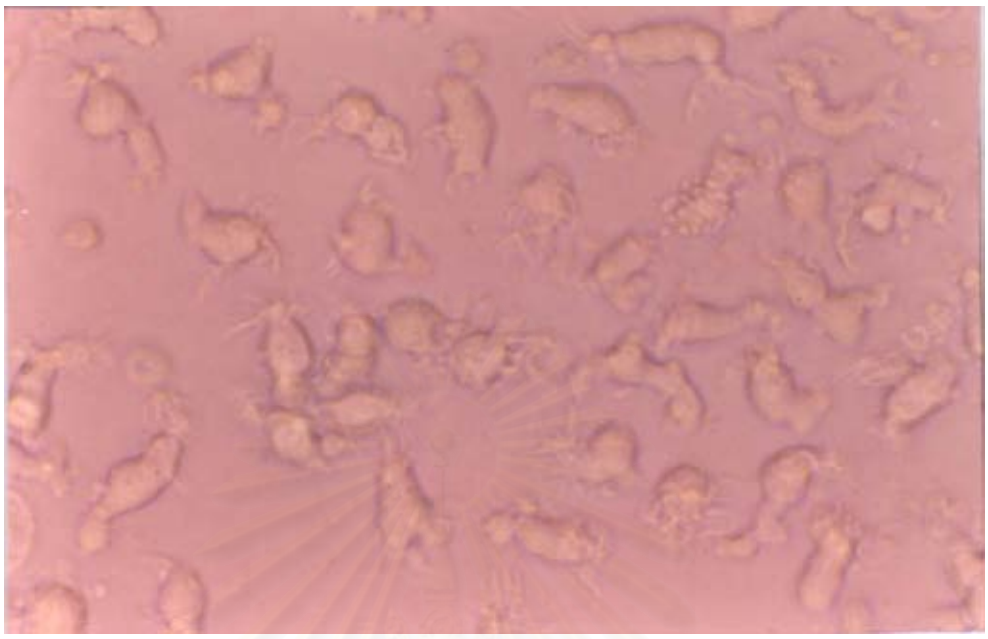


Figure 2A. Morphology of $CD14^{+}$ monocytes that had been cultured with cytokines, GM-CSF and IL-4 for 6 days. (400x)

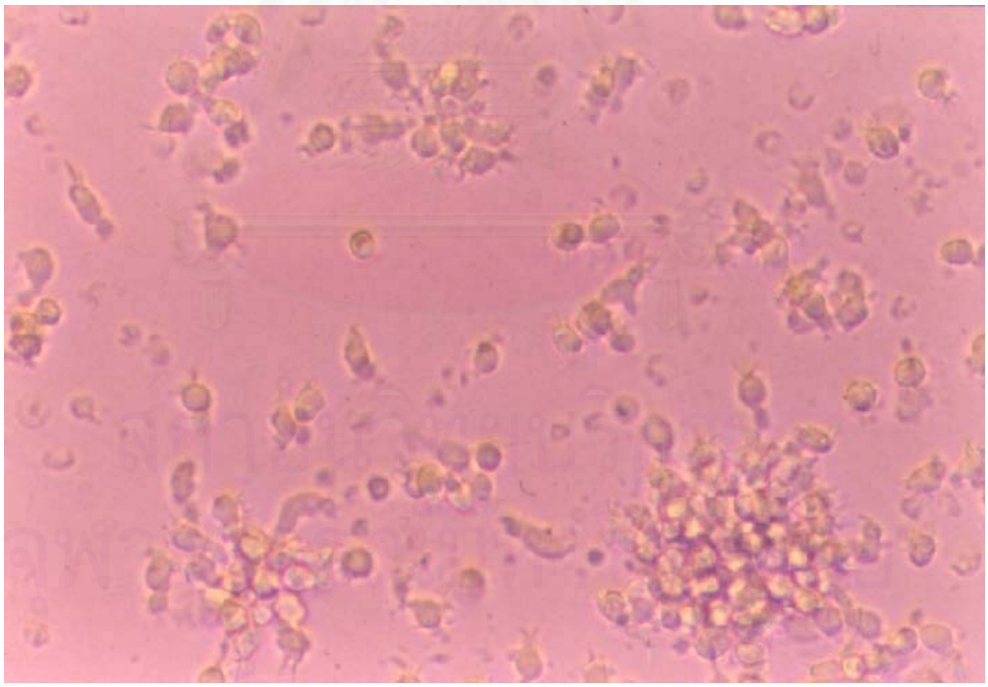


Figure 2B. Small clusters of cells were observed in monocyte-derived DC culture. (200x)

