ลิโพพอลิแซ็กคาไรด์ของพอร์ไฟโรโมแนสจิงจิวาลิสและผลึกคอเลสเตอรอลในการเหนี่ยวนำการกระตุ้นอินเฟลมมาโซมในเซลล์แมคโครฟาจมนุษย์

นายมหัทธน พูลเกษร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาปริทันตศาสตร์ ภาควิชาปริทันตวิทยา
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

Porphyromonas gingivalis lipopolysaccharide and cholesterol crystals induce inflammasome activation in human macrophages

Mr. Mahatana Poolgesorn

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Periodontics
Department of Periodontology
Faculty of Dentistry
Chulalongkorn University
Academic Year 2011

Thesis Title	*PORPHYROMONAS GINGIVALIS* LIPOPOLYSACCHARIDE AND CHOLESTEROL CRYSTALS INDUCE INFLAMMASOME ACTIVATION IN HUMAN MACROPHAGES
By	Mr. Mahatana Poolgesorn
Field of Study	Periodontics
Thesis Advisor	Associate Professor Rangsini Mahanonda, Ph.D.
Thesis Co-advisor	Sathit Pichyangkul, Ph.D.

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

…… Dean of the Faculty of Dentistry
(Associate Professor Wacharaporn Tasachan)

THESIS COMMITTEE

…… Chairman
(Associate Professor Naulchavee Hongprasong)

…… Thesis Advisor
(Associate Professor Rangsini Mahanonda, Ph.D.)

…… Thesis Co-advisor
(Sathit Pichyangkul, Ph.D.)

…… External examiner
(Professor Stitaya Sirisinha, Ph.D.)

มหัทธน พูลเกษร : ลิโพพอลิแซ็กคาไรด์ของพอร์ไฟโรโมแนสจิงจิวาลิสและผลึกคอเลสเตอรอลในการเหนี่ยวนำการกระตุ้นอินเฟลมมาโซมในเซลล์แมคโครฟาจมนุษย์. (*PORPHYROMONAS GINGIVALIS* LIPOPOLYSACCHARIDE AND CHOLESTEROL CRYSTALS INDUCE INFLAMMASOME ACTIVATION IN HUMAN MACROPHAGES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. รังสินี มหานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. สาธิต พิชญางกูร, 55 หน้า.

ช่องปากได้รับความสนใจว่าเป็นแหล่งของการติดเชื้อในอวัยวะอื่นๆของร่างกายมายาวนาน โรคปริทันต์เป็นโรคอักเสบเรื้อรังที่เกิดจากเชื้อแบคทีเรียแกรมลบ เช่น *Porphyromonas gingivalis* และผลผลิตของเชื้อ โรคหลอดเลือดแดงแข็ง (atherosclerosis) เป็นโรคหัวใจและหลอดเลือดที่สำคัญและเป็นสาเหตุการตายอันดับต้นๆในกลุ่มประชากรทั่วโลก ลักษณะของโรคเกี่ยวข้องกับการอักเสบเรื้อรังและสัมพันธ์กับการสะสมคอเลสเตอรอลในผนังหลอดเลือด ระบบภูมิคุ้มกันชนิดอินเนตจากเนื้อเยื่อหลอดเลือดมีบทบาทสำคัญในกระบวนการเกิดและดำเนินของโรคหลอดเลือดแดงแข็ง ลักษณะสำคัญของรอยโรคหลอดเลือดแดงแข็งคือ lipid-laden macrophages หรือโฟมเซลล์ (foam cell) ซึ่งเป็นแหล่งสำคัญของ proinflammatory cytokines รวมทั้ง interleukin-1ß (IL-1ß) ซึ่งเกิดจากการกระตุ้นผ่าน NLRP3 อินเฟลมมาโซม ในปัจจุบันยังไม่มีการศึกษาใดที่อธิบายความสัมพันธ์ของบทบาทเชื้อก่อโรคปริทันต์ต่อโรคหลอดเลือดแดงแข็งในแง่มุมของการกระตุ้นอินเฟลมมาโซม ดังนั้นในการศึกษานี้ จึงทำการศึกษาถึงผลของ *P. gingivalis* LPS และผลึกคอเลสเตอรอลต่อการกระตุ้นเซลล์แมคโครฟาจมนุษย์ในลักษณะของการกระตุ้น NLRP3 อินเฟลมมาโซมซึ่งวัดผลจากการสร้าง IL-1ß ระหว่างเซลล์แมคโครฟาจที่กระตุ้นโดยใช้ M-CSF (M2 macrophage) และเซลล์แมคโครฟาจที่กระตุ้นโดยใช้ GM-CSF (M1 macrophage) ผลการศึกษาแสดงให้เห็นว่า *P. gingivalis* LPS และผลึกคอเลสเตอรอลสามารถกระตุ้นให้มีการหลั่ง IL-1ß จากเซลล์แมคโครฟาจทั้งสองชนิดได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับเซลล์แมคโครฟาจที่เลี้ยงในอาหารเลี้ยงเซลล์ ($p<0.05$) และระดับของ IL-1ß สัมพันธ์กับความเข้มข้นของผลึกคอเลสเตอรอล ไม่มีความแตกต่างทางสถิติระหว่างระดับของ IL-1ß ที่สร้างจาก M1 macrophage เมื่อเทียบกับ M2 macrophage ในทุกความเข้มข้นของผลึกคอเลสเตอรอล ($p>0.05$) การศึกษานี้ถือเป็นการศึกษาแรกที่แสดงให้เห็นถึงบทบาทการกระตุ้น NLRP3 inflammasome ในเซลล์แมคโครฟาจทั้งสองชนิดโดยใช้ *P. gingivalis* LPS และผลึกคอเลสเตอรอล ซึ่งอาจเป็นไปได้ว่ากลไกดังกล่าวน่าจะเกี่ยวข้องกับการสนับสนุนให้เกิดโรคหลอดเลือดแดงแข็งได้

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

สาขาวิชา.....ปริทันตศาสตร์..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2554..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5276122032 : MAJOR PERIODONTICS

KEYWORDS: *P. GINGIVALIS* LPS / INFLAMMASOMES / ATHEROSCLEROSIS / HUMAN MACROPHAGE / CHOLESTEROL CRYSTALS

MAHATANA POOLGESORN: *PORPHYROMONAS GINGIVALIS* LIPOPOLYSACCHARIDE AND CHOLESTEROL CRYSTALS INDUCE INFLAMMASOME ACTIVATION IN HUMAN MACROPHAGES.

ADVISOR: ASSOC. PROF. RANGSINI MAHANONDA, Ph.D,

CO-ADVISOR : SATHIT PICHYANGKUL, Ph.D, 55 pp.

Human mouth has long been recognized as a focal infection and being connected to the systemic health. Periodontal disease is a chronic inflammatory disease caused by Gram-negative bacteria and their products such as *Porphyromonas gingivalis*. Atherosclerosis, the major cardiovascular disease, is a leading cause of death worldwide. It is a chronic inflammatory disease associated with cholesterol deposition in the arterial walls. The innate immune response of the vascular tissue is known to play important role in atherosclerosis. A hallmark of the atherosclerotic lesion is lipid-laden macrophages or foam cells which are a source of various proinflammatory cytokines including interleukin-1ß (IL-1ß) produced by inflammasome activation. To the best of our knowledge, no previous studies have assessed the role of periodontal infection in atherosclerosis in term of inflammasome activation. In this study, we examined the effects of *P. gingivalis* LPS and cholesterol crystals on human monocyte derived macrophage in term of NLRP3 inflammasome activation by IL-1ß production and compared the magnitude of inflammasome activation as measured by IL-1ß production between macrophages derived by M-CSF(M2 macrophage) and those derived by GM-CSF(M1 macrophage). The results showed that *P. gingivalis* LPS and cholesterol crystals induced dose-dependent IL-1ß secretion from both M1 macrophage and M2 macrophage via NLRP3 inflammasome activation when compared with control ($p<0.05$). No significant differences in IL-1ß production at each concentration of cholesterol crystals were observed between two types of macrophages ($p > 0.05$). Our results represent the first demonstration that NLRP3 inflammasome activation in human M1 and M2 macrophage via *P. gingivalis* LPS and cholesterol crystals may be, in part, a possible mechanism of which promote atherosclerosis.

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

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

Academic year……2011…… Co-advisor's Signature…….

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my advisor, Associate Professor Dr. Rangsini Mahanonda, for her guidance, encouragement, supervision, suggestion and kindness throughout the course of my master degree program. I am extremely indebted to my co-advisor, Dr. Sathit Pichyangkul, Department of Immunology and Medical Component, AFRIMS, for providing the laboratory facilities and his grateful guidance, supervision, valuable technical advice and correction of this thesis. I wish to thank my thesis committee members; Associate Professor Naulchavee Hongprasong and Professor Dr. Stitaya Sirisinha for their suggestions and kindness in being committee members.

Sincere appreciation is expressed to Dr. Chantrakorn Champaiboon and Mr. Noppadol Sa-Ard-Iam for their assistance in setting the experiments and preparing this manuscript. I also would like to thank Ms. Pimprapa Rerkyen for kind advice and technical assistance.

I would like to acknowledge research grant from the NRCT's research fund for the partial financial support for this study. My sincere appreciation is also extended to the staff of Periodontology Department, Faculty of Dentistry, Chulalongkorn University for their kindness, guidance and encouragement. Finally, I would like most sincerely to thank my father, my mother, my brothers and my friends for their love, caring, understanding and encouragement.

CONTENTS

Page

Chapter

LIST OF TABLES

LIST OF FIGURES

Figures Page

LIST OF ABBREVIATIONS

ApoE	Apolipoprotein E
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	Adenosine triphosphate
CC	cholesterol crystal
CD	Cluster of differentiation
DAMPs	Danger-associated molecular patterns
E. coli	*Escherichia coli*
GM-CSF	Granulocyte macrophage colony-stimulating factor
HSPs	Heat shock proteins
IL	Interleukin
IL-1ß	Interleukin-1ß
K^+	Potassium ion
KDO	3-Deoxy-D-manno-oct-2-ulosonic acid
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
M1 macrophage	GM-Macrophage, pro-inflammatory macrophage
M2 macrophage	M-Macrophage, anti-inflammatory macrophage
MDP	Muramyl dipeptide
ml	Milliliter
mM	Millimolar

MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MSU	Monosodium urate
M-CSF	Monocyte colony-stimulating factor
ng	nanogram
NHANES	National Health and Nutrition Examination Survey
NLRP3	Nucleotide-binding domain leucine-rich repeated containing family, pyrin domain containing 3
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptor
NO	Nitric oxide
P2X7	Purinergic receptor, ligand-gated ion channel, 7
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
pg	picogram
P. gingivalis	*Porphyromonas gingivalis*
PPARγ	Peroxisome proliferator-activated receptor γ
PRRs	Pattern recognition receptors

ROS	Reactive oxygen species
TGF-ß	Transforming growth factor-ß
Th1	T helper 1
Th2	T helper 2
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRX	Thioredoxin
TXNIP	Thioredoxin-interacting protein
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very-low-density lipoprotein
µg	Microgram
µm	Micrometre

CHAPTER I

INTRODUCTION

1.1 Background of the present study

Human mouth has long been recognized as a focal infection and being connected to the systemic health since the nineteenth century (Hansson, 2009). Recently in the general conclusion of the 2010 European workshop in periodontal health and cardiovascular disease consensus document stated that based on epidemiological evidence, there is a moderate but significant association between periodontitis and cardiovascular disease (Bouchard et al., 2010). However, the underlying mechanisms between the two diseases is still lacking which is remained to be determined.

Periodontal disease is one of the most common chronic inflammatory disease in humans. It affects the tooth attachment apparatus called periodontium. The etiologic agent is bacteria in dental plaque. Bacterial plaque and their components trigger host inflammatory responses which subsequent lead to periodontal destruction such as bone loss. *Porphyromonas gingivalis* (*P. gingivalis*) is one of the key periodontal pathogens which is frequently found in many forms of periodontal diseases such as chronic and aggressive periodontitis. It is one of the most studied organisms in periodontal disease due to its virulence factors such as fimbriae and lipopolysaccharide (LPS). *P. gingivalis* LPS is a potent stimulator for human immune systems and able to enhance periodontal bone destruction (Jain and Darveau, 2010).

Cardiovascular disease, in which atherosclerosis is the major underlying cause, is currently the leading cause of death in many societies. Atherosclerosis is a chronic

inflammatory disease which involves thickening or hardening of the large and medium-sized arteries. Accumulation of lipids within macrophage (lipid-laden macrophage) with foam cell appearance in the intima is recognized as a hallmark of the early atherosclerotic lesion. These foam cells differentiate from recruited blood monocytes. Mature plaque can cause clinical complications such as flow-limiting stenoses, but the most severe end-stage complications or events include myocardial infarction, stroke, critical limb ischaemia which follow the rupture of a plaque and thrombosis (Libby, 2002).

A hundred years ago, atherosclerosis was considered as a lipid disease (Hansson, 2009). Now it becomes clear that the disease is a multi-factorial in nature. Besides lipid, the importance of the role of infection and inflammation in the initiation and progression of atherosclerosis is widely accepted (Ross, 1999). Distant chronic infection such as periodontitis is one of the candidates. Ulcerated inflamed periodontal pockets in periodontitis may be a pathway for plaque micro-organisms and their products such as LPS to enter blood vessels in the connective tissue and into systemic circulation, thus subsequently activate inflammatory response of the artery. This is supported by the observations that key periodontal pathogens such as *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* could be detected in human atherosclerotic lesion (Haraszthy et al., 2000). In addition, previous study using monocyte-derived macrophages demonstrated that live *P. gingivalis* promoted foam cell formation in the presence of low-density lipoprotein (Giacona et al, 2004).

Cholesterol, an indispensable lipid in vertebrates, is effectively insoluble in aqueous environments. When it is abundant, it crystallizes from a liquid to a solid state, forming cholesterol crystals. A novel link between cholesterol crystals and inflammation in atherosclerotic lesion was shown by their ability to activate caspase-1-activating nucleotide-binding domain leucine-rich repeated containing family, pyrin domain containing 3 (NLRP3)

inflammasome, which results in cleavage and secretion of Interleukin-1 (IL-1) family cytokines, in human and mouse macrophages (Rajamaki et al.,2010, Duewell et al.,2010).

