บทบาทของวิถีสัญญาณ Notch ในการควบคุมการทำงานของแมคโครฟาจ

นางสาวชญานิษฐ์ บูรณรักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

THE ROLES OF NOTCH SIGNALING PATHWAY IN REGULATING MACROPHAGE FUNCTIONS

Miss Chayanit Buranaruk

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Industrial Microbiology
Department of Microbiology
Faculty of Science
Chulalongkorn University
Academic Year 2007

Thesis Title	THE ROLES OF NOTCH SIGNALING PATHWAY IN REGULATING MACROPHAGE FUNCTIONS
By	Miss Chayanit Buranaruk
Field of Study	Industrial Microbiology
Thesis Advisor	Assistant Professor Tanapat Palaga, Ph.D.

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

..... Dean of the Faculty of Science

(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Assistant Professor Suthep Thaniyavarn, Ph.D.)

..... Thesis Advisor

(Assistant Professor Tanapat Palaga, Ph.D.)

..... Member

(Associate Professor Sirirat Rengpipat, Ph.D.)

..... Member

(Associate Professor Chintana Chirathaworn, Ph.D.)

ชญานิษฐ์ บูรณรักษ์ : บทบาทของวิถีสัญญาณ Notch ในการควบคุมการทำงานของแมคโครฟาจ. (THE ROLES OF NOTCH SIGNALING PATHWAY IN REGULATING MACROPHAGE FUNCTIONS) อ. ที่ปรึกษา : ผศ. ดร. ธนาภัทร ปาลกะ, 111 หน้า

Notch รีเซพเตอร์ คือโปรตีนบนผิวเซลล์ที่มีการอนุรักษ์ไว้ในระดับสูงระหว่างวิวัฒนาการของสัตว์ชั้นสูง วิถีสัญญาณ Notch มีหน้าที่ควบคุมการเจริญ การแปรสภาพเพื่อทำหน้าที่จำเพาะ และการตายของเซลล์แบบอะพอพโทซิส Notch มีการแสดงออกในเซลล์ของระบบสร้างเลือด และระบบภูมิคุ้มกัน รวมถึงแมคโครฟาจด้วย แมคโครฟาจเป็นเซลล์เม็ดเลือดขาวที่ทำหน้าที่สำคัญทั้งในระบบภูมิคุ้มกันโดยกำเนิด และภูมิคุ้มกันแบบจำเพาะ โดยมีหน้าที่กำจัดสิ่งแปลกปลอมโดยการกลืนกิน การผลิตและหลั่งไซโตไคน์ และการนำเสนอแอนติเจนต่อทีเซลล์ แมคโครฟาจที่ถูกกระตุ้นแบ่งออกเป็น 3 ชนิดตามชนิดของตัวกระตุ้นและลักษณะการตอบสนอง คือ Ca-Mϕ, AA-Mϕ และ Mϕ-II ในงานวิจัยนี้จึงศึกษาบทบาทของวิถีสัญญาณ Notch ต่อการทำงานของแมคโครฟาจและความสัมพันธ์ระหว่างการแสดงออกของ Notch ในแมคโครฟาจทั้ง 3 ชนิด ในการทดลองพบว่า มีการแสดงออกของทั้ง Notch รีเซพเตอร์ และจีนเป้าหมายของวิถีสัญญาณ Notch คือ *Hes1* ในแมคโครฟาจที่ถูกกระตุ้น โดย *Notch1* และ *Hes1* มีการแสดงออกเพิ่มขึ้นในแมคโครฟาจที่ถูกกระตุ้นแบบ Ca-Mϕ นอกจากนี้ แมคโครฟาจที่ถูกระตุ้นชนิดนี้ยังมีระดับของการแสดงออกของ *IL-12p40*, *iNOS*, *Arg1*, *IL-10*, *TNFα* และ MHC class II ที่แตกต่างกัน และเมื่อทำการเพิ่มการแสดงออกของ Notch1 หรือกดการแสดงออกของวิถีสัญญาณ Notch โดยสารกดแกมมาซีครีเตส พบว่ามีผลต่อการแสดงออกของ *IL-12p40*, *iNOS*, *Arg1*, *IL-10*, *TNFα*, MHC class II และการผลิตไนตริกออกไซด์ และ TNFα แต่ไม่มีผลต่อการฆ่าแบคทีเรียทั้งแกรมบวกและแกรมลบ ดังนั้น Notch 1 น่าจะเกี่ยวข้องกับการตอบสนองของแมคโครฟาจที่ถูกกระตุ้นในการสร้างไซโตไคน์เหล่านี้ จากการศึกษาการแสดงออกของ Notch1 ในแมคโครฟาจที่ถูกกระตุ้นทั้ง 3 ชนิด พบว่า Ca-Mϕ มีการแสดงออกของ Notch 1 สูงสุด แต่ไม่พบการแสดงออกใน AA-Mϕ ซึ่งเมื่อทำการศึกษาการแสดงออกของจีนโดยวิธี RT-PCR พบว่าแมคโครฟาจที่ถูกกระตุ้นทั้ง 3 ชนิด มีการแสดงออกของจีนโดยเฉพาะไซโตไคน์ที่แตกต่างกัน ดังนั้น Notch1 น่าจะมีส่วนเกี่ยวข้องในการควบคุมการทำงานของแมคโครฟาจที่ถูกกระตุ้นแบบ Ca-Mϕ ดังนั้น วิถีสัญญาณ Notch น่าจะเป็นทางเลือกหนึ่งในการควบคุมการทำงานของแมคโครฟาจ เช่น การบำบัดโรคที่เกิดจากการอักเสบเรื้อรังที่เกิดจากการกระตุ้นแมคโครฟาจ

ภาควิชาจุลชีววิทยา
สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม
ปีการศึกษา 2550

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....

4872257723 : MAJOR INDUSTRIAL MICROBIOLOGY

KEY WORD: MACROPHAGE / NOTCH / Ca-Mϕ / AA-Mϕ / Mϕ-II

CHAYANIT BURANARUK: THE ROLES OF NOTCH SIGNALING PATHWAY IN REGULATING MACROPHAGE FUNCTIONS. THESIS ADVISOR: ASST. PROF. TANAPAT PALAGA, Ph.D., 111 pp.

Notch receptors are evolutionarily conserved transmembrane proteins found in metazoans. Notch receptors are expressed in both hematopoietic and immune cells including macrophages. Notch signaling is involved in regulation of proliferation, differentiation and apoptosis. Macrophages are white blood cells playing important roles in both innate and acquired immunity. Macrophages destroy antigen by phagocytosis, and mediate immune responses by cytokine secretion and antigen presentation. Activated macrophages are divided into 3 types, Ca-Mϕ, AA-Mϕ and Mϕ-II, based on stimuli and phenotypic responses. The purposes of this study are to investigate the roles of Notch signaling in regulating macrophage functions and the relationship between expression profiles of Notch1 in three different types of activated macrophages. Expression of Notch receptors and Notch target gene, *Hes1* in activated macrophages were observed and both *Notch1* and *Hes1* were upregulated in Ca-Mϕ. In addition, Ca-Mϕ was differentially expressed *IL-12p40*, *iNOS*, *Arg1*, *IL-10*, *TNFα* and MHC class II. Notch1 overexpression or inhibition of Notch signaling using γ-secretase inhibitor altered expression of *IL-12p40*, *iNOS*, *Arg1*, *IL-10*, *TNFα*, MHC class II and nitric oxide and TNFα production but did not affect the ability of macrophages to kill both Gram positive and negative bacteria. Thus, Notch1 may involve in the responses of macrophages followed this stimulation. Furthermore, Ca-Mϕ showed the highest Notch1 expression among three different types of macrophages. This result suggests that Notch1 may function specifically in regulating Ca-Mϕ function. Therefore, Notch signaling may be a novel target for intervention with macrophage activity such as in chronic inflammatory condition arising from hyperactivation of macrophages.

Department: Microbiology Student's signature: Chayanit Buranaruk

Field of study: Industrial Microbiology Advisor's signature: Tanapat Palaga

Academic year: 2007

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Assistant Professor Dr. Tanapat Palaga for the excellent instructions, guidance and support throughout this thesis.

My gratitude is also extended to Assistant Professor Dr. Suthep Thaniyavarn, Associate Professor Dr. Sirirat Rengpipat and Associate Professor Dr. Chintana Chirathaworn for serving as thesis committee.

I wish to thank Dr. Cynthia Baldwin (U. of Massachusetts at Amherst, USA) for Rabbit Anti-SRBC IgG, Max Plank Institute of Infection Biology, Germany for *Escherichia coli* DH5α, Professor Todd E. Golde (Mayo Clinic College of Medicine, USA) for IL-CHO, Professor Barbara A. Osborne (U. of Massachusetts at Amherst, USA) for plasmid pcDNA3 and plasmid pcDNA3-ICN1 and Associate Professor Dr. Sirirat Rengpipat for serving *Staphylococcus aureus* ATCC 25923. I also thank Noppadol Sa-Ard-Iam for his help on FACS analysis.

I would like to thank Department of Microbiology, Faculty of Science, Chulalongkorn University for providing some research facilities.

I wish to thank The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) for supporting my thesis.

I would like to thank all of my 403 laboratory members for their assistance and friendship.

Finally, I would like to express my deepest gratitude to my parents for their love, understanding and encouragement extended throughout my study.

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1.	%	Percentage
2.	°C	Degree Celsius
3.	μg	Microgram
4.	μm	Micrometer
5.	μM	Micromolar
6.	/	Per
7.	:	Ratio
8.	+ve	Positive control
9.	×	Fold
10.	∞	Infinity
11.	A	Absorbance
12.	Ab	Antibody
13.	*Arg1*	Arginase1
14.	bp	Base pair
15.	cDNA	Complementary DNA
16.	CFU	Colony forming unit
17.	CO_2	Carbon dioxide
18.	dATP	Deoxyadenosine triphosphate
19.	dCTP	Deoxycytidine triphosphate
20.	dGTP	Deoxyguanosine triphosphate
21.	DNA	Deoxyribonucleic acid
22.	dNTP	dATP, dCTP, dGTP and dTTP
23.	dTTP	Deoxythymidine triphosphate
24.	E-IgG	Erythrocyte opsonized with IgG
25.	ELISA	Enzyme-linked immunosorbent assay

26.	g (centrifugation speed)	Gravity
27.	GSI	Gamma secretase inhibitor
28.	hr	Hour
29.	*Hes1*	Hairy/Enhancer of Split1
30.	HPLC	High performance liquid chromatography
31.	HRP	Horse radish peroxidase
32.	IFNγ	Interferon gamma
33.	IgG	Immunoglobulin G
34.	IL	Interleukin
35.	*iNos*	Inducible nitric oxide syntase
36.	kDa	Kilo Dalton
37.	LB	Luria Bertani
38.	LPS	Lipopolysaccharide
39.	m	Murine
40.	mA	Milliampere
41.	mg	Milligram
42.	MHC class II	Major histocompatibility complex class II
43.	ml	Milliliter
44.	mM	Millimolar
45.	mϕ	Macrophage
46.	nm	Nanometer
47.	No.	Number
48.	OD	Optical density
49.	PAGE	Polyacrylamide gel electrophoresis
50.	PBS	Phosphate buffer saline
51.	PBST	Phosphate buffer saline – Tween
52.	pcDNA3	Plasmid cDNA3

53.	pcDNA3 ICN1	Plasmid cDNA3 with intracellular Notch1
54.	PCR	Polymerase chain reaction
55.	psi	Pound per square inch
56.	PVDF	Polyvinylidine fluoride
57.	r	Recombinant
58.	RNA	Ribonucleic acid
59.	rpm	Revolution per minute
60.	RT	Reverse transcription
61.	SDS	Sodium dodecyl sulfate
62.	T_0	Time 0 h
63.	T_{24}	Time 24 h
64.	*TNFα*	Tumor necrosis factor alpha
65.	U	Unit
66.	v	Volume
67.	-ve	Negative control
68.	w	Weight
69.	α	Alpha
70.	β	Beta
71.	γ	Gamma

CHAPTER I

BACKGROUND

Notch signaling plays a key role in several cellular processes including differentiation, proliferation and apoptosis. Notch receptor family proteins are highly conserved transmembrane receptors that express in both vertebrates and invertebrates (Kojika and Griffin, 2001). Four distinct Notch receptor genes have been identified in mammals, *i.e. Notch 1-4* (Logeat *et al.*, 1998). Notch ligands are also transmembrane proteins which are expressed on cell surface. Five different Notch ligands have been characterized in mammals, *i.e.* Jagged 1, 2 and Delta1, 3 and 4 (Allman *et al*., 2002). After receptor ligand interaction, Notch receptors undergo two proteolytic cleavage steps that release the intracellular Notch (ICN) from the plasma membrane. ICN translocates to the nucleus and forms complex with various transcriptional mediator proteins and subsequently activates Notch target genes such as *Hes1* (Maillard *et al*., 2003). The functions of Notch signaling have reported hematopoietic and lymphoid development (Radtke *et al*., 2004), promotion of self-renewal of stem cells (Kunisato *et al*., 2003) and regulation of lineage specification (Pui *et al*., 1999). In human, mutations of genes of the Notch receptors, Notch ligands or protein components associated with Notch signaling are linked to human diseases such as tumor formation (Nam *et al*., 2002).

Macrophages are phagocytic cells providing host protection in both innate and acquired immunity. Macrophages recognize, engulf and destroy many pathogens including bacteria, pathogenic protozoa, fungi and helminthes (Kaufmann *et al*., 2004). Upon phagocytosis, macrophages degrade proteins and process antigens for presentation on MHC molecules, where T cells recognize the antigen and undergo clonal expansion (Madigan *et al*., 2003). Macrophages also produce and secrete many cytokines, such as TNFα, IL-12 and IL-10, and reactive oxygen and nitrogen species to support their ability for killing pathogens

(Kawai and Akira, 2005). Macrophages presented in distinct microenvironment exhibit a wide variety of functions and phenotypes (Stout *et al*., 2004). Activated macrophages have been divided into 3 categories based on stimuli, *i.e.* classically activated macrophages (Ca-Mϕ), alternatively activated macrophages (AA-Mϕ) and type-II activated macrophages (Mϕ-II). These cells are stimulated by distinct stimuli and expressed distinctive phenotypes (Edwards *et al*., 2006).

Notch signaling has been identified to be expressed and play roles in antigen presenting cells such as macrophages and dendritic cells (Yamaguchi *et al*., 2002). Notch signaling also regulates monocytes development and macrophage maturations (Nomaguchi *et al*., 2001). Macrophages express both Notch receptors and Notch ligands with varying degree (Palaga *et al*., 2008). *Hes1*, one of Notch target genes, was detected in activated macrophages suggesting that Notch signaling is activated during macrophage stimulation (Palaga *et al*., 2008).

Although there are reports on the involvement of Notch signaling in regulating macrophage function, the exact roles this signaling play during inflammation are largely unknown. In addition, the involvement of Notch signaling in differentiation of three distinctive population are unexplored.

Objectives

The purposes of this research are to investigate the roles of Notch signaling in regulating macrophage functions and to observe the relationship between three populations of activated macrophages and Notch signaling.

CHAPTER II

LITERATURE REVIEWS

2.1 Notch signaling

2.1.1 Notch receptors and Notch ligands

Proteins in the Notch receptor family are evolutionarily conserved transmembrane proteins playing essential roles in regulating cell fate determination during metazoan development, including hematopoiesis and immune system development (Kojika and Griffin, 2001).

There are four mammalian Notch receptors which are encoded by four different genes, *i.e. Notch 1-4.* Notch receptors are single-pass transmembrane proteins that are cleaved within the trans-golgi network by furin-like convertase during biosynthesis into two pieces which constitute a heterodimeric cell-surface receptor (Logeat *et al*., 1998).

Structure of Notch proteins are depicted in Figure 2.1. The extracellular domain of Notch (ECN) contains 29-36 tandem epidermal growth factor (EGF)-like repeats, varying among Notch receptors. Some EGF-like repeats are responsible for interacting with Notch ligands. There are also three LIN12/Notch repeats, locating in the proximity to the transmembrane domain, that prevent ligand-independent signaling (Allman *et al*., 2002). The intracellular portion of Notch (ICN) contains several functional domains which mediates Notch signal transduction. These include the membrane-proximal RBP-j-associated molecule (RAM) domain and ankyrin repeats (ANK) that interact with downstream effector proteins, two nuclear localization signal (NLS) motifs, and a C-terminal proline-glutamate serine-

threonine-rich (PEST) domain regulating protein stability (Rogers *et al*., 1986). Notch1-2, but not 3-4, contain a C-terminal transcriptional activation domain (TAD) (Maillard *et al*., 2003). The RAM domain is the binding site for C promoter binding factor-1 (CBF-1) (also known as recombination signal binding protein-J kappa (RBP-Jκ)) (Tamura *et al*., 1995), and the ANK repeats domain can also interact with CBF-1. ANK repeats are also binding sites for Deltex and MAML which negatively modulate Notch signaling (Matsuno *et al*., 1995 and Wu *et al*., 2000).

Five mammalian Notch ligands have been identified, *i.e.* Jagged 1, Jagged 2, Delta-like (Dll) 1, Dll 3 and Dll 4. Like Notch receptors, these ligands are transmembrane proteins which are composed of EGF-like repeats and a unique N-terminal DSL domain (for Delta, Serrate and *C. elegans* homolog Lag2) and cysteine-rich region (CR) found in only Jagged 1 and 2. The intracellular domains of the ligands are small (75-210 residues) and not highly conserved, but they are also important for signal initiation (Allman *et al*., 2002).

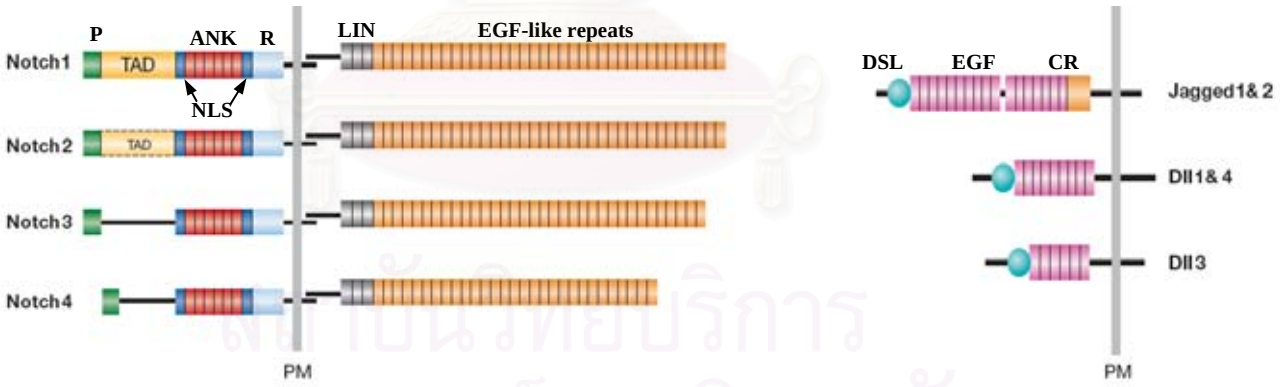


Figure 2.1 Structure of Notch receptors and Notch ligands. For Notch protein, the ECN of Notch 1-2 consist of 36 EGF-like repeats and 3 LIN12/Notch repeats. Notch 3-4 have 34 and 29 EGF-like repeats, respectively. ICN consists of a RAM domain, 6 ANK repeats, 2 NLS and PEST domain. Notch ligands are composed of DSL, EGF and CR (found in Jagged 1 and 2). PM represents plasma membrane. (Radtke *et al*., 2005).

2.1.2 Activation of Notch Signaling

Notch signaling is initiated upon engagement of Notch receptors with their ligands presented on the same cells or between neighboring cells. Notch receptor is proteolytically cleaved by two enzymes, resulting in the release of ICN. The first cleavage occurs at the extracellular domain mediated by a TNFα converting enzyme (TACE) proximal to the LIN domain. The second cleavage takes place within the transmembrane domain mediated by a multiprotein complex with γ-secretase activity whose components include presenilin and nicastrin (Nam *et al*., 2002). Following cleavages, ICN is released and translocates to the nucleus, where it binds the transcription factor CBF1/Supessor of Hairless (Su(H)/LAG1 (CSL) (for CBF-1/RBP-Jκ in mammals)). ICN displaces co-repressors (CoR), recruits co-activators (CoA), and activates transcription of its target genes. Mastermind-like family proteins (MAML) form a complex with ICN and CSL and act as scaffolding protein for multiprotein complex formation, and act as potent transcriptional coactivators. (Maillard *et al*. (2003) and Allman *et al*. (2002)).

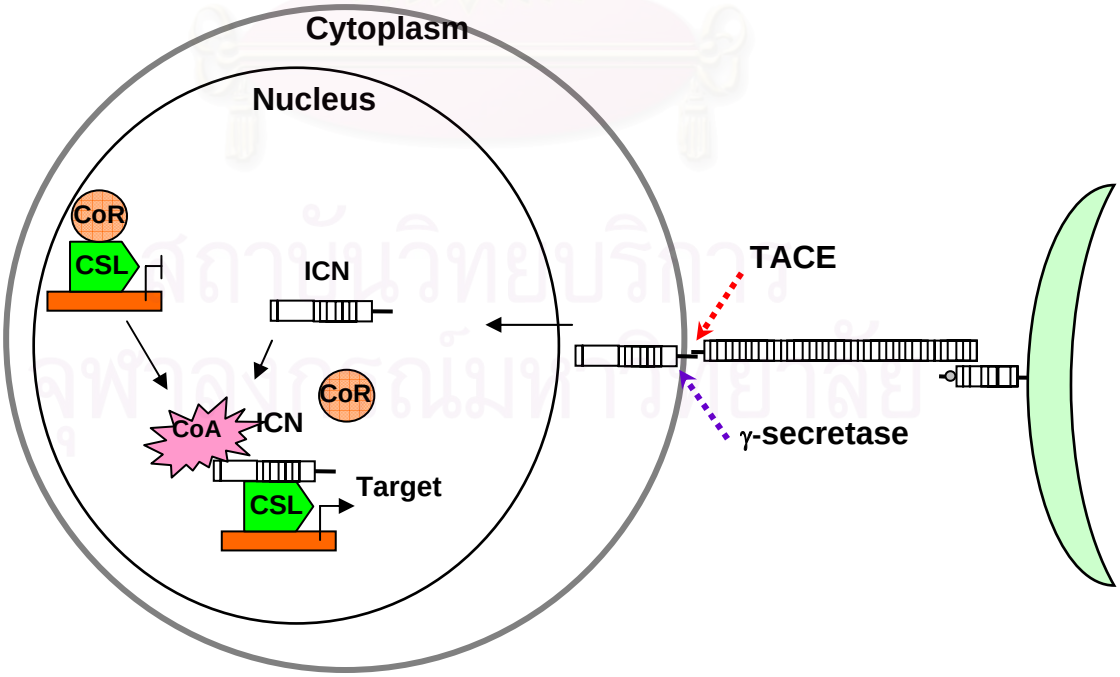


Figure 2.2 Notch signaling. Notch signaling is initiated after receptor-ligand interaction, which induces two sequential proteolytic cleavages. The first cleavage within the extracellular domain is mediated by the metalloprotease TACE. The second cleavage occurs within the transmembrane domain and is mediated by the γ-secretase activity. This interaction leads to transcriptional activation of Notch target genes by displacement of CoR and simultaneous recruitment of CoA (Nam *et al*., 2002).

2.1.3 Notch target genes

The effect of Notch activation directly increased transcription of Notch target genes. One of the most important targets of Notch signaling is a family of basic helix-loop-helix (bHLH) transcriptional repressor known as Hairy and Enhancer of Split 1 (Hes1) (Osborne and Miele (1999)). Hes1 functionally antagonizes bHLH genes such as *Mash1* which plays role in neurogenesis (Kageyama *et al*., 2005). In addition, Hes5, a member of the Hes family, Hey (hairy/enhancer-of-split related with YRPW) (Allman *et al*., 2002), pTα, a pre TCRα gene, and Notch1 itself are also downstream targets of Notch signaling (Maillard *et al*., 2003). Furthermore, Deltex, a positive regulator of Notch signaling, which functions by antagonizing the interaction between Notch and Su(H) (Matsuno *et al*., 1995), is also identified to be a Notch target gene (Yamamoto *et al*., 2001). Oswald and colleagues (1998) found that CBF-1 normally repress NF-κB2 expression in the absent of Notch signaling. Truncated Notch1 strongly induce NF-κB2 promoter activity. Thus, it is possible that NF-κB2 may be a Notch target gene.

2.1.4 Gamma-secretase inhibitor (γ-secretase inhibitor; GSI)

γ-secretase, a large protease complex located on the membrane, is composed of a catalytic subunit (presenilin (ps) 1 or 2) and accessory subunit (presenilin enhancer-2 (pen-2), anterior pharynx-defective phenotype-1 (aph-1) and nicastrin (Shih and Wang, 2007) (Figure

2.3). Presenilin is depicted as the catalytic subunit containing separate binding and catalytic sites (Tain *et al*., 2003).