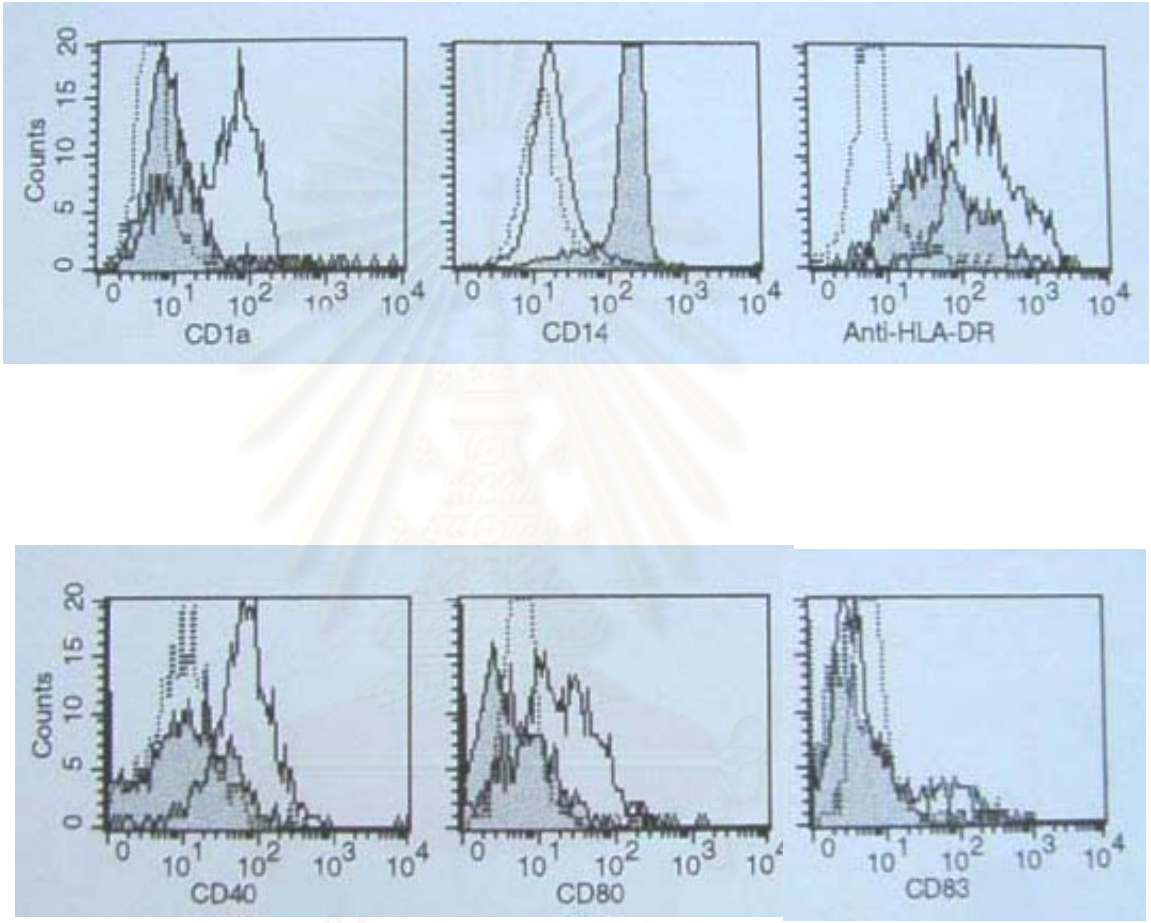


Figure 3. Phenotypic analysis of monocyte-derived DCs in comparison with monocytes. (dot line : IgG, gray area : monocyte, solid line : monocyte-derived dendritic cell)

4.2 Activation of DCs by LPS derived from *A. actinomycetemcomitans*

Six day cultured immature DCs were stimulated with varying concentrations of *A. actinomycetemcomitans* LPS (0, 100, 300 and 1,000 ng/ml). After 24 h., cell activation was evaluated. Fig.4 depicts the apperance of *A. actinomycetemcomitans* LPS-stimulated DCs. More clusters of cells were evident as compared to unstimulated cells. Flow cytometry analysis indicated that LPS from *A. actinomycetemcomitans* induced the up-regulation of HLA-DR, CD40 and CD80. Furthermore, *A. actinomycetemcomitans* LPS induced the expression of CD83, a maturation marker of DCs. Similar results were observed in 4 separate donors (Fig. 5A, 5B, 5C and 5D).

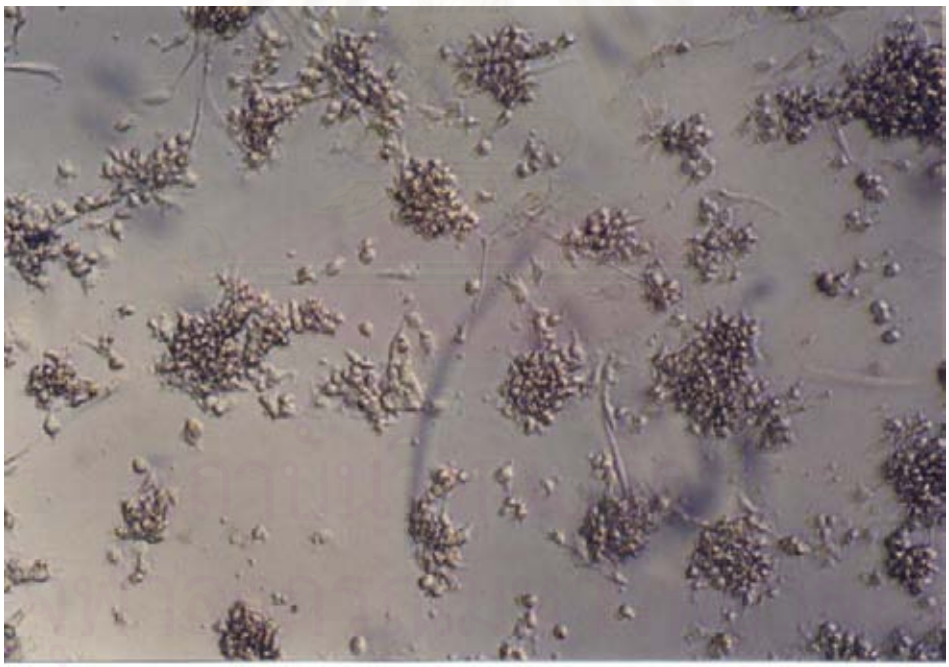


Figure 4 Morphology of monocyte-derived dendritic cells that had been cultured with *A. actinomycetemcomitans* LPS for 6 days (100x). More cluster of cells were evident as compared to unstimulated cells (Fig. 2B).