The inflammasomes are large multiprotein complex which play a key role in the innate immunity by regulating the maturation and secretion of cytokine IL-1ß (Martinon et al., 2009). The inflammasomes belong to the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family of pattern recognition receptors, an intra-cellular sensor. The NLRP3 inflammasome (also known as NALP3 and cryopyrin) is the most fully studied of the inflammasomes. It comprises the NLR protein NLRP3, the adaptor ASC (Apoptosis-associated speck-like protein containing a caspase recruitment domain) and pro-caspase-1. Even though, the mechanisms of NLRP3 inflammasome activation are still not clear, the general consensus is that maturation and release of IL-1ß requires two distinct signals. The first signal is through Toll-like receptors (TLRs) or by cytokines, such as tumor necrosis factor or IL-1ß itself which leads to synthesis of pro-IL-1ß. The second signal results in assembly of the NLRP3 inflammasome, caspase-1 activation and IL-1ß secretion (Chen and Pedra, 2010).

IL-1ß causes a wide variety of biological effects associated with infection, inflammation and autoimmune processes. IL-1ß, a pro-inflammatory cytokine, is implicated in recruitment of inflammatory cells to a site of infection or injury. IL-1ß is produced as inactive precursors, pro-IL-1ß, and share a common maturation mechanism that requires caspase-1. Activation of caspase-1 occurs within the inflammasome following its assembly.

In the present study, we were extended the previous study of cholesterol crystal-induced NLRP3 inflammasome activation in human macrophage, which may contribute to inflammatory response in atherosclerosis. We used an *in vitro* model of human monocyte-derived macrophage to investigate inflammasome activation by a key periodontal pathogen - *P.gingivalis* LPS, a TLR ligand, and cholesterol crystals in order to explore the link

between periodontitis and atherosclerosis. Moreover, macrophage are phenotypically diverse. M1 and M2 macrophages had been described; macrophages cultivated with granulocyte macrophage colony-stimulating factor (GM-CSF) as M1 macrophage (also GM-Mac, pro-inflammatory macrophage) and with monocyte colony-stimulating factor (M-CSF) as M2 macrophage (also M-Mac, anti-inflammatory macrophage) (Brocheriou et al., 2010). Therefore, in this study, we compared inflammasome activation between human monocyte derived macrophages derived by M-CSF and those derived by GM-CSF.

1.2 Objectives

In this study we examined the effects of *P. gingivalis* LPS and cholesterol crystals on human monocyte derived macrophage in term of NLRP3 inflammasome activation by IL-1ß production and compared the magnitude of inflammasome activation as measured by IL-1ß production between macrophages derived by M-CSF (M2 macrophage) and those derived by GM-CSF (M1 macrophage).

1.3 Hypothesis

1.3.1 *P.gingivalis* LPS and cholesterol crystals stimulate inflammasome activation in monocyte-derived macrophages.

1.3.2 M-CSF derived macrophage (M2 macrophage) produces different level of IL-1ß when compare with GM-CSF derived macrophage (M1 macrophage) upon activation by *P.gingivalis* LPS and cholesterol crystals.

1.4 Field of research

Innate immune activation via inflammasome in human macrophages by *P.gingivalis* LPS and cholesterol crystals.

1.5 Criteria inclusions

1.5.1 Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult subjects who had no taken any antibiotics or anti-inflammatory drugs within the past 3 months prior to blood donation.

1.5.2 Analysis of cell surface markers was determined by flow cytometry.

1.5.3 The responses of monocyte-derived macrophages to *P. gingivalis* LPS, cholesterol crystals, *E. coli* LPS and ATP were measured by ELISA.

1.6 Limitation of research

This study cannot investigate many human peripheral blood mononuclear cell samples due to high expenses.

1.7 Application and expectation of research

1.7.1 New scientific information : how *P.gingivalis* LPS affects human monocyte-derived macrophages. A better understanding about the link between *P.gingivalis* LPS and atherosclerosis.

1.7.2 Publication in the national peered-review journal.

1.8 Keywords

P. gingivalis LPS, Inflammasomes, Atherosclerosis, Human macrophage, cholesterol crystals

CHAPTER II

LITERATURE REVIEW

2.1 Periodontal disease

Periodontal disease is a chronic inflammatory disease affecting the periodontium that consists of gingiva, periodontal ligament, cementum, and alveolar bone. It is initiated by specific bacterial pathogens with progressive destruction of attachment apparatus of the tooth (Pollanen et al., 2003). The disease initiation and progression involve chronic inflammation of the periodontium with the specific response of the host that seems to be a major role of disease development. Periodontal disease also affects people at all ages, however, the serious destruction of the periodontium is more common in adult. Over the past decades, periodontal disease has accumulated supporting evidence that it is a potential risk factor for several systemic diseases including diabetes mellitus and atherosclerosis (Buhlin et al., 2009).

The destructive mechanisms of the disease may involve many modalities of both the host defense and microbial virulence. Bacteria and its components trigger inflammation and subsequent destruction of periodontal tissue (Schwartz et al., 1997). The inflammatory process involves activation of broad axis of innate immunity, specifically by up-regulation of proinflammatory cytokines including members of the interleukin 1 family such as IL-1ß and IL-18 (Taylor, 2010), which are present in the diseased tissue, and their unbalanced productions appear to mediate periodontal tissue destruction. Despite identification of over 700 different bacterial species in the oral cavity (Hamlet, 2010), only a relative few organisms are linked to periodontal disease. However, *Porphyromonas gingivalis* (*P. gingivalis*) is one of the most studied organisms linked to any forms of periodontal disease

(Taylor, 2010). *P. gingivalis* is a gram-negative, non-motile, a saccharolytic rod bacterial anaerobe and has been involved with increasing risk of periodontal disease severity and progression. *P.gingivalis* produces a large groups of virulence factors, including lipopolysaccharide (LPS).

LPS also known as bacterial endotoxin is outer membrane molecule essentials for alll gram-negative bacteria (Erridge et al., 2002). It is described as a nonproteinaceous, heat-stable, endotoxic microbial cell wall components that consists of highly variable as well as very high conserved segments. It is known as a complicated glycolipid molecule consisted of a hydrophilic polysaccharide part and a hydrophobic portion known as lipid A. Figure 1 shows general overview of LPS.

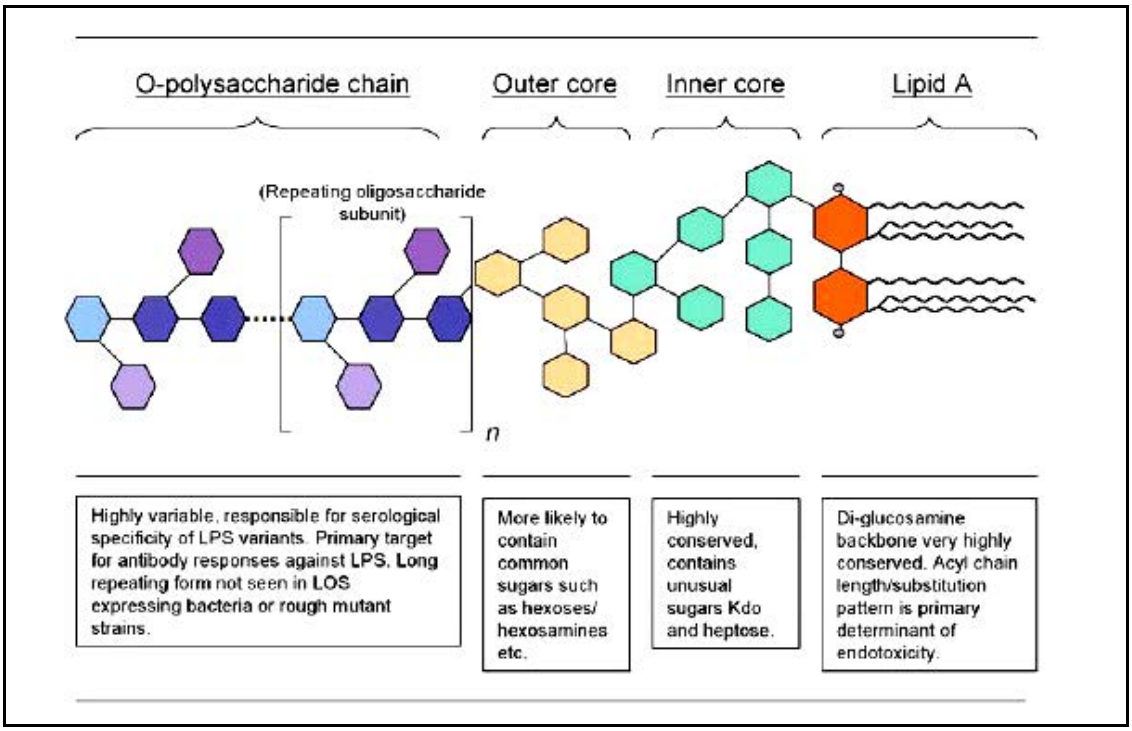


Figure 1. General overview of gram-negative lipopolysaccharide.
LOS=lipo-oligopolysaccharide (Erridge et al., 2002).

Lipid A is the most conserved part of protein structure and also known as endotoxically active part of the molecule. Lipid A moiety is typically conserved within bacterial species, however, there is often heterogeneity in the number and type of secondary fatty acids expression(Dixon and Darveau, 2005). The inner core that proximal to lipid A highly contains proportion of unusual sugar, particularly 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) and heptose (Wang, 2002). The outer core that is extended from bacterial surface is consists of more common hexose sugars i.e. glucose, galactose, and N-acetyl glucosamine. The outer core shows more various component than inner core. All inner and outer core sugar residues have a chance of substitution with charged groups such as phosphate, pyrophosphate, and 2-aminoethylpyrophosphate. Some species contain a common part that consists of specific components like O-antigen (Erridge et al., 2002). The repeating portions of the O-polysaccharide part consist from 1 to 8 glycosyl

residues and differ between strains by identification of the sugars, sequence, chemical molecule linkage, and substitution. The outermost part of the LPS is the O-polysaccharide. It is expressed on bacteria and is the main antigen that targeted by host antibody responses.

P. gingivalis lipid A structure expresses less level of endotoxin activity when compare with LPS from another gram negative bacteria and lacks of phosphate group or acyl chain in some type of lipid A structure. The 5-acyl chains with mono-phosphorylated lipid A from *P.gingivalis* has different response from the hexa-acyl diphosphorylated lipid-A from *Escherichia coli* (*E.coli*) while recognized by the TLR4 (Hirai, 2003). Alternatively, other studies suggested that *P. gingivalis* LPS also shows as the strong inducer of various biological responses, such as polyclonal B-cell activation, bone resorption, inhibition of bone formation, and fibroblast proliferation (Wang and Ohura, 2002). The LPS from *P. gingivalis* is recognized for TLR2 (Jain and Darveau, 2010). The reaction with TLR is the first signal of inflammasome activation that leads to IL-1ß secretion after stimulation with the second signals such as ATP and cholesterol crystals. Figure 2 shows *E. coli* and *P. gingivalis* Lipid A. The heterogeneity of *P. gingivalis* Lipid A may involved the variation of innate immune response and further investigation also required for more understanding about the role of *P. gingivalis* Lipid A in human immune response especially by TLR recognition (Jain and Darveau, 2010).

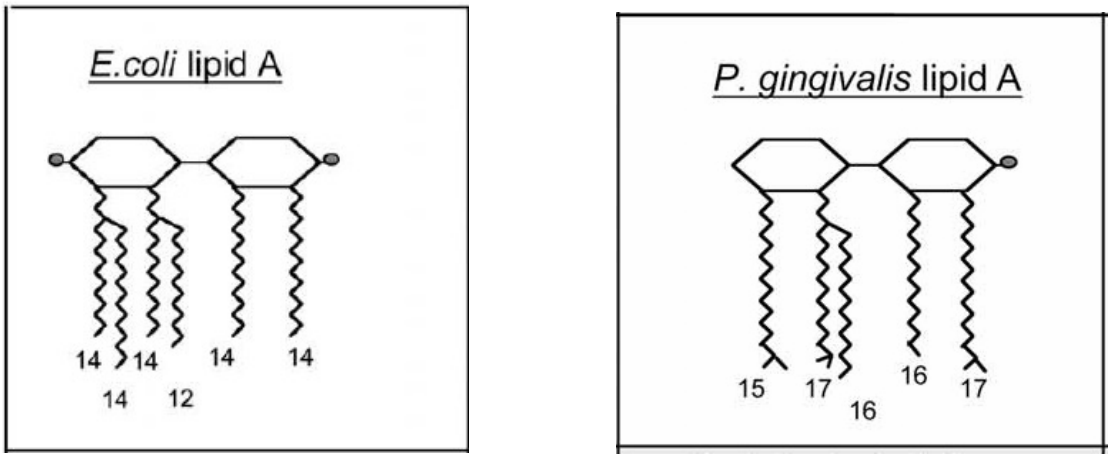


Figure 2. *E. coli* and *P. gingivalis* Lipid A (Erridge et al., 2002).

2.2 Atherosclerosis

Atherosclerosis is the cardiovascular disease associated with elevated circulating lipids and arterial vessel lipid accumulation, actually involves a progressive inflammatory response. The inflammation within coronary atherosclerotic plaques is increasingly thought to be crucial determinants of the clinical course of patients (Libby, 2002). The pathogenesis of this disease initiated by hypercholesterolemia that stimulates vascular endothelium to express vascular cell adhesion molecule-1 (VCAM-1) for adhering mononuclear leukocytes and monocytes (Libby et al., 2010). In physiologic condition, endothelial cells resist to prolonged contact with leukocyte and monocyte, but undergo inflammatory activation, they increase the expression of various leukocyte adhesion molecules. Monocyte seems to have a major role by infiltrating into tunica intima and change to be tissue macrophage. In the atheromatic plaque, macrophage expresses scavenger receptors that bind internalized lipoprotein particles modified by glycation or oxidation i.e. oxidized LDL. The process gives rise to the arterial foam cell, a hallmark of the atheromatic lesion, which is resulted of accumulation of lipid droplets in the cytoplasm and appears under microscope (Klinkner et al., 1995). Macrophage has many functions related to atherosclerosis and disease

complications. The foam cell secretes large arrays of pro-inflammatory cytokines that amplify the local inflammatory response in the lesion, such as reactive oxygen species and IL-1ß. The activated mononuclear phagocyte has an important role in the thrombotic complications of atherosclerosis by producing matrix metalloproteinases (MMPs) that enhance degradation of extracellular matrix and lend the strength to the plaque's fibrous cap (Libby, 2002). After plaque rupture, it allows the blood to contact another macrophage products, the potent pro-coagulant protein tissue factor. Macrophages in the core of the plaque can die and produce a necrotic core of the atherosclerotic lesion (Tabas, 2010).