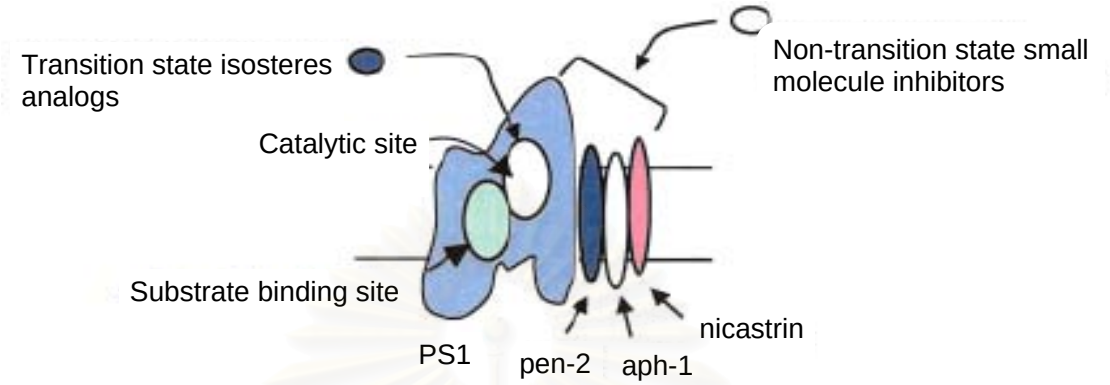


Figure 2.3 γ-Secretase is a high molecular weight multi-component protein complex containing at least presenilin, nicastrin, aph-1, and pen-2 (from Tain *et al*., 2003).

The amyloid β-protein (Aβ) deposited in Alzheimer's disease (AD) derived from the amyloid β-protein precursor (APP) by two proteolytic cleavages. An initial β-secretase cleavage at the N-terminus of Aβ sequence is then followed by γ-secretase cleavage at the C-terminus of Aβ (McLendon *et al*., 2000).

Inhibitors for γ-secretase have been investigated for blocking the generation of Aβ peptide for Alzheimer's disease therapy. Because, ICN is also released by γ-secretase cleavage resulting in Notch signaling activation, GSI is able to prevent Notch receptor activation. An original GSI, z-IL-CHO, was shown to have Notch1-dependent antineoplastic activity in Ras-transformed fibroblasts (Weijzen *et al*., 2002). Moreover, tripeptide GSI (z-Leu-Leu-Nle-CHO) was reported to suppress tumor growth in cell lines and/or xenografts in mice from melanoma and Kaposi sarcoma (Curry *et al*., 2005). Treatment with dipeptide GSI N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-5-phenylglycine t-butyl ester (DAPT) resulted in inhibition of proliferation of human acute T cell leukemia cell line and human liver

hepatoblastoma cell line but not affected to apoptosis in both cell lines. By contrast, when ICN1 was overexpressed in both cell lines, GSI did not affect proliferation of both cells. Moreover, decreased expression of *Hes1* and increased expression of Notch1 in both cells were detected after treatment with DAPT for 4 days (Suwanjunee *et al*., 2008).

Small peptide based (peptidomimetic) inhibitors were the first reported inhibitors of γ-secretase activity (Rochette and Murphy, 2002). Peptide aldehyde, such as z-IL-CHO (IC50 ~ 10 μM (McLendon *et al*., 2000)) was used in recent studies for inhibiting Notch pathway. For examples, blockade of Notch signaling by using IL-CHO resulted in inhibition of TCR-induced Notch1 expression in splenocytes, and IL-CHO completely blocked CD4 and CD8 T cell proliferation (Palaga *et al*., 2003). Furthermore, IL-CHO also blocked Notch1 expression in activated bone marrow macrophages. In the presence of IL-CHO, NO production was suppressed, but MHC class II was enhanced in activated bone marrow macrophages (Palaga *et al*., 2008).

2.1.5 Functions of Notch Signaling

2.1.5.1 Notch signaling during development

2.1.5.1.1 Notch signaling in hematopoiesis

The Notch pathway plays a central role in cell fate decisions and commitments that affects the development and function of many organs, including hematopoiesis and the immune systems.

Hematopoietic stem cells and stromal cells both express Notch receptors and Notch ligands (Figure 2.4). Notch signaling affects the survival, proliferation and fate choice of precursor cells in hematopoiesis. (Radtke *et al*., 2004)

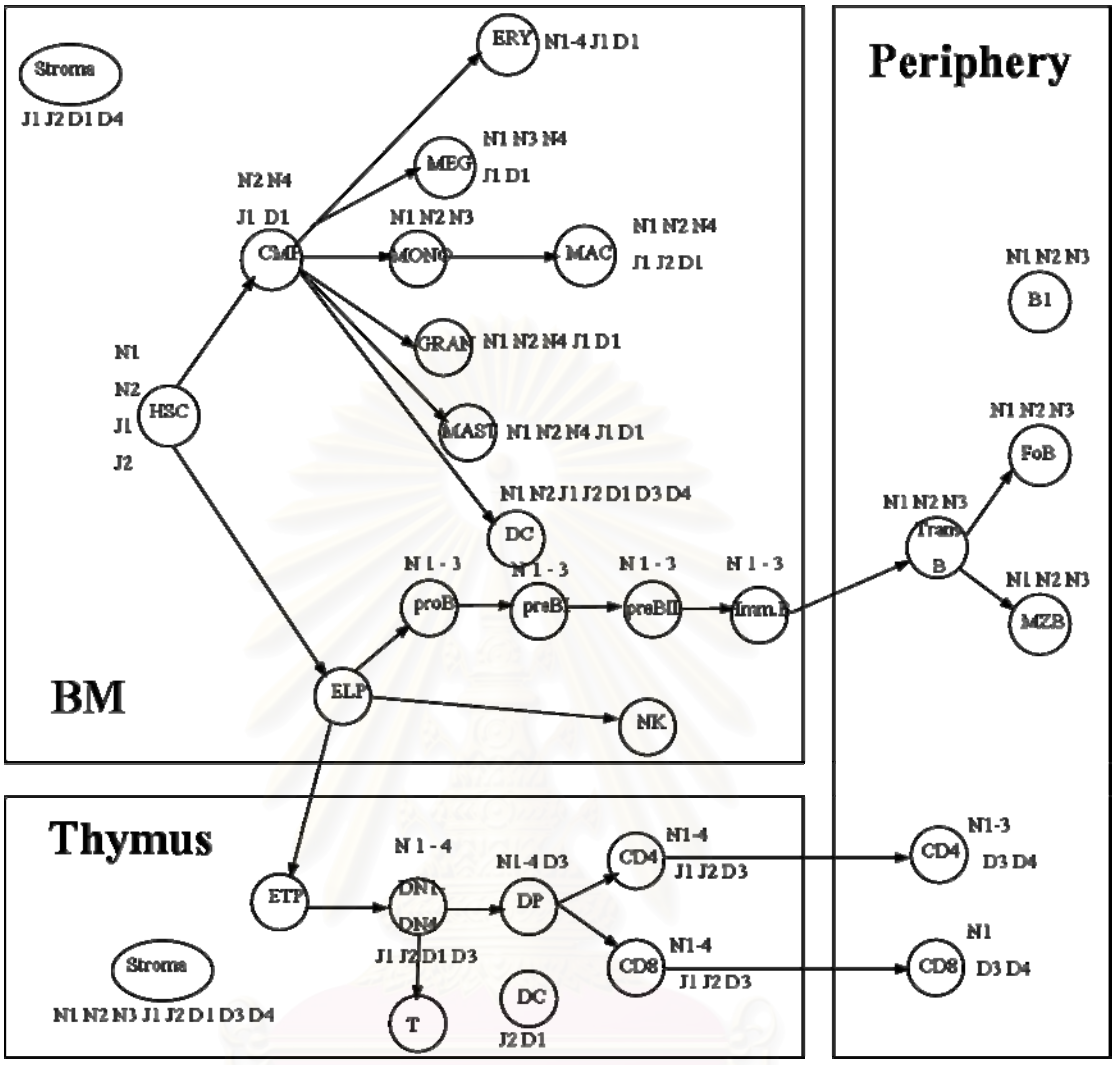


Figure 2.4 Expression of Notch receptors and Notch ligands in hematopoiesis. Abbreviations; N, Notch; J, Jagged; D, Deltal-like; HSC, hematopoietic stem cells; BM, bone marrow; CMP, common myeloid progenitor; ELP, early lymphoid progenitor; Ery, erythroblast; Meg, megakaryocyte; Mono, monocyte; Mac, macrophage; Gran, granulocyte; DC, dendritic cell; Mast, mast cell; Imm.B, immature B cell; Trans.B, transitory B cell; NK, natural killer cell; ETP, early thymic progenitor; DN, double negative (After Radtke *et al*., 2004)

Kunisato and colleagues (2003) found that Hes1 played a significant role in mouse long-term hematopoietic reconstituting cells expansion *ex vivo*. $CD34^{low/-}$ cells

represent the most highly purified population of hematopoietic stem cells (HSC) in the adult bone marrow. *Hes1*-transduced HSC increased production of progenies. Moreover, the ratios of the Hoechst dye-staining for defining side population and $CD34^{low/-}$ cells in *Hes1* overexpressing cells were significantly higher than those in competitor derived non-transduced cells in the bone marrow of each recipient mouse.

Using $Notch1^{-/-}$ and $Notch2^{-/-}$ mouse embryos, hematopoietc cell development and angiogenesis were severely impaired in $Notch1^{-/-}$ embryo, but not in the $Notch2^{-/-}$ embryo. Thus, Notch1 is essential for generating hematopoietic stem cells from endothelial cells (Kumono *et al*., 2003).

Notch signaling regulates osteoclast (the monocyte/macrophage linage) development. By using an immobilized Delta1, osteoclast development from bone marrow, spleen, and peritoneal cavity tissue was inhibited. When ICN1 was introduced in stromal cells by transfection, expression of several molecules responsible for osteoclast development was found to be increased (Yamada *et al*., 2003).

Notch signaling is selectively activated in neurosphere-initiating multipotent neural stem cells *in vitro* and in radial glia during forebrain development (Kohyama *et al*., 2005).

Prolonged *in vitro* culture of murine bone marrow linage negative cells in medium supplemented with early acting cytokines and with immobilized Jagged1 resulted in robust expansion of serially transplantable hematopoietic stem cells with long-term repopulating ability (Kertesz *et al*., 2005).

Introducing Notch1 expression into mesodermal cell lineages, including endothelial, neural, cardiac muscle and hematopoietic cells, resulted in profound

alterations in the generation of all of these cells. When Notch signaling was activated in mesodermal cells, generation of cardiac muscle, endothelial and hematopoietic cells are inhibited, favoring the generation of muscle cells (Schroeder *et al*., 2006).

Delta1 acted synergistically with IL-6R/IL-6 fusion protein (FP6) to enhance the generation of erythroid cells from the erythroid progenitors. In contrast, Delta1 antagonized the effects of IL-6 and FP6 on the development of monocytic and granulocytic cells from myeloid progenitors (Yamamura *et al*., 2007).

2.1.5.1.2 Notch signaling in B cell lineage

In addition, Notch associated with B cell was reported (Pui *et al*., 1999). ICN1 transgenic mice had defective B cell development and ICN1 had been shown to promote cell-cycle arrest and apoptosis in chicken B cells rather than producing B cell malignancy (Morimura *et al*., 2000). Moreover, Notch2 is a predominant Notch expressed on B-lineage cells (Kojika and Griffin, 2001).

Mastermind-like 1 (MAML1) deficiency in mice abolished the development of splenic marginal zone B cells, a subset strictly dependent on Notch2, a CSL protein and Delta1 ligand. MAML1 deficiency also caused a partially impaired development of early thymocytes, while not affecting the generation of definitive hematopoiesis, processes that were dependent on Notch1 (Oyama *et al*., 2007).

Notch signaling promoted the ubiquitination and degradation of E47 protein in aged B cell precursors. The transcriptional regulator E47, encoded by the E2A gene, is crucial to B lymphopoiesis. The reduced levels of E47 proteins were resulted from increased proteosome-mediated protein turnover (King *et al*., 2007).

2.1.5.1.3 Notch signaling in T cell lineage

Notch signaling is also involved in several steps of lymphoid development. Notch receptors are presented in both CD4 and CD8 peripheral T cells (Maillard *et al*., 2003).

Among hematopoietic cells, Notch1 is most clearly involved in regulation of T cell development. Notch1 promotes T cell maturation, protects lymphocyte from TCR-mediated apoptosis, and regulates CD4 T cell mediated immune response (Kojika and Griffin, 2001).

Furthermore, Notch signaling is associated with peripheral T cells. Stimulation of T cell through TCR increased in Notch protein, whereas loss-of-function of Notch signaling using IL-CHO inhibited T cell proliferation (Palaga *et al*., 2003).

The extracellular domain of Delta1 fused to the Fc domain of human IgG1 increased the number of progenitors capable of short-term lymphoid and myeloid reconstitution and increase the number of T cell progenitors (Dallas *et al*., 2007).

Notch1 signaling has complicated effects on molecules involved in T cell development and that activation of Notch1 may protect thymocytes form death by neglect mediated by glucocorticoid. Moreover, Notch1 expression results in the repression of pro-apoptosis nuclear hormone receptor *Nur77*-induced transcription. Thus, Notch1 may involve in apoptosis inhibition in certain cell types (Osborne and Miele, 1999)

2.1.5.2 Notch and human diseases

A variety of human diseases result from mutations in genes encoding Notch receptors and their ligands. These diseases range from cancer to neurodegenerative disorders. T cell acute lymphoblastic leukemias (T-ALL) was first recognized when a recurrent t(7;9)q(34;q34.3) chromosomal translocation, which creates a truncated, constitutively activated human Notch1. Mutations in human *Notch3* at the EGF repeants in an extracellular domain lead to the development of the hereditary disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). Alagille's syndrome, an autosomal dominant disease, results from frameshift mutations of *Jagged1* that lead to developmental defects in several organs including liver, heart, eye, skeleton and/or kidney. Homozygous mutations in *Delta3* have been linked to a developmental effect of the axial skeleton called spondylocostal dysostosis. (Nam *et al*., 2002)

Deregulated expression of Notch receptors, ligands and their target genes is observed in solid tumors, including cervical, head and neck, endometrial, renal, lung, pancreatic, ovarian, breast and prostate carcinomas, osteosacroma, mesothelioma, gliomas and medulloblastomas. Hodgkin's lymphomas, anaplastic large-cell non- Hodgkin's lymphomas, some acute myeloid leukemias, B cell chronic lymphoid leukemias and multiple myeloma also show deregulated expression of Notch receptors or ligands (Miele, 2006).

Notch1 signaling in tumor cells by its ligand Jagged1, regulated growth and survival of both B cell-derived Hodgkin and Reed-Sternberg cells and in tumor cells of T cell derived anaplastic large cell lymphoma (Jundt *et al*., 2002).

High-level expression of Notch1 and Jagged1 is associated with poor prognosis in breast cancer and with metastasis in prostate cancer (Miele, 2006). Therefore,

Notch and its signaling genes are considered to be proto-oncogene in various cell and tissue types.

2.2 Macrophages

A basic way in which the body reacts to infection, irritation or other injury is inflammation, the key feature of which being redness, warmth, swelling and pain. Inflammation is recognized as a type of nonspecific immune response. The inflammatory response directs components of an immune system to the site of injury or infection, and is manifest by increased blood supply and vascular permeability which allows chemotactic peptide, neutrophils and mononuclear cells to leave the intravascular component. Microorganisms are engulfed by phagocytic cells particularly macrophages in an attempt to contain the infection in a small tissue space. The response includes attraction of phagocytes in a chemotactic gradient of microbial products, movement of phagocytes to the inflammatory site and elimination of antigen by phagocytosis.

Macrophages provide innate and acquired immunity for every tissue in the body. Macrophages are derived from myeloid precursors in bone marrow (BM), spleen and fetal liver (Stout *et al*. 2004). Optimal proliferation and differentiation of macrophages from pluripotent progenitors require the presence of a combination of polypeptide growth factors. These growth factors include macrophage colony-stimulating factor (M-CSF or CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-3, stem cell factor (SCF), IL-1, leukemia inhibitory factor (LIF) and interferon-γ (IFNγ) (Kaufmann *et al.*, 2004). Inexperienced macrophages, termed monocytes, leave the unique environment of the BM and enter to the blood, where they are exposed to a plethora of agents, including cytokines, chemokines and immunoglobulins, which are capable of impacting their functional and phenotypic characteristics (Stout *et al*., 2004).

2.2.1 Macrophage functions

Macrophage functions include phagocytosis and killing of pathogens, antigen presentation to T and B lymphocytes, and secretion of a large number of diverse mediators, including prostaglandins and leukotrienes, complement components, coagulation factors, proteolytic enzymes and the production of reactive oxygen and nitrogen species (Kota *et al*., 2006).

2.2.1.1 Phagocytosis (Madigan *et al*., 2003)

The primary function of macrophages is to engulf and destroy pathogens. In this process, macrophages can act as antigen-presenting cells (APCs) and generate the peptide antigens that activate T lymphocytes and the specific immune response.

Macrophages are usually motile and move by amoeboid action. Most have granular inclusions called lysosomes, which contain bactericidal substances such as hydrogen peroxide, lysozyme, proteases, phosphatases, nucleases and lipases. Macrophages can trap a pathogen on their surfaces. The cytoplasmic membrane of macrophages then engulfs the pathogen. The entire complex pinches off and eventually fuses with the lysosomes, forming a new inclusion called a phagolysosome. The toxic substances and enzymes inside the phagolysosome usually kill and digest the engulfed microorganisms. Ingestion of a pathogen stimulates the macrophages to become more efficient, enhancing their ability to engulf and destroy pathogens (Figure 2.5).

Toxic oxygen containing compounds including hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), hydroxyl radicals ($OH^{\cdot}$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and nitric oxide (NO) are found in phagolysosome. Macrophages use the toxic oxygen to kill ingested bacterial cells. In some cases, pathogens have developed mechanisms

for neutralizing the effects of toxic phagocyte products, for killing the phagocyte, or for avoiding phagocytosis. For example, *Staphylococcus aureus* produces pigment compounds called carotenoids, which quench singlet oxygen and prevent killing. Intracellular pathogen such as *Mycobacterium tuberculosis* grows and persists within macrophage. They apparently use cell wall glycolipids to scavenge toxic oxygen compounds. These glycolipids remove hydroxyl radicals and superoxide anions, the most lethal toxic oxygen species produced by macrophages. In addition, some pathogens evade killing by macrophages through inhibition of phagolysosome formation.

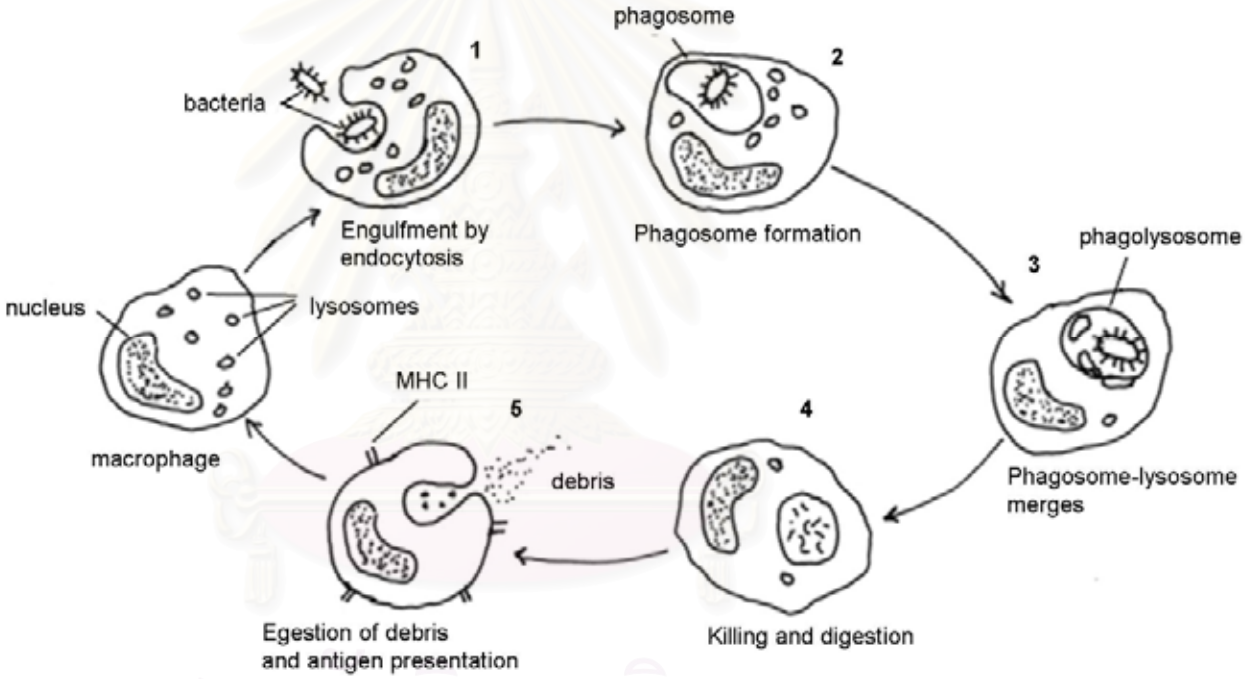


Figure 2.5 Phases of phagocytosis. Step 1 and 2 microbe or antigen is phagocytosed by macrophage into the phagosome. Step 3, the phagosome containing antigen is fused with lysosome containing digestive enzymes to form phagolysosome. Step4, the antigen inside the phagolysosome is digested. Then, step 5, the digestible materials and residual bodies are discharged from the macrophage and presented to T helper cells.

(www.textbookofbacteriology.net/innate.html)

2.2.1.2 Antigen presentation (Madigan *et al*., 2003)

Macrophages take up and degrade (process) antigen (Ag). Processed peptide Ag then becomes embedded, or bound, to the MHC protein, and complex is passed through the cytoplasmic membrane and presented on the cell surface.

Two distinct Ag-processing schemes are known, one for class I scheme, Ags that are manufactured by host degradation reactions in non-phagocytic cells are bound by class I protein in the endoplasmic reticulum. The class I protein is found in all nucleated cells. The actual processed peptide is about 10 amino acid long. This method of Ag processing is very important in virus infections, where the host cell manufactures and degrades viral proteins. The complex of processed protein and MHC class I move to the cell surface where it is presented to peptide-specific T cells through the T cell receptors (TCR). Next, the TCR on the surface of the T cell interacts with both Ag (non-self) and MHC (self) sites. This cell-cell interaction induces specialized T-cytotoxic cell to produce cytotoxic proteins that kill the virus-infected target cell. (Figure 2.6 A)

A second Ag presentation scheme involves the class II molecule. The class II protein is found in B cells, macrophages and dendritic cells. In this case, class II proteins, complete with a self peptide called li, or invariant chain, line the cell vacuoles (lysosomes) that degrade antigen phagocytosed by APCs. The phagosome containing the foreign Ag fuses with the lysosomes forming a phagolysosome, and the Ags are digested by proteolytic enzymes along with the li. The foreign peptides, generally about 11 to 15 amino acids in length, are then bound by the newly opened class II Ag-binding site, and the whole complex is eventually expressed on the external cytoplasmic membrane where it is presented to specialized T-helper, or T_H cells. The T_H cell is activated by contact with foreign Ag and

secretes molecules that either stimulate Ab production or secrete a battery of inflammatory cytokines. (Figure 2.6 B)

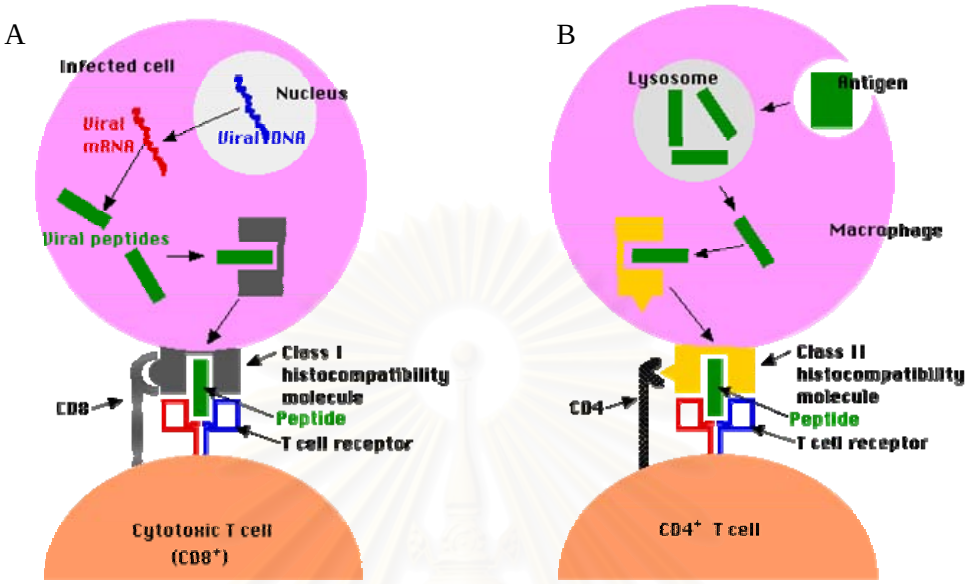


Figure 2.6 Antigen presentation process. (A) and (B) are antigen presentation to cytotoxic T cell and helper T cell, respectively. (users.rcn.com/.../A/AntigenPresentation.html)

2.2.2 Macrophage recognition and activation

2.2.2.1 Toll-like receptors (TLRs) (Pasare and Medzhitov, 2003 and Franchini *et al*., 2005)

The innate immune system is capable of recognition conserved microbial structures or products of microbial metabolism (Pathogen-associated molecular patterns, PAMPs) such as lipid, carbohydrate, peptide and nucleic acid structures. PAMPs are sensed by pattern recognition receptors (PRR) expressed by a variety of cells, particularly those in innate immune system such as macrophages. The families of TLRs are responsible for initiating acute inflammatory responses against invading pathogen by induction of antimicrobial genes and inflammatory cytokines (IL-1β, IL-6, TNF, etc.). TLR signaling leads

to phagocyte activation and direct killing of the infectious agents. Mammalian TLRs are a family of at lease 12 membrane proteins that trigger innate immune response through nuclear factor-κB (NF-κB)-dependent and interferon (IFN)-regulatory factor (IRF)-dependent signaling pathway (Figure 2.7 and 2.8).