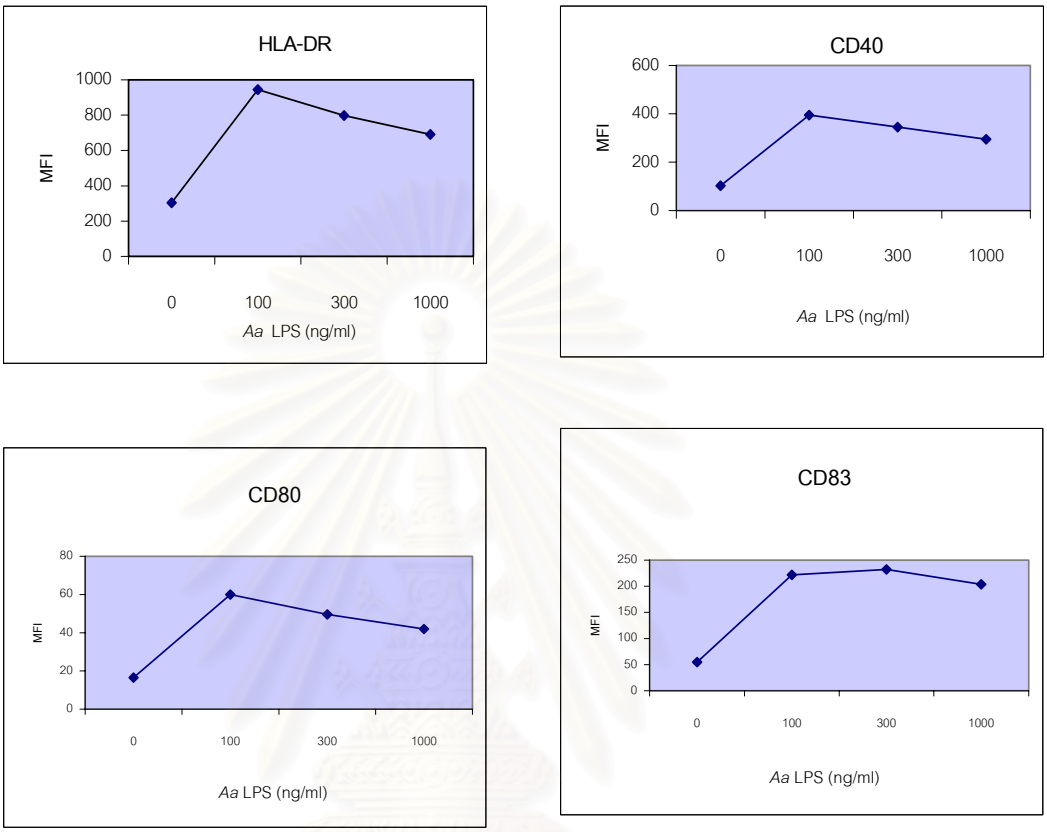


Figure 5A. Donor1: Analysis of co-stimulatory molecule and CD83 expression by flow cytometry.

Aa LPS = *Actinobacillus actinomycetemcomitans* LPS

MFI = mean fluorescence intensity

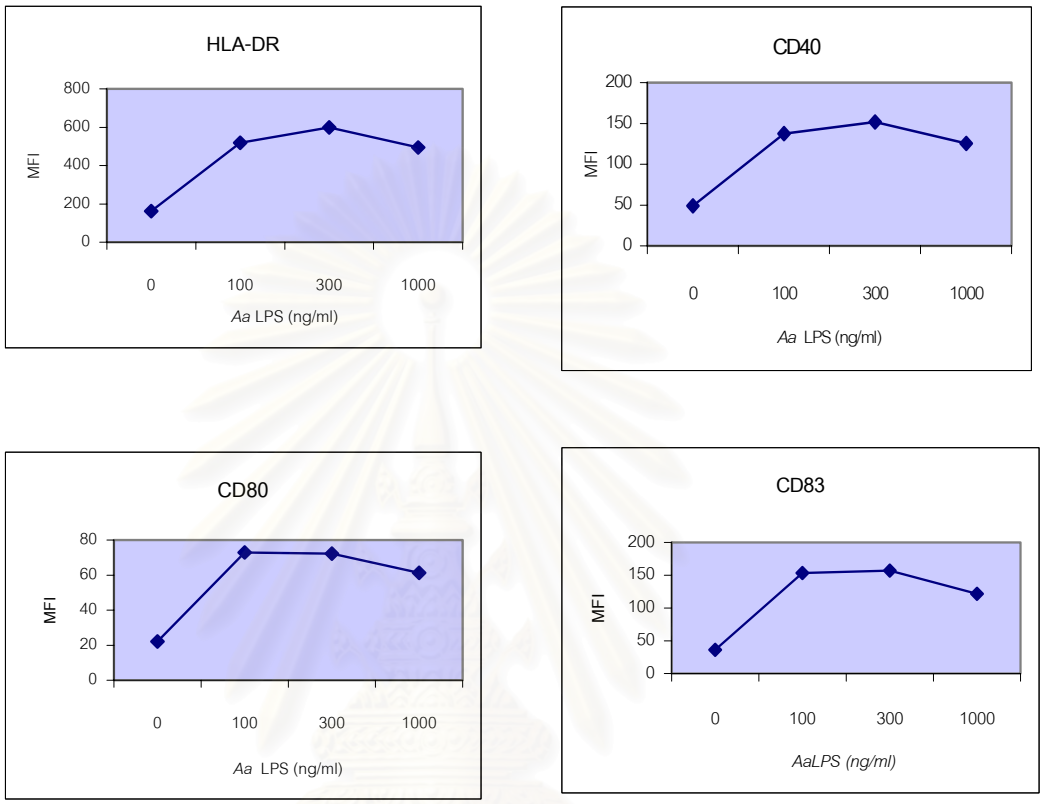


Figure 5B. Donor2: Analysis of co-stimulatory molecule and CD83 expression by flow cytometry.