After formation of fatty streak, atheromatic plaque typically evolves into the more complex lesion and leads to clinical manifestations. According to the traditional incidents, fatty streaks evolve into complicated atheroma through the multiplication of smooth muscle cells, which are accumulated in the plaque and produce an abundant extracellular matrix. When the lesion becomes more bulky, arterial lumen narrows till it hampers flow and leads to clinical complications i.e. acute myocardial infarction.

Accumulating evidences have implicated specific infectious microbial agents including Cytomegalovirus, *Chlamydia pneumoniae* and *Helicobacter pylori* might contribute to initial atherogenesis. Atheromatic plaques are responsible not for acute infections, but rather for chronic infections. So that periodontal disease, a chronic inflammatory infection of the periodontal tissue, is one of the appropriate candidate for the risk factor of atherosclerosis (Gibson III et al., 2006).

2.3 Association between periodontal disease and atherosclerosis

The majority of epidemiological studies have shown the presence of a significant association between periodontal disease and cardiovascular disease. Such studies usually report a moderately increased risk i.e. odds ratio of periodontal disease in coronary heart

disease is 1.5 and stroke is 2.8 (Tonetti, 2009). Another study shows relative risk of periodontal disease to nonhemorrhagic stroke and cerebrovascular accidents is 1.41 and 1.66, respectively (Hujoel et al., 2000). In addition, periodontitis was found to be a principal independent predictor of carotid atherosclerosis (Soder et al., 2005). The consensus document from European workshop of periodontal health and cardiovascular disease concludes the relationship between periodontal disease and cardiovascular disease that although it has a low significant relationship but almost of the epidemiological studies have a significant relationship (Bouchard et al., 2010). Despite epidemiological evidences, more understanding about the underlying mechanism between the two diseases is still lacking and further research is required. Atherosclerosis is a multi-factorial in nature. Traditional risk factors include hypercholesterolemia, diabetes mellitus, cigarette smoking, and infection. Both diseases share many of the same risk factors and have common characteristic of chronic inflammation as disease hallmark. From early studies, infections such as *Chlamydia pneumoniae*, Cytomegalovirus, herpes simplex virus, *Helicobacter pylori* and periodontal pathogens are candidates for triggering and perpetuating the inflammatory responses of the arterial wall (O'Connor et al., 2001).

Possible mechanisms of periodontal pathogens in the formation of atherosclerosis contribute directly by bacterial invasion to the endothelial cell such as *P. gingivalis* attachment with fimbriae (Zhou et al., 2005). Then the infected endothelial cell will release abundance of adhesion molecules and cytokines. Low level bacteremia also occurred by invasion of bacteria and its product via ulcerated inflamed periodontal pocket (Gibson III et al., 2006). *P. gingivalis* mediates up-regulation of Toll-like receptors and may contribute significantly to the process of atherosclerotic formation (Gibson III et al., 2006). The indirect effects that initiate the formation of atherosclerosis may come from bacterial components such as LPS (Kamisaki and Wada, 2010). It stimulates the release of various cytokines from inflammatory cells and effect various tissues especially the recruitment of macrophage, resulting in the generation of foam cells and atherosclerotic formation.

Macrophage plays a major role in the formation of atherosclerosis. After inflammation of endothelial tissue, adhesion molecules such as VCAM-1, are expressed by endothelial cell to stimulate monocyte chemotaxis and diapediasis into tunica intima. Monocytes further differentiate to be tissue macrophages which express scavenger receptors. Scavenger receptors that bind internalized lipoprotein particles is a key process to give rise of the arterial foam cell which comes from the result of accumulation of lipid droplets in the cytoplasm. Foam cell or lipid-laden macrophage, the hallmark of atheromatic lesion, were localized to the sites of activated endothelium and comprise the primary cell of the fatty streak (Ross, 1999). Foam cell secretes pro-inflammatory cytokines that amplify the local inflammatory response in the lesions. The activated mononuclear phagocytes have a key role in the thrombotic complication of atherosclerosis by producing matrix metalloproteinases (MMPs) that stimulate degradation of extracellular matrix and weaken strength to the plaque's fibrous cap. When the plaque rupture, it may occludes the artery and leads to serious cardiovascular and cerebrovascular events (Tabas, 2010).

Since endothelial cells produce IL-8 in response to invasive *P.gingivalis* (Nassar, 2002), later studies also showed that *P.gingivalis* and its components could stimulate monocytes to produce various chemokines that might be the signal for recruitment of inflammatory cells to the site of *P.gingivalis* infection. *P.gingivalis* stimulated foam cell formation via TLR but the mechanism was poorly understood (Giacona et al., 2004). The cytokine profiles after stimulation of human monocyte and macrophage with *P.gingivalis* was also investigated. *P. gingivalis* whole cells could stimulate IL-1ß production while fimbriae or LPS do not have this effects when used as only one signal (Zhou et al., 2006).

2.4 Macrophages

Macrophage is known as the marrow-derived phagocytic cell and also essential for human tissue homeostasis. It is found in all tissue in the human body. Human macrophage

is about 21 μm in diameter. It could be identified by specific expression of a number of surface protein markers including CD14, CD16, CD 36, and CD163 detected by flow cytometry or immunohistochemical staining (Waldo et al., 2008; Verreck et al., 2006; Lucas and Greaves, 2001). Activated monocyte translocates by action of amoeboid movement. When a monocyte entering damaged tissue through the endothelium of a blood vessel, it undergoes a several process to become macrophage. Monocyte is attracted to a damaged site by chemical substances through leukocyte chemotaxis, triggered by a various of stimuli including damaged cells, pathogens and cytokines released by macrophages . At some area of the body such as the testis, macrophages have been shown a capacity of repopulation in the organ through proliferation. Macrophages could survive longer in the human body up to a maximum of several months when compared with neutrophils (Lucas and Greaves, 2001). The identification markers of macrophage in the difference from monocyte are resistance to apoptosis, increasing of mitochondria and lysosomes, active phagocytosis, and up-regulation of scavenger receptors with less number of CD14 (Daigneault et al., 2010).

Tissue macrophage is derived from circulating monocytes that is recruited to tissue by inflammatory signals. It shows great variation in morphological structure and undertake a range of physiological functions. Specific antibodies have been developed and allow the detection of tissue-resident macrophage and macrophage recruited to sites of infection and inflammation. Some of the antibodies such as CD 68 recognized all macrophages while others recognize only a tissue macrophage (Waldo et al., 2008).

Tissue remodeling during development and wound repair also require macrophage. Various cytokines are secreted such as growth factors and proteases. Key proinflammatory cytokines secreted by macrophages include IL-1ß, tumor necrosis factor alpha (TNF-α), IL-10, and IL-12. Not only various cytokine and enzyme secretion, but also expression of

scavenger receptors for its function. Important scavenger receptors such as CD36 and CD68 that bind oxidized LDL (Lucas and Greaves, 2001).

In atherosclerosis, monocyte and macrophage are present at all stages of atherogenesis and have various functions in disease initiation and progression.The role of monocyte and macrophage in the disease involve IL-1ß production that required the signal from TLR activation. Human blood monocyte expresses TLR1, TLR2, TLR4, TLR5, TLR6, TLR8, and TLR9 mRNA with TLR2 and TLR4 being the most highly expressed (Cole et al., 2010). Surface expression of TLR2 and TLR4 has been confirmed by flow cytometry (Brocheriou et al., 2010). Despite the description of the subsets of human monocytes, no previous study has examined the differential expression of TLRs on different monocyte subsets. The functional consequences of TLR activation in atherosclerosis involve in both human innate and adaptive immune response. Plaque macrophage displays features of activation and can exacerbates numerous effects on other vascular cells via releasing of a proinflammatory mediators including IL-1ß but the exact mechanism is also unknown. The variations and key functions of monocytes and macrophages in any phases of atherogenesis highlights the need for more understanding of the human innate immune receptors expressed by these cells and their activation, particular in relation to the different subsets of macrophage that have been described (Cole et al., 2010).

From recent knowledge, macrophages are phenotypically diverse and their heterogeneity depends upon local environment and stimuli in atheromatic lesions (Tabas, 2010). Granulocyte macrophage colony-stimulating factor (GM-CSF) and monocyte colony-stimulating factor (M-CSF) produced by various cells in response to injury are the major survival factors for the macrophage lineage with capacity to activate and induce their differentiation. Initially, macrophage subpopulation described as M1 and M2 macrophage phenotypes were described by analogy to Th1 and Th2 lymphocyte subsets. (Brocheriou et al., 2010). Macrophage cultivated with GM-CSF is M1 macrophage which is also called GM-

macrophage and M-CSF is M2 macrophage which is also called M-macrophage (Martinez et al., 2006). The difference between M1 and M2 macrophage is showed in the table 1.

Table 1 : Macrophage phenotype characteristics (Boyle, 2005; Waldo et al., 2008; Verreck et al., 2006)

Characteristics	M1 macrophage	M2 macrophage
Synonym	Classically activated macrophage	Alternatively activated macrophage
Th polarization	Th1	Th2
Stimulating factor	GM-CSF	M-CSF
Morphology	Round shape	Elongated shape
Cell surface markers	$CD14^{+}$, $CD16^{-}$, $CD36^{-}$,$CD163^{-}$	$CD14^{+}$, $CD16^{+}$, $CD36^{+}$,$CD163^{+}$
Predominated stage in atheromatous development	Fatty streaks dominant	Fibro-fatty plaques dominant

2.5 Inflammasomes

The human innate immune system is the first line of defense mechanisms of body. Innate immunity has a key functions in controlling infection and eliminating pathogens, as well as the T- and B- cell responses of adaptive immunity. A number of classes of germline-encoded pattern recognition receptors (PRRs) are grouped by membrane associated PRRs and cytosolic PRRs (Chen and Pedra, 2010). NOD-like receptor (NLR) family is a large family of cytosolic PRRs. The major function of the NLRs appears to be regulated the production of proinflammatory cytokines IL-1ß and IL-18 (Bryant and Fitzgerald, 2009). In human, the NLR family is composed of 22 members. They are classified into subfamilies

according to their N-terminal effector domains. Some NLR family members also form multiprotein complexes, are called inflammasomes that capable for activation the cysteine protease caspase-1 in order to response with a wide range of stimuli including both microbial substances and self-molecules. NLRs induce the recruitment of the adaptor molecule ASC (apoptosis associated speck-like protein containing CARD), leading to the processing and activation of pro-IL-1ß an IL-18 through caspase-1 (Martinon, 2009).

NLRP3 (nucleotide-binding domain leucine-rich repeat containing (NLR) family, pyrin domain containing 3) is activated by diverse stimuli i.e. crystal structures, and adenosine triphosphate (ATP). LPS and extracellular ATP stimulate caspase-1 in a NLRP3 dependent fashion (Chen and Pedra, 2010). LPS activation by the response of TLR2 and TLR4 also recognized as the first signal for proinflammatory cytokine production. The second signal stimulation comes from ATP activation via Purinergic receptor, ligand-gated ion channel, 7(P2X7) receptor. Pannexin-1 forms a larger pore of the P2X7 purinergic receptor upon activation and is important for caspase-1 activation (Hornung et al., 2008). Caspase-1 further changes proinflammatory IL-1ß to become active IL-1ß and secretes throughout the cell membrane.

IL-1ß is one of the most important proinflammatory cytokine in immune-mediated human disease. This cytokine induces the synthesis and expression of other important mediators. IL-1ß also triggers a series of molecular and cellular responses, including tissue inflammation, adaptive immunity and repair (Taylor, 2010). IL-1ß stimulates the synthesis of prostaglandin E2, platelet activating factor and nitrous oxide by activating the enzymes responsible for their synthesis. IL-1ß enhances vascular changes associated with inflammation and facilitation of blood flow to the site of infection or damage.

At the specific site of atheromatic plaque, monocyte-derived macrophage could secretes a range of cytokines including IL-1ß. The level of IL-1ß in blood circulation and

atherosclerotic lesion has been shown to be correlate with disease severity. Knocking out of IL-1ß gene in atherosclerosis-prone Apolipoprotein E (ApoE)-/- mice leads to attenuation of disease development (Libby, 2002). The production of IL-1ß requires 2 separate signals. First, induction of IL-1ß mRNA via stimulation of pattern recognition receptors i.e. TLR4 stimulation by *E.coli* LPS. A group of toll-like receptors (TLRs) has been defined recently as a key component of PRR machinery. Recently, 9 human TLRs have been identified (Cole et al., 2010). According to the recent knowledge, we already considered atherosclerosis as an inflammatory disease. The role of innate immune response in atherosclerosis is very important. It is also clear the TLRs expression in atherosclerosis could be observed and their expression involves multiple inflammatory and noninflammatory cells such as monocytes, macrophages, dendritic cells, B and T lymphocytes, endothelial cells, and smooth muscle cells. The increasing of TLR expression by cells during the process of atherosclerosis may results in enhanced signaling through the TLRs and thus exacerbates of cell activation and proatherogenic downstream pathways (Erridge and Nicolaou, 2010).