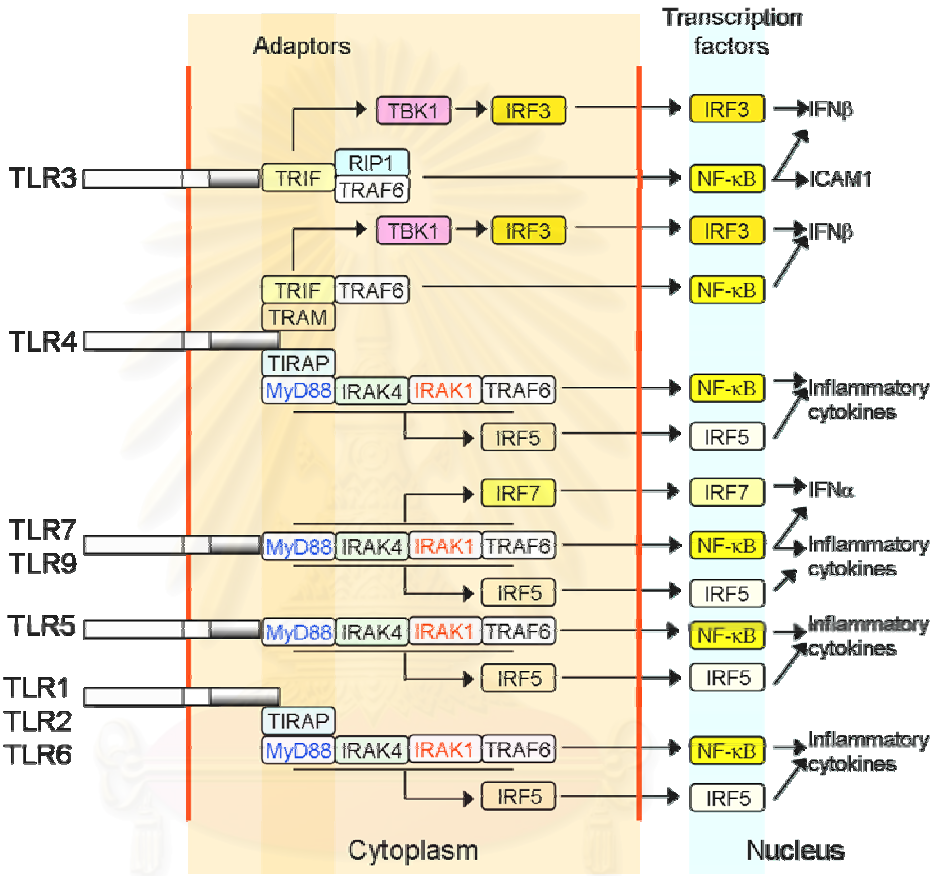


Figure 2.7 Schematic representation of TLR-signaling pathways. All TLRs except for TLR3 share the MyD88-dependent pathway that activates NF-κB and subsequently induces genes encoding inflammatory cytokines (After Kawai and Akira, 2005).

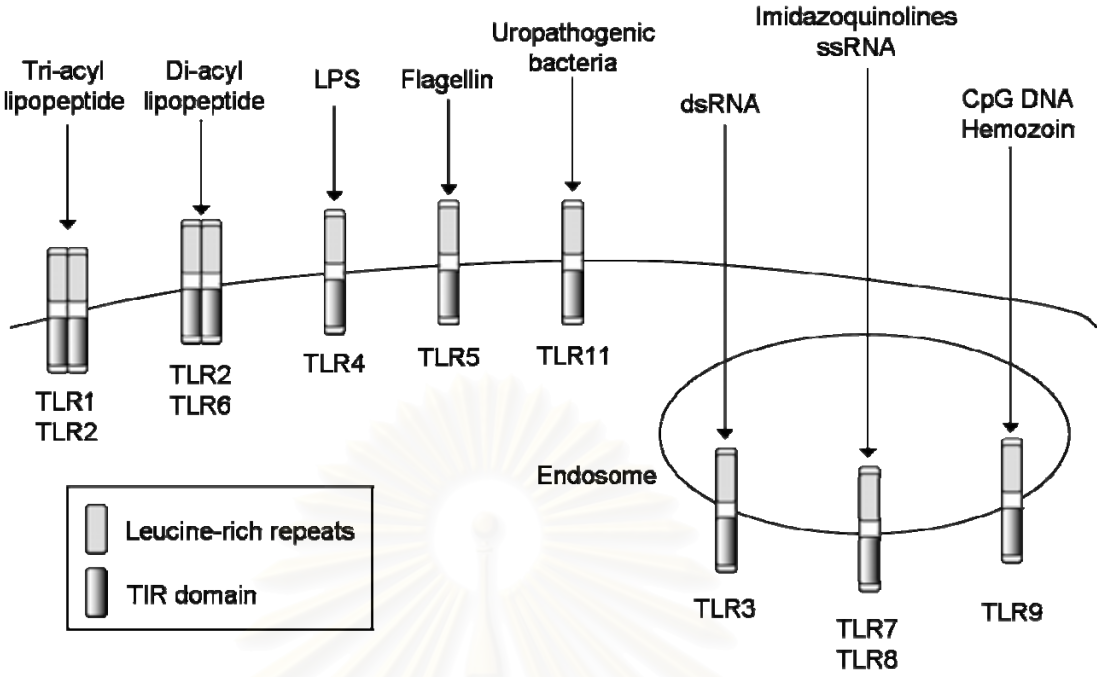


Figure 2.8 Structures and ligands for Toll-like receptors. TLR2, in collaboration with TLR1 or TLR6, discriminates between the molecular structures of triacyl and diacyl lipopeptides, respectively. TLR4 recognizes bacterial LPS. TLR5 recognizes bacterial flagellin. TLR11 recognizes uropathigenic bacteria products. TLR3, TLR7, TLR8 and TLR9 reside in endosomal compartments and recognize nucleic acids; TLR3 recognizes viral dsRNA, whereas TLR7 and TLR8 recognize viral ssRNA. TLR9 recognizes bacterial and viral CpG DNA motifs. TLR9 also recognizes non-nucleic acids, such as hemozoin. (After Kawai and Akira, 2005)

2.2.2.2 Lipopolysaccharide (LPS) (Fujihara *et al*., 2003)

Bacterial endotoxin (lipopolysaccharide, LPS) is a complex glycolipid composed of a hydrophilic polysaccharide moiety and a hydrophobic domain known as lipid A (Figure 2.9). LPS is a major component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation. LPS activates monocytes and macrophages to produce proinflammatory cytokines such as tumor necrosis factor-α (TNFα),

IL-1, IL-6, IL-8 and IL-12. In response to LPS, macrophages also secrete a wide variety of other biological response mediators including platelet-activating factor, prostaglandins, enzymes, and free radicals, such as nitric oxide. Production of these inflammatory cytokines and mediators by monocytes/macrophages contributes to the efficient control of growth and dissemination of invading pathogens. However, excessive and uncontrolled production of these inflammatory cytokines and mediators may lead to serious systemic complications such as microcirculatory dysfunction, tissue damage and septic shock with a high mortality.

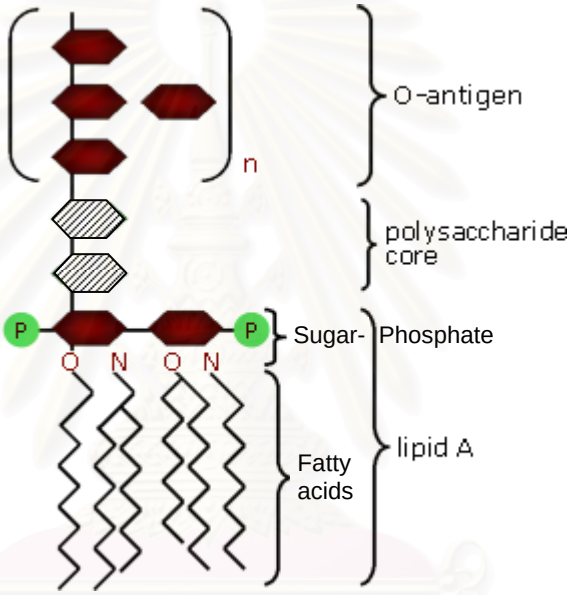


Figure 2.9 A complex structure of LPS. LPS is composed of a highly variable polysaccharide region (O-Antigen), a less variable oligosaccharide region (core), and a relatively conserved lipid region (Lipid A). (www.spectraldx.com/endotoxinmarker.htm)

The LPS receptor complex is composed of 3 proteins, CD14, TLR4 and myeloid differentiation protein-2 (MD-2). CD14 membrane-associated protein expressed at the surface of cells, especially macrophages. The LPS first binds to a serum LPS binding protein (LBP), which in turn transfers the LPS monomer from the bacterial cell wall

to membrane-bound CD14 on macrophage, subsequently causing LPS responses through TLR4 pathway (Figure 2.10).

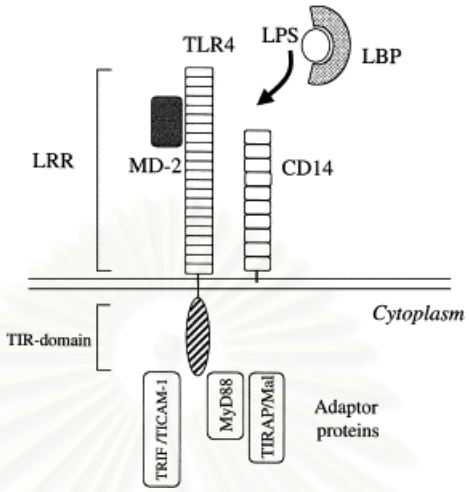


Figure 2.10 The LPS receptor complex on macrophages. LPS is recognized by a complex of three proteins: CD14, TLR4, and MD-2. A serum LBP transfers LPS to CD14. CD14 concentrates LPS and presents it to TLR4-MD-2. MD-2 plays a role in LPS recognition and regulates the cellular distribution of TLR4. TLR4 also plays a role in LPS recognition and functions as the signal-transducing receptor for LPS. Three adaptor proteins including MyD88, TIRAP (also known as Mal), and TRIF (also known as TICAM-1) are responsible for the TLR4-mediated signaling. (Fujihara *et al*., 2003)

RAW264.7 macrophage cell line treated with 1 μg/ml of LPS, increased in cell size and acquired distinct dendritic morphology. In flow cytometric studies, the cell surface markers known to be expressed on dendritic cells and involved in antigen presentation and T cell activation (B7.1, B7.2, CD40, MHC class II antigen and CD1d) were also markedly upregulated upon LPS treatment in macrophages. (Saxena *et al*., 2003)

Actinobacillus actinomycetemcomitans, a gram-negative oral bacterium, plays a crucial role in the development of periodontal disease. LPS from *A.*

actinomycetemcomitans could stimulate arginase activity in RAW264.7, but was less potent than LPS from *E. coli*. (Sosroseno *et al*., 2006).

LPS could also induce expression of IL-10, an anti-inflammatory cytokine, in macrophages. IL-10 biosynthesis depends not only on the activation of transcription factors, such as Sp1 (Brightbill *et al*., 2000), c-Maf (Cao *et al*., 2005) and NF-κB1 (p50) (Cao *et al*., 2006) but also on covalent modifications to the histones associated with the IL-10 promoter. These modifications render the IL-10 promoter accessible to the transcription factors that bind there (Zhang *et al*., 2006).

2.2.2.3 Interferon-γ (IFNγ) (Schroder *et al*., 2004)

IFNγ is the type II interferon. Initially, it was believed that T helper cells, cytotoxic lymphocytes and NK cells exclusively produce IFNγ. However, other cells, such as B cells, NKT cells and professional APCs secrete IFNγ. IFNγ production by monocytes/macrophage and dendritic cell acting locally may be important in cell self-activation and activation of nearby cells. IFNγ secretion by NK cells and professional APCs is important in early host defense against infection, whereas T cells become the major source of IFNγ in the adaptive immune response.

IFNγ primary signals through the Jak-Stat (Janus kinases (Jaks) and Signal transducers and activators of transcription (Stat)) pathway, a pathway used by over 50 cytokines, growth factors and hormone to affect gene regulation. Jak-Stat signaling involves sequential receptor recruitment and activation of members of the Jaks and the Stats to control transcription of target gene via specific response elements.

Kota *et al.* (2006) analyzed the gene expression profiles by micro array in RAW 264.7 macrophage cell line stimulated with IFNγ for 4 h. They found that IFNγ can induce gene involved in antimicrobial and antiviral responses, antigen presentation, chemokine and cytokine signaling, and inhibition of cell growth. IFNγ could also suppress cell cycle control, DNA repair and lipid metabolism.

IFNγ influences LPS dependent signaling capabilities by promoting ligands-receptor interactions as well as downstream signaling machinery. IFNγ augmented mRNA and surface expression of TLR4 and LPS-binding ability in macrophages. Furthermore, IFNγ counteracted the LPS-induced downregulation of TLR4. Expression of the accessory component MD-2 and of the adaptor protein MyD88 was also increased. IFNγ-primed monocytes showed increased responsiveness to LPS in term of phosphorylation of the IL-1 receptor-associated kinase (IRAK; a downstream of the MyD88), NF-κB DNA binding activity, TNFα and IL-12 production. (Bosisio *et al*., 2002 and Schroder *et al*., 2004)

2.2.3 Three functionally different populations of macrophages

Environmental factors (including cytokines, chemokines, hormones, TLR agonists) differentially regulate macrophage response patterns such that macrophages resident in different tissues display different patterns of functions (Stout *et al*., 2004). Recently, activated macrophages are divided into 3 groups of population. Classically activated macrophages (Ca-Mϕ) are primed with IFNγ and stimulated with LPS. Type II activated macrophages (Mϕ-II) are similarly primed but stimulated with LPS plus immune complexes. Alternatively activated macrophages (AA-Mϕ) are stimulated with IL-4 (Edwards *et al*., 2006) (Table 2.1).

In Ca-Mϕ activation, exposure of macrophages to IFNγ primes the cells to respond to further stimulation by TNFα or an inducer of TNFα, frequently LPS or other bacterial

products. Ca-Mϕ secrete various cytokines and chemokines including TNFα, IL-12, IL-6 and CCL2; they upregulate expression of MHC class II and produce NO and O_2^-. These cells are particularly important for killing and degrading intracellular pathogens (Scotton *et al.* (2005), Mantovani *et al*. (2005) and Edwards *et al*. (2006)).

AA-Mϕ arise in the immune response to the Th2 cytokines IL-4/or IL-13. These cells are functionally and biochemically distinct from Ca-Mϕ. They fail to produce NO, but they upregulate mannose receptor expression and enhance the captivity for endocytosis of macrophages (Goerdt *et al*., 1999). One distinct feature of AA-Mϕ is the expression of *Arginase1* (*Arg1*), which can shift arginine utilization from the production of NO by *iNos* in Ca-Mϕ. This shift in arginine metabolism contributes to wound healing by synthesis of matrix compound (Zhang *et al*., 2007). These cells are associated with parasitic diseases and the production of the extracellular matrix (ECM) (Edwards *et al*., 2006).

In murine bone marrow macrophages, *Arg1* expression and arginase activity were upregulated upon Th2 stimulation (stimulated by IL-4 or IL-10), whereas *Arg2* was never detected (Munder *et al*., 1999).

Peritoneal macrophages stimulated with IL-4 plus IL-13 produced Arg1, which decreased the expression of the TCR CD3ζ chain and impair T cell responses (Rodriguez *et al*., 2004).

The third population of activated macrophages is Mϕ-II which are generated by activating macrophages by FcγR ligation using immune complexes (IC) followed by stimulation of TLR, CD40 or CD44 (Scotton *et al*., 2005). These cells produce many of the cytokines seen in Ca-Mϕ (e.g., TNFα and IL-6), but they switch off IL-12 production and secrete large number of IL-10. The IL-10 secreted by these cells made them potent, anti-inflammatory cells. When these macrophages present Ag to naïve T cells, they stimulated the

production of Th2-liked T cells, which produced high levels of IL-4 (Anderson and Mosser, 2002).Thus, these cells can contribute to resolution of inflammation because they produce a high levels of IL-10, they are highly phagocytic and can remove cellular debris, and they can present Ag to T cells and induce them to produce IL-4 (Zhang *et al*., 2007). These cells may play a role in visceral leishmaniasis.

The region responsive to IL-12 inhibition following FcγR ligation in Mϕ-II lies in the inhibition of binding of a PU.1-containing complex to the Ets site of the IL-12 promoter (Grazia Cappiello *et al*., 2000).

When macrophages encounter IC, the histones are phosphorylated in response to MAPK activation. However, this phosphorylation does not result in *IL-10* expression. Macrophages exposed to IC alone produce no IL-10, despite the activation of ERK and the phosphorylation of histones. Rather, transcription of IL-10 gene requires a second signal provided by TLR stimulation. This stimulation activates the necessary transcription factors, allowing them to bind to the phosphorylated chromatin (Zhang *et al*., 2006).

Table 2.1 Comparison among activated macrophage populations (Edwards *et al.*, 2006). Phenotypes, biochemical markers and functions of each activated macrophages are different as shown in the table.

	Ca-Mϕ	AA-Mϕ	Mϕ-II
Stimuli	IFNγ and LPS	IL-4 and/or IL-13	IFNγ, LPS and IC
Phenotypes	IL-12^{high}, IL-10^{low}	IL-12^{low}, IL-10^{low}	IL-12^{low}, IL-10^{high}
Reactive mediators	NO, reactive oxygen species (iNos)	Arginase	NO, reactive oxygen species (iNos)
Biochemical markers	*IL-12p40*	*FIZZ1*, *YM1/2*, mannose receptor	*SPHK1*
Functions	1. kill microorganisms and tumor cells 2. produce proinflammatory cytokines 3. amplify Th1 immune responses 4. APC	1. provide immunity during helminth infections 2. production of ECM	1. Th2-like T cell stimulation 2. effective APC 3. support T cell proliferation

Edwards and his colleagues (2006) found that *SPHK1* was shown to be upregulated specifically in Mϕ-II, relative to Ca-Mϕ. Thus, high *SPHK1* expression may be useful to identify Mϕ-II. *SPHK1* encodes sphingosine kinases which catalyze the production of sphingosine-1 phosphate from sphingosine. SPHK1, an isoform of mice SPHK, has been shown to be necessary for C5a-triggered, intracellular Ca^{2+} signals (Melendez and Ibrahim, 2004). SPHK1 activation can also protect LPS-activated macrophages from apoptosis (Wu *et al.*, 2004).

FIZZI (found in inflammatory zone 1) is a secreted protein that has been associated with allergic and pulmonary inflammation. Moreover, FIZZ1 may have a role in respiratory epithelial cell maintenance and response to injury (Holcomb *et al*., 2000).

Chitin is a common element in organisms including parasites, fungi and bacteria, but does not occur in mammalian tissues, allowing for selective antimicrobial activity of chitinase. Ym1 and Ym2 synthesized by activated macrophages are homologous to chitinase and have chitinase activity (Song *et al*., 2007). Ym1 was originally described as an eosinophil chemotactic factor produced by CD8 lymphocytes. Ym1 produces large crystals in the lungs of mice with chronic lung pathology. One possibility is that Ym1 is an effector molecule which involves in encapsulation of chitin-bearing pathogens such as yeast, fungi or nematodes. Alternatively, it may function to interact with extracellular matrix components consistent with a role for Th2 driven macrophages in wound healing. (Nair *et al*., 2003)

Raes and colleagues (2002) opened perspectives for new insights into the functional properties of AA-Mϕ and establish FIZZ1 and Ym1 as markers for AA-Mϕ. They used suppression subtractive hybridization to identify genes that are expressed differentially in AA-Mϕ versus Ca-Mϕ elicited during infection with this *Trypanosoma brucei brucei* variant. They showed that *FIZZ1* and *Ym1* are induced strongly in *in vivo*- and *in vitro*-elicited AA-Mϕ as compared with Ca-Mϕ. The *in vivo* induction of *FIZZ1* and *Ym1* in macrophages depends on IL-4 and that *in vitro*, IFNγ antagonizes the effect of IL-4 on the expression of *FIZZ1* and *Ym1*.

In macrophages, L-arginine can be metabolized by three different pathways that result in the production of : (i) L-citrulline and nitric oxide by inducible nitric oxide synthase (iNos); (ii) ureum and L-ornithine by arginase; and/or (iii) agmatine by arginine decarboxylase (ADC) (Figure 2.11). The cross regulation of the iNos-arginase balance by Th1 mediators, such as IFNγ and LPS, and by Th2 cytokines, such as IL-4 and IL-13, suggest that

the measure of NO level and arginase activity in distinct macrophage populations reflects their activation state, Ca-Mϕ and AA-Mϕ (Ginderachter *et al*., 2006).

In addition, intermediates of the L-arginine metabolism affect the activity of iNOS or arginase. For instance, agmatine is an inhibitor of iNOS. However, it is not known whether the production of agmatine, which is induced by IL-4 and blocked by LPS, influences the function and state of activation of macrophages. Similarly, L-hydroxy-arginine (LOHA), an intermediate of NO production, is an inhibitor of arginase activity (Figure 2.11).

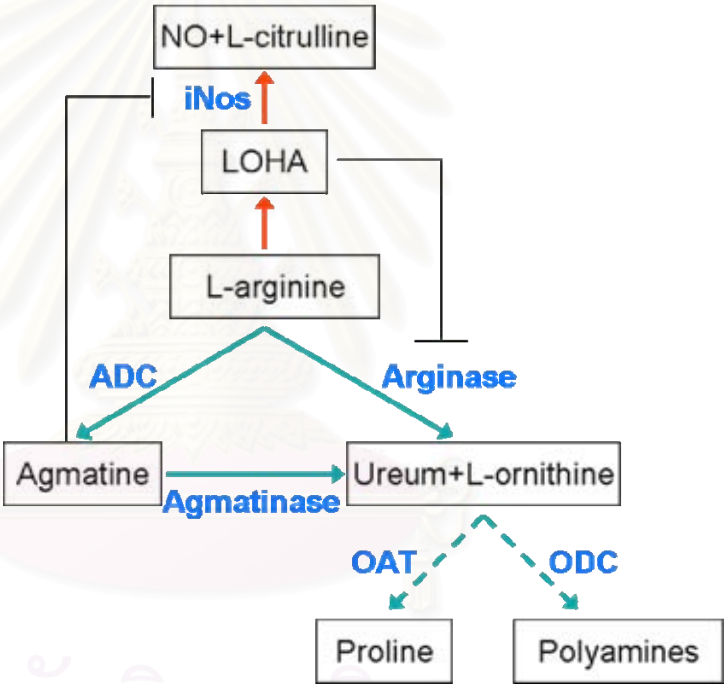


Figure 2.11 L-arginine metabolism in macrophages. Key: broken arrows, hypothetical pathway; green arrows, pathway induced by IL-4 and/or IL-13; red arrows, pathway induced by IFNγ and/or TNFα. Abbreviations: LOHA, L-hydroxy-arginine; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase (After Noel *et al*., 2004).

2.2.4 Tumor-associated macrophages (TAMs)

Macrophages within the tumor microenvironment facilitate angiogenesis and extracellular matrix breakdown and remodeling and promote tumor cell motility. They are named tumor-associated macrophages (TAMs) (Condeelis and Pollard, 2006). TAMs isolated from various murine tumors and from human ovarian cancers express low levels of inflammatory cytokine receptors. TAMs have little cytotoxicity for tumor cells and they actually promote tumor-cell proliferation. They are also poor producers of NO which are resulted from accumulation of the NF-κB inhibitory p50 homodimer. TAMs express high levels of the mannose receptor and are poor at Ag presentation. TAMs also express IL-10^{high} and IL-12^{low} phenotypes (Mantovani *et al*., 2002). Moreover, tumor cells can invade and egress into the blood vessel (intravasation) resulted from direct communication between macrophages and tumor cells (Condeelis and Pollard, 2006). Thus, macrophages are an important drug target for cancer therapy.