Aa LPS = *Actinobacillus actinomycetemcomitans* LPS

MFI = mean fluorescence intensity

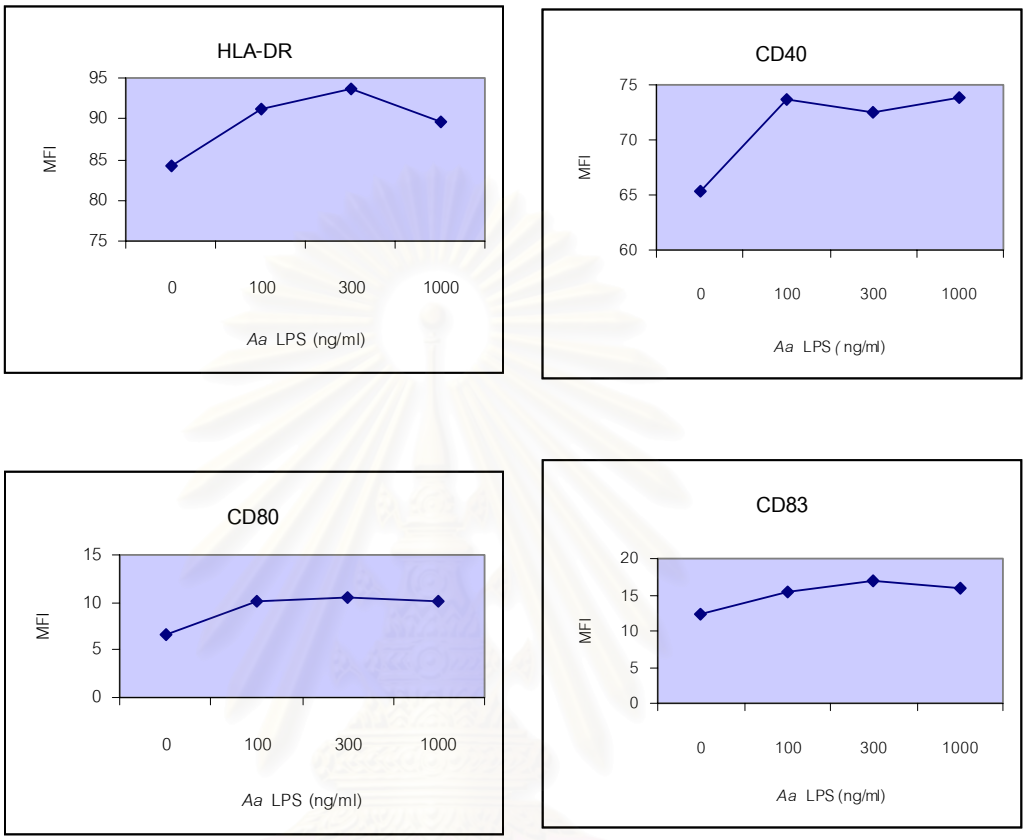


Figure 5C. Donor3: Analysis of co-stimulatory molecule and CD83 expression by flow cytometry.

Aa LPS = *Actinobacillus actinomycetemcomitans* LPS

MFI = mean fluorescence intensity

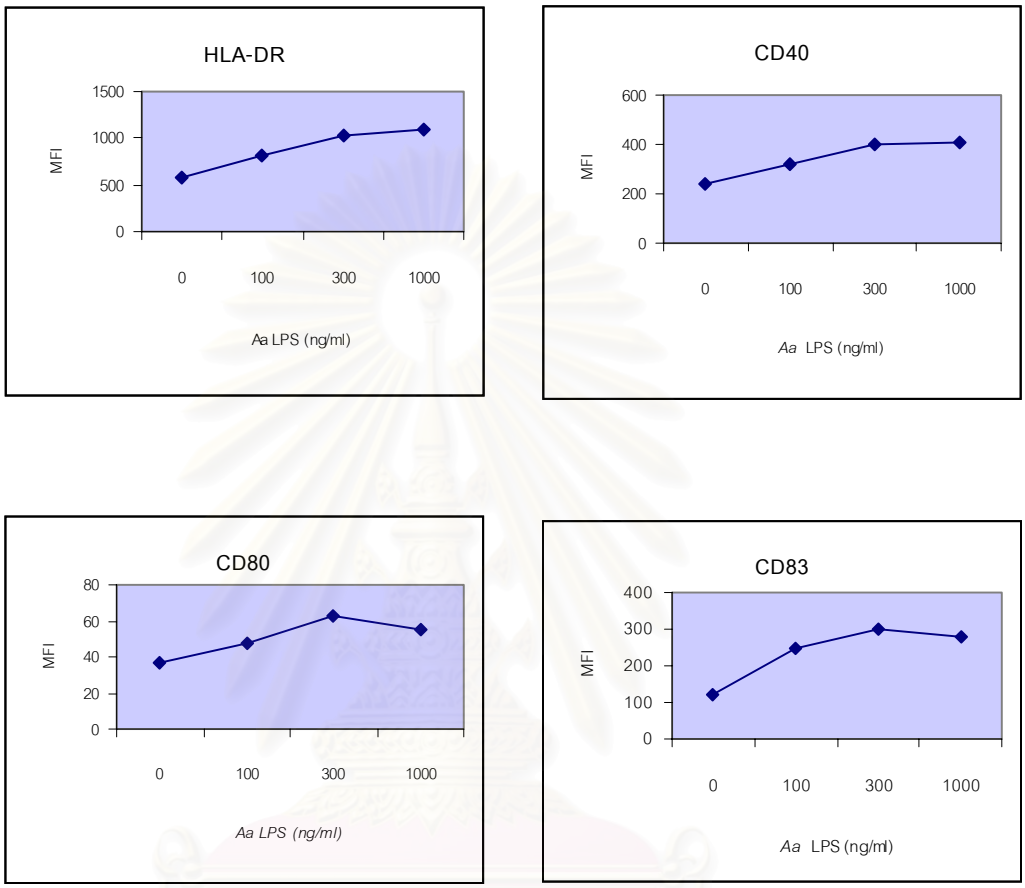


Figure 5D. Donor4: Analysis of co-stimulatory molecule and CD83 expression by flow cytometry.

Aa LPS = *Actinobacillus actinomycetemcomitans* LPS

MFI = mean fluorescence intensity

4.3 Cytokine production by monocyte-derived DCs stimulated with *A. actinomycetemcomitans* LPS.

We next examined the cytokine production from *A. actinomycetemcomitans* LPS - stimulated immature DCs. Six day cultured DCs were stimulated with 1,000 ng/ml of *A. actinomycetemcomitans* LPS. After 24 h., culture supernatants were harvested and then assayed for cytokine production. *A. actinomycetemcomitans* LPS consistently induced TNF-α but not IL-12 p70 production. Induction of IL-10 was observed in one of 4 donors.