Both endogenous and exogenous TLRs ligands have been described. TLR2 is essential for the recognition of mainly bacterial lipoproteins, and lipotheicoic acid while TLR4 is predominantly activated by LPS. Many exogenous TLR ligands are expressed in atheromatic lesions such as infectious agents. Human atheromatic plaque contains numerous bacterial components, including peptidoglycan or exogenous bacterial heat shock proteins (HSPs). The endogenous ligand is also important. The atheromatic plaque is characterized by accumulation of lipoproteins, extracellular matrix and cellular debris from necrotic cells in necrotic core. Product degradations of extracellular matrix macromolecules or HSPs have been showed and found to function as TLR ligands (Cole et al., 2010). Lipid is a strong ligand for both TLR2 and TLR4. Saturated fatty acid shows this capacity of delivering a TLR4 signal but the polyunsaturated fatty acid blocks the activation of TLR4. ApoCIII, a component of the very-low-density lipoprotein (VLDL), was also found to be recognized by TLR2 and induced proinflammatory signal in monocyte (Cole et al., 2010).

Second signal is required for activation of caspase-1. Caspase-1 is activated by NLRP3 inflammasome, a complex protein that activate by various substances such as ATP, uric acid, cholesterol crystal. After IL-1ß secretion, the biological effects will occur (Kankkunen et al., 2010; Martinon et al., 2009).

NLRP3 inflammasome activation mechanism is currently unclear. Very recently, 3 models are proposed by Tschopp and Schroder (2010).

1. The channel model is mediated by several key substances such as extracellular ATP (Figure 3). It triggers P2X7 ATP-gated ion channel, which allows rapid potassium ion (K^+) efflux from the cell and recruitment the pore formation of the pannexin 1 hemichannel. Bacterial muramyl dipeptide (MDP) has been phagocytosed and translocates from acidified phagocytic vesicles into the cytoplasm through the pathway that depends on pannexin 1 function. Moreover, bacterial pore-forming proteins such as the α-toxin of *Staphylococcus aureus* also activates K^+ efflux through bacterial pore-forming channel. But some NLRP3 agonists, such as Monosodium urate (MSU) crystals or the particulate asbestos, are too large for cytoplasmic translocation through any type of channel or pore.

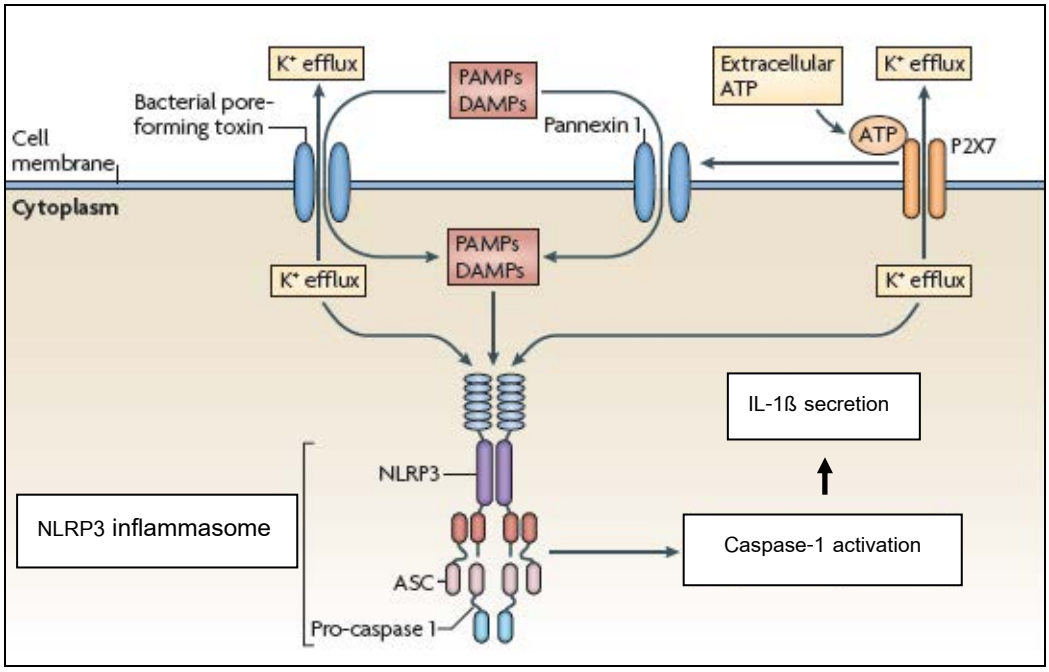


Figure 3. The channel model of NLRP3 inflammasome activation. After the stimulation of pyrin domain-containing 3 (NLRP3) inflammasome by extracellular ATP, leads to formation of the associated pannexin 1 pore and K^+ efflux from P2X7 receptor. This pore, which also formed by the bacterial toxins, allows cytoplasmic entry of extracellular substance that is the direct NLRP3 ligands and also allows K+ efflux from the affected cell. ASC, apoptosis-associated speck like protein containing a CARD; DAMPs, damage-associated molecular patterns; IL, interleukin; PAMPs, pathogen-associated molecular patterns (Tschopp and Schroder, 2010).

2. The lysosome rupture model is shown as an alternative model of inflammasome activation that involves the size of the activators (Figure 4). According to this model, a large particulate activators such as alum or silica cannot clear completely following phagocytosis and leads to phagosomal destabilization and lysosome rupture. The releasing of the lysosomal protein, which called cathepsin B, into cytoplasm triggers NLRP3 inflammasome activation. Conversely, there are some studied showed normal NLRP3 inflammasome activity in cathepsin B-deficient mice.

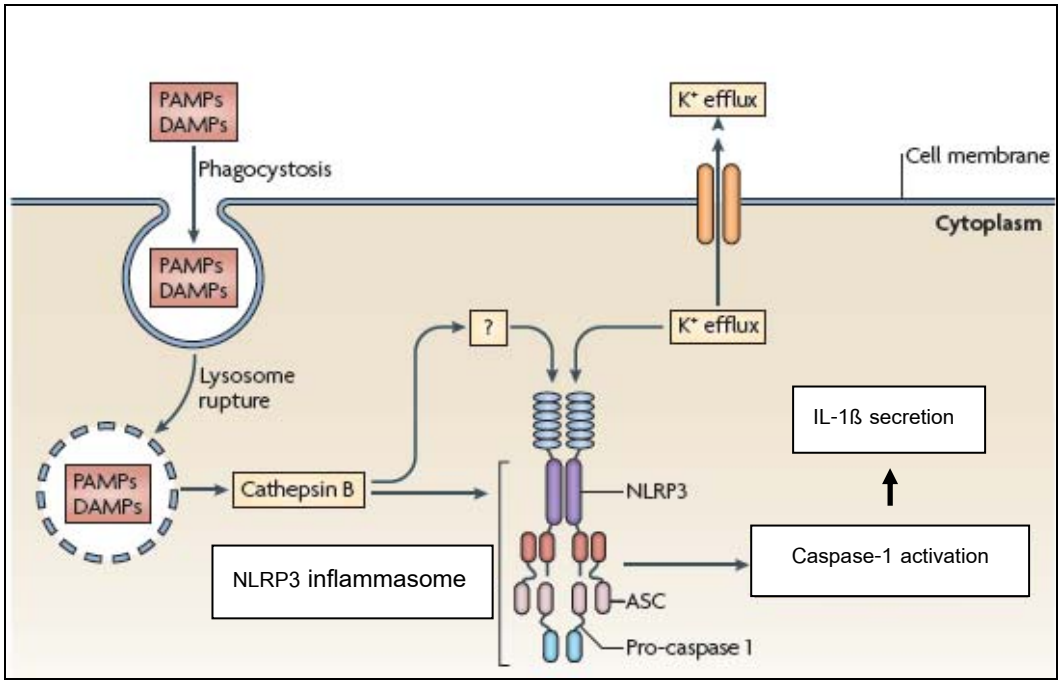


Figure 4. The lysosome rupture model of NLRP3 inflammasome activation. Particulate or crystalline structural substances (such as cholesterol crystals and silica) are phagocytosed, resulting in lysosomal rupture and the cytoplasmic releasing of cathepsin B. Cathepsin B, either attack directly or through cleaving an undefined substrate, induces activation of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome. ASC, apoptosis-associated speck-like protein containing a CARD; DAMPs, damage-associated molecular patterns; IL, interleukin; PAMPs, pathogen-associated molecular patterns (Tschopp and Schroder, 2010).

3. The reactive oxygen species (ROS) model is recognized as the third model by proposing a mechanism for NLRP3 activation (Figure 5). The inflammasome activators including ATP and particulate activators trigger the generation of short-lived ROS, and the treatment with ROS scavengers blocks NLRP3 activation. ROS might triggers thioredoxin-interacting protein (TXNIP) that normally bound with oxidoreductase thioredoxin (TRX). After binding of TXNIP with NLRP3, further leading to NLRP3 activation. Although, there are many

stimuli that induce the production of ROS do not activate the NLRP3 inflammasome. Furthermore, most of the evidence for an important role of ROS is based on pharmacological inhibitors that are used at high doses, which have variable effects. However, these 3 models of NLRP3 activation, which have been reported in by Tschopp and Schroder (2010) remain unclear and require further investigation for the precise mechanism.

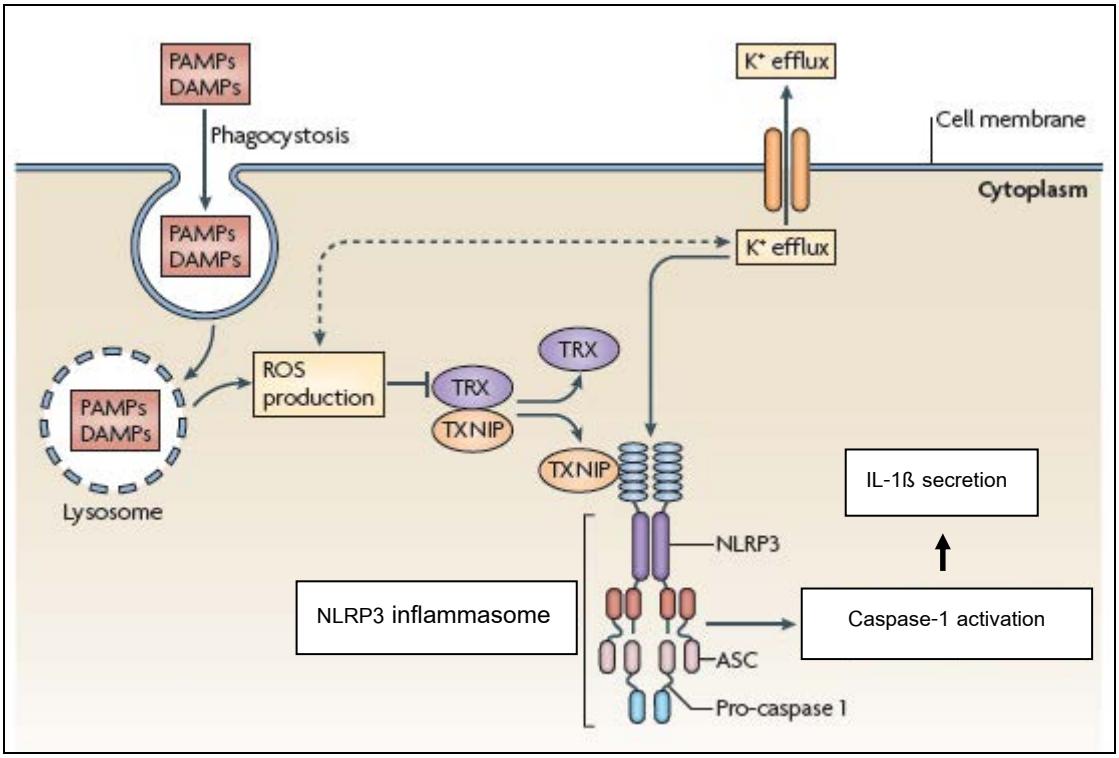


Figure 5. The reactive oxygen species model of NLRP3 inflammasome activation. ROS production results in NLRP3 inflammasome activation by release of the ROS-sensitive NLRP3 ligand thioredoxin-interacting protein (TXNIP) from its inhibitor thioredoxin (TRX). ASC, apoptosis-associated speck-like protein containing a CARD; DAMPs, damage-associated molecular patterns; IL, interleukin; PAMPs, pathogen-associated molecular patterns (Tschopp and Schroder, 2010).

For the present study, we purposed a hypothetical model (Figure 6) of IL-1ß production by PAMPs via TLR as signal 1 and cholesterol crystals or ATP as signal 2.

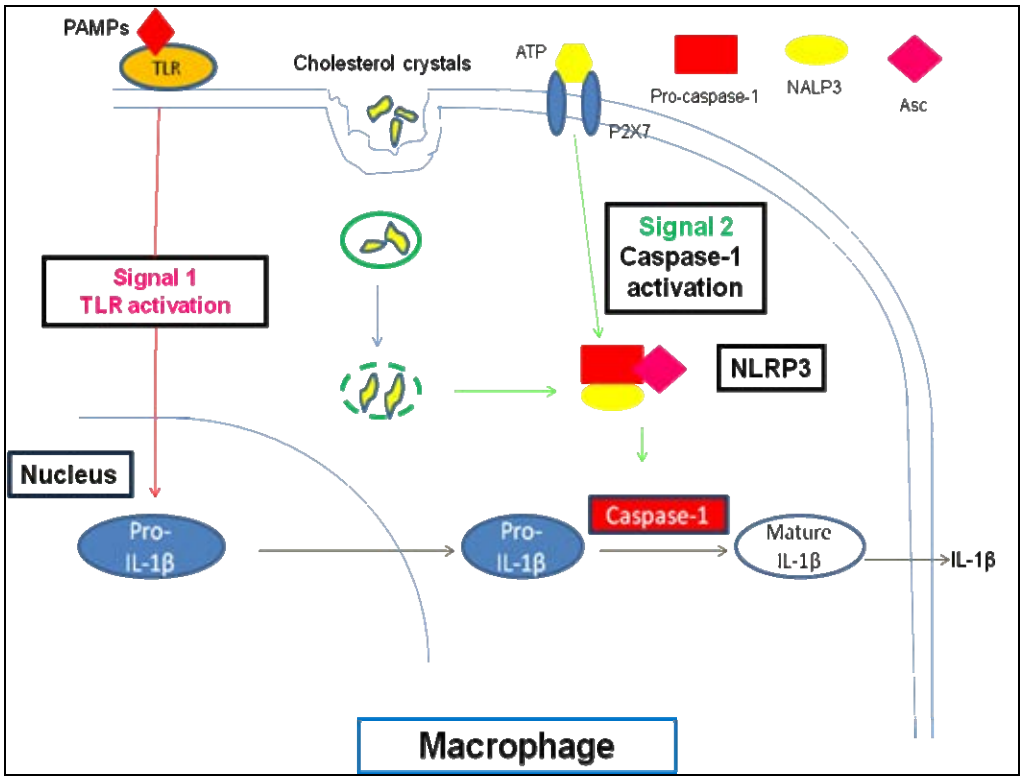


Figure 6. Activation of the NLRP3 (Nucleotide-binding domain leucine-rich repeated containing family, pyrin domain containing 3) inflammasome regulates IL-1ß (interleukin-1 beta) production in macrophage (simplified from Chen and Pedra (2010)). Generation of IL-1ß requires two signals. Signal 1: TLR (Toll-like receptor) activation, often from PRRs (Pattern recognition receptors) such as TLRs which results in pro- IL-1ß production. Signal 2: caspase-1 activation, NLRP3 inflammasome can be activated by DAMPs (Danger-associated molecular patterns) such as ATP (adenosine triphosphate), crystals such as cholesterol crystals. NLRP3 oligomerization and assembly of ASC (Apoptosis associated speck-like protein containing a CARD) and pro-caspase-1 trigger activation of caspase-1. Active caspase-1 then cleaves pro- IL-1ß, resulting in the maturation and secretion of IL-1ß.