2.3 Notch and Macrophages

In monocytes, B and T cells, high amount of Notch1 and Notch2 were detected. Immobilized truncated form of Delta1 induced apoptosis in monocytes in the presence of M-CSF but not GM-CSF. Thus, Notch protein has multiple functions during hematopoiesis (Ohishi *et al*., 2000).

Macrophage precursors expressed high levels of *Notch1* as analyzed by RT-PCR, while maturing macrophages expressed high levels of both Notch1 and Notch4. Moreover, *Jagged1* transcript was detected at a high level in terminally maturing cells including mast cells and megakaryocytes (Singh *et al*., 2000).

Bone marrow macrophages constitutively produced Jagged1 and upregulated Jagged1 in the presence of cytokines, M-CSF and IL-3. Thus, macrophages in the hematopoietic microenvironment play a role in hematopoiesis through production of Jagged1 (Nomaguchi *et al*., 2001).

The expression pattern of three Notch ligands in macrophages and dendritic cells was investigated by immunofluorescence cell signaling method and RT-PCR. It was found that three Notch ligands, Jagged1, Jagged2 and Delta1 were expressed in macrophages and dendritic cells (Yamaguchi *et al*., 2002).

In 2002, Weijzen and colleagues found that Jagged1 may be associated with maturation of human dendritic cells. Co-culture of dendritic cells (DC) with cells expressing Jagged1 induced DC maturation marker, IL-12 production, T cell proliferative responses and IFNγ production.

Delta1 and Jagged1 have differential effects on myeloid bipotent and unipotent progenitors and differentially regulate the development of granulocytic and monocytic cell linages. Jagged1 increased the number of bipotent macrophages and unipotent progenitors, whereas Delta1 reduced the number of bipotent macrophages and differentiated monocytic cells (Neves *et al*., 2006).

Monslave and colleagues (2006) determined Notch receptors expression during macrophage activation. They reported that untreated macrophages express Notch1, Notch2, Notch4 as well as their ligands Jagged1 and Jagged2. Upon LPS and/or IFNγ stimulation, Notch1 and Jagged1 were increased. ICN1 overexpressing macrophages and subsequent signaling following macrophage activation modulated gene expression patterns known to affect the function of mature macrophages, such as increase in IRF1 and decrease in NO production.

Expression of Delta4 is increased in macrophage exposed to proinflammatory stimuli such as LPS and IL-1β. Soluble Delta4 bound to human macrophages. Co-incubation of macrophages with Delta4 expressing cells triggered Notch activation; increased the transcription of *iNos* and other proinflammatory genes. Notch3 selectively increased during macrophage differentiation. Notch3 knockdown during macrophage differentiation decreased the inflammation genes such as *iNos* (Fung *et al*., 2007).

TLR agonists similarly triggered upregulation of Notch1 in bone marrow macrophages and macrophage-like cell line RAW 264.7 through NF-κB activation which resulted in proinflammatory responses (Palaga *et al*., 2008).

Although, Notch signaling has been reported to be expressed and involved in regulating macrophage functions. However, regulation of three populations of activated macrophage remains unclear. In this study, the role of Notch signaling to regulate functions of activated macrophages was investigated. This study will open a new insight between Notch signaling and macrophages, and a new way to manipulate diseases associated with macrophages.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

1.	-20˚C Freezer model MDF-U332	Sanyo, Japan
2.	37˚C Incubator	Memmert, Germany
3.	4˚C Refrigerator	Mitsubishi Electric, Japan
4.	5% CO_2 Incubator model 311	Thermo Electron Corperation, USA
5.	500 ml Bottle Top Filter w/33 mm Neck 0.2 μm	Hycon, Germany
6.	-70˚C Deep Freezer model ULT1780	Forma Scientific, USA
7.	Autoclave model MLS 3020	Sanyo, Japan
8.	Balance	Metler Toledo, Switzerland
9.	Bench-Top Centrifuge model Stratagene	Profuge, USA
10.	Centrifuge : Rotafix 32	Hettich, Germany
11.	Centrifuge Tube 15 and 50 ml	Corning Incorperation, USA
12.	Cryotube	Corning Incorperation, USA
13.	Cuvette 0.4 cm	BioRad, USA
14.	DNA Thermal Cycle : 2400	Perkin-Elmer, USA
15.	Fluorescence Microscope	Nikon, Japan
16.	Gel Documentation and Quantity One 4.4.1	BioRad, USA
17.	Haemocytometer	Boeco, Germany
18.	Heat Block : Thermomixer Compact	Eppendorf, Germany
19.	High Performance Chemilumunescence Film : Amersham Hyperfilm™ ECL	Amersham Biosciences, England

20.	Hot Air Oven model D06063	Memmert, Germany
21.	Inverted Microscope	Olympus, USA
22.	Laminar Flow Cabinet model H1	Lab Survice LTD part, Thailand
23.	Liquid Nitrogen Tank 34 HC Taylor Wharton Cryogenic	Harsco Corperation, USA
24.	Magnetic Stirrer	Clifton, USA
25.	Microcentrifuge Tube 1.5 ml	Axygen Scientific, USA
26.	Micropipette P2, P20, P100 and P1000	Gilson, France
27.	Microplate Reader : Elx 800	Bio-Tek instrument, Canada
28.	Mini Gel Electrophoresis Unit for DNA,RNA and Proteins Mupid-2 Advance	Cosmo Bio, Japan
29.	PCR Tube 200 µl	Corning Incorperation, USA
30.	Petri Dish	Hycon, Germany
31.	pH-meter model S20-K	Metler Toledo, Switzerland
32.	Pipette Aid	Drummond, USA
33.	Polyvinylidine Fluoride (PVDF) membrane	GE Healthcare, USA
34.	Power Supply	BioRad, USA
35.	Protein III System for SDS-PAGE	BioRad, USA
36.	Refrigerated Centrifuge model 1920	Kubota, Japan
37.	RNase-free Tip 2, 10, 100 and 1000 µl	Corning Incorperation, USA
38.	Semi-dry Electrophoretic Transfer Cell : Trans-Blot® SD	BioRad, USA
39.	Spectrophotometer model Lambda 25	Perkin-Elmer, USA
40.	Syringe 1 ml	Nipro, Japan
41.	Syringe Fliter 0.20 µm	Corning Incorperation, USA
42.	Tissue Culture Plate 12, 24 and 96 well	Nunc™, Denmark
43.	Ultra-Pure Water Purification System	Elga, England

44.	USB High-Definition CCD Camera : Actcam-2000SH	Artray, Japan
45.	Vortex mixer model G560E	Scientific Industries, USA
46.	Water bath	Memmert, Germany

3.1.2 Chemicals, Antibodies and Kits

1.	β-mercapto-ethanol	Sigma Aldrich, USA
2.	100 bp DNA Ladder	Fermentas, Canada
3.	100 mM dNTP Mix	Fermentas, Canada
4.	Absolute ethanol	Merck, Germany
5.	Absolute methanol	Merck, Germany
6.	Acrylamide and Bis-acrylamide solution	BioRad, USA
7.	Agar	
8.	Agarose Gel	Research Organics, USA
9.	Ammonium persulfate	Bio Basic Inc., Canada
10.	Ampicillin	Bio Basic Inc., Canada
11.	Antibiotic G418 Sulfate	Bio Basic Inc., Canada
12.	Rabbit Anti-SRBC IgG	Obtained from Dr.Cynthia Baldwin (U. of Massachusetts at Amherst, USA)
13.	Anti-Mouse I-Ab MHC biotin conjugate	Caltag Laboratories, USA
14.	Bactotryptone	Becton, Dickinson and company, France
15.	BCA (bicinchoninic acid)^{TM} protein assay	PIERCE, USA
16.	Bovine Serum Albumin (BSA)	Sigma Aldrich, USA
17.	Bromphenol blue	Sigma Aldrich, USA

18.	Chloroform	Lab-Scan, Ireland
19.	DEPC (diethylpyrocarbonate)	Sigma Aldrich, USA
20.	Dimethylsulfoxide (DMSO)	Sigma Aldrich, USA
21.	Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany
22.	Dithiothreitol (DTT)	USB Corperation, USA
23.	Donkey anti-rabbit IgG-HRP	GE Healthcare, USA
24.	EDTA (ethylenediaminetetraacetic acid)	Merck, Germany
25.	*Escherichia coli* DH5α	Max Plank Institute of Infection Biology, Germany
26.	Ethidium Bromide	Sigma Aldrich, USA
27.	Fetal Bovine Serum (FBS)	Hyclone, England
28.	Film Developer and Fixer	J.nasen Co.,Ltd., Thailand
29.	FuGene6 Reagent	Roche, Germany
30.	Glycerol	Cario ERBA, France
31.	HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	Hyclone, England
32.	HPLC grade water	Merck, Germany
33.	Hydrochloric acid (HCl)	Merck, Germany
34.	Hydrogen peroxide	Merck, Germany
35.	IL-CHO	Gifted from Professor Todd E. Golde
36.	Isopropanol	Merck, Germany
37.	Lipopolysaccharide from *E. coli* serotype O26:B6	Sigma Aldrich, USA
38.	Luminol	Fluka
39.	M-MuLV Reverse Transcriptase	Fermentas, Canada
40.	Mouse anti-Actin antibody	Chemicon International, USA
41.	NED (N-1-naphthylethylenediamine	Sigma Aldrich, USA

	dihydrochloride)	
42.	Nonidet P-40	Bio Basic Inc., Canada
43.	Paraformaldehyde	Sigma Aldrich, USA
44.	p-coumaric acid	Sigma Aldrich, USA
45.	Penicillin	General Drugs House Co., Ltd., Thailand
46.	Plasmid pcDNA3	Gifted from Professor Barbara A. Osborne
47.	Plasmid pcDNA3 – ICN1	Gifted from Professor Barbara A. Osborne
48.	Potassium chloride (KCl)	Merck, Germany
49.	Potassium di-hidrogen phosphate (KH_2PO_4)	Merck, Germany
50.	Prestained molecular weight marker	Fermentas, Canada
51.	Protease Inhibitor Cocktail Tablets	Roche, Germany
52.	QIA prep spin miniprep and midiprep kit	Qiagen, Germany
53.	Rabbit anti-Cleaved Notch1 antibody	Cell Signal Technology, USA
54.	Rabbit anti-Notch1 antibody	Santa Cruz Biotechnology, USA
55.	Random Hexamer Primer	Fermentas, Canada
56.	Recombinant mouse interferon-γ	R&D systems, Inc., USA
57.	Recombinant mouse interleukin-4	R&D systems, Inc., USA
58.	RibolockTM Ribonuclease Inhibitor	Fermentas, Canada
59.	RPMI 1640	Hyclone, England
60.	SDS (Sodium dodecyl sulfate)	Amersham Biosciences, England
61.	Sheep anti-mouse IgG-HRP	Amersham Biosciences, England
62.	Sheep red blood cell	National Laboratory Animal Center, Mahidol University, Thailand
63.	Sodium azide	Merck, Germany

64.	Sodium chloride (NaCl)	Merck, Germany
65.	Sodium hydrogen carbonate ($NaHCO_3$)	Sigma Aldrich, USA
66.	Sodium nitrite	Carlo Erba
67.	Sodium pyruvate	Hyclone, England
68.	*Staphylococcus aureus* ATCC 25923	ATCC, USA
69.	Streptavidin PE	BioLegend, USA
70.	Streptomycin	M & H Manufacturing Co., Ltd., Thailand
71.	Sulfanilamide	BDH Chemicals Ltd., England
72.	Taq Polymerase	Fermentas, Canada
73.	TEMED (N, N, N', N'-Tetramethyl ethylenediamide)	Bio Basic Inc., Canada
74.	TNFα ELISA kit	eBioscience, England
75.	Trisma Base (tris [hydroxymethyl] aminomethane, $CH_4H_{11}NO_3$)	Sigma Aldrich, USA
76.	Triton X-100	Amersham Biosciences, England
77.	Trizol reagent	Invitrogen, England
78.	Trypan Blue 0.5% w/v	Biochrom AG, Germany
79.	Tween 20	Research Organics, USA
80.	Yeast Extract	Bio Springer, France

3.2 Oligonucleotide Primers

Table 3.1 Oligonucleotide primers used in experiments

Primer	Gene Bank Association Number	Sequence (5′→3′)	References
Murine Notch1 forward	AF508809	GTGAGGGTGATGTCAATG	Palaga *et al.*, 2008
Murine Notch1 reverse		TGAAGTTGAGGGAGCAGT	
Murine Notch2 forward	NM_010928	TGGAGGTAAATGAATGCCAGAGC	Palaga *et al.*, 2008
Murine Notch2 reverse		TGTAGCGATTGATGCCGTCC	
Murine Notch3 forward	NM_008716	ACACTGGGAGTTCTCTGT	Palaga *et al.*, 2008
Murine Notch3 reverse		GTCTGCTGGCATGGGATA	
Murine Notch4 forward	NM_010929	CACCTCCTGCCATAACACCTTG	Palaga *et al.*, 2008
Murine Notch4 reverse		ACACAGTCATCTGGGTTCATCATCT CAC	
Murine Hes1 forward	NM_008235	CCGGTCTACACCAGCAACAGT	Palaga *et al.*, 2008
Murine Hes1 reverse		CACATGGAGTCCGAAGTGAGC	
β-actin forward	NM_001101	ACCAACTGGGACGACATGGAGAA	Palaga *et al.*, 2008
β-actin reverse		GTGGTGGTGAAGCTGTAGCC	
TNFα forward	NM_013693	CCTGTAGCCCACGTCGTAGC	Lee *et al.*, 2007
TNFα reverse		TTGACCTCAGCGCTGAGTTG	
iNos forward	NM_010927	CCCTTCCGAAGTTTCTGGCAGCAGC	Lee *et al.*, 2007
iNos reverse		GGCTGTCAGAGCCTCGTGGCTTTGG	
IL-12p40 forward	S82420S3	AACCTCACCTGTGACACGCC	Tada *et al.*, 2000
IL-12p40 reverse		CAAGTCCATGTTTCTTTGCACC	

Primer	Gene Bank Association Number	Sequence (5′→3′)	References
IL-10 forward	NM_010548	TCAAACAAAGGACCAGCTGGACAA CATACTGC	Palaga *et al.*, 2008
IL-10 reverse		CTGTCTAGGTCCTGGAGTCCAGCAG ACTCAA	
Arg1 forward	NM_007482	CAGAAGAATGGAAGAGTCAG	Edwards *et al.*, 2006
Arg1 reverse		CAGATATGCAGGGAGTCACC	
SPHK1 forward	NM_011451	ACAGCAGTGTGCAGTTGATGA	Edwards *et al.*, 2006
SPHK1 reverse		TGTCGTCACACGTCAACTACT	

3.3 Antibodies for Western blot

Table 3.2 Antibodies used in Western blot

Antigen	Working Dilution of Primary Antibodies	Working Dilution of Secondary Antibodies
Notch1	Rabbit anti-Notch1 1 : 1000	Donkey anti-rabbit IgG-HRP 1 : 4000
Actin	Mouse anti-Actin 1 : 5000	Sheep anti-mouse IgG-HRP 1 : 5000

3.4 Cell line and media

Macrophage-like cell line RAW 264.7 (ATCC TIB-71) was obtained from American Type Culture Collection (USA) and maintained in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 0.4 mg/ml streptomycin, 1% sodium pyruvate and 1% HEPES at 37˚C in humidified 5% CO_2 incubator.

3.5 Cell culture and treatment

3.5.1 Cell culture

RAW 264.7 was detached from a tissue culture dish by repetitive pipetting in PBS. Single cell suspension was centrifuged at 1000 rpm for 5 min. After centrifugation, PBS layer was discarded and RPMI 1640 complete media was added. Cell viability was assessed by trypan blue dye exclusion method using a haemocytometer. The cell number was calculated according to the following formula.

Cell number (cell/ml) = number of counted cell in 16-large squares × 2 × 10^4

After calculation, cell was diluted to appropriate cell concentration and plated in tissue culture plates for further experiments.

3.5.2 Cell storage

Freeze media were prepared by adding 10% DMSO (v/v) to RPMI 1640 complete media. After collecting cells by centrifugation, cells were resuspended in 1 ml cold freeze media and stored in cryotubes. The frozen cells were immediately stored in -80˚C refrigerator overnight and moved for long term storage in liquid nitrogen the next day.

3.5.3 Thawed cell

Frozen cells in cryogenic vials in liquid nitrogen were thawed in 37˚C water bath. Cell suspensions were added to 9 ml serum-free media and centrifuged at 1000 rpm for 5 min. Freezing media in serum-free media was removed and RPMI 1640 complete media was added. Cells in complete media were plated in a tissue culture dish for experiments.

3.6 RNA extraction

RAW264.7 (1×10^6 cell/ml in 0.5 ml culture) was plated in 24-well tissue culture plate overnight. The next day cells were stimulated with 100 ng/ml LPS with or without 10 ng/ml IFNγ for indicated time. At indicated time, culture supernatant was removed and 1 ml of TriZol reagent was added for RNA extraction. Cells were incubated with TriZol reagent for 5 min at room temperature. The samples were transferred to microcentrifuge tubes and 0.2 ml of chloroform was added. The tubes were shook vigorously by hands for 15 sec and incubated at room temperature for 2-3 min. The samples were centrifuged at 12000xg for 15 min at 2-8˚C. Following centrifugation, a colorless upper aqueous phase was transferred to fresh tubes. RNA was precipitated from the phase by gently mixing with 0.5 ml of isopropanol. The sample was incubated at room temperature for 10 min and centrifuged at 12000xg for 10 min at 2-8˚C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellets were washed once with 1 ml of ice cold 75% ethanol in 0.01% DEPC water. The sample was mixed by vortexing and centrifuged at 7500xg for 5 min at 2-8˚C. RNA pellets were left for air dry for 5-10 min. RNA was dissolved in 20 µl of 0.01% DEPC water and incubated for 10 min at 60˚C. RNA samples were stored at –70˚C until further analysis.

3.6.1 Quantification of RNA via spectrophotometry

RNA was diluted to 100-fold dilution in 0.01% DEPC water. The diluted RNA was subjected to read at absorbance of 260 and 280 nm in spectrophotometer. An OD_{260} of 1.0 corresponds to a concentration of 40 µg/ml single stranded RNA (Ausubel *et al*., 1999). The concentration of RNA was calculated in µg/ml by using the following equation.

$$\text{RNA } (\mu g/ml) = OD_{260} \times 40 \times \text{dilution factor}$$

The purity of RNA was evaluated from a ratio of OD_{260}/ OD_{280}. The ratio of appropriately purified RNA was in the range of 1.8-2.0.

3.7 cDNA synthesis by reverse transcriptase

Obtained RNA 1 μg was used for converting to cDNA. RNA 1 μg was mixed with random hexamer (0.2 μg), and the final volume was adjusted by 0.01% DEPC water to 11 μl. The RNA mixture was incubated at 70°C for 5 min and 4°C for 5 min in DNA Thermal Cycle (Perkin-Elmer, USA). Then, 5×Reverse transcriptase buffer, 10 mM dNTP mix and RNase Inhibitor were added to the mixture at the final concentration 1×, 1 mM and 20 U, respectively. The final volume of the reaction mixture was adjusted to 19 μl by 0.01% DEPC water followed by incubation at room temperature for 5 min. Reverse transcriptase was finally added to final concentration 200 U, and the reaction was incubated at 25°C for 10 min, 42°C for 60 min, 70°C for 10 min and 4°C for infinity. The cDNA was stored for long period at -80°C refrigerator.

3.8 Polymerase chain reaction (PCR)

The obtained cDNAs were used as templates to amplify *Notch1-4*, *TNFα*, *iNos*, *IL-10*, *Arg1*, *IL-12p40*, *Hes1*and *β-actin*. The components of PCR are shown in Table 3.3.

Table 3.3 Components of PCR.

Component	Final concentration
10×Taq buffer	1×
10 mM dNTP mix	0.64 mM
25 mM $MgCl_2$	2 mM
10 mM forward primer	0.2 µM
10 mM revers primer	0.2 µM
Taq polymerase	25 U
HPLC water	To total volume 25 µl
cDNA	2.5 µl
Total volume	25 µl

β-actin was used as loading control. RT-PCR without reverse transcriptase was used as negative control, and mRNA from thymus was used as positive control.

The PCR condition is as follows:

Hot start	94˚C	5 min	
Denaturation	94˚C	1 min	number of cycle
Annealing	X˚C	1 min	
Extension	72˚C	1 min	
Final extension	72˚C	10 min	

Annealing temperatures, number of cycles and sizes of expected PCR product are shown in Table 3.4.

Table 3.4 Annealing temperatures, number of cycles and sizes of expected PCR product

Gene	Annealing temperature (˚C)	number of cycles	sizes of expected PCR product (bp)
Murine *Notch1*	55	30	660
Murine *Notch2*	55		522
Murine *Notch3*	55		467
Murine *Notch4*	60		239
Murine *Hes1*	60		89
β-actin	55	25	380
TNFα	55		374
iNos	60	30	497
IL-12p40	65		689
IL-10	58		421
Arg1	56		250
SPHK1	56		200

The PCR reaction was carried out using DNA thermal cycle (Perkin-Elmer, USA). The PCR products were analyzed on 2% agarose gel by electrophoresis, except those for *Hes1* which was analyzed on 5% polyacrylamide gel, and visualized after staining with ethidium bromide using Gel Documentation System.

3.9 Western blot

3.9.1 Protein extraction and quantification

RAW 264.7 (1.5×10^5 cell/ml in 0.5 ml culture) was plated on 24-well tissue culture plate overnight. Cells were stimulated with 100 ng/ml LPS and 10 ng/ml IFNγ for indicated time. At indicated time, proteins from stimulated cell were extracted as described by Palaga *et al*. (2003). In brief, culture supernatant was removed and cells were washed twice with 1 ml of PBS and 250 µl of Buffer A (Appendix A). Cells were lysed by adding 30 µl of Buffer B (Appendix A) right on the plates. After mixing by micropipette, cell lysates were transferred to microcentrifuge tubes and subjected to centrifuge at 5000 rpm for 5 min at room temperature. The supernatant was transferred to a fresh microcentrifuge tubes and stored at –80˚C until further analysis.

Amounts of protein were measured using BCA protein assay kit, according to manufacturer's instruction. The working reagent composed of reagent A: reagent B at the ratio of 50: 1 was prepared and BSA (1mg/ml) was used as a standard protein. BSA and samples were diluted in deionized water in 96-well microtiter plate. The concentrations of standard BSA were 0, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml. The samples were diluted to 1:10 (1 µl of sample in 9 µl of sterile deionized water) Two hundred µl of working reagent was added to each well, and the plates were incubated for 30 min at 37˚C. After incubation, absorbance at 540 nm (A_{540} nm) was monitored via microplate reader.

3.9.2 SDS-PAGE

SDS-polyacrylamide gel sized 0.15×8.4×8.4 cm^3 was prepared following formula described in Appendix A. Protein samples (30 µg) were mixed with 30 µl of 2×Laemmli buffer (Appendix A) and adjusted to 60 µl using sterile deionized water. The mixture of protein samples were heated at 100˚C for 5 min. Heated samples and prestained molecular weight markers were loaded onto the gels. Protein samples were separated at 100 volt for 90 min in Western blot running buffer (Appendix A) using Protein III system.

3.9.3 Protein blotting

After protein separation, the stacking gel was removed and size of the separating gel was measured. The gels were equilibrated in transfer buffer (Appendix A) for 5 min. During gel equilibration, 6 pieces of Whatman filter paper and a PVDF membrane were cut to the same size as the separating gels. Then, PVDF membrane was pre-wetted in absolute methanol and washed once with deionized water. PVDF membrane was immersed in transfer buffer for 5 min. Gels, PVDF membrane and filter papers were assembled in a semi-dry transfer apparatus and air bubbles were eliminated by gently rolling a glass test tube on the filter paper. After that, transfer buffer was dropped on the filter paper. The semi-dry transfer apparatus was carried out under the following condition, 60 mA for 90 min for one gel.

3.9.4 Antibody Probing

Total proteins were transferred from acrylamide gels onto PVDF membrane. The PVDF membranes were blocked twice in blocking solution (Appendix A) for 5 min each. After blocking, PVDF membranes were probed with 4 ml of specific primary antibody diluted in blocking solution (according to working dilutions shown in table 3.2) at 4˚C overnight.

After that, the probed membranes were further incubated on a rocker for 30 min at room temperature. After incubation, the primary antibody solution was discarded, and the membrane was washed with PBST (Appendix A) for 5 min twice and 15 min twice. After washing, PBST was discarded, and 4 ml of diluted secondary antibody conjugated with horse-radish peroxidase was added. PVDF membrane was incubated with the secondary antibody for 1 hr before washing with PBST for 5 min twice and 15 min twice.