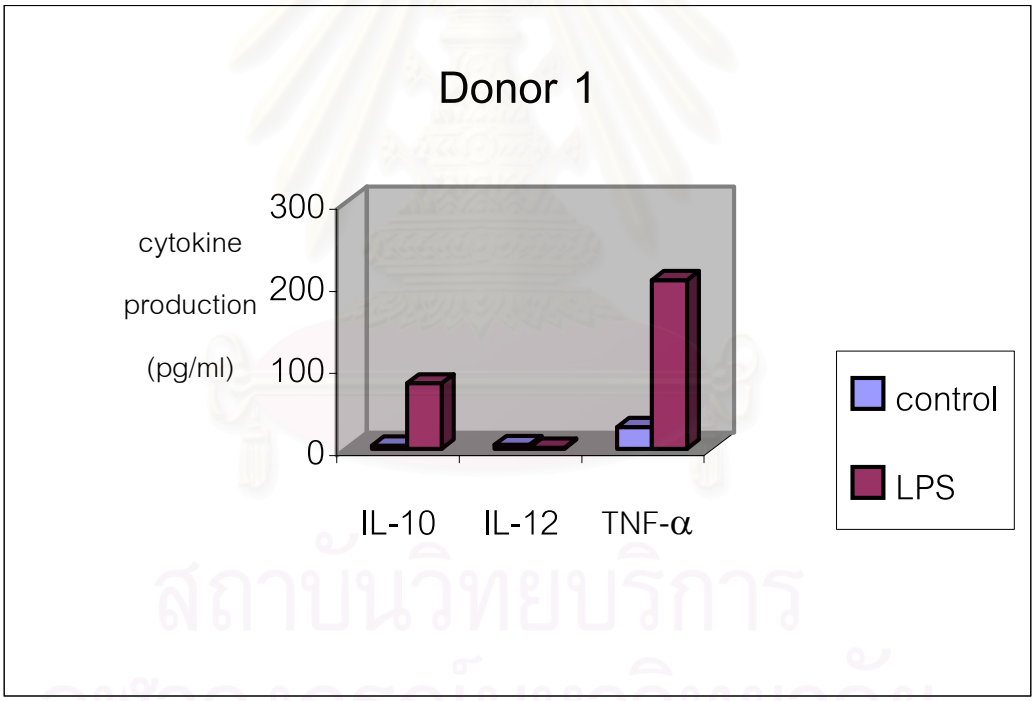


Figure 6A. Donor1: *A. actinomycetemcomitans* (*Aa*) LPS stimulates cytokine production from monocyte-derived DCs. DCs were cultured with *A. actinomycetemcomitans* LPS (1,000 ng/ml). Culture supernatants were collected after 24 h. of incubation and then assessed for cytokine production.

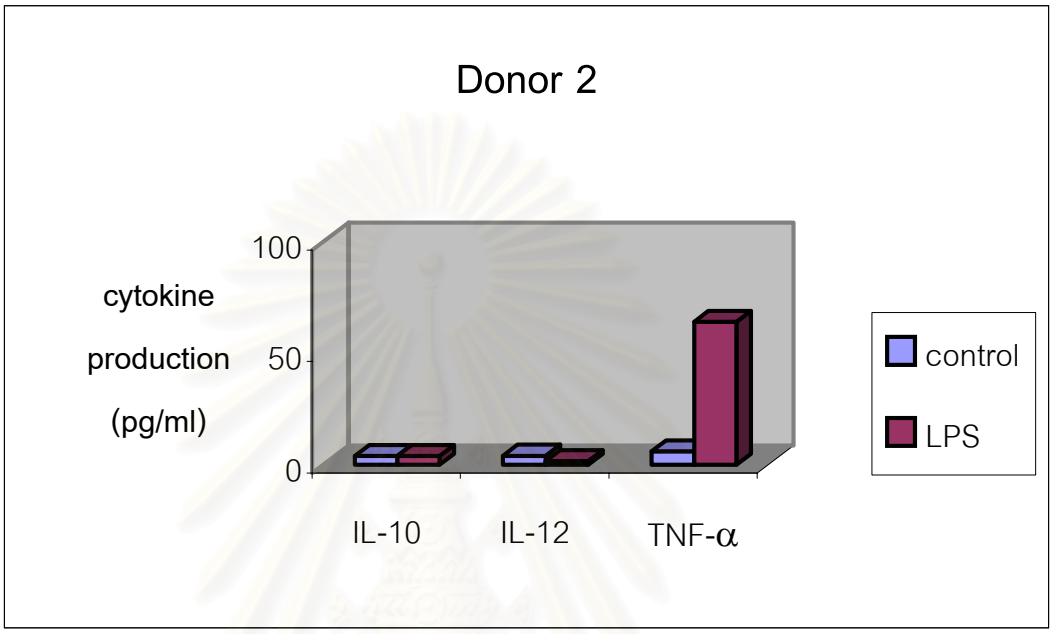


Figure 6B. Donor2: *A. actinomycetemcomitans* (*Aa*) LPS stimulate cytokine production from monocyte-derived DCs. DCs were cultured with *A. actinomycetemcomitans* LPS (1,000 ng/ml). Culture supernatants were collected after 24 h. of incubation and then assessed for cytokine production.