2.6 Cholesterol crystals

Cholesterol, an insoluble lipid, plays a major role of the localized cellular inflammation by activating the formation of the atheromatic plaque and plaque rupture. The process is initiated by entering of low-density lipoprotein or very-low-density lipoprotein into the arterial intimal layer, resulting in entrapment of cholesterol caused by interaction with cellular matrix components (Libby, 2002). This step follows by the entry of recruited monocyte from blood circulation and transforming into activated macrophages that further taking up the cholesterol. As cholesterol builds up in the macrophages, they become foam cells or lipid-laden macrophage that eventually form in early fatty streaks and leading to the development of the necrotic core (Libby, 2002). The danger signal triggered by intracellular crystals initiates a local inflammatory response via NLRP3 inflammasome protein and leads to IL-1ß secretion (Rajamaki et al., 2010).

The role of cholesterol crystals in the formation of foam cell was investigated by Duewell et al. (2010) study. The results demonstrated that cholesterol crystal was detected in LPS-primed mouse macrophage and subendothelial layer. Duewell study showed the role of cholesterol crystals that involved not only in late stage of disease but also in the early stage of atherosclerotic progression. The danger signal from cholesterol crystal enhanced the first signal from LPS to stimulate IL-1ß secretion (Duewell et al., 2010).

The mechanism from cholesterol crystal is not well understood. Rajamaki et al. (2010) proposed the mechanism of cholesterol crystals induced inflammasome activation (Figure 7). After phagocytosed by macrophage, cholesterol crystals causing lysosomal destabilization and leakage of cathepsin B to cytoplasm, where the enzyme indirectly activates the NLRP3 inflammasome. Lowering of potassium concentration caused by phagocytosed cholesterol crystals are required for NLRP3 activation (Rajamaki et al., 2010).

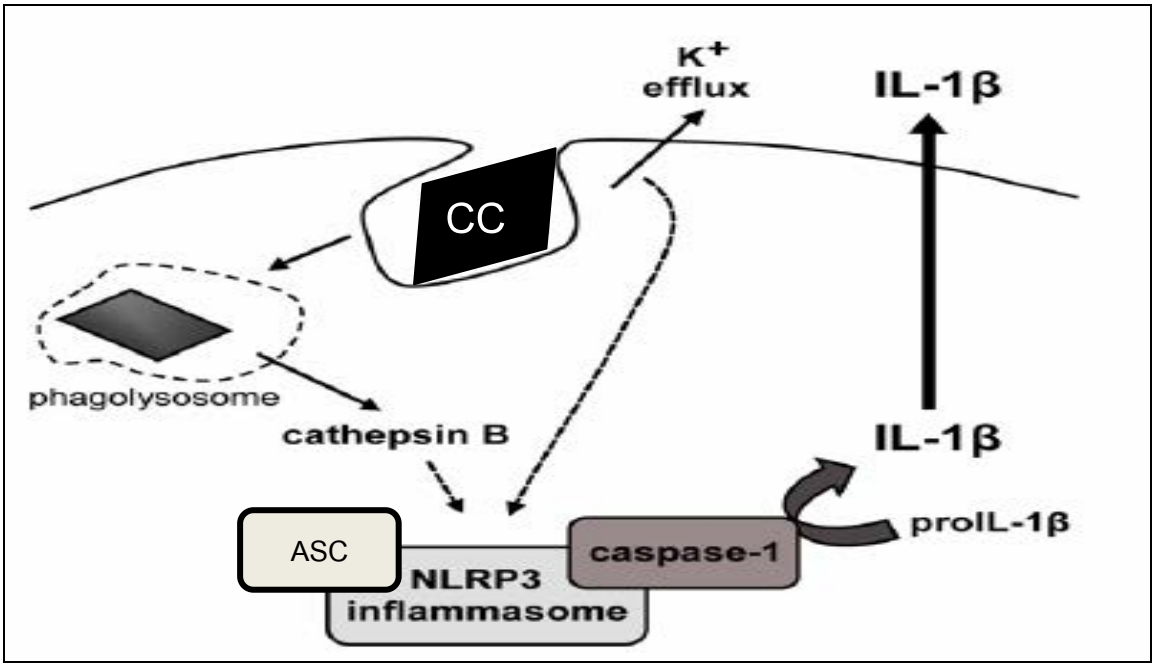


Figure 7. Proposed mechanism of cholesterol crystal-induced inflammasome activation. Cholesterol crystal (CC) is phagocytosed by macrophages, leading to lysosomal destabilization and leakage of cathepsin B into cytoplasm. Cathepsin B indirectly activates the NLRP3 inflammasome. The reduction of intracellular potassium concentration level caused by phagocytosed CC may also required for NLRP3 activation. ASC, apoptosis-associated speck-like protein containing a CARD (Rajamaki et al., 2010).

CHAPTER III

MATERIALS AND METHODS

3.1 Medium

Macrophage serum free medium (AIM-V, Invitrogen, Carlsbad, CA, USA) was used for monocyte-derived macrophages culture throughout the study. RPMI-1640 medium (Invitrogen) was used for PBMC separation.

3.2 Reagents

We used specific ultrapure TLR2 ligand (*P. gingivalis* LPS) and TLR 4 ligand (*E. coli* LPS) obtained from Invitrogen. The recombinant human M-CSF and GM-CSF were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Tissue culture grade cholesterol was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Histopaque 1.077 was purchased from Sigma-Aldrich. Adenosine triphosphate (ATP) was purchased from Invitrogen. Human anti-CD14 microbeads were purchased from Millenyi Biotech (Bergisch Gladbach, Germany). Mouse anti-human CD14, anti-human CD16, anti-human CD36, anti-human CD163, anti-human TLR2, anti-human TLR4, and mouse IgG1 monoclonal antibodies were obtained from BD Bioscience (San Jose, CA, USA). The endotoxin-free water was purchased from Cambrex (Walkersville, MD, USA). Ethanol and acetone were purchased from Merck (Darmstadt, Germany).

3.3 Subject selection and ethical considerations

Blood samples were collected from 4 healthy volunteers . Since the project involved handling of human blood samples, an ethical approval would be necessary. This ethical approval was obtained from the Ethics committee of the faculty of dentistry, Chulalongkorn university. An informed consent was obtained from the participants, since their blood samples were used for the project. Also, the identities, locations of patients and all data were kept securely confidential. An informed consent was ensured that the participants were explained as fully as possible regarding the project, why it is undertaken and what would be the findings. Healthy volunteers were assured that they were safe and there was no risk from the study.

3.4 Preparation and purification of human monocyte-derived macrophage

Heparinized peripheral blood was obtained from healthy human donors after applied informed consents. Sixty ml of blood from healthy volunteer was collected by nurse at the examination room on 4^{th} floor, Somdejya 93^{th} building. Blood sample was collected for 1 to 2 times. Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation using Histopaque-1077 (Sigma-Aldrich). $CD14^{+}$ monocytes were isolated by positive selection from PBMCs using human CD14 magnetic cell sorting (MACS) system (Millenyi Biotech) according to the manufacturer's instructions, yielding an average 98% purify. Highly pure M1 and M2 macrophage were obtained after 6 days of culturing in macrophage serum free medium supplemented with 10 ng/ml rhGM-CSF (R&D systems) or with 50 ng/ml rhM-CSF (R&D systems), respectively. The cultures were maintained at $37^{\circ}C$ in humidified atmosphere of 5% CO_2. Every third day, half the volume of medium was replaced with fresh medium containing the same amount of cytokines. The phenotypes of human monocyte-derived macrophage and their subsets (M1 macrophage or M2 macrophage) were analyzed by flow cytometry.

3.5 Flow cytometric analysis

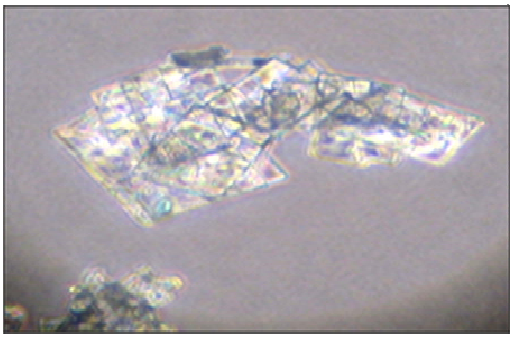
The phenotypes of monocyte-derived macrophage were analyzed by staining the cells that had been cultured for 6 days with mononclonal antibodies (mAbs) anti-CD14, CD16, CD36, CD163, TLR2, and TLR4. Mouse isotype mAbs always used as control. Cell was stained at 4 °C for 30 minutes, washed in PBS and then reconstituted with 1% paraformaldehyde. Normally, 5,000-10,000 cells were analyzed by FACSCalibur (Becton Dickinson).

Table 2 : Monoclonal antibodies used for flow cytometric analysis

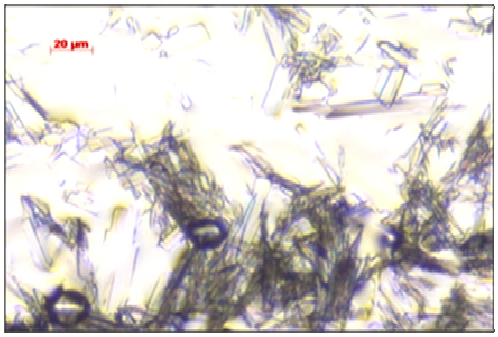
Monoclonal antibody	Function, Ligands and associated molecule
CD14	LPS receptor for LPS/LBS complex, TLR2, TLR4
CD16	Receptor for IgG Fc
CD 36	Scavenger receptor for Oxidized LDL, Thrombospondin, Collagen
CD163	Scavenger receptor for CSNK2B
TLR 2	Bacterial components i.e. Lipoteichoic acid, host cells i.e. HSP70
TLR 4	Bacterial components i.e. LPS, host cells i.e. fibrinogen
Mouse IgG-1	-

3.6 Preparation of cholesterol crystals

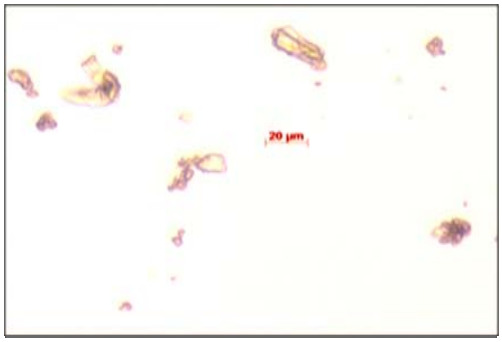
In the present study we followed the preparation of cholesterol crystal by Duewell et al (2010). In brief, tissue culture grade cholesterol was solubilized in hot acetone and crystallized by cooling. After six cycles of recrystallization, the final crystallization performed in the presence of 10% endotoxin-free water to obtain hydrated cholesterol crystals. Cholesterol crystals size were varied with a microtube tissue grinder, and stored at -20°C until used. Endotoxin-free cholesterol crystals were used in the study. Figure 8 shows morphology of cholesterol crystal preparation under microscope.



A: Cholesterol crystal powder



B: 6th recrystalllization



C: Grinded cholesterol crystals

Figure 8. Cholesterol crystals morphology under microscope. Tissue-culture grade cholesterol powders showed cuboid shape under microscope(A) (10X). After preparation following Duewell et al. (2010) technique, cholesterol crystals from 6th recrystallization showed a rosette aggregation with various size(B). Cholesterol crystals were grinded with a microtube tissue grinder demonstrated a rhombic shape with a repeated size between 10 - 20 µm(C).

3.7 Treatment of cells with cholesterol crystals and *Porphyromonas gingivalis* LPS

Primary macrophages ($5x10^5$ mononuclear cells/ml), M1 macrophage and M2 macrophage (from 4 subjects), were cultured with 1.) *P. gingivalis* LPS 5 µg/ml or 2.) *E.coli* LPS 5 µg/ml, (positive control for first signal of inflammasome activation) at 37 °C under 5% CO_2 in serum-free culture medium in 96 well plates at Day 0.

At Day 1, 1.) cholesterol crystals (different concentrations : 500, 1000, 2000 µg/ml) or 2.) ATP (5 mM, postitive control for second signal of inflammasome activation) were added into the culture. Medium control was used as negative control.

At Day 2, culture supernatants were collected for IL-1ß analysis.

3.8 Analysis of cytokine secretion

The level of IL-1ß production was measured by commercial ELISA kit (R&D Systems) according to the manufacturer's protocols.