3.9.5 Signal detection by chemiluminescence and autoradiography

The substrate of horse-radish peroxidase was prepared according to formula shown in Appendix A. Briefly, solution A was mixed with solution B and incubated with PVDF membranes for 1 min. Then, membranes were wrapped by a plastic wrap and placed on a X-ray film cassette in order to expose to x-ray film for indicated time in the dark room. Exposure time for Notch1 and β-actin (as loading control) was 5 min and 10 sec, respectively. Exposed film was developed in developer for 5 sec, washed with tap water, fixed with fixer for 4 min and finally washed with tap water.

3.10 Measurement of TNFα by ELISA

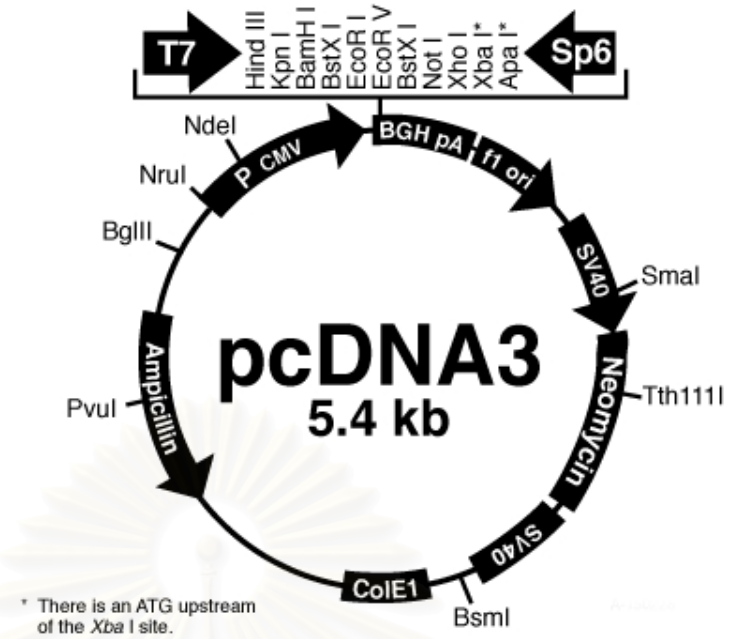
Samples for TNFα detection were obtained from culture supernatant of stimulated RAW 264.7 (1.5×10^5 cell/ml in 0.5 ml culture) for indicated times. TNFα ELISA was performed according to the manufacturer's instruction. Briefly, 96-well plate was coated with 100 µl of capture antibody diluted in 1×coating buffer. The plate was sealed and incubated overnight at 4˚C. After incubation, the wells were aspirated and washed 3 times with 200 µl/well of wash buffer. The plate was inverted and blotted on a thick tissue paper to remove residual buffer. The wells were blocked with 200µl/well of 1×assay diluent and incubated at room temperature for 1 hr. The wells were aspirated and washed as in previous step. Standard TNFα (100µl/well) was added to the appropriated wells in triplicate. Two-fold serial dilutions

of the top standards to make the standard curve were performed. The samples (100µl/well) were diluted 1 : 20 fold dilution and added in triplicate to the appropriate wells. The plate was sealed and incubated at room temperature for 2 hr. The wells were aspirated and washed 5 times as in previous step. Detection antibody diluted in 1×assay diluent (100µl/well) was added to the wells. The plate was sealed and incubated at room temperature for 1 hr. The wells were aspirated and washed 5 times as in previous step. Avidin-HRP diluted in 1×assay diluent (100 µl/well) was added. The plate was sealed and incubated at room temperature for 30 min. The wells were aspirated and washed 7 times. In this step, the wells were soaked in wash buffer for 1-2 min prior to aspiration. Next, 100µl/well of substrate solution was added to each well. The plate was incubated at room temperature for 15 min. Stop solution (1 M H_2SO_4) (50 µl) was added to each well. Then, the plate was read at 450 nm using microplate reader.

3.11 Overexpression of activated Notch1 (ICN1) in RAW 264.7 by transfection

Overexpression of ICN1 in RAW 264.7 was carried out by using pcDNA and pcDNA3 containing ICN1 at amino acid region 1759 to 2556. These two plasmids were provided by Professor Barbara Osborne, University of Massachusetts, USA. The plasmid constructions are shown in Figure 3.1. Stable transfection was used for overexpression by FuGene6 reagent. The methods of overexpression are described as following.

A



B

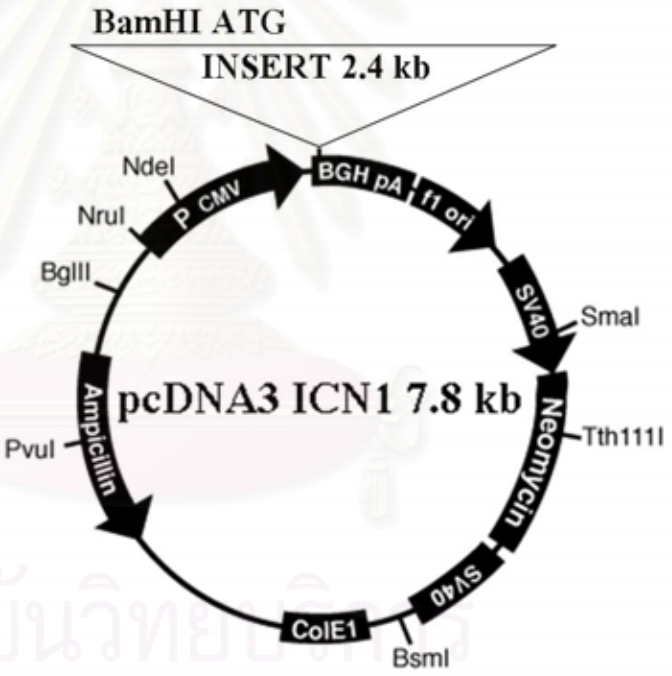


Figure 3.1 Restriction enzyme map of plasmids used in experiment

Figure 3.1 A. Restriction enzyme map of plasmid pcDNA3

Figure 3.1 B. Restriction enzyme map of plasmid pcDNA3 ICN1 plasmid containing ICN1 region between amino acid 1759 to 2556 downstream of Cytomegalo virus (CMV) promoter

3.11.1 Plasmid preparation

3.11.1.1 Transformation of plasmid into competent cell by heat-shock technique

Competent *E. coli* DH5α from -80˚C was thawed on ice. Next, 50 µl of the competent cell was added to the microcentrifuge tube containing 1 µl of plasmid. The reaction was incubated on ice for 30 min. Then, the reaction was heated shock in 42˚C water bath for 90 sec and 4˚C for 2 min. After incubation, all volume of competent cell and plasmid were transferred to 1 ml of LB broth (Appendix A) and incubated at room temperature for 1 hr in a 200 rpm-shaker for *E. coli* amplification. After that, bacterial culture (50 µl) was cultured on LB agar plate containing 50µg/ml ampicillin by spread plate. The plate was incubated at 37˚C for 24 hr. The formed colony was observed for next experiments.

3.11.1.2 Plasmid isolation

An *E. coli* colony was picked in order to culture overnight in 2 ml LB broth containing 50 µg/ml ampicillin at 37˚C. After that, the culture (1.5 ml) was sterilely poured to microcentrifuge tube, and cell was harvested by centrifugation at 3000 rpm for 30 sec. Then, the supernatant was discarded and the plasmid was extracted using QIA prep spin miniprep or midiprep kit according to manufacturer's instructions. The obtained plasmid was resuspended in HPLC water and stored at -20˚C.

3.11.1.3 Plasmid quantitative assay

Plasmid stored at -20˚C was diluted in deionized water at 50 fold dilution. Then, the diluted plasmid was examined by measuring the optical density at 260 and 280 nm. An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml plasmid DNA (Ausubel *et al.*,

1999). Therefore, the concentration of plasmid was calculated in µg/ml by using the following equation.

$$\text{Plasmid (µg/ml)} = OD_{260} \times 50 \times \text{dilution factor}$$

The purity of plasmid can be evaluated from a ratio of OD_{260}/ OD_{280}. The ratio of appropriately purified plasmid was 1.8-2.0.

3.11.2 Transfection using FuGene6 transfection reagent

RAW 264.7 (2×10^5 cell/ml in 0.5 ml culture) was plated overnight on 24-well plate. After that, FuGene6 reagent was directly added into serum-free RPMI 1640 without antibiotic at the ratio of serum-free media : FuGene6 reagent 3 : 1 (18.8 µl : 1.2 µl) in a clean microcentrifuge tube. Then, the tube was gently tapped and incubated at room temperature for 15 min. Next, 0.4 µg of plasmid solution (pcDNA ICN1 and pcDNA as an control empty vector) was added to the prediluted FuGene6 reagent. Then, the tube was gently tapped and incubated at room temperature for 15 min. After that, the complex mixture was distributively dropped to RAW 264.7. Then, the plate was swirled and incubated for 48 hr. After incubation, the culture supernatant was removed and replaced by 0.5 ml of complete RPMI 1640 containing 300 µg/ml G418 for transfected cell screening. The transfected cell can resist and grow in the selective media. Western blot was used to detect the efficacy of overexpression.

3.12 Generation of three types of activated macrophages

Alternatively activated macrophage (AA-Mϕ) was prepared by stimulating RAW 264.7 with 100 ng/ml recombinant IL-4 (rIL-4) for 4 hr. Moreover, classically activated macrophage (Ca-Mϕ) and type II activated macrophage (Mϕ-II) were prepared by priming

RAW 264.7 with 10 ng/ml IFNγ overnight. Ca-Mϕ was washed and stimulated with 100 ng/ml LPS for 4 hr. Mϕ-II received LPS along with immune complex consisting of erythrocyte opsonized with IgG (E-IgG) at ratio 1 : 10 (macrophage : E-IgG) for 4 hr.

3.13 Determination of nitric oxide by Griess reaction

RAW 264.7 (1×10^6 cell/ml in 0.2 ml culture) were plated on 96-well plate overnight. After that, cells were treated with 100 ng/ml LPS and 10 ng/ml IFNγ for 0, 6, 12 and 24 hr. Culture supernatant was collected for nitric oxide determination by Griess reaction. In brief, standard nitrite solution 100μM was prepared by diluting 0.1 M nitrite standard 1 : 1000 in the complete RPMI 1640. Nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0 μM) was generated by 6 serial 2-fold dilutions (50 μl/well) in triplicate down the 96-well plate. Experimental samples were added to the same plate in duplicate. Fifty μl of sulfanilamide solution was dispensed to all wells. Then, the plate was incubated for 5-10 min at room temperature in dark condition. Next, 50 μl of the NED solution was dispensed to all wells and incubated for 5-10 min at room temperature in dark condition. Finally, the plate was measured using microplate reader with a filter 540 nm.

3.14 Measurement of bactericidal activities by total plate count technique

RAW 264.7 (2×10^5 cell/ml in 1 ml of complete RPMI 1640 without antibiotic) were plated on 12-well plate overnight. Bacterial glycerol stock (*E. coli* or *S. aureus*) was thawed and diluted in PBS to final concentration 2×10^6 cell/0.1 ml. The diluted bacteria (100 μl) was added to macrophage culture at MOI 1 : 10 (macrophage : bacteria) for 1 hr. The remaining diluted bacteria was diluted to 10^{-5} and 10^{-6} subjected to spread plate in triplicate. After 1 hr of infection (T_0), the culture supernatant including diluted bacterial solution was discarded, and macrophage was washed twice with 1 ml of PBS. Then, macrophage was lysed using 100

μl of 0.1% Triton X-100 in cold PBS and incubated for 5 min. After that, intracellular bacteria were released from macrophage and the CFU of intracellular bacteria at T_0 was determined by plate count technique in triplicate. For T_{24} observation, after 1 hr of infection, 1 ml of complete media (containing antibiotic) was added and incubated for 24 hr. After that, macrophage was lysed as described above. Then, the CFU of intracellular bacteria at T_{24} was observed in triplicate.

3.15 Determination of MHC class II expression by flow cytometry

RAW 264.7 (2.5×10^5 cell/ml in 1 ml of complete RPMI) were cultured on 35 mm × 10 mm cell culture dishes overnight. These cells were then treated with 100 ng/ml LPS and 10 ng/ml IFNγ for 24 hr. After that, cells were harvested with 1 ml of cold 5 mM EDTA in PBS for 5 min, and scrapped gently. The harvested cells were then centrifuged at 3000 rpm for 5 min and resuspended in 200 μl of staining buffer (Appendix A). Next, 0.5 μg of biotin conjugated anti-Mouse I-Ab MHC was added into the cell solution and incubated on ice for 15 min. The cell solution was centrifuged at 3000 rpm for 5 min and resuspended in 200 μl of staining buffer. Next, 0.5 μg of Streptavidin PE was added to the cell solution and incubated on ice for 15 min in dark condition. Next, cells were washed twice by centrifugation at 3000 rpm for 5 min in staining buffer. Finally, cells were fixed with 4% paraformaldehyde 500 μl subjected to further analysis. The negative control are cells stained with Streptavidin PE alone and unstained cells.

3.16 Measurement of cell viability upon GSI treatment by crystal violet staining

RAW 264.7 (1×10^5 cell/ml in 100 μl culture) was plated in triplicate in 96-well plate overnight. After that, cell was pre-treated with 25 μM and 50 μM of IL-CHO (Gifted from Professor Todd E. Golde) 0.5 and 1% of DMSO as mock control for 1 hr. Then, cell was

stimulated with 100 ng/ml LPS and 10 ng/ml IFNγ for 12 and 24 hr. Next, the culture supernatant was removed. Eighty µl of 0.2% (w/v) crystal violet in 20% (v/v) methanol diluted in PBS was added and incubated for 15 min. Next, cell was washed with deionized water 3 times. The crystal violet was solubilized in 100 µl of 1% (w/v) SDS. Finally, the plate was read at OD 540 nm. The complete RPMI 1640 was used as a blank control. The percentage of cell viability was calculated according to the following formula.

$$\text{The percentage of cell viability} = \frac{\text{OD of treated cells} \times 100}{\text{OD of control untreated cells}}$$

3.17 Statistical analysis

To calculate the statistical differences between control and samples, Student's paired t-test was used. Values of $p<0.05$ were considered significant.

CHAPTER IV

RESULTS

4.1 Expression of *Notch1-4, Hes1* and genes encoding effector molecules in stimulated RAW 264.7 by RT-PCR

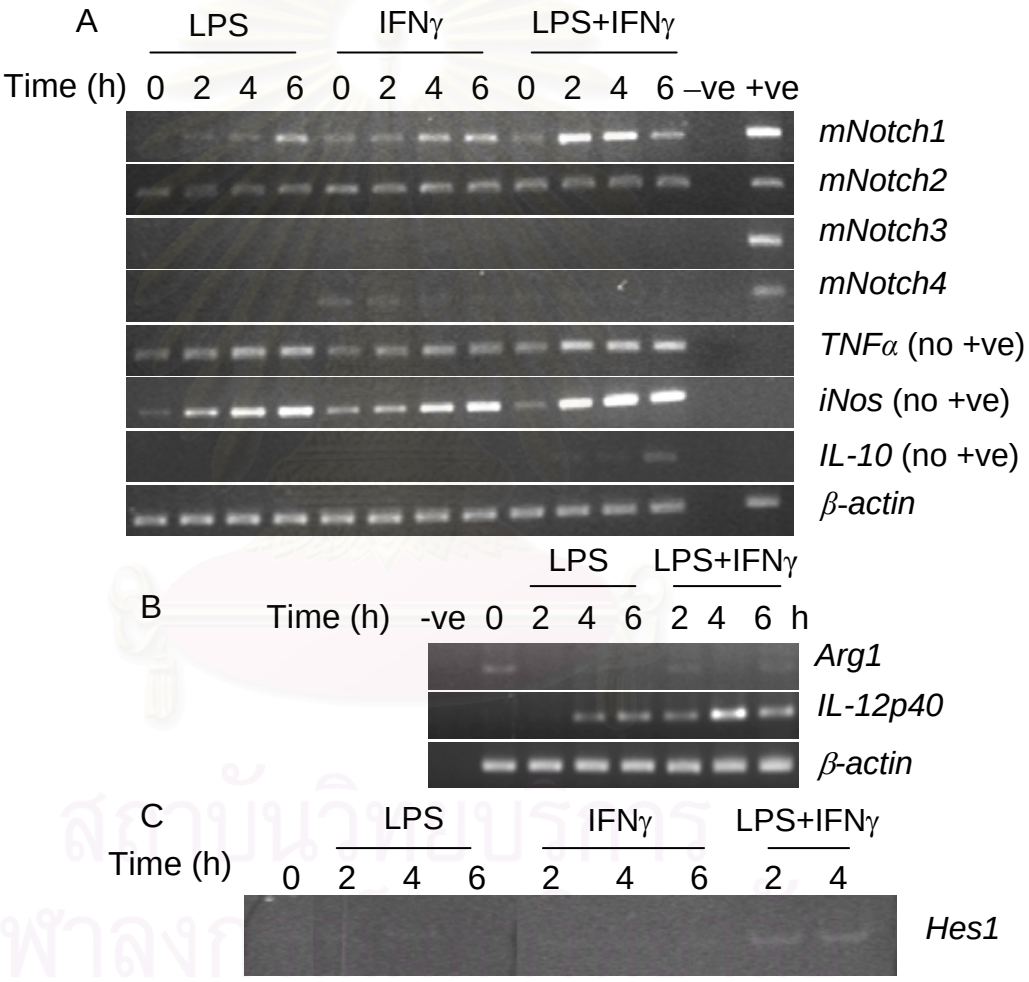


Figure 4.1 Activated RAW 264.7 expressed different Notch receptors, Notch target gene and cytokines. RT-PCR was performed to examine the expression of *mNotch1*, *mNotch2*, *mNotch3*, *mNotch4*, *TNFa*, *iNos*, *IL-10* (A), *Arg1*, *IL-12p40* (B) and *Hes1* (C) in RAW 264.7 treated with LPS (100 ng/ml) with or without IFNγ (10 ng/ml) for the indicated durations.

Expression of *β-actin* was used as loading control. RT-PCR without reverse transcriptase was used as negative control (-ve), and total RNA from murine thymus was used as positive control (+ve).

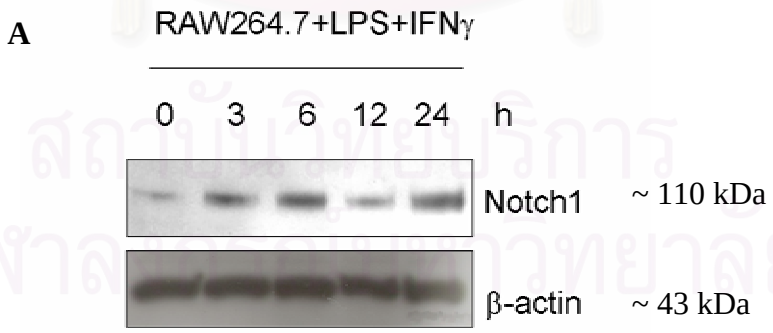
To investigate Notch receptors and *Hes1* expression in RAW 264.7 cell line, RT-PCR was performed using specific primers for *Notch1*-4 and *Hes1* genes. Stimulation with LPS with or without IFNγ significantly upregulated *Notch1* expression which peaked at 2 and 4 hr after stimulation. Expression of *Notch2* was detected in all types of stimulation, and the stimulation did not affect the expression level. Expression of *Notch4* was downregulated at 3 hr after treatment with IFNγ alone. However, *Notch3* expression was undetectable at any time point of stimulation (Figure 4.1A). In addition, the Notch target gene, *Hes1*, was also upregulated after simulation with LPS and IFNγ while LPS alone or IFNγ alone induced *Hes1* expression for shorter duration and to a lesser extent (Figure 4.1C). Upregulation of *Hes1* strongly suggested that Notch signaling is activated in stimulated RAW 264.7 cell.

To examine changes in cytokine expression during stimulation of macrophages in relation to activation of Notch signaling, RT-PCR was performed using specific primers for *TNFα, iNos, IL-10, Arg1* and *IL-12p40*. RAW 264.7 treated with LPS with or without IFNγ differently expressed both pro- and anti-inflammatory cytokines. Genes encoded molecules of proinflammatory nature such as *TNFα* and *iNos* were strongly induced at all time point after stimulation (Figure 4.1A). Treatment with LPS in the presence or absence of IFNγ induced higher *TNFα* and *iNos* expression than treatment with IFNγ alone, while LPS plus IFNγ triggered the highest expression of *TNFα* and *iNos*. *IL-10*, an anti-inflammatory cytokine, was upregulated in RAW 264.7 only when treated with LPS in the presence of IFNγ beginning at 2 hr and peaked at 6 hr after stimulation (Figure 4.1A). Expression of *Arginase1* (*Arg1*) was also detected in unstimulated RAW 264.7 and its expression was downregulated by treatment with LPS with or without IFNγ (Figure 4.1B). *IL-12p40*, a pro-inflammatory cytokine involved in Th1 type immune response, was upregulated upon stimulation and peaked at 4 hr

after treatment with LPS plus IFNγ (Figure 4.1B). Because *Notch1* was upregulated upon RAW 264.7 stimulation and Notch signaling is activated as suggested by the result of *Hes1* expression, we hypothesized that *Notch1* and Notch signaling may be involved in regulating macrophage effector functions.

4.2 Expression of Notch1 expression in stimulated RAW 264.7 examined by Western blot

To confirm Notch1 upregulation in activated RAW 264.7 at the protein level, Western blot using antibody specific for intracellular domain of Notch1 was performed. As shown in Figure 4.2, Notch1 was upregulated in RAW 264.7 upon stimulation with LPS and IFNγ at 0, 3 and 6 hr, but slightly downregulated at 12 hr before another round of upregulation was seen at 24 hr after stimulation. When RAW 264.7 cell line was stimulated with LPS alone similar Notch1 upregulation was observed (data not shown). This result indicates that Notch1 is upregulated in activated RAW 264.7 at both transcriptional and translational level.



B

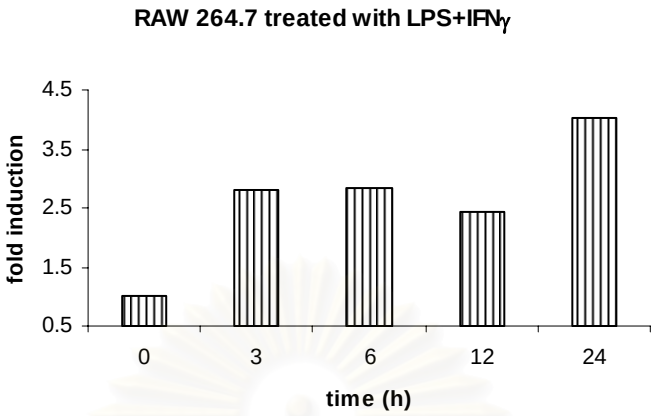


Figure 4.2 (A) Notch1 is upregulated upon LPS and IFNγ stimulation. RAW 264.7 was treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for indicated times, and cell lysates were analyzed for Notch1 expression by Western blot. β-actin was used as loading control. (B) The band intensities from result of Western blot in (A) were quantitated using Quanti One software by normalizing to β-actin expression.

4.3 Notch1 expression in three types of activated RAW 264.7

Ca-Mϕ exhibited the highest expression of Notch1 while Mϕ-II showed moderate level of Notch1 expression. AA-Mϕ did not show inducible Notch1 profile. This result suggests that Notch1 may involve in distinctive characters of activated macrophage populations, particularly cytokine expressions.

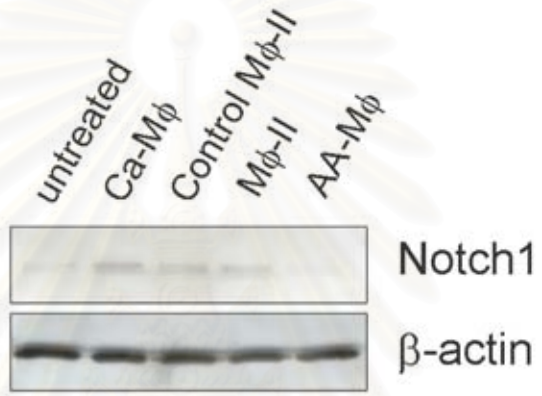


Figure 4.3 Activated macrophages expressed Notch1 at different level. Classically activated macrophages (Ca-Mϕ) and Type II – activated macrophages (Mϕ-II) were prepared by priming RAW 264.7 overnight with 10 ng/ml IFNγ. Ca-Mϕ were washed and stimulated with 100 ng/ml LPS for 4 hr. Mϕ-II received LPS along with immune complexes (IC) consisting erythrocytes opsonized with IgG (E-IgG) for 4 hr. Control of Mϕ-II received E-IgG alone after priming with IFNγ overnight. Alternatively activated macrophages (AA-Mϕ) were prepared by stimulating RAW 264.7 with 100 U/ml recombinant IL-4 for 4 hr. Cell lysates were analyzed for Notch1 expression by Western blot. β-actin was used as loading control.