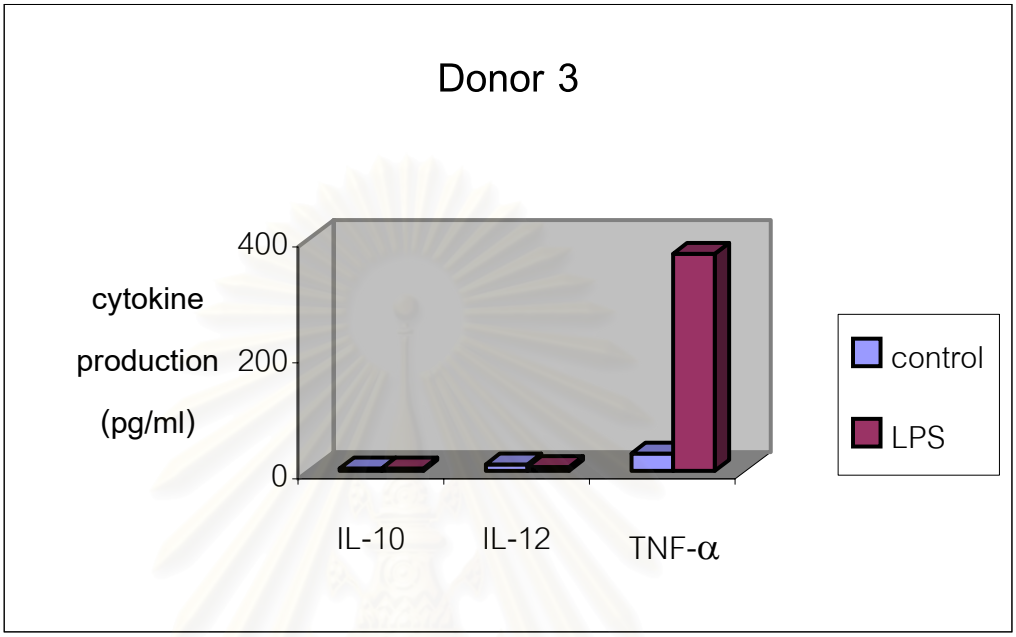


Figure 6C. Donor3: *A. actinomycetemcomitans* (*Aa*) LPS stimulates cytokine production from monocyte-derived DCs. DCs were cultured with *A. actinomycetemcomitans* LPS (1,000 ng/ml). Culture supernatants were collected after 24 h. of incubation and then assessed for cytokine production.

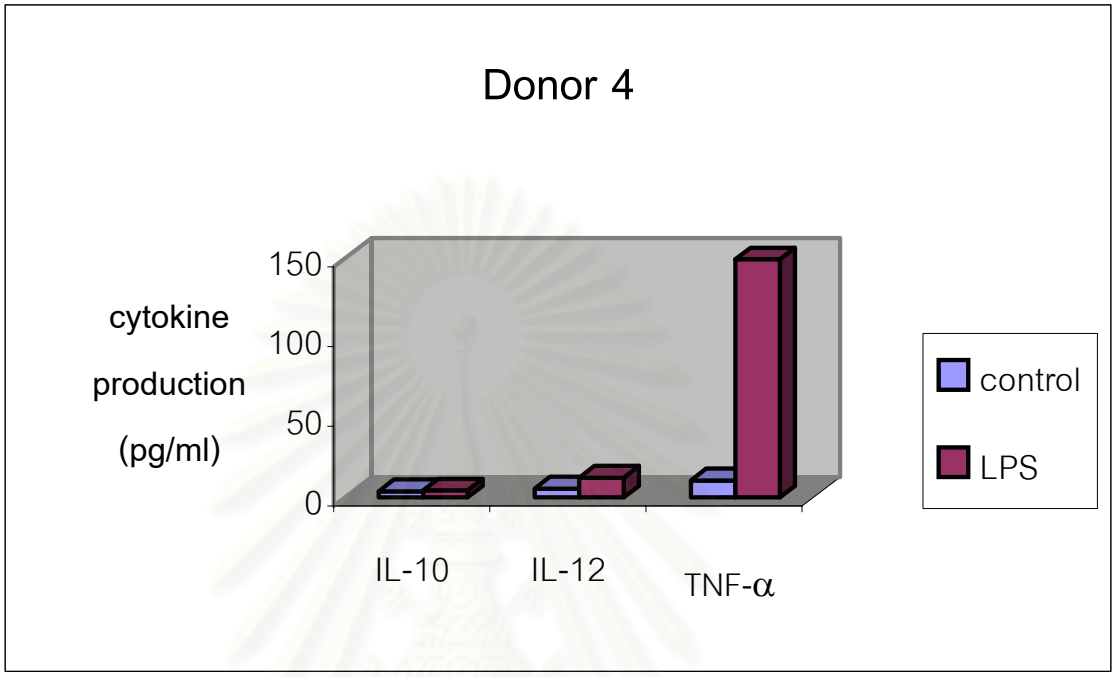


Figure 6D. Donor4: *A. actinomycetemcomitans* (*Aa*) LPS stimulates cytokine production from monocyte-derived DCs. DCs were cultured with *A. actinomycetemcomitans* LPS (1,000 ng/ml). Culture supernatants were collected after 24 h. of incubation and then assessed for cytokine production.

4.4 Intracellular cytokine staining in MLR

To examine the role of *A. actinomycetemcomitans* LPS-stimulated DCs in naïve T helper cell differentiation, allogeneic naïve $CD4^+$ T cells were cultured for 6 days with *A. actinomycetemcomitans* LPS-treated DCs. The activated T cells were then restimulated with PMA and ionomycin. Intracellular cytokine staining showed that *A. actinomycetemcomitans* LPS-treated DCs induced $CD4^+$ T cells to differentiate into Th1 cells associated with the ability to produce IFN-γ. No production of IL-4 and IL-10 was observed.

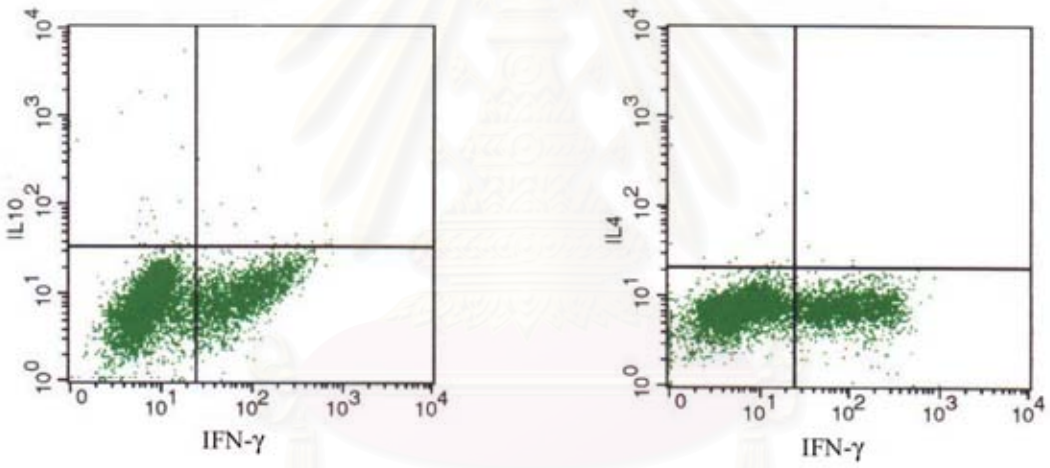


Figure 7 *A. actinomycetemcomitans* LPS - treated DCs predominantly induced IFN-γ production from allogeneic T cells. *A. actinomycetemcomitans* LPS - treated DCs were used to prime allogeneic naïve $CD4^+$ T cells for 6 days in mixed leukocyte reaction and production of IFN-γ, IL-4 and IL-10 was measured by intracellular cytokine staining.