3.9 Statistical analysis

The data were analyzed using the computer program SPSS vesion 17.0 (SPSS Inc., Chicago, IL, USA). Results were presented as means ± SE. Mann-Whitney's non-parametric rank sum test was used to determine the combination effect of *P. gingivalis* LPS and cholesterol crystals on the level of IL-1ß production and the difference between M1 macrophage and M2 macrophage. Kruskal – Wallis one way analysis of variance by rank test was used to determine the concentration effects of cholesterol crystals on the level of IL-1ß production and Mann-Whitney's non-parametric rank sum test was used to determine the different between group of cholesterol crystal concentration after Kruskal – Wallis one way analysis of variance by rank test were significance . A critical level of 0.05 was employed. Thus, *p* values less than 0.05 were regarded as statistically significance.

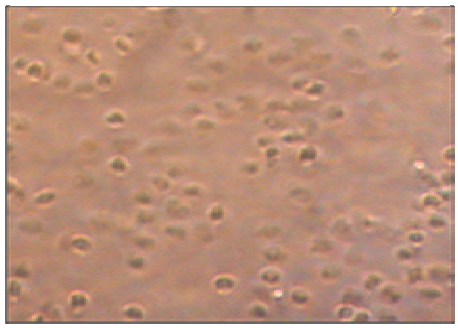
CHAPTER IV

RESULTS

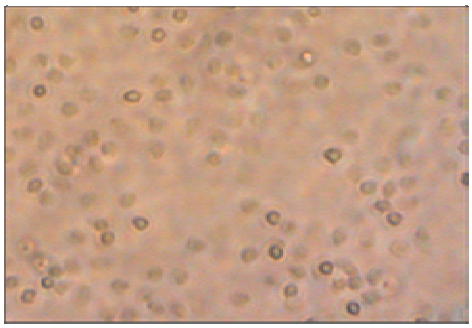
4.1 M1 and M2 macrophages

Human macrophage subpopulations: M1 and M2 macrophage were established from four healthy adults. They were differentiated from $CD14^+$ monocytes (98% purify) in response to cytokine stimulants GM-CSF or M-CSF. Figure 9 demonstrates $CD14^+$ monocytes at Day 0, which were obtained from PBMCs by positive sorting. These cells had an ovoid or kidney-shaped nucleus with round shape and were not much varied in size. Monocyte-derived macrophages, M1 and M2 macrophages showed different morphologies after 6 days incubation with GM-CSF and M-CSF, respectively. M1 consistently had a classical adherent “fried egg” morphology (Figure 10). M2, on the contrary, primarily appeared as adherent cells with a stretched, spindle-like morphology (Figure 10).

In addition to morphology, phenotypic analysis of relevant cell surface markers, M1 and M2 monocytes and macrophages by flow cytometry was shown in Figure 12. Monocytes showed higher expression of CD14, CD36, TLR2, and TLR4 than both M1 and M2 macrophages. M2 macrophage showed higher expression of CD16 and CD 163 than M1 macrophage and monocytes. Both macrophages expressed similar levels of TLR2 and TLR4 but M1 macrophage expressed much lower levels of CD14, CD16, and CD36 as compared to M2 macrophage.

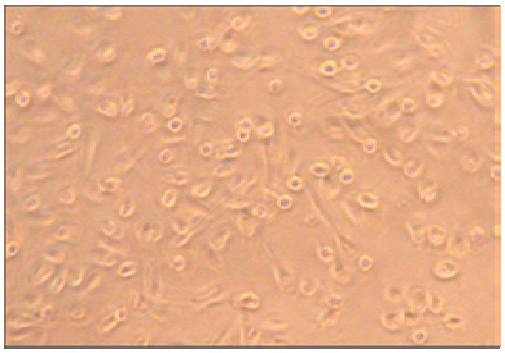


M1 macrophage

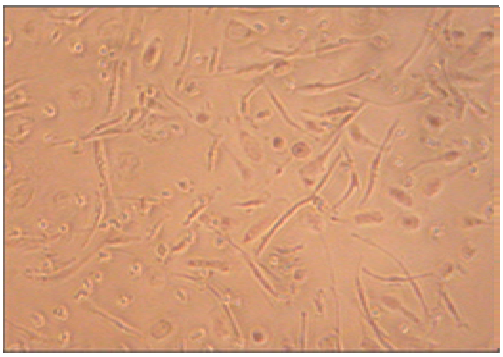


M2 macrophage

Figure 9. Morphology of monocytic cells at Day 0 before priming with GM-CSF and M-CSF. The cell condition was observed under microscope (10x).



A: M1 macrophage



B: M2 macrophage

Figure 10. Morphology of differentiated macrophages. A: Human monocyte cultured for 6 days in the presence of GM-CSF (M1 macrophage). B: Human monocyte cultured for 6 days in the presence of M-CSF (M2 macrophage). The cell condition was observed under microscope (10x).

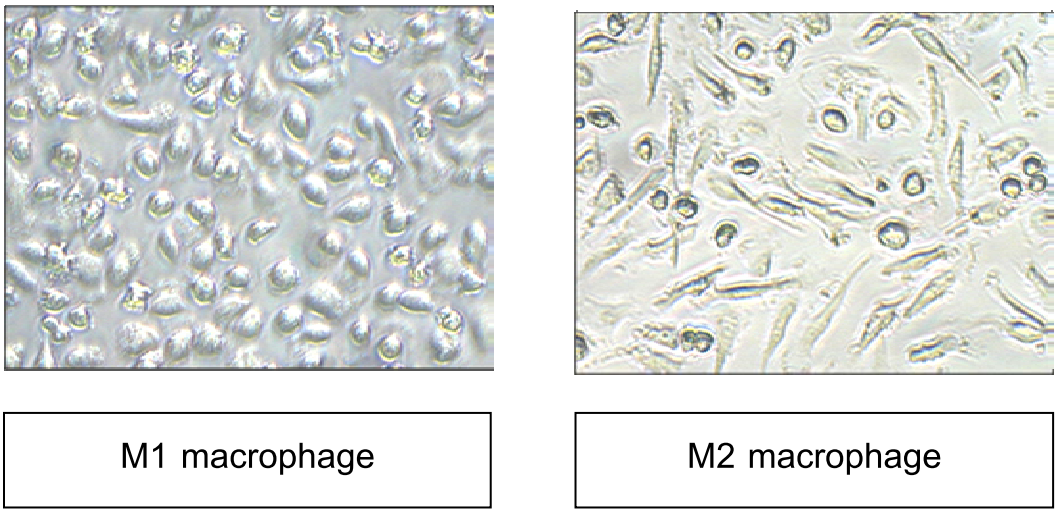


Figure 11. Morphology of monocyte-derived macrophages accumulate cholesteryl esters when incubated with cholesterol crystals observed under microscope (10x).

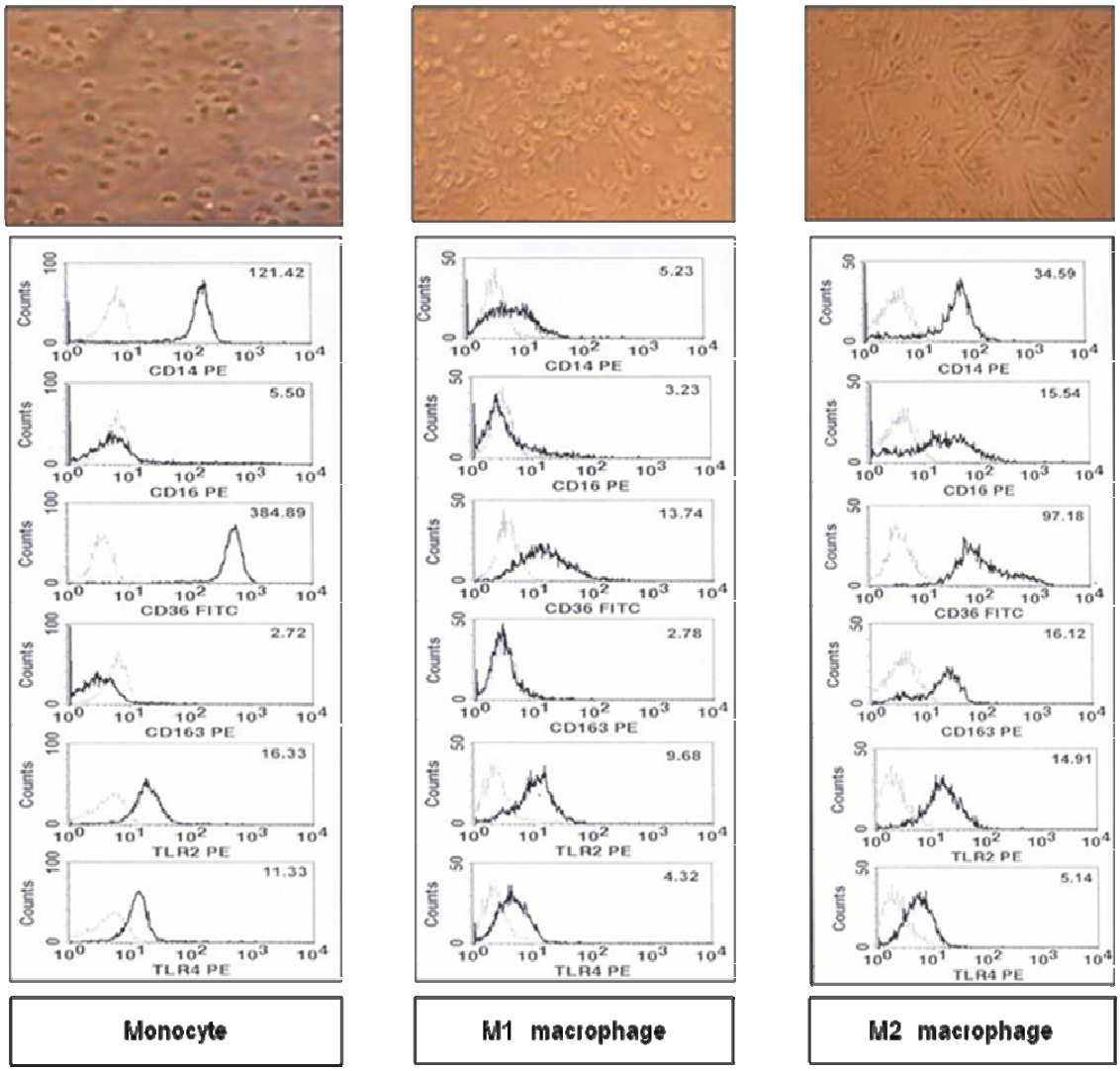


Figure 12. Representative microscopic image of monocyte and macrophage with their phenotypes analysed by flow cytometry. The cell condition was observe under microscope (10x). Histogram in each columns are results from flow cytometric analysis. The x-axis and the y-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates surface markers mean fluorescence intensity (MFI).

4.2 Inflammasome activation of M1 and M2 macrophage by *P. gingivalis LPS* and cholesterol crystals

It is known that inflammasome activation requires two signals. First signal is via TLR stimulation and the second signal is caspase-1 activation. In this study, we investigated the ability of key periodontal pathogens, *P. gingivalis* LPS, a TLR2 ligand and cholesterol crystals to induce inflammasome activation in human macrophages. IL-1ß protein, a product of inflammasome activation was measured by ELISA. Human primary M1 and M2 macrophages were incubated with *P. gingivalis* LPS (first signal) for 24 hours and cholesterol crystals (second signal) at different concentrations (500, 1000, 2000 µg/ml) was added into these TLR-ligand primed cultures which were incubated for 24 hours. Figure 11 demonstrates addition of cholesterol crystals led to accumulation of cholesterol crystal in cytoplasm of *P. gingivalis* LPS primed M1 and M2 macrophages. The culture supernatants were evaluated for the amount of IL-1ß protein, a product of inflammasome activation by ELISA. Un-stimulated macrophages (medium only) were used as a control. *E. coli* LPS, a TLR4 ligand and ATP were used as a positive control inducer for inflammasome activation.

We found that *P. gingivalis* LPS and cholesterol crystals induced IL-1ß protein secretion from both human primary macrophages, M1 and M2 (Figure 13 and 14). Varying concentrations of cholesterol crystals (500 µg/ml, 1,000 µg/ml, and 2,000 µg/ml) induced IL-1ß production both types of macrophages, which were primed with either *P. gingivalis* LPS or *E. coli* LPS, in a dose dependent manner (Figure 13 and 14, the data of IL-1ß production was shown in Appendix). Cholesterol crystal at each concentration when combined with *P. gingivalis* LPS significantly induced IL-1ß production from M1 and M2 macrophages ($p < 0.05$) as compared to the controls. Both types of macrophages showed good response to a positive control inducers *E. coli* LPS and ATP. In culture with single stimulator, *P. gingivalis* LPS or *E. coli* LPS, some IL-1ß could also be observed (Figure 13 and 14). But there was no

cytokine detected in the cultures with cholesterol crystal alone or ATP alone (data not shown).

P. gingivalis LPS and *E. coli* LPS are considered as bacterial PAMPs which could induce TLR activation. We compared the ability of these two bacterial PAMPs (TLR2 ligand vs TLR4 ligand) to induce inflammasome activation in macrophages. At each concentration of cholesterol crystals, there was a trend of higher IL-1ß production in M2 cultures primed with *P. gingivalis* LPS than those primed with *E. coli*. However, no significant differences were found. Unlike, M2 culture, similar levels of IL-ß could be observed.

Specific type of macrophages may contribute to the observed high IL-1ß levels in atherosclerotic lesions (Galea et al., 1996). We compared the magnitude of IL-1ß production from different types of macrophages induced by inflammasome activation. IL-1ß production from activated M1 macrophage consistently higher than activated M2 macrophage under the same culture condition (*P. gingvalis* LPS + cholesterol crystals, *E. coli* LPS + cholesterol crystals, *P. gingvalis* LPS + ATP, *E. coli* LPS + ATP), however such differences did not reach the statistical significance ($p > 0.05$) (Figure 13 and 14).