4.4 Gene expression in differentially stimulated RAW 264.7

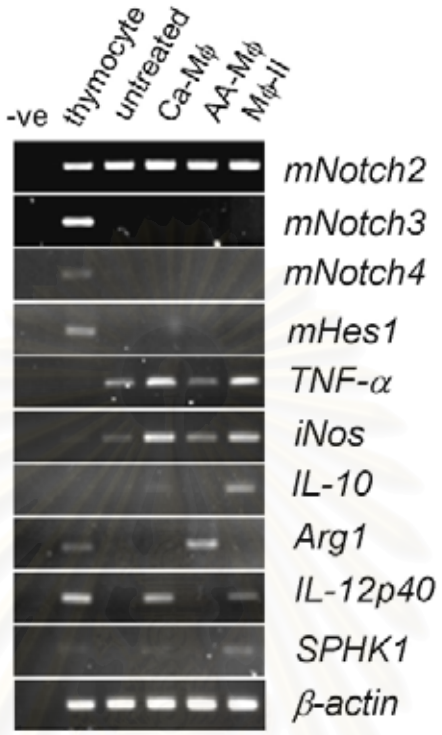


Figure 4.4 Differentially stimulated macrophages exhibited distinct gene expression profiles. RAW 264.7 was stimulated as described in Figure 4.3. Gene expressions were analyzed by RT-PCR. *β-actin* was used as loading control. RT-PCR without reverse transcriptase was used as negative control (-ve), and mRNA from thymus was used as positive control (+ve).

Since Notch1 was expressed in activated macrophages at the highest in Ca-Mϕ with low or undetectable in AA-Mϕ assessed by Western blot, expressions of genes of other Notch receptors and other signature molecules were subsequently observed by RT-PCR. All types of activated macrophages exhibited different gene expression profiles. While *Notch2* was expressed at a constant level in all types of activated macrophages, *Notch3*, *Notch4* and *Hes1* were undetectable in all cell types.

Ca-Mϕ strongly upregulated *TNFα*, *iNos*, *IL-10* and *IL-12p40* (Figure 4.4). In contrast, AA-Mϕ expressed *TNFα* and *iNos* at low level while *Arg1*was specifically detected only in this type of macrophages (Figure 4.4). To examine TNFα production by AA-Mϕ, ELISA was performed for measuring this cytokine (Figure 4.5). AA-Mϕ produced low level of TNFα at all time point of stimulation as compared with unstimulated RAW 264.7 (Figure 4.9). Moreover, AA-Mϕ also produced low amount of NO (data not shown). In addition, Mϕ-II expressed *TNFα*, *iNos*, *IL-10*, *IL-12p40* and *SPHK1*, found in Mϕ-II only (Figure 4.4).

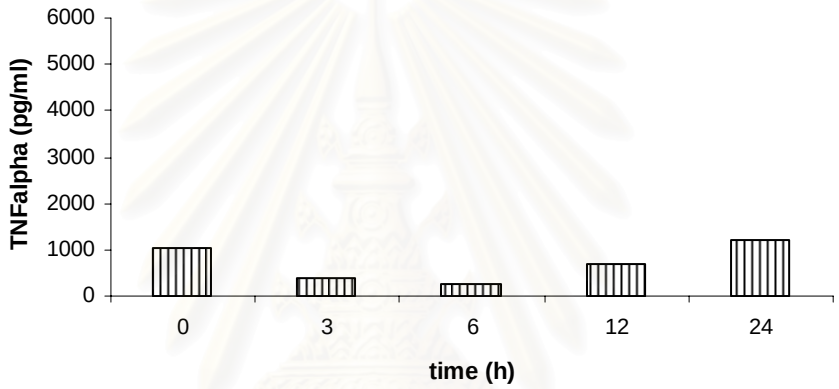
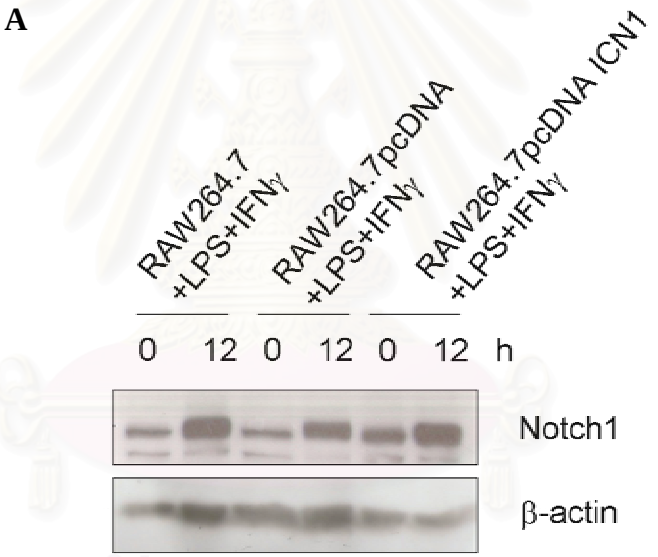


Figure 4.5 AA-Mϕ produce low amount of TNFα production as compared with untreated RAW 264.7 in Figure 4.9. AA-Mϕ were prepared by stimulating RAW 264.7 with 100 U/ml recombinant IL-4 for indicated time, and culture supernatants were analyzed for TNFα production by ELISA. The results represented triplicate experiments.

4.5 Generating ICN1 overexpressing RAW 264.7

The results obtained in this study so far strongly suggest that Notch1 may play an important role in regulating macrophage functions. To study the role of Notch1 in regulating macrophage functions, overexpression of activated Notch1 (ICN1) in RAW 264.7 cell line was employed.

Stable ICN1 overexpressing RAW 264.7 expressed higher Notch1 expression at 0 and 12 hr after stimulation (~2 and 4 fold, respectively) as compared with their controls (Figure 4.6). Therefore, RAW 264.7 cell line stably overexpressing ICN1 was obtained.



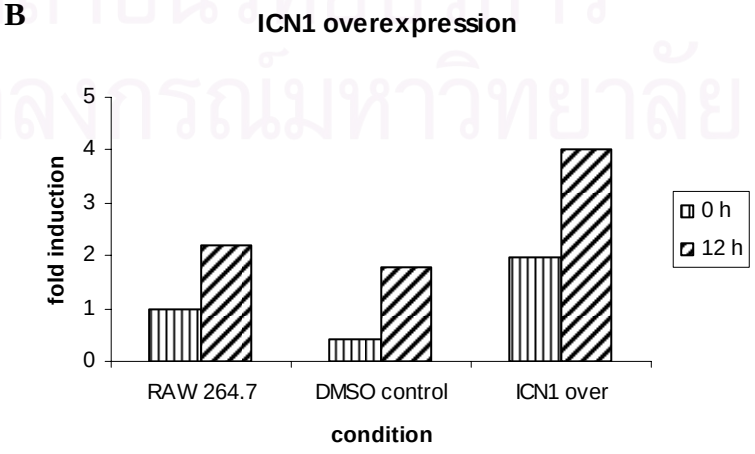


Figure 4.6 (A) Higher Notch1 expression in RAW 264.7 stably transfected with pcDNA ICN1.RAW 264.7 was transfected with pcDNA ICN1 plasmid containing neomycin resistant gene. An empty vector, pcDNA, was used as control. The stable clones were selected using G418 as described in materials and methods. Cells were similarly treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for indicated times, and cell lysates were analyzed expression of Notch1 by Western blot. β-actin was used as loading control. (B) Band intensities from result of Western blot in (A) were quantitated using Quanti One software by normalizing to β-actin expression.

4.6 Morphology of stimulated ICN1 overexpressing RAW 264.7

The morphological changes of macrophages were also observed under inverted microscope with or without stimulation. In a resting stage, ICN1 overexpressing RAW 264.7 appeared more dendritic cell-like than untransfected RAW 264.7 and empty vector control, which were mostly round shape with occasional flat appearance (Figure 4.7 A, C, E). The dendrite was obviously increased upon stimulation for 12 hr in all cell types, and the most dendritic character appeared in activated ICN1 overexpressing cell (Figure 4.7 B, D, F).

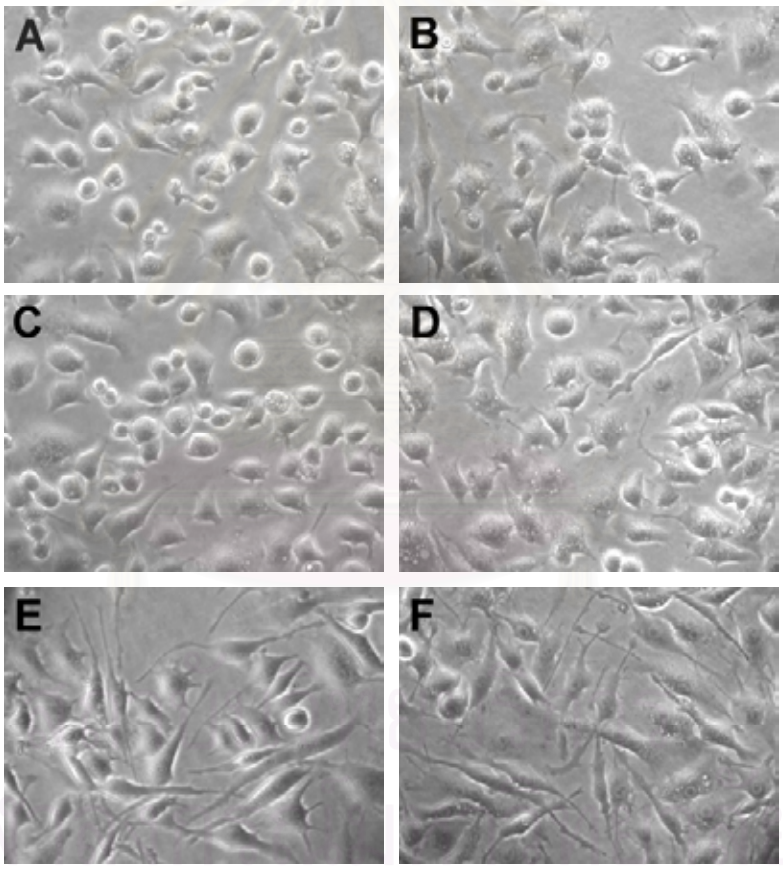


Figure 4.7 ICN1 overexpressing RAW 264.7 exhibited distinct cell morphology.
A, C, and E showed untreated RAW 264.7, untreated RAW 264.7 pcDNA and untreated RAW 264.7 pcDNA ICN1, respectively. Panel B, D and F show RAW 264.7, RAW 264.7 pcDNA and RAW 264.7 pcDNA ICN1 upon stimulation with LPS (100 ng/ml) and IFNγ (10 ng/ml) for 12 hr.

4.7 Effects of ICN1 overexpression on phenotypes of RAW 264.7

To examine the effects of ICN1 overexpression on phenotypes of RAW 264.7, expression of genes encoding some effector molecules were analyzed by RT-PCR.

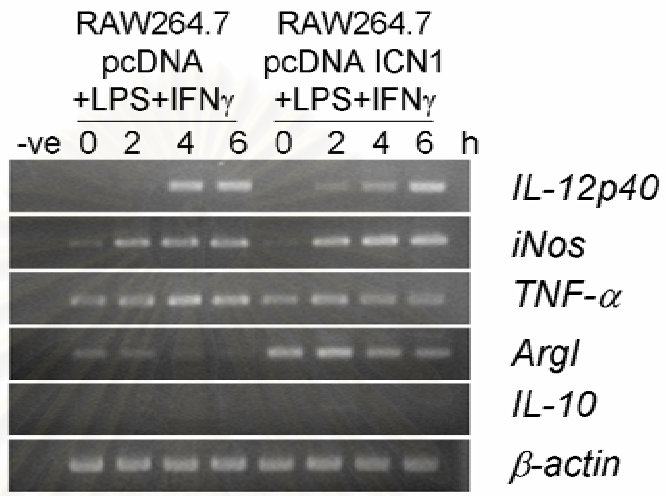


Figure 4.8 Differential gene expression profiles in ICN1 overexpresing RAW 264.7.
RAW 264.7 pcDNA and RAW 264.7 pcDNA ICN1 cells were stimulated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for indicated time. Semi-qunatitative RT-PCR was performed to examine the expression level of of *IL-12p40*, *iNos*, *TNFa*, *Arg1* and *IL-10* mRNA. *β-actin* mRNA was used to normalize loading. RT-PCR without reverse transcriptase was used as negative control (-ve).

IL-12p40 expression in ICN1 overexpressing cells was upregulated at 2 hr while that in empty vector control expressed at 4 hr after treatment. In addition, *iNos* expression in ICN1 overexpressing cell was slightly higher than expression in empty vector control (Figure 4.8). Moreover, Nitric oxide production detected by Griess reaction in ICN1 overexpressing cell showed significantly higher concentration at 24 hr as compared with control (Figure 4.10). These results suggest that overexpression of Notch positively regulates pro-inflammatory response. *Arg1* expression in ICN1 overexpressing cell was dramatically stronger than

expression in empty vector control even after stimulation. Although mRNA level of *TNFα* in ICN1 overexpressing cells was slightly lower than that in empty vector control (Figure 4.8), TNFα cytokine measured by ELISA in ICN1 overexpressing cells was significantly higher at 24 hr as compared with control, suggesting post transcriptional regulation may be involved (Figure 4.9). Moreover, *IL-10* expression in ICN1 overexpressing cells was slightly increased after 2 hr of stimulation (Figure 4.8). Although ICN1 overexpressing cells expressed many cytokines necessary for bacterial clearance, their activity for killing *E. coli* and *S. aureus* displayed about 80 – 90 % efficiency similar to empty vector control (Figure 4.11). In addition, untreated ICN1 overexpressing cells showed lower MHC class II level as compared with control. In the presence of LPS and IFNγ, ICN1 overexpressing cells also downregulated comparable level of MHC class II molecule as compared with control (Figure 4.12).

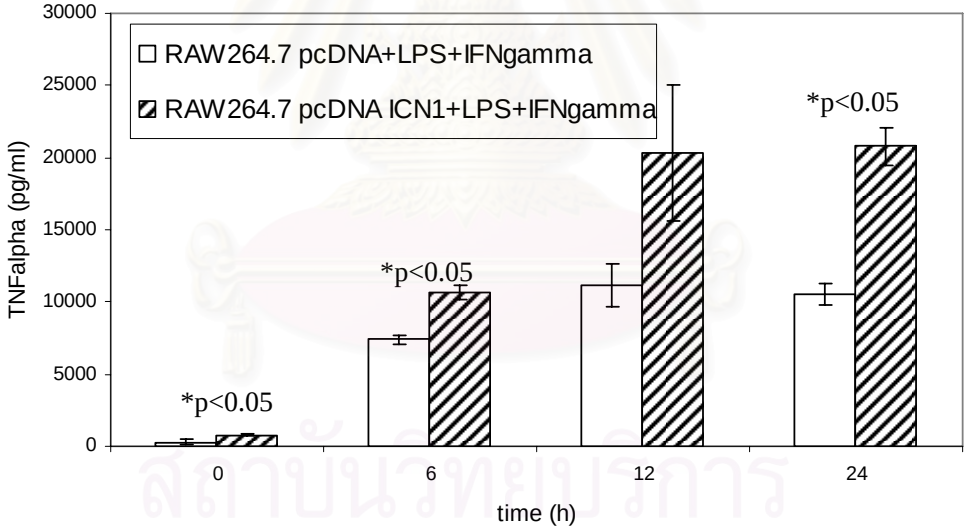


Figure 4.9 ICN1 overexpressing RAW 264.7 produces higher TNFα production.

RAW 264.7 pcDNA and RAW 264.7 pcDNA ICN1 were treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for indicated time, and culture supernatants were analyzed for TNFα production by ELISA. The results represent triplicate ± SD.

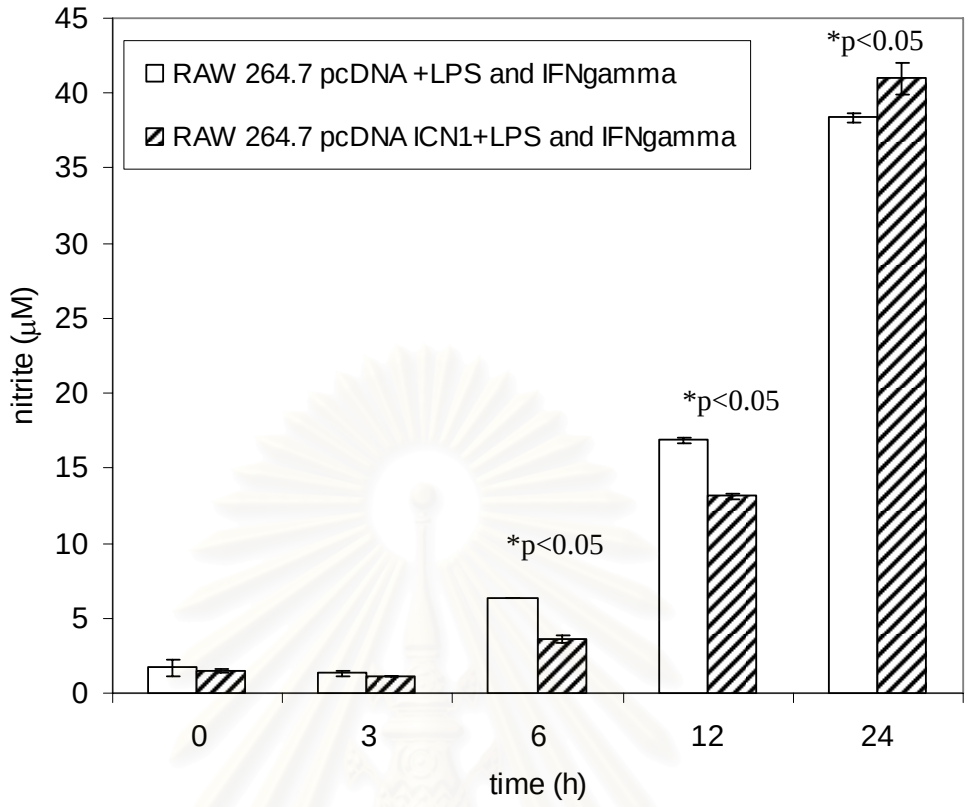


Figure 4.10 ICN1 overexpressing RAW 264.7 produces more NO.

RAW 264.7 pcDNA and RAW 264.7 pcDNA ICN1 were treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for indicated time and culture supernatants were analyzed for nitrite concentration by Griess reagent. The results represented triplicate ± SD.

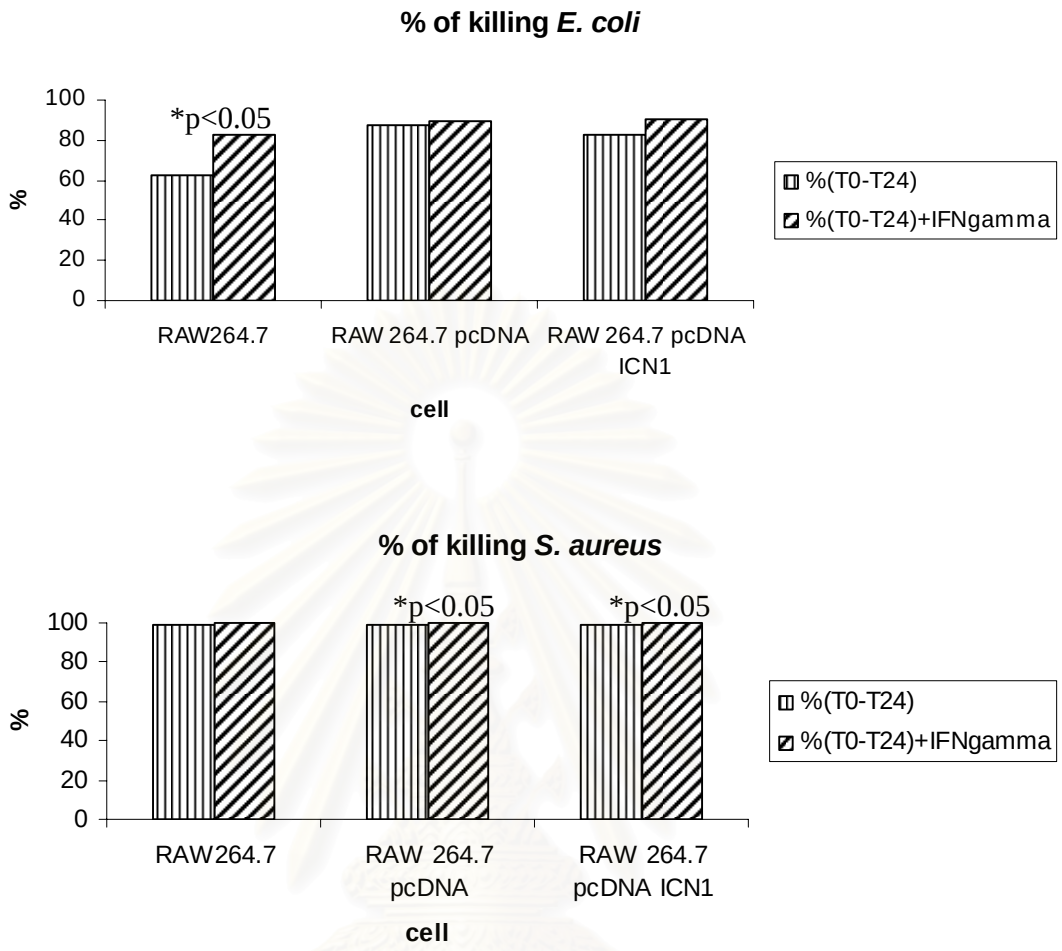


Figure 4.11 ICN1 overexpressing RAW 264.7 exhibit similar bactericidal activity as empty vector control. RAW 264.7, RAW 264.7 pcDNA and RAW 264.7 pcDNA ICN1 were infected with *E. coli* or *S. aureus* at ratio 10:1 (bacteria: macrophages) for 1 hr. After infection, infected macrophages were washed. Then, media containing antibiotic was added and incubated for 0 and 24 hr. After incubation, infected macrophages were lysed to release intracellular bacteria. The intracellular bacteria were analyzed by total plate count technique. Number of bacteria after 1 hr of infection was use as control.

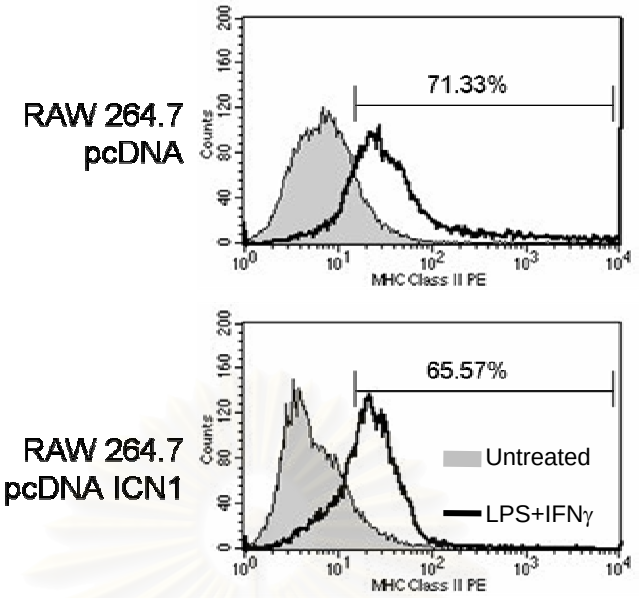


Figure 4.12 Effects of ICN1 overexpression on MHC class II expression in RAW 264.7. RAW 264.7 pcDNA and RAW 264.7 pcDNA ICN1 (2.5×10^5 cell/ml in 1 ml of complete RPMI) were stimulated with or without LPS and IFNγ for 24 hr. Cell surface expression of MHC class II were determined by FACS analysis.

4.8 Effects of IL-CHO on cell viability of RAW 264.7

To further investigate the role Notch1 plays in regulating macrophage functions, GSI, IL-CHO, was employed to inhibit Notch signaling. Notch signaling occurs through the proteolytic release of its cytoplasmic domain of all Notch receptors by multi-subunit enzyme, γ-secretase. Using GSI for inhibiting Notch receptor processing can circumvent the problem of functional redundancy among family member.

Upon evaluation of an appropriate concentration of IL-CHO, we found that treated RAW 264.7 with 25 μM and 50 μM of IL-CHO for 12 hr resulted in decreasing cell viability to 89% and 62%, respectively, when untreated RAW 264.7 was set as 100%. Treatment with 25 μM and 50 μM IL-CHO for 24 hr resulted in decreasing in % cell viability to 87% and 32%, respectively (Figure 4.13). Thus, treatment with 25 μM of IL-CHO was chosen for using in further experiments.