CHAPTER 5

DISCUSSION

Antigen-presenting DCs are continuously produced from hematopoietic stem cells in the bone marrow and are widely distributed as immature DCs into both lymphoid and non-lymphoid tissues. The toll-like receptors (TLRs) which are expressed on DCs recognize conserved PAMPs found in microbial carbohydrated, lipids, protein and nucleic acid (Akira et al., 2001) The binding of PAMPs to TLRs triggers a cascade of cellular signals resulting in up-regulation of co-stimulatory molecule expression and production of cytokines.

DCs can be divided into subsets that differ in phenotype, function and localization. In human skin two subsets of immature DCs are found: Langerhans cells in the epidermis and interstitial DCs in the dermis (Bancherau and Steinman, 1998). In human blood, two subsets of DCs have been identified: $CD11c^+$ immature DCs (myeloid) and $CD11c^-$ DCs (plasmacytoid DCs) (Liu et al., 2001). DCs have the capacity to induce different types of T cell mediated immune response. The functional plasticity of DCs is depended on multiple factors in directing T cell responses. Certain characteristics of the pathogens play an important role in DC induced T cell differentiation. For example, viruses stimulate IFN-α from $CD11c^-$ DCs and induce their differentiation into DCs that elicit IFN-γ and IL-10-producing T cells; (Kadowaki et al., 2000), however, IL-3 induces their differentiation into Th2-inducing DCs (Rissoan et al., 1999). Different forms of the fungus instruct DCs to induce either Th1 or Th2 response. Yeast stage of *Candida albicans* stimulated DCs to produce IL-12 and induce Th1 response. However, the hyphae stage of *Candida albicans* stimulates DCs to produce IL-2

and induce Th2 response (de'Ostiani et al., 2000). Several viruses can suppress DC functions. Both HIV and measle viruses infect DC and suppress the ability to prime T cell response (Cameron et al., 1992). Anti-inflammatory molecules such as IL-10, transforming growth factor-β, prostaglandin E_2 and steroids stimulate DCs to induce Th2 differentiation or to inhibit Th1 differentiation (King et al., 1990; Kalinski et al. 1999). Tissue origin of DCs could also effect Th differentiation. DCs isolated from Payer's patch (Iwasaki, 1999), respiratory tract (Stumbles, 1998) and liver (Khanna et al., 2000) preferentially induce Th2 response. In contrast, $CD11c^+$ DCs isolated from the spleen preferentially induce Th1 response. These functional differences among different tissue DCs may result from difference in the tissue cytokine microenvironment.

Taken all together, these observations clearly indicate that the functional plasticity of DCs in priming Th response depends on multi factors including their lineage, maturation stage, activation signal and microenvironment.

A. actinomycetemcomitans is well known as one of the major putative periodontopathic bacteria. The most recent international workshop for a classification of periodontal diseases and conditions (AAP, 1999) added *A. actinomycetemcomitans* as a common microbiological feature of plaque in aggressive periodontitis, a group with rapid periodontal attachment loss and bone resorption. Subgingival colonization of *A. actinomycetemcomitans* has been demonstrated to increase the relative risk for periodontal disease progression three to four folds (Haffajee and Socransky, 1994). *A. actinomycetemcomitans* produces a multitude of virulent products such as collagenase, LPS, leukotoxin, polyclonal B cell activators, alkaline and acid phosphatase, (Wilson et al., 1996). Among these products, LPS derived from *A. actinomycetemcomitans* has been

considered to be involved in the pathogenesis of alveolar bone in periodontitis due to the ability to induce *in vivo* and *in vitro* bone resorption (Iino and Hopps, 1984; Ishihara et al., 1991; Nishihara et al., 1995; Ueda et al., 1998) or promote osteoclastogenesis (Kikuchi et al., 2001). Being potent stimulators for immune cells such as monocytes/macrophages and B cells, *A. actinomycetemcomitans* LPS was shown to induce monocyte/macrophage secretory function in culture. These cytokines and mediators include collagenases, IL-1α, IL-1β, IL-6, IL-8 and TNF-α, all of which mediate a variety of important immune, inflammatory and connective tissue effects involving in chronically inflamed periodontal tissues (Wahl, 1974; Cury et al., 1988; Saglie et al., 1990; Zadeh et al., 1999).

Immature DCs are present in small number (<2%) in most tissues, makes it so difficult to isolate and perform *in vitro* study. Recent advance in tissue culture demonstrated that DCs can be derived from monocytes *in vitro* by culturing with cytokines: GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). In the present study we isolated monocyte from human blood by positive sorting of $CD14^+$ cells using flow cytometry. The purity of the sorted cells was always > 95%. The sorted $CD14^+$ monocytes were cultured in medium containing 10% heat inactivated autologous serum and 50 ng/ml of GM-CSF and 50 ng/ml of IL-4 for 5-7 days. Differentiation of monocytes to DCs was analyzed by cell morphology and cell phenotypes. Upon cultured with GM-CSF and IL-4 for 6 days, cells became non-adherent, clustered, displayed different extracts of veiled and dendritic morphology. Phenotypic analysis indicated that they became $CD14^-$ and increasing $CD1a^+$ but they remained characteristics of immature DCs as CD83 expression was minimal.

These immature DCs when stimulated with *A. actinomycetemcomitans* LPS *in vitro* for 24 h. led to cell maturation associated the enhanced expression of

co-stimulatory molecule (CD40 and CD80), maturation marker (CD83) and HLA-DR. Unlike *Escherichia coli* LPS which is known to be potent for cytokine production (Jarrossay et al., 2001), *A. actinomycetemcomitans* LPS at 1,000 ng/ml induced minimally production of cytokines. Moderate amount of TNF-α was consistently observed, however, we could not detect any IL-12 production. TNF-α is known as a cytokines that involves DC maturation. Its presence in the cultures may help support the growth of DCs.