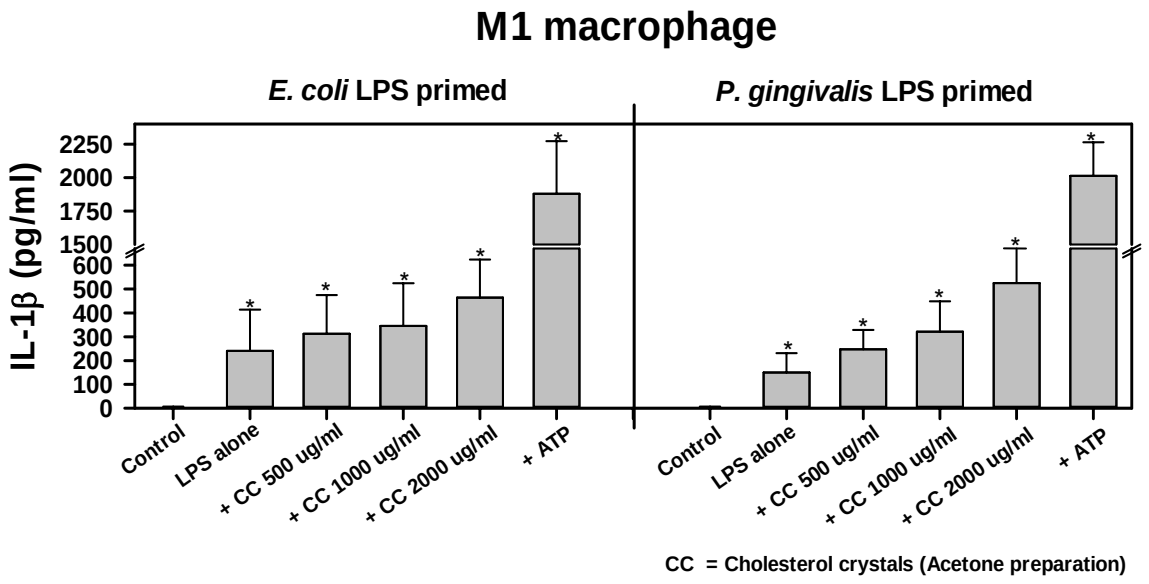


Figure 13. NLRP3 inflammasome activation in M1 macrophage. IL-1ß production was measured in cell cultured supernatant by ELISA. M1 macrophage was stimulated with the following ligands : *P. gingivalis* LPS (TLR2 ligand) and *E. coli* LPS (TLR4 ligand). Cholesterol crystals in various concentration were added at 24 h later. ATP was used as a positive control. Control was untreated cell. Cell cultured supernatant from M1 macrophage and control were harvested after 24 h. Data represent the mean ± SEM (n=4, $*p < 0.05$, compared with unstimulated control).

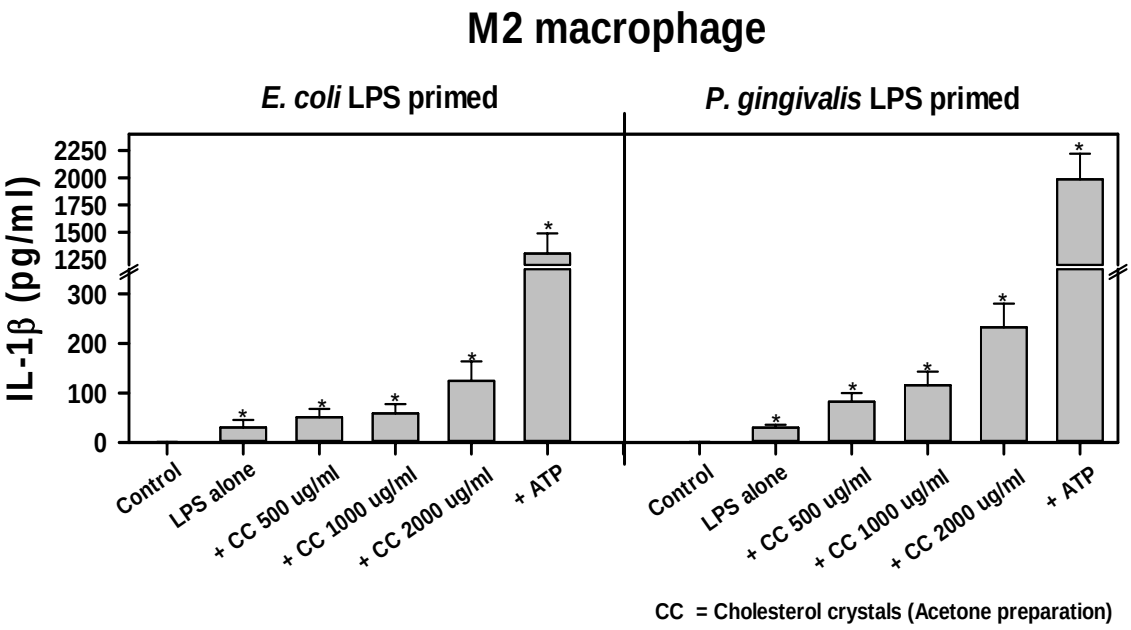


Figure 14. NLRP3 inflammasome activation in M2 macrophage. IL-1ß production was measured in cell cultured supernatant by ELISA. M2 macrophage was stimulated with the following ligands : *P. gingivalis* LPS (TLR2 ligand) and *E. coli* LPS (TLR4 ligand). Cholesterol crystals in various concentration were added at 24 h later. ATP was used as a positive control. Control was untreated cell. Cell cultured supernatant from M2 macrophage and control were harvested after 24 h. Data represent the mean ± SEM (n=4, $*p < 0.05$, compared with unstimulated control).

CHAPTER V

DISCUSSION AND CONCLUSION

Acitvated macrophage is recognized as a hallmark of atherosclerotic lesions. Macrophage activation through PRR, including TLRs and NLRs, the latter of which components of the inflammasome may significantly contribute to inflammatory outcome in atherosclerosis. In this study, the role of *P. gingivalis* LPS and cholesterol crystals in inflammasome activation was investigated. By using an *in vitro* primary human macrophage model, we found that *P. gingivalis* LPS and cholesterol crystals induced IL-1ß secretion.

Our finding is in line with the original study by Duewell et al. (2010) which demonstrated cholesterol crystal-activated inflammasome in mouse macrophage. They proposed that such activation is important in the development of atherosclerotic lesion due to appearance of cholesterol crystals in early diet-induced atherosclerotic lesions as well as the high pro-inflammatory cytokine IL-1ß released from activated mouse macrophage. Rajamaki et al. (2010) further used primary human macrophage model for inflammasome activation. Similar to our results, the cholesterol crystals were uptake by the macrophage primed with *E coli* LPS. The concentrations of cholesterol crystals used in their study were in the same range as ours (500-2000 µg/ml) which was shown to induce dose-dependent secretion of IL-1ß from the macrophages. These *in vitro* data of IL-1ß producing macrophage supports autopsy data that macrophages were major source of IL-1ß in atherosclerotic lesion (Galea et al., 1996).

Macrophages are phenotypically diverse and their heterogeneity depends upon stimuli (Tabas, 2010). A recent report demonstrated that two subpopulation of macrophages, M1 and M2 macrophages, were presence in human atherosclerotic lesions

(Brocheriou et al., 2010). However, the role of M1 and M2 in atherosclerosis is not clear. In this study, we established human primary M1 and M2 macrophages from purified $CD14^+$ cell culture in the presence of GM-CSF and M-CSF, respectively. These M1 and M2 macrophages showed similar profiles of cell morphology and cell surface phenotypes as in previous studies (Verreck et al., 2006; Brocheriou et al., 2010; Waldo et al., 2008). We found that both M1 and M2 macrophages produce IL-1ß after stimulation with *P. gingivalis* LPS and cholesterol crystals. M1 tended to produce higher levels of IL-1ß than M2, however this difference did not reach the statistical significance ($p > 0.05$). It would be of interest to investigate the expression of other mediators associated inflammasome activation including IL-18 and IL-33. Our data agree with other studies which demonstrated that M1 macrophage produced higher levels of IL-1ß than M2 macrophage in stimulated condition such as incubation with *Mycobacterium* sonicate and CD40L (Verreck et al., 2006).

Generally, inflammasome activation requires TLR signal and inflammasome signal. We observed in our study that both TLR2 (*P. gingivalis* LPS) and TLR4 (*E. coli* LPS) when combined with cholesterol crystals induce similar levels of IL-1ß in both M1 and M2 macrophages. In general, M1 is recognized as inflammatory macrophage (linked with Th1 cytokine) whereas M2 macrophages are recognized as anti-inflammatory macrophage (linked with Th2 cytokines) (Shimada, 2009). There are little data concerning the physiological function of M1 and M2 macrophages. Morris et al. (2011) and Laoui et al. (2011) hypothesized the association of M1 and M2 with inflammation in obesity and the progression of cancer, respectively. In atherosclerosis, Bouhel et al. (2007) described the presence of M1 in the core lesion whereas M2 was located in periphery. Further research is required to understand the immune modulating activity of M1 and M2 in pathology process of atherosclerosis.

In conclusion, our study provides some insights into the effect of *P. gingivalis* LPS and cholesterol crystals in inflammasome activation of human macrophage. We found that both M1 and M2 macrophages are able to secrete IL-1ß after stimulation with *P. gingivalis* LPS and cholesterol crystals. It is known that atherosclerosis, a chronic inflammatory disease, consistently generate DAMPs such as extracellular ATP from apoptotic cells (Libby, 2002; Libby et al., 2010; Tabas, 2010). Further research of the combination effect of TLRs, DAMPs and cholesterol crystals on inflammasome activation in macrophages may be of interest.

REFERENCES

Abela, G.S. 2010. Cholesterol crystals piercing the arterial plaque and intima trigger local and systemic inflammation. J Clin Lipid. 4:156-164.

Barnett, M.L. 2006. The oral-systemic disease connection : An update for the practicing dentist. JADA. 137:5S-6S.

Bostanci, N., Emingil, G., Saygan, B., Turkoglu, O., Atilla, G., Curtis, M.A., and Belibasakis, G.N. 2009. Expression and regulation of the NALP3 inflammasome complex in periodontal diseases. Clin Exp Immunol. 157:415-422.

Bouchard, P., Boutouyrie, P., D'Aiuto, F., Deanfield, J., Deliargyris, E., Fernandez-Aviles, F., et al. 2010. European workshop in periodontal health and cardiovascular disease consensus document. Eur Heart J. 12(suppl B):B13-B22.

Bouhel, M.A., Derodas, B., Rigamonti, E., Dievart, R., Brozek, J., Haulon, S., et al. 2007. PPARγ activation primes human monocytes into alternative M2 macrophages with anti- inflammatory properties. Cell Metb. 6:137-143.

Boyle, J.J. 2005. Macrophage activation in atherosclerosis : Pathogenesis and pharmacology of plaque rupture. Curr Vas Pharmacol. 3:63-68.

Brocheriou, I., Maouche, S., Durand, H., Braunersreuther, V., Naour, G.L., Gratchev, A., et al. 2010. Antagonistic regulation of macrophage phenotype by M-CSF and GM-CSF: Implication in atherosclerosis. In press. Atherosclerosis.

Bryant, C., and Fitzgerald, K.A. 2009. Molecular mechanisms involved in inflammasome activation. Trends Cell Biol. 19:455-464.

Buhlin, K., Hultin, M., Norderyd, O., Persson, L., Pockley, A.G., Rabe, P., Klinge, B., and Bustafsson, A. 2009. Risk factors for atherosclerosis in cases with severe periodontitis. J Clin Periodontol. 36:541-549.

Chen, G., and Pedra, J.H.F. 2010. The Inflammasome in Host Defense. Sensors. 10: 97-111.

Cole, J.E., Georgiou, E., and Monaco, C. 2010. The expression and functions of Toll-like receptors in atherosclerosis. Mediators Inflamm. 1-18.

Daigneault, M., Preston. J.A., Marriott, H.M., Whyte, M., and Dockrell, D.H. 2010. The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte- Derived Macrophages. Plos ONE. 5:1-10.

Dixon, D. R., and R. P. Darveau. 2005. Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid A structure. J Dent Res. 84:584-595.

Duewell, P., Kono, H., Rayner, K.J., Sirois, C.M., Vladimer, G., Bauernfeind, F.G., et al. 2010. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature. 464:1357-1362.

Duewell, P., Kono, H., Rayner, K.J., Sirois, C.M., Vladimer, G., Bauernfeind, F.G., et al. 2010. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals (Supplementary information). Nature. 1-6.

Erridge, C., Bennett-Guerrero, E., and Poxton, I.R. 2002. Structure and function of lipopolysaccharides. Microbes and Infection. 4:837-851.

Erridge, C., and Nicolaou, G. 2010. Toll-like receptor-dependent lipid body formation in macrophage foam cell formation. Curr Opin Lipidol. 21:427-433.

Galea, J., Armstrong, J., Gadsdon, P., Holden, H., Francis, S.E., et al. 1996. Interleukin-1ß in coronary arteries of patients with ischemic heart disease. Arterioscler Thromb Vasc Biol. 16:1000-1006.

Giacona, M.B., Papapanou, P.N., Lamster, I.B., Rong, L.L., D'Agati, V.D., Schmidt, A.M., and Lalla, E. 2004. *Porphyromonas gingivalis* induces its uptake by human macrophages and promotes foam cell formation in vitro. FEMS Lett. 241:95-101.

Gibson III, F.C., Yumoto, H., Takahashi, Y., Chou, H.H., and Genco, C.A. 2006. Innate immune signaling and *Porphyromonas gingivalis*-associated Atherosclerosis. J Dent Res. 85:106-121.

Hamlet, S.M. 2010. Quantitative analysis of periodontal pathogens by ELISA and real-time polymerase chain reaction. Methods Mol. Biol. 666:125-40.

Hansson, G.K. 2009. Atherosclerosis- An immune disease. The Anitschkov Lecture 2007. Atherosclerosis. 202:2-10.

Haraszthy V.I., Zambon J.J., Trevisan M., Zeid M., and Genco R.J. 2000. Identification of periodontal pathogens in atheromatous plaques. J Periodontol. 71:1554-1560.

Hirai, K., Yoshizawa, H., Hasegawa, H., Ueda, O., Shibata, Y., and Fujimura, S. 2003. Comparison of ability of apoptosis induction by lipopolysaccharide of *Porphyromonas gingivalis* with *Escherichia coli*. Eur J Med Res. 8:208-11.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H., Rock, K.L., Fitzgerald, K.A., and Latz, E. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol. 9:847-856.

Hujoel, P.P., Drangsholt, M., and Spickeman, C. 2000. Periodontal diseases and coronary heart disease risk. JAMA. 284,1406-1410.