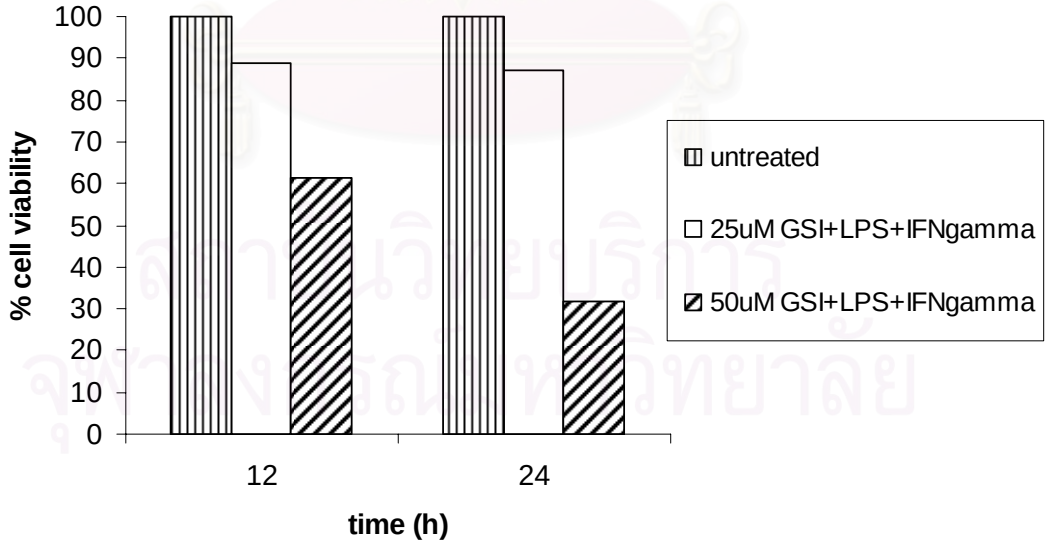


Figure 4.13 Percentage of cell viability of RAW 264.7 treatment with 25 and 50 μM IL-CHO

4.9 Effects of GSI treatment of macrophage cell morphology

The morphological changes of macrophage were observed under inverted microscope after 1 hr of pre-treatment of cells with IL-CHO. Cells cultured in the presence of IL-CHO appeared more flat, round and deattached from the surface than cells cultured with vehicle control (Figure 4.14). Thus, inhibition of Notch signaling using GSI altered biological functions of macrophages in contrast to ICN1 overexpressing RAW 264.7.

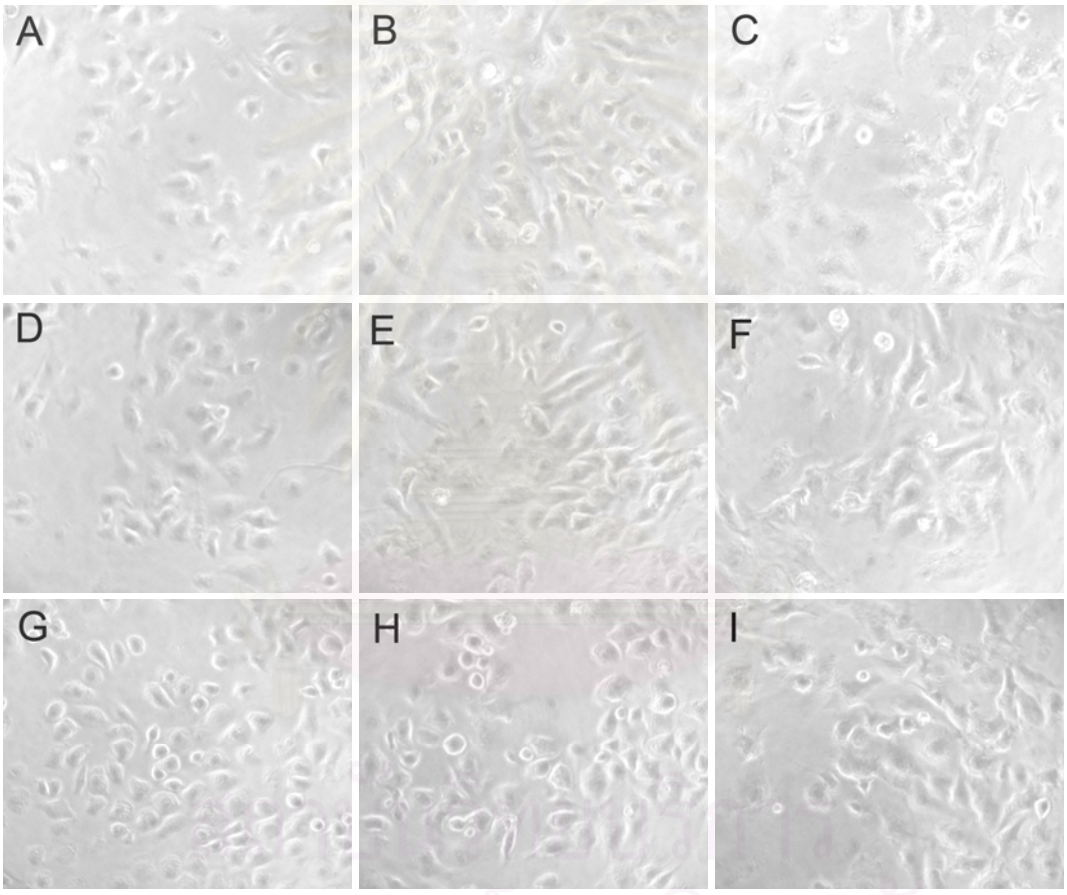


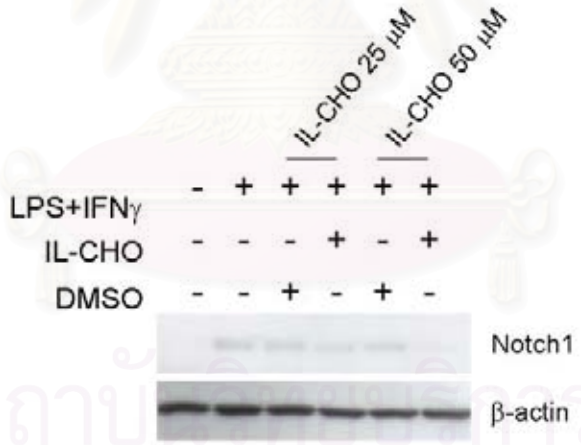
Figure 4.14 Morphological changes of RAW264.7 cell line upon IL-CHO treatment
Panel A, D and G represent untreated RAW 264.7, 0.5% DMSO treated RAW 264.7 for 1 hr and 25 μM IL-CHO treated RAW 264.7 for 1 hr, respectively. Panel B, E and H represent RAW 264.7, 0.5% DMSO treated RAW 264.7 and 25 μM IL-CHO treated RAW 264.7 upon stimulation with LPS (100 ng/ml) and IFNγ (10 ng/ml) for 12 hr. Panel C, F and I represent

RAW 264.7, 0.5% DMSO treated RAW 264.7 and 25 μM IL-CHO treated RAW 264.7 upon stimulation with LPS (100 ng/ml) and IFNγ (10 ng/ml) for 24 hr.

4.10 Effects of GSI on Notch1 expression

Several studies reported that expression of Notch1 was under control of Notch signaling and inhibition of Notch signaling by GSI also decreased Notch1 expression (Palaga *et al*., 2003). Notch1 expression in both 25 μM and 50 μM IL-CHO treated RAW 264.7 was downregulated as compared with their DMSO mock controls (Figure 4.15). However, treatment with 25 μM IL-CHO exhibited %cell viability higher than treatment with 50 μM IL-CHO (Figure 4.13) and downregulation seen in this experiment may result from cell death. Therefore, 25 μM IL-CHO was sufficient for suppressing Notch activation and Notch1 expression.

A



B

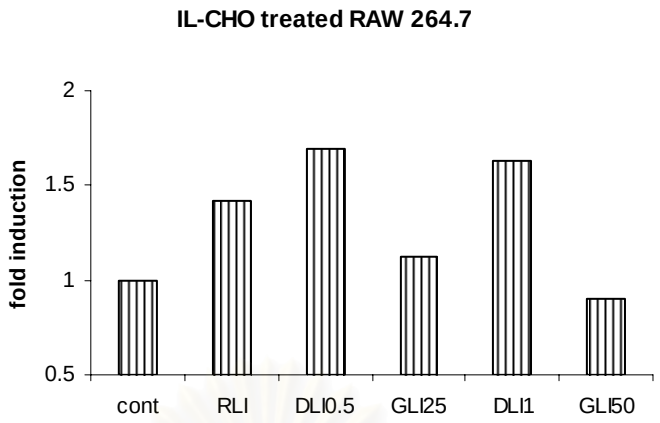


Figure 4.15 Effect of GSI on Notch1 expression.

A. RAW 264.7 was pre-treated with 25 and 50 μM IL-CHO or DMSO mock control for 1 hr and treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for 24 hr. Cell lysates were analyzed for Notch1 expression by Western blot. β-actin was used as loading control.

B. Band densities of the result obtained in A were quantiated by the Quanti One software by normalizing to β–actin.

4.11 Effects of IL-CHO on phenotypes of macrophage

To investigate the effects of IL-CHO on phenotypes of macrophage, RAW 264.7 were pre-treated with IL-CHO for 1 hr and treated with LPS and IFNγ for 3 and 6 hr. Expression of various effector molecules were observed by RT-PCR.

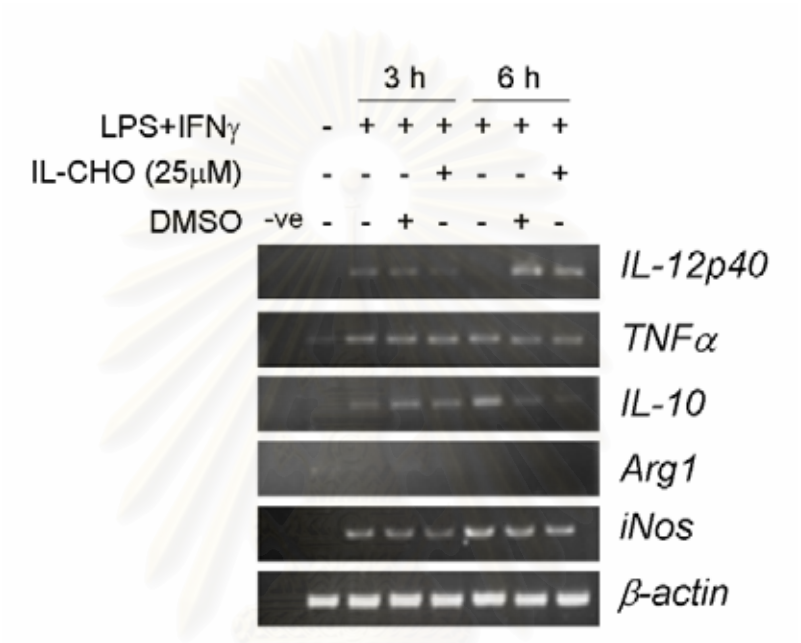


Figure 4.16 Differential gene expression profiles in RAW 264.7 treated with 25 μM IL-CHO. RAW 264.7 was pre-treated with 25 μM IL-CHO or DMSO mock control for 1 hr and treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for 3 and 6 hr. RT-PCR was performed to examine the expression of *IL-12p40*, *iNos*, *TNFa*, *Arg1* and *IL-10* mRNA levels. *β-actin* mRNA was used as loading control. RT-PCR without reverse transcriptase was used as negative control (-ve).

Macrophages incubated in the presence of GSI exhibited distinct cytokine expression profiles. *IL-12p40* expression was downregulated upon 25 μM IL-CHO stimulation for 3 hr as compared with controls. However, the expression level of *IL-12p40* after 6 hr of stimulation was inconclusive because DMSO vehicle alone may affect *IL-12p40* gene expression level

during macrophage stimulation (Figure 4.16). Expression of *TNFa* after 3 and 6 hr of stimulation with IL-CHO were similar to that of untreated controls (Figure 4.16). In addition, production of TNFα at 3 and 6 hr after IL-CHO treatment as measured by ELISA showed similar results (Figure 4.17). In contrast to *TNFα* expression, *iNos* expression at 6 hr of stimulation was stronger than expression at 3 hr of stimulation, but the level of gene expression of IL-CHO treated samples were not different as compared with their controls (Figure 4.16). However, NO production by IL-CHO treated RAW 264.7 at 12 and 24 hr after stimulation was significantly decreased when compared with DMSO control (Figure 4.18). Moreover, *Arg1* expression in IL-CHO treated was not detected in all time points examined in this study (Figure 4.16). Furthermore, *IL-10* expression was downregulated after 3 hr of stimulation. The higher IL-10 expression was found at 6 hr of stimulation with LPS and IFNγ, but the expression was decreased upon treatment with DMSO and IL-CHO (Figure 4.16). Although IL-CHO treated RAW 264.7 downregulated many cytokines necessary for bacterial clearance, such as NO production and *IL*-12*p40*, their activity for killing *E. coli* and *S. aureus* displayed about 80 – 90 % efficiency remained similar to that of cells with mock treatment (Figure 4.19). In addition, inhibition of Notch signaling in RAW 264.7 by GSI showed slightly increase in MHC class II level as compared with controls. However, in the presence of LPS and IFNγ, cell surface expression of MHC class II was enhanced in all cell types and the highest expression was observed in GSI treatment RAW 264.7 (Figure 4.20). The expression of several cytokines produced by IL-CHO treated RAW 264.7 exhibited different patterns, therefore Notch signaling may involve in regulating macrophage functions.

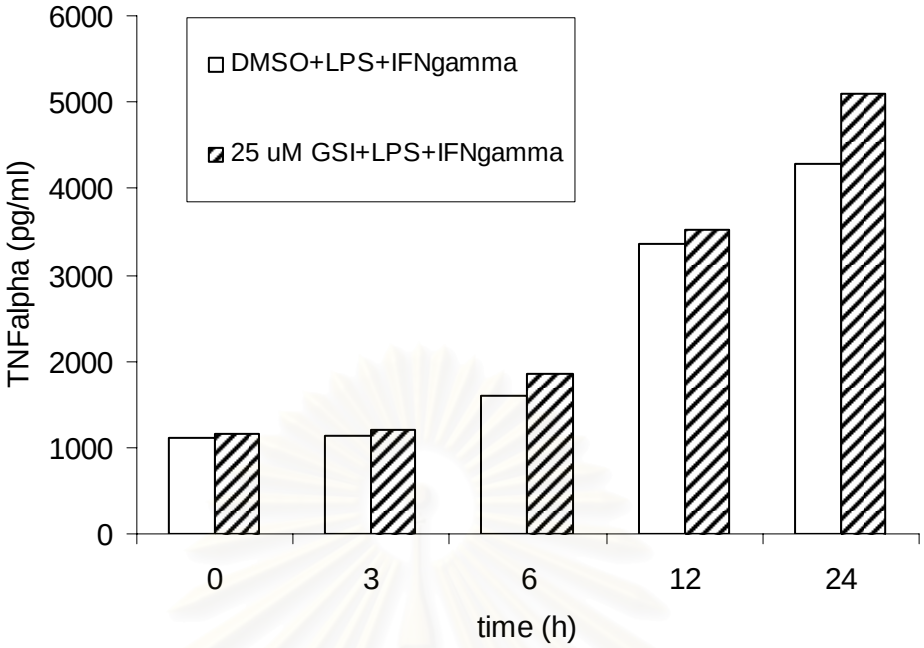


Figure 4.17 RAW 264.7 treated with IL-CHO produce similar amount of TNFα.

RAW 264.7 was pre-treated with 25 μM IL-CHO or DMSO mock control for 1 hr and treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for indicated time. Then, culture supernatants were analyzed for TNFα production by ELISA. The results represented duplicate experiments.

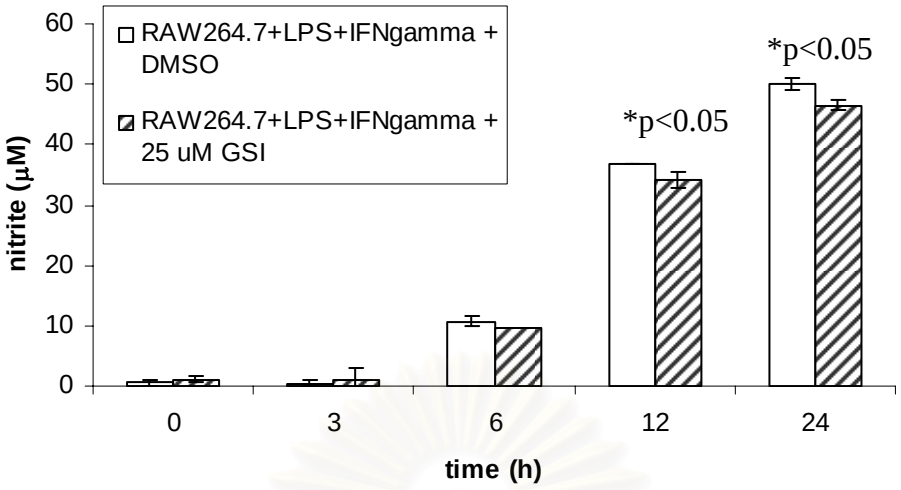


Figure 4.18 RAW 264.7 treated with IL-CHO exhibit significantly lower NO production. RAW 264.7 was pre-treated with 25 μM IL-CHO or DMSO mock control for 1 hr and treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for indicated time. Culture supernatants were analyzed for NO production by Griess reaction. The results represented triplicate ± SD.

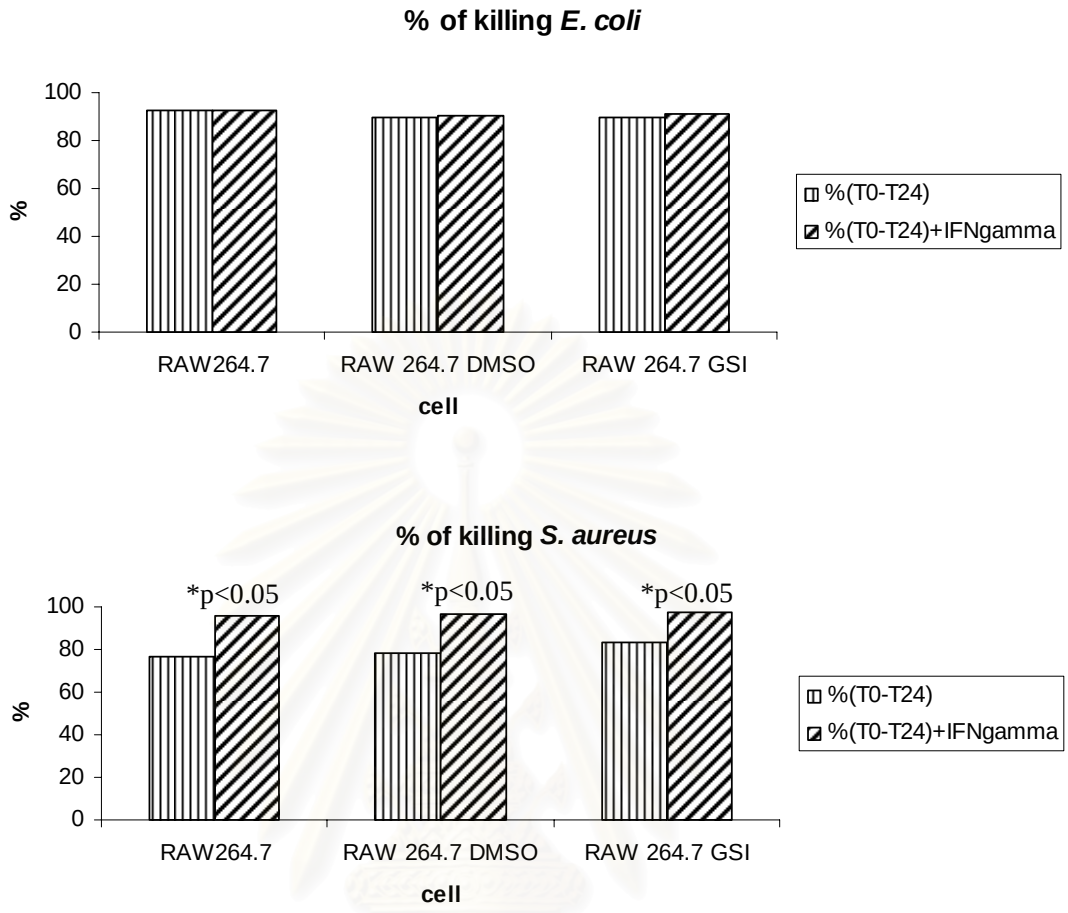


Figure 4.19 GSI treated RAW 264.7 exhibit similar bactericidal activity as controls.

RAW 264.7 was pre-treated with 25 μM IL-CHO or DMSO mock control for 1 hr and infected with *E. coli* or *S. aureus* at ratio of 10:1 (bacteria:macrophage) for 1 hr. After infection, infected macrophages were washed. Then, media containing antibiotic was added and incubated for 0 and 24 hr. After incubation, infected macrophages were lysed to release intracellular bacteria. The intracellular bacteria were analyzed by total plate count technique. Number of bacteria after 1 hr of infection was used as control.

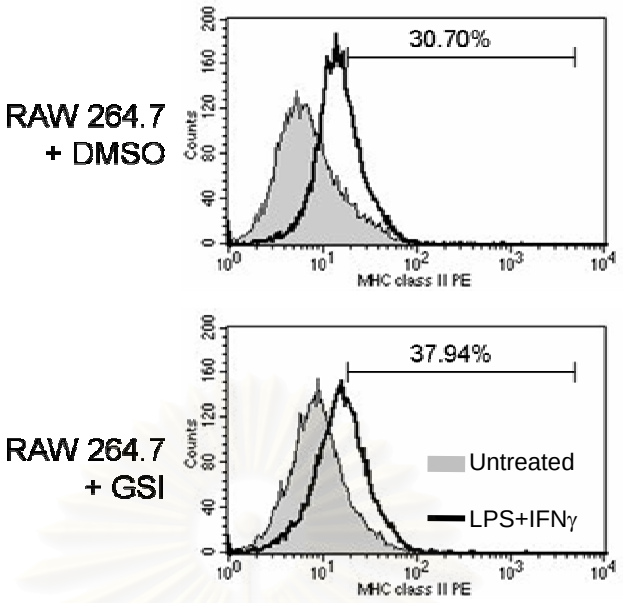


Figure 4.20 Effects of GSI treatment on MHC class II expression in RAW 264.7.

RAW 264.7 (2.5×10^5 cell/ml in 1 ml of complete RPMI) were cultured overnight before being pre-treated with 25 μM IL-CHO or DMSO mock control for 1 hr. Cells were treated with LPS (100 ng/ml) and IFNγ (10 ng/ml) for 24 hr. Cell surface expression of MHC Class II were analyzed by FACS analysis.

CHAPTER V

DISCUSSION

It has been well documented that Notch signaling plays an essential role in cell fate decisions in hematopoiesis (Radtke *et al*., 2004). Notch signaling is also required for development of T and B cells as well as macrophages (Maillard *et al*., 2003, Pui *et al*., 1999 and Ohishi *et al*., 2000). In previous studies, relationship between Notch signaling and macrophages have been reported. Monslave *et al*. (2006) reported that Notch receptors are expressed during macrophage activation. In addition, expression of Dlk 4 is increased in macrophage exposed to proinflammatory stimuli such as LPS and IL-1β. Co-incubation of macrophages with Dlk 4-expressing cells triggered Notch activation and Notch3 was selectively increased during macrophage differentiation (Fung *et al*., 2007). TLR agonists similarly triggered upregulation of Notch1 in bone marrow macrophages and macrophage-like cell line RAW 264.7 through NF-κB activation which resulted in proinflammatory responses (Palaga *et al*., 2008). Thus, Notch signaling is involved in macrophage functions.

Macrophages are one of the important cells in the immune system. The major functions of macrophages include phagocytosis, antigen presentation and cytokine secretion (Madigan *et al*., 2003). Currently, activated macrophages have been classified into 3 types, Ca-Mϕ, AA-Mϕ and Mϕ-II based on their stimuli and their phenotypic responses (Edwards *et al*., 2006).

Although, the role of Notch signaling in macrophages functions was established, the role of Notch signaling in these 3 activated macrophage populations was unclear. In this study, Notch signaling was found to be expressed in activated macrophages. Previously, *Notch1, 2, 4* and *Hes1* expressions were detected in activated macrophages (Monslave *et al*., 2006 and

Palaga *et al*., 2008). Therefore, the results obtained in this study are consistent with and compliment the previous studies. In addition, we found that in RAW 264.7, only *Notch1* was upregulated upon macrophages stimulation suggesting that Notch1 may involve in activated macrophages.

Macrophages are able to produce a wide range of cytokines and reactive oxygen and nitrogen species upon infection (Kaufmann *et al*., 2004). In this study, 3 types of activated macrophages exhibited different cytokine expressions and Notch1 expression. Induction of cytokine expressions in macrophages mainly depend on engagement of TLR with specific microbial components (Franchini *et al*., 2005). Palaga *et al.* (2008) established that Notch signaling is activated through TLR pathway via MyD88. Therefore, expressions of cytokines in activated macrophages may associate with Notch signaling, particularly Notch1 expression.

Edwards *et al.* (2006) categorized activated macrophages into 3 types. They uncovered biochemical markers for each activated macrophage including *SPHK1*, a gene encoding sphingosine kinases which catalyze the production of sphingosine-1 phosphate from sphingosine. SPHK1 has been shown to be necessary for C5a-triggered, intracellular Ca^{2+} signals (Melendez and Ibrahim, 2004). In this study, we also found that SPHK1 was selectively expressed in macrophages stimulated with LPS in the presence of an immune complex. More importantly, Notch1 is significantly expressed in classically activated macrophages whereas IL-4-stimulated macrophages showed undetectable level of expression. Type II macrophages expressed intermediate level of Notch1. Thus, the results indirectly imply that Notch1 may involve in effector functions of these three distinctively activated macrophages.

To observe whether Notch1 is essential in macrophage functions, activated Notch1 overexpression was employed. ICN1 overexpression was an effective method for determining the effects of Notch1 on phenotypes of interested cells. Suwanjunee *et al*. (2008) reported that

ICN1 overexpressing cells by transient and stable transfection expressed higher level of Notch1 as compared with controls. In this study, ICN1 overexpression in RAW 264.7 was accomplished by stable transfection. Interestingly, overexpression of ICN1 did not yield high level of protein, suggesting that high level of ICN1 may be toxic to macrophages.

Further method for determining the effects of Notch1 on phenotypes of macrophages is Notch loss-of-function by pharmaceutical approach. Palaga *et al.* (2008) reported that 25 μM IL-CHO can inhibit both cleaved Notch1 and Notch1 in bone marrow macrophages. In this experiment, 25 μM IL-CHO was an appropriate concentration for using in RAW 264.7.

Tohda *et al.* (2005) found that stimulation by Notch ligand tended to alter blast cells into cells into more differentiated morphology, including macrophage-like morphology and increase in differentiation markers. In contrast, inhibition of Notch signaling in bone marrow macrophages rendered them to be round (Palaga *et al.*, 2008). In the present study, ICN1 overexpressing macrophages appeared to contain more dendrite-like structures protruding from cell membranes than GSI treatment cells, suggesting that Notch1 may involve in controlling morphological changes of macrophages.

Activated macrophages normally produce a great number of cytokines depending on stimuli (Fujihara *et al.*, 2003). IL-12p40 is a potent cytokine primarily produced by APC. In macrophages, IL-12p40 is induced by IFNγ and LPS through PU.1/ETS2, p50 and c-Rel, and C/EBPβ and AP1 transcription factors (Trinchiei, 2003). IL-12p40 was highly produced in Ca-Mϕ and moderately induced in Mϕ-II but not AA-Mϕ (Edwards *et al.*, 2006). In this study, ICN1 overexpressing macrophages upregulated *IL-12p40* expression whereas GSI treated cells downregulated *IL-12p40* at 3 hr of stimulation. However, this effect at later time point (6 hr) may be due to the effect of vehicle DMSO. The impact of manipulating Notch signaling on IL-12p40 is intriguing in light of the importance of this cytokine subunit. IL-12p40 is a common subunit for IL-12 and IL-23, both are signature cytokine for Th1 and Th17 type

immune response, respectively (Goriely and Goldman, 2007). Therefore, it implies that by manipulating Notch signaling, one may be able to tip the balance of an immune response.

iNos is a gene responsible for the increase production of NO through oxidation of L-arginine to L-citrullin. NO is a key cytotoxic chemical mediators for microbicidal processes (Helene *et al.*, 2007). *iNos* induction occurs by LPS, IFNγ, IL-13 priming (Helene *et al.*, 2007), CpG DNA (Utaisincharoen *et al.*, 2002) and Fcγ ligation followed by IFNγ priming (Edwards *et al.*, 2006). iNos was expressed in both Ca-Mϕ and Mϕ-II but not AA-Mϕ (Edwards *et al.*, 2006). In this study, ICN1 overexpressing cells exhibited greater amount of *iNos* expression and NO production. On the other hand, GSI treated macrophages expressed unaltered *iNos* expression but downregulated NO production. These results suggest that Notch signaling may play an important role in maximum *iNos* induction but it is not required for iNos expression.

Previously, Notch signaling inhibition by IL-CHO in bone marrow macrophages did not alter *TNFα* expression but the amount of TNFα cytokine was markedly decrease at early stage of stimulation. Thus, Notch signaling regulates TNFα production at the post-translational level (Palaga *et al.*, 2008). In this study, ICN1 overexpressing cells exhibited unaltered *TNFα* mRNA expression but increase in TNFα cytokine production was detected. Moreover, GSI treated cells also expressed unaltered *TNFα* mRNA and TNFα cytokine. This result suggested that Notch1 may regulate TNFα production at the post-translational level. In addition, the discrepancies between this study and others may be due to different in cell types used for investigation.

Arginase plays a crucial role in wound healing by enhancing fibroblast proliferation and collagen production as well as T cell proliferation (Sosroseno *et al.*, 2006). Arginase participates in the regulation of NO synthesis by competing for substrate L-arginine (Munder *et al.*, 1999). *Arg1* is expressed in several conditions including IFNγ, IL-4 or IL-10 stimulated

bone marrow macrophages (Munder *et al.*, 1999). Moreover, *Arg1* is expressed in AA-Mϕ induced by IL-4. In this study, ICN1 overexpressing cell upregulated *Arg1*, but GSI treated macrophages downregulated *Arg1*. From the results, DMSO may have a negative effect on the expression of this gene.

Macrophages treated with LPS express IL-10 through several transcription factors including Sp1 (Brightbill *et al.*, 2002), c-Maf (Cao *et al.*, 2005) and NF-κB1 (Cao *et al.* 2006). Moreover, IL-10 is expressed at higher level in Mϕ-II which is induced by Fcγ ligation following IFNγ priming (Edwards *et al.*, 2006). In this study, ICN1 overexpressing cells upregulated IL-10 slightly.

One of effective strategies for pathogen elimination of macrophages is to produce toxic substances in phagolysosome such as toxic oxygen species and lysozyme (Madigan *et al*., 2003). Moreover, proinflammatory cytokines produced by activated macrophages are secreted to enhance the killing ability (Stout *et al.*, 2004). In this study, although, ICN1 overexpressing macrophages expressed higher toxic substances, NO than GSI treatment cells, the ability to kill *E. coli* and *S. aureus* was not differ. However, ICN1 may involve in induction of *iNos*, potential killer molecules and enzymes produce by activated macrophages are complicated. Inside the lysosome, H_2O_2, lysozyme, proteases, phosphatases, nucleases and lipases are generated (Madigan *et al.*, 2003). The production of these enzymes may not depend on Notch signaling as we did not observe any difference in bacterial killing in our study. Treatment with IFNγ increased the ability of killing because IFNγ can induce TNFα and iNos expression (Figure 4.1). Activity of macrophages for killing *S. aureus* in both ICN1 overexpressing cells and GSI treatment cells is higher than *E. coli*. Takeuchi *et al.* (1999) found that TLR-2 deficient mice respond to LPS to the same extent as wild-type mice. TLR2 and TLR4 deficient macrophages lacked the response to *S. aureus* peptidoglycan and gram +ve lipoteichoic acids, respectively. Thus, two mains components of gram +ve bacteria can

stimulate TLR2 and TLR4. On the other hand, LPS, a main component of gram –ve bacteria can induce TLR4. Moreover, priming with peptidoglycan affects a release of cytokine resulting in upregulation of CD14, TLR2 and TLR4 on monocytes (Hadley *et al.* 2005). These results suggest that gram +ve is more potent to induce macrophages for killing than gram –ve.

Activated macrophages are able to present Ag to T cells through MHC molecules. MHC class II was markedly upregulated on LPS-treated RAW 264.7 cells and dendritic cells (Saxena *et al.*, 2003 and Weijzen *et al.*, 2002). Moreover, Edwards and co-workers have shown that Mϕ-II had the highest expression of MHC class II, and Ca-Mϕ showed moderate level of MHC class II. In contrast, AA-Mϕ minimally upregulated MHC class II (Edwards *et al.*, 2006). In this study, the effects of Notch signaling on the MHC class II expression in RAW 264.7 were analyzed by FACS. We found that inhibition of Notch signaling in activated RAW 264.7 increased in MHC class II expression. Moreover, activated ICN1 overexpressing cells exhibited MHC class II downregulation. Interestingly, untreated ICN1 overexpressing cells also decreased in MHC class II expression. This study is consistent with previous report from Palaga *et al.* (2008). They reported that GSI treated bone marrow macrophages enhanced expression of MHC class II. However, this study is in contrast with the findings of Monsalve *et al.* (2006). They found that ICN1 overexpressing RAW 264.7 significantly upregulated MHC class II. The difference between the results obtained in our study and those from Monsalve may occur because of the differences of the ICN1 region used in their study. It is suggested that, from these results, Notch1 may affect on MHC class II expression. Thus, Notch signaling may play a vital role in regulating macrophage functions as antigen presenting cells.

In conclusion, we have investigated the expression profiles of Notch receptors in RAW 264.7 cell lines and found that Notch1 is selectively upregulated upon LPS and IFNγ

stimulation. In addition, Notch1 is expressed at the highest level under classically activated conditions. When Notch signaling is manipulated through overexpression of ICN1 or GSI treatment, complicated phenotypes were observed. The exact roles of Notch signaling in activation of macrophages deserve to be investigated more in details. This data may yield an insight into mechanism of pro-inflammatory and anti-inflammatory macrophages in chronic inflammatory diseases such as autoimmune disorders and atherosclerosis.

CHAPTER VI

CONCLUSIONS

1. *Notch1*, *2*, *4* and *Hes1* expressions were detected in activated RAW 264.7 by RT-PCR. Only Notch1 was upregulated upon stimulation of RAW264.7 cell line, suggesting that Notch1 may involve in activated macrophages.

2. Three types of activated macrophages exhibited different gene expressions and, more importantly Notch1 expression. Ca-Mϕ exhibited the highest expression of Notch1 while Mϕ-II showed intermediate level of Notch1 expression. AA-Mϕ did not show inducible Notch1 expression profile.

3. Notch1 overexpression or Notch signaling inhibition using γ-secretase inhibitor affected the expression of *IL-12p40*, *iNOS*, *Arg1*, *IL-10*, *TNFα* and nitric oxide and TNFα production but did not affect the ability of macrophages to kill Gram positive and Gram negative bacteria.

4. Concentration of IL-CHO at 25 μM is sufficient for suppressing Notch activation and Notch1 expression in RAW264.7 cell line. DMSO, a diluent of IL-CHO, may interefere with some gene expression such as *IL-12p40* and *IL-10*.

5. Notch signaling may involve in regulating MHC class II expression, particularly Notch1.

Suggestions for the future work

1. Due to GSI and DMSO may have negative effects on macrophage viability and some gene expressions, siRNA technique for specific Notch1 silencing should be employed in investigating the role of Notch1 in macrophages.

2. Due to there is difference in Notch1 expression in Ca-Mϕ and Mϕ-II, it is possible that Notch signaling may regulate macrophage differentiation. Therefore, it is interesting to observe the phenotypic changes upon ICN1 overexpression and Notch1 inhibition followed by IFNγ and immune complex treatment.

3. Although Notch1 was not detected in AA-Mϕ, the effects of ICN1 overexpression in RAW 264.7 followed by IL-4 treatment on phenotypes of these macrophages should be investigated.

REFERENCES

Allman, D., Aster, J. C., and Pear, W. S. (2002). Notch signaling in hematopoiesis and early lymphocyte development. Immunol Rev *187*, 75-86.

Anderson, C. F., and Mosser, D. M. (2002). A novel phenotype for an activated macrophage: the type 2 activated macrophage. J Leukoc Biol *72*, 101-106.

Bosisio, D., Polentarutti, N., Sironi, M., Bernasconi, S., Miyake, K., Webb, G. R., Martin, M. U., Mantovani, A., and Muzio, M. (2002). Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. Blood *99*, 3427-3431.

Brightbill, H. D., Plevy, S. E., Modlin, R. L., and Smale, S. T. (2000). A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. J Immunol *164*, 1940-1951.

Cao, S., Liu, J., Song, L., and Ma, X. (2005). The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. J Immunol *174*, 3484-3492.

Cao, S., Zhang, X., Edwards, J. P., and Mosser, D. M. (2006). NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. J Biol Chem *281*, 26041-26050.

Condeelis, J., and Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell *124*, 263-266.

Curry, C. L., Reed, L. L., Golde, T. E., Miele, L., Nickoloff, B. J., and Foreman, K. E. (2005). Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. Oncogene *24*, 6333-6344.

Dallas, M. H., Varnum-Finney, B., Martin, P. J., and Bernstein, I. D. (2007). Enhanced T-cell reconstitution by hematopoietic progenitors expanded ex vivo using the Notch ligand Delta1. Blood *109*, 3579-3587.

Edwards, J. P., Zhang, X., Frauwirth, K. A., and Mosser, D. M. (2006). Biochemical and functional characterization of three activated macrophage populations. J Leukoc Biol *80*, 1298-1307.

Franchini, M., Schweizer, M., Matzener, P., Magkouras, I., Sauter, K. S., Mirkovitch, J., Peterhans, E., and Jungi, T. W. (2006). Evidence for dissociation of TLR mRNA expression and TLR agonist-mediated functions in bovine macrophages. Vet Immunol Immunopathol *110*, 37-49.

Fujihara, M., Muroi, M., Tanamoto, K., Suzuki, T., Azuma, H., and Ikeda, H. (2003). Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol Ther *100*, 171-194.

Fung, E., Tang, S. M., Canner, J. P., Morishige, K., Arboleda-Velasquez, J. F., Cardoso, A. A., Carlesso, N., Aster, J. C., and Aikawa, M. (2007). Delta-like 4 induces notch signaling in macrophages: implications for inflammation. Circulation *115*, 2948-2956.

Goerdt, S., and Orfanos, C. E. (1999). Other functions, other genes: alternative activation of antigen-presenting cells. Immunity *10*, 137-142.

Goriely, S., and Goldman, M. (2007). The interleukin-12 family: new players in transplantation immunity? Am J Transplant *7*, 278-284.

Grazia Cappiello, M., Sutterwala, F. S., Trinchieri, G., Mosser, D. M., and Ma, X. (2001). Suppression of Il-12 transcription in macrophages following Fc gamma receptor ligation. J Immunol *166*, 4498-4506.

Hadley, J. S., Wang, J. E., Foster, S. J., Thiemermann, C., and Hinds, C. J. (2005). Peptidoglycan of Staphylococcus aureus upregulates monocyte expression of CD14, Toll-like receptor 2 (TLR2), and TLR4 in human blood: possible implications for priming of lipopolysaccharide signaling. Infect Immun *73*, 7613-7619.

Holcomb, I.N., Kabakoff, R.C., Chan, B., Baker, T.W., Gurney, A., Henzel, W., Nelson, C., Lowman, H.B., Wright, B.D., Skelton, N.J., *et al.* (2000). FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. EMBO J *19*, 4046-4055.

Jundt, F., Anagnostopoulos, I., Forster, R., Mathas, S., Stein, H., and Dorken, B. (2002). Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. Blood *99*, 3398-3403.

Kageyama, R., Ohtsuka, T., Hatakeyama, J., and Ohsawa, R. (2005). Roles of bHLH genes in neural stem cell differentiation. Exp Cell Res *306*, 343-348.

Kaufmann, S., Gordon, S.,Medzhitov, R. (2004). The Innate Immune Response to Infection. Berlin, Germany：ASM Press,

Kawai, T., and Akira, S. (2005). Pathogen recognition with Toll-like receptors. Curr Opin Immunol *17*, 338-344.

Kertesz, Z., Vas, V., Kiss, J., Urban, V. S., Pozsonyi, E., Kozma, A., Paloczi, K., and Uher, F. (2006). In vitro expansion of long-term repopulating hematopoietic stem cells in the presence of immobilized Jagged-1 and early acting cytokines. Cell Biol Int *30*, 401-405.

King, A. M., Van der Put, E., Blomberg, B. B., and Riley, R. L. (2007). Accelerated Notch-dependent degradation of E47 proteins in aged B cell precursors is associated with increased ERK MAPK activation. J Immunol *178*, 3521-3529.

Kohyama, J., Tokunaga, A., Fujita, Y., Miyoshi, H., Nagai, T., Miyawaki, A., Nakao, K., Matsuzaki, Y., and Okano, H. (2005). Visualization of spatiotemporal activation of Notch signaling: live monitoring and significance in neural development. Dev Biol *286*, 311-325.

Kojika, S., and Griffin, J. D. (2001). Notch receptors and hematopoiesis. Exp Hematol *29*, 1041-1052.

Kota, R. S., Rutledge, J. C., Gohil, K., Kumar, A., Enelow, R. I., and Ramana, C. V. (2006). Regulation of gene expression in RAW 264.7 macrophage cell line by interferon-gamma. Biochem Biophys Res Commun *342*, 1137-1146.

Kunisato, A., Chiba, S., Nakagami-Yamaguchi, E., Kumano, K., Saito, T., Masuda, S., Yamaguchi, T., Osawa, M., Kageyama, R., Nakauchi, H., *et al.* (2003). HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. Blood *101*, 1777-1783.

Lee, Y. G., Kim, J. Y., Lee, J. Y., Byeon, S. E., Hong, E. K., Lee, J., Rhee, M. H., Park, H. J., and Cho, J. Y. (2007). Regulatory effects of Codonopsis lanceolata on macrophage-mediated immune responses. J Ethnopharmacol *112*, 180-188.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G., and Israel, A. (1998). The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc Natl Acad Sci U S A *95*, 8108-8112.

Madigan, M. T., Martinko, J. M., Parker, J. (2003). Brock Biology of Microorganisms. Vol 10th Edition. New Jersey : Pearson Education, Inc.,

Maillard, I., Adler, S. H., and Pear, W. S. (2003). Notch and the immune system. Immunity *19*, 781-791.

Mantovani, A., Sica, A., and Locati, M. (2005). Macrophage polarization comes of age. Immunity *23*, 344-346.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol *23*, 549-555.

Matsuno, K., Diederich, R. J., Go, M. J., Blaumueller, C. M., and Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. Development *121*, 2633-2644.

McLendon, C., Xin, T., Ziani-Cherif, C., Murphy, M. P., Findlay, K. A., Lewis, P. A., Pinnix, I., Sambamurti, K., Wang, R., Fauq, A., and Golde, T. E. (2000). Cell-free assays for gamma-secretase activity. Faseb J *14*, 2383-2386.

Melendez, A. J., and Ibrahim, F. B. (2004). Antisense knockdown of sphingosine kinase 1 in human macrophages inhibits C5a receptor-dependent signal transduction, Ca2+ signals, enzyme release, cytokine production, and chemotaxis. J Immunol *173*, 1596-1603.

Miele, L. (2006). Notch signaling. Clin Cancer Res *12*, 1074-1079.

Monsalve, E., Perez, M. A., Rubio, A., Ruiz-Hidalgo, M. J., Baladron, V., Garcia-Ramirez, J. J., Gomez, J. C., Laborda, J., and Diaz-Guerra, M. J. (2006). Notch-1 up-regulation and signaling following macrophage activation modulates gene expression patterns known to affect antigen-presenting capacity and cytotoxic activity. J Immunol *176*, 5362-5373.

Morimura, T., Goitsuka, R., Zhang, Y., Saito, I., Reth, M., and Kitamura, D. (2000). Cell cycle arrest and apoptosis induced by Notch1 in B cells. J Biol Chem *275*, 36523-36531.

Munder, M., Eichmann, K., Moran, J. M., Centeno, F., Soler, G., and Modolell, M. (1999). Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J Immunol *163*, 3771-3777.

Nam, Y., Aster, J. C., and Blacklow, S. C. (2002). Notch signaling as a therapeutic target. Curr Opin Chem Biol *6*, 501-509.

Neves, H., Weerkamp, F., Gomes, A. C., Naber, B. A., Gameiro, P., Becker, J. D., Lucio, P., Clode, N., van Dongen, J. J., Staal, F. J., and Parreira, L. (2006). Effects of Delta1 and Jagged1 on early human hematopoiesis: correlation with expression of notch signaling-related genes in CD34+ cells. Stem Cells *24*, 1328-1337.

Noel, W., Raes, G., Hassanzadeh Ghassabeh, G., De Baetselier, P., and Beschin, A. (2004). Alternatively activated macrophages during parasite infections. Trends Parasitol *20*, 126-133.

Nomaguchi, K., Suzu, S., Yamada, M., Hayasawa, H., and Motoyoshi, K. (2001). Expression of Jagged1 gene in macrophages and its regulation by hematopoietic growth factors. Exp Hematol *29*, 850-855.

Ohishi, K., Katayama, N., Shiku, H., Varnum-Finney, B., and Bernstein, I. D. (2003). Notch signalling in hematopoiesis. Semin Cell Dev Biol *14*, 143-150.

Ohishi, K., Varnum-Finney, B., Flowers, D., Anasetti, C., Myerson, D., and Bernstein, I. D. (2000). Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. Blood *95*, 2847-2854.

Osborne, B., and Miele, L. (1999). Notch and the immune system. Immunity *11*, 653-663.

Oswald, F., Liptay, S., Adler, G., and Schmid, R. M. (1998). NF-kappaB2 is a putative target gene of activated Notch-1 via RBP-Jkappa. Mol Cell Biol *18*, 2077-2088.

Oyama, T., Harigaya, K., Muradil, A., Hozumi, K., Habu, S., Oguro, H., Iwama, A., Matsuno, K., Sakamoto, R., Sato, M., *et al.* (2007). Mastermind-1 is required for Notch signal-dependent steps in lymphocyte development in vivo. Proc Natl Acad Sci U S A *104*, 9764-9769.

Palaga, T., Miele, L., Golde, T. E., and Osborne, B. A. (2003). TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. J Immunol *171*, 3019-3024.

Palaga, T., Buranaruk, C., Rengpipat, S., Fauq, A. H., Golde, T. E., Kaufmann, S. H., and Osborne, B. A. (2008). Notch signaling is activated by TLR stimulation and regulates macrophage functions. Eur J Immunol *38*, 174-183.

Pasare, C., and Medzhitov, R. (2004). Toll-like receptors and acquired immunity. Semin Immunol *16*, 23-26.

Pui, J. C., Allman, D., Xu, L., DeRocco, S., Karnell, F. G., Bakkour, S., Lee, J. Y., Kadesch, T., Hardy, R. R., Aster, J. C., and Pear, W. S. (1999). Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity *11*, 299-308.

Radtke, F., Wilson, A., Mancini, S. J., and MacDonald, H. R. (2004). Notch regulation of lymphocyte development and function. Nat Immunol *5*, 247-253.

Rochette, M. J., and Murphy, M. P. (2002). Gamma-secretase: substrates and inhibitors. Mol Neurobiol *26*, 81-95.

Rodriguez, P. C., Quiceno, D. G., Zabaleta, J., Ortiz, B., Zea, A. H., Piazuelo, M. B., Delgado, A., Correa, P., Brayer, J., Sotomayor, E. M., *et al.* (2004). Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res *64*, 5839-5849.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science *234*, 364-368.

Saxena, R. K., Vallyathan, V., and Lewis, D. M. (2003). Evidence for lipopolysaccharide-induced differentiation of RAW264.7 murine macrophage cell line into dendritic like cells. J Biosci *28*, 129-134.

Schroder, K., Hertzog, P. J., Ravasi, T., and Hume, D. A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol *75*, 163-189.

Schroder, K., Sweet, M. J., and Hume, D. A. (2006). Signal integration between IFNgamma and TLR signalling pathways in macrophages. Immunobiology *211*, 511-524.

Schroeder, T., Meier-Stiegen, F., Schwanbeck, R., Eilken, H., Nishikawa, S., Hasler, R., Schreiber, S., Bornkamm, G. W., Nishikawa, S., and Just, U. (2006). Activated Notch1 alters differentiation of embryonic stem cells into mesodermal cell lineages at multiple stages of development. Mech Dev *123*, 570-579.

Scotton, C. J., Martinez, F. O., Smelt, M. J., Sironi, M., Locati, M., Mantovani, A., and Sozzani, S. (2005). Transcriptional profiling reveals complex regulation of the monocyte IL-1 beta system by IL-13. J Immunol *174*, 834-845.

Shih Ie, M., and Wang, T. L. (2007). Notch signaling, gamma-secretase inhibitors, and cancer therapy. Cancer Res *67*, 1879-1882.

Singh, N., Phillips, R. A., Iscove, N. N., and Egan, S. E. (2000). Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis. Exp Hematol *28*, 527-534.

Song, H. M., Jang, A. S., Ahn, M. H., Takizawa, H., Lee, S. H., Kwon, J. H., Lee, Y. M., Rhim, T. Y., and Park, C. S. (2008). Ym1 and Ym2 expression in a mouse model exposed to diesel exhaust particles. Environ Toxicol *23*, 110-116.

Sosroseno, W., Musa, M., Ravichandran, M., Fikri Ibrahim, M., Bird, P. S., and Seymour, G. J. (2006). Arginase activity in a murine macrophage cell line (RAW264.7) stimulated with lipopolysaccharide from *Actinobacillus actinomycetemcomitans*. Oral Microbiol Immunol