Recognition of bacterial LPS is known to be mediated by TLRs on DCs. *E. coli* LPS and *P. gingivalis* LPS have been shown to bind to TLR4 (Poltorak et al., 1998; Qureshi et al., 1999) and TLR2 (Underhill and Ozinsky, 2002) respectively. It would be of interest for the future study to evaluate the particular type of TLR on DCs that recognizes *A. actinomycetemcomitans* LPS.

Due to the very low frequency of antigen-specific T cells, it is very difficult to set up the experiment in order to test antigen-specific T cell stimulatory effect of DCs in human. At present, the most common and established assay used to test the antigen presenting ability of DCs is allogeneic MLR (Caux et al., 1995). The results of MLR in this study imply that *A. actinomycetemcomitans* LPS treated-DCs possibly promote Th1 response as indicated by predominant production of IFN-γ but not IL-4 and IL-10 by allogeneic T cells. It should be noted that a single experiment of MLR with *A. actinomycetemcomitans* LPS treated-DCs was carried out and the experiment of DC controls (untreated-DCs) was not performed. It was due to the very low number of sorted naïve $CD4^+$ T cells and the limit amounts of five mAbs required for this specific cell sorting. Therefore, more studies are required to confirm these findings.

The observed Th1 response in MLR is unlikely to be mediated by IL-12 since this cytokine production was not detected in the culture supernatants of *A. actinomycetemcomitans* LPS treated-DCs. The mechanisms underlying Th1 and Th2 response are very complex. Other factors such as expression of co-stimulatory molecule, CD80 on DCs (Schipers et al., 1998) and IFN-α (Kadowaki et al., 2000) has also been proposed to play role in favor of Th1 response.

In conclusion, our study demonstrated that we were able to generate DCs from monocytes using established procedure in which flow cytometrically sorted $CD14^+$ monocytes were cultured with GM-CSF and IL-4 for 6 days. *In vitro* generated DCs were at immature stage. They became mature when stimulated with *A. actinomycetemcomitans* LPS and capable to drive Th1 response. It is too early to draw any conclusion concerning the effect of *A. actinomycetemcomitans* on DC-induced Th response. In addition to LPS, other components derived from *A. actinomycetemcomitans* such as lipid, lipoprotein and DNA may also have effects on DCs as well. The use of DCs generated by these techniques will enhance further evaluation of oral plaque bacteria and their products on DCs.

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APPENDIX

Table 2. Donor 1 : Co-stimulatory molecule and CD83 expression on monocyte derived DCs after *A. actinomycetemcomitans* LPS stimulation.

Donor 1	MFI			
	LPS (ng/ml)			
	0	100	300	1000
HLA-DR	304.3	945.3	797.7	691.3
CD40	102.5	394.8	344.8	294.9
CD80	16.5	60	49.6	41.9
CD83	54.9	221.9	232.3	203.8

Table 3. Donor 2 : Co-stimulatory molecule and CD83 expression on monocyte derived DCs after *A. actinomycetemcomitans* LPS stimulation.

Donor 2	MFI			
	LPS (ng/ml)			
	0	100	300	1000
HLA-DR	161.7	518.2	598.6	494.8
CD40	49	137.4	151.9	125.4
CD80	22.1	72.9	72.3	61.2
CD83	36.4	153.6	157.2	121.8

Table 4. Donor 3 : Co-stimulatory molecule and CD83 expression on monocyte derived DCs after *A. actinomycetemcomitans* LPS stimulation.

Donor 3	MFI			
	LPS (ng/ml)			
	0	100	300	1000
HLA-DR	84.3	91.1	93.6	89.6
CD40	65.3	73.6	72.6	73.8
CD80	6.7	10.1	10.5	10.1
CD83	12.3	15.3	16.9	15.8

Table 5. . Donor 4 : Co-stimulatory molecule and CD83 expression on monocyte derived DCs after *A. actinomycetemcomitans* LPS stimulation.

Donor 4	MFI			
	LPS (ng/ml)			
	0	100	300	1000
HLA-DR	576.4	816.8	1032.7	1086.5
CD40	241	317.1	403.1	404.6
CD80	36.2	48.1	62.5	55.6
CD83	118.8	248	299.2	278.4

Table 6. Donor 1 : Cytokine production from monocyte-derived DCs with and without *A. actinomycetemcomitans* LPS stimulation. Sensitivity : TNF-α = 4.5 pg/ml, IL-10 = 3.9 pg/ml, IL-12 = 4 pg/ml.

	Cytokine production (pg/ml)	
	LPS (ng/ml)	
	0	1000
IL-10	3.9	79.6
IL-12	44.8	not detected
TNF-α	26.5	205.5

Table 7. Donor 2 : Cytokine production from monocyte-derived DCs with and without *A. actinomycetemcomitans* LPS stimulation. Sensitivity : TNF-α = 4.5 pg/ml, IL-10 = 3.9 pg/ml, IL-12 = 4 pg/ml.

	Cytokine production (pg/ml)	
	LPS (ng/ml)	
	0	1000
IL-10	3.9	3.9
IL-12	4	not detected
TNF-α	6	64.1

Table 8. Donor 3 : Cytokine production from monocyte-derived DCs with and without *A. actinomycetemcomitans* LPS stimulation. Sensitivity : TNF-α = 4.5 pg/ml, IL-10 = 3.9 pg/ml, IL-12 = 4 pg/ml.

	Cytokine production (pg/ml)	
	LPS (ng/ml)	
	0	1000
IL-10	3.9	3.9
IL-12	10.7	6.4
TNF-α	29.8	374.7

Table 9. Donor 3 : Cytokine production from monocyte-derived DCs with and without *A. actinomycetemcomitans* LPS stimulation. Sensitivity : TNF-α = 4.5 pg/ml, IL-10 = 3.9 pg/ml, IL-12 = 4 pg/ml.

	Cytokine production (pg/ml)	
	LPS (ng/ml)	
	0	1000
IL-10	3.9	4.4
IL-12	5.6	12.4
TNF-α	10.7	149.4

BIOGRAPHY

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