Jain, S., and Darveau, R.P. 2010. Contribution of *Porphyromonas gingivalis* lipopolysaccharide to periodontitis. Periodontol 2000. 54:53-70.

Kankkunen, P., Teirila, L., Rintahaka, J., Alenius, H., Wolff, H., and Matikainen, S. 2010. (1,3)-ß-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. J Immunol. 184:6335-6342.

Kamisak,i Y., and Wada, K. 2010. Molecular dissection of *Porphyromonas gingivalis*-related arteriosclerosis: a novel mechanism of vascular disease. Periodontol 2000. 54:222-234.

Klinkner, A.M., Waites, C.R., Kerns, W.D., and Bugelski, P.J. 1995. Evidence of Foam Cell and Cholesterol Crystal Formation in Macrophages Incubated with Oxidized LDL by Fluorescence and Electron Microscopy. J Histochem Cytochem. 43:1071-1078.

Kozarov, E., Sweier, D., Shelburne, C., Progulske-fox, A., and Lopatin, D. 2006. Detection of bacterial DNA in atheromatous plaques by quantitative PCR. Microbes and Infection. 8:687- 693.

Laoui, D., Overmeire, E.V., Movahedi, K., Bossche, J.V., Schouppe, E., Mommer, C., et al. 2011. Mononuclear phagocyte heterogeneity in cancer : Different subsets and activation states reaching out at the tumor site. Im Bio. In press.

Libby, P. 2002. Inflammation in atherosclerosis. Nature. 420:868-874.

Libby, P., Okamoto, Y., Rocha, V.Z., and Folco, E. 2010. Inflammation in Atherosclerosis : Transition From Theory to Practice. Circ J. 74:213-220.

Lucas, A.D., and Greaves, D.R. 2001. Atherosclerosis : role of chemokines and macrophages. Expert reviews in molecular medicine. Exp. Rev. Mol. Med. 5:1-18.

Martinez, F.O., Gordon, S., Locati, M., and Mantovani, A. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol. 177:7303-7311.

Martinon, F., Mayor, A., and Tschopp, J. 2009. The Inflammasomes: Guardians of the Body. Annu Rev Immunol. 27:229-265.

Martinon, F., Petrilli, V., Mayor, A., Tardivel, A., and Tschopp, J. 2006. Gout-associated uric acid crystals activate NALP3 inflammasome. Nature. 440:237-241.

Martinon, F. 2009. Mechanisms of uric acid crystal-mediated autoinflammation. Immunol Rev. 233:218-232.

Morris, D.L., Singer, K., and Lumeng, C.N. 2011. Adipose tissue macrophages:phenotypic plasticity and diversity in lean and obese states. Curr Opin Clin Nutr Metab Care. 14:341-346.

Nassar, H., Chou, H., Khlagatian, M., Gibson III, F.C., Van Dyke, T.E., and Genco, C.A. 2002. Role for fimbriae and Lysine specific cysteine proteinase gingipain K in expression of interleukin-8 and monocyte chemoattractant protein in *Porphyromonas gingivalis* – infected endothelial cells. Infect Immun. 70:268-276.

O'Connor, S., Taylor, C., Campbell, L.A., Epstein, S., and Libby, P. 2001. Potential infectious etiologies of atherosclerosis : a multifactorial perspective. Emerg Infect Dis. 7:780-788.

Pollanen, M.T., Salonen, J.I., and Uitto, V.J. 2003. Structure and function of the tooth-epithelial interface in health and disease. Periodontol 2000. 31:12-31.

Pussinen, P.J., Vilkuna-Rautiainen, T., Alfthan, G., Palosuo, T., Jauhiainen, M., Sundvall, J., et al. 2004. Severe Periodontitis Enhances Macrophage Activation via Increased Serum Lipopolysaccharide. Arterioscler Thromb Vasc Biol. 24:2174-2180.

Rajamaki, K., Lappalainen, J., Oorni, K., Vallmaki, E., Matikainen, S., Petri, T., et al. 2010.

Cholesterol Crystals Activate the NLRP3 Inflammasome in Human Macrophages: A Novel Link between Cholesterol Metabolism an Inflammation. PloS ONE. 5:1-9.

Ross, R. 1999. Atherosclerosis-an inflammatory disease. N Engl J Med. 340:115-126.

Sakai, M., Kobori, S., Miyazaki, A., and Horiuchi, S. 2000. Macrophage proliferation and atherosclerosis. Curr Opin Lipidol. 11:503-509.

Schwartz, Z., Goultschin, J., Dean, D.D., and Boyan, B.D. 1997. Mechanisms of alveolar bone destruction in periodontitis. Periodontol 2000. 14:158-172.

Shimada, K. 2009. Immune system and atherosclerotic disease. Heterogenity of leukocyte subsets participating in the pathogenesis of atherosclerosis. Circ J. 73:994-1001.

Small, D.M. 1988. George Lyman Duff memorial lecture. Progression and regression of atherosclerotic lesions. Insights from lipid physical biochemistry. Arterioscler Thromb Vasc Biol. 8:103-129.

Soder, P.O., Soder, B., Nowak, J., and Jogestrand, T. 2005. Early carotid atherosclerosis in subjects with periodontal diseases. Stroke. 36:1195-1200.

Tabas, I. 2010. Macrophage death and defective inflammation resolution in atherosclerosis. Nat Rev Immunol. 10:36-46.

Taylor, J.J. 2010. Cytokine regulation of immune responses to *Porphyromonas gingivalis*. Periodontol 2000. 54:160-194.

Tonetti, M.S. 2009. Periodontitis and risk for atherosclerosis: an update on intervention trials. J Clin Periodontol. 36(Suppl. 10): 15-19.

Tschopp, J., and Schroder, K. 2010. NLRP3 inflammasome activation : The convergence of multiple signaling pathways on ROS production?. Nat Rev Immunol. 10:210-5.

Verreck, F.A.W., Boer, T., Langenberg, D.M.L., Zanden, L., and Ottenhoff, T. 2006. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and Interferon gamma and CD40L-mediated costimulation. J Leukoc Biol. 79:1-9.

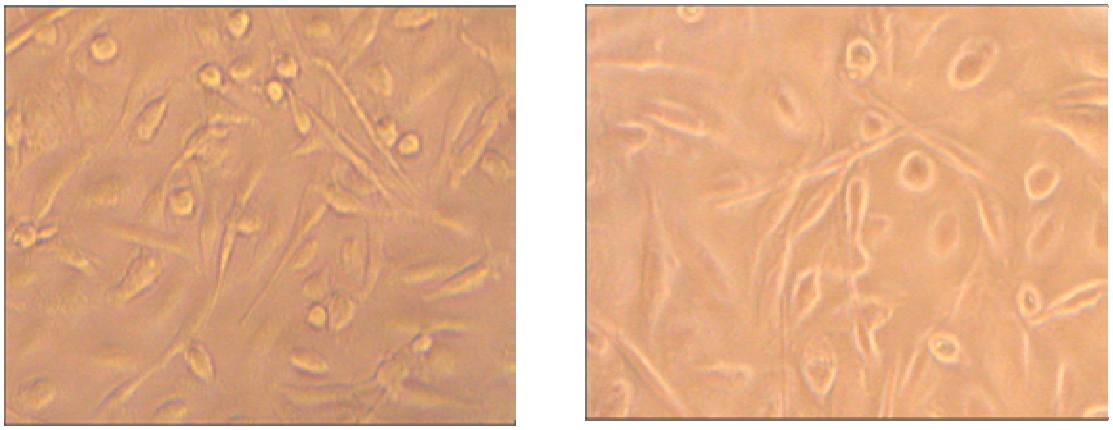
Waldo, S.W., Li, Y., Buono, C., Zhao, B., Billings, E.M., Chang, J., and Kruth, H.S. 2008. Heterogenity of human macrophages in culture and in atherosclerotic plaques. Am J Pathol. 172: 1112-1126.

Wang, P. L., and Ohura, K. 2002. *Porphyromonas gingivalis* lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. Crit Rev Oral Biol Med. 13:132-42.

Zhou, Q., Desta, T., Fenton, M., Graves, D.T., and Amar, S. 2005. Cytokine Profiling of Macrophages Exposed to *Porphyromonas gingivalis*, Its Lipopolysacccharide, or Its FimA Protein. Infect Immun. 73:935-943.

APPENDIX

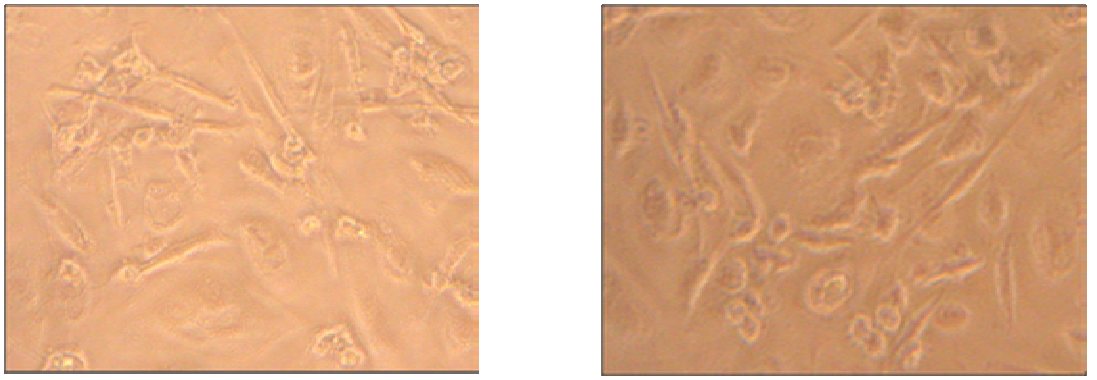
A : M1 macrophage



M1 with *E. coli* LPS

M1 with *P. gingivalis* LPS

B: M2 macrophage



M2 with *E. coli* LPS

M2 with *P. gingivalis* LPS

Figure 15. Morphology of differentiated macrophage after priming with *E.coli* LPS and *P.gingivalis* LPS. The cell condition was observed under microscope (20x).

Table 3 : IL-1ß productions by M1 macrophages after stimulating with *E. coli* LPS and cholesterol crystals (ATP as positive control) (n=4)

Group of Stimulations	IL-1ß production (pg/ml)			
	1	2	3	4
Control	6.40	4.63	9.40	1.00
E. coli LPS	758.37	98.28	20.38	84.89
ATP	5.59	5.91	7.13	1.00
CC-2000 µg/ml	13.24	7.72	3.63	1.00
CC-1000 µg/ml	23.74	7.22	0.73	1.00
CC-500 µg/ml	25.83	8.56	2.01	1.00
E. coli LPS +ATP	2306.66	2358.09	712.53	2142.81
E. coli LPS + CC2000 µg/ml	888.13	460.23	113.54	394.82
E. coli LPS + CC1000 µg/ml	857.25	305.10	40.51	177.99
E. coli LPS + CC500 µg/ml	772.97	283.76	17.50	175.13

Table 4 : IL-1ß productions by M1 macrophages after stimulating with *P. gingivalis* LPS and cholesterol crystals (ATP as positive control) (n=4)

Group of Stimulations	IL-1ß production (pg/ml)			
	1	2	3	4
Control	6.40	4.63	9.40	1.00
P. gingivalis LPS	389.04	106.80	72.09	33.06
ATP	5.59	5.91	7.13	1.00
CC-2000 µg/ml	13.24	7.72	3.63	1.00
CC-1000 µg/ml	23.74	7.22	0.73	1.00
CC-500 µg/ml	25.83	8.56	2.01	1.00
P. gingivalis LPS + ATP	2308.13	2390.50	1290.86	2066.90
P. gingivalis LPS + CC2000 µg/ml	904.79	677.75	306.94	213.20
P. gingivalis LPS + CC 1000 µg/ml	687.62	139.33	304.82	153.04
P. gingivalis LPS + CC 500 µg/ml	462.91	132.83	281.58	112.97

Table 5 : IL-1ß productions by M2 macrophages after stimulating with *E. coli* LPS and cholesterol crystals (ATP as positive control) (n=4)

Group of M-macrophage	IL-1ß production (pg/ml)			
	1	2	3	4
Control	0.44	0.01	1.59	1.00
E. coli LPS	76.05	14.49	12.74	17.78
ATP	2.20	0.57	4.42	1.00
CC-2000 µg/ml	14.31	0.82	17.86	4.05
CC-1000 µg/ml	2.06	0.21	4.02	1.00
CC-500 µg/ml	0.57	0.02	0.01	1.00
E. coli LPS +ATP	1042.55	1692.96	938.80	1541.57
E. coli LPS + CC2000 µg/ml	172.77	87.19	32.09	204.88
E. coli LPS + CC1000 µg/ml	108.38	29.30	27.71	68.42
E. coli LPS + CC500 µg/ml	90.72	20.16	25.50	67.72

Table 6 : IL-1ß productions by M2 macrophages after stimulating with *P. gingivalis* LPS and cholesterol crystals (ATP as positive control) (n=4)

Group of M-macrophage	IL-1ß production (pg/ml)			
	1	2	3	4
Control	0.44	0.01	1.59	1.00
P. gingivalis LPS	43.03	25.83	35.26	15.44
ATP	2.20	0.57	4.42	1.00
CC-2000 µg/ml	14.31	0.82	17.86	4.05
CC-1000 µg/ml	2.06	0.21	4.02	1.00
CC-500 µg/ml	0.57	0.02	0.01	1.00
P. gingivalis LPS + ATP	2261.24	2259.78	1287.90	2140.36
P. gingivalis LPS + CC2000 µg/ml	287.56	158.06	145.44	339.57
P. gingivalis LPS + CC 1000 µg/ml	154.06	54.14	85.90	168.92
P. gingivalis LPS + CC 500 µg/ml	128.22	44.65	81.72	75.43

BIOGRAPHY

Mr. Mahatana Poolgesorn was born on 18th of April 1978 in Nakhon Sri Thammarat. He graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Thammasat University in 2006, and became a staff member of the Faculty of Dentistry, Thammasat University, Pathumthani. He studied in Master degree program in Periodontology at Graduate School, Chulalongkorn University in 2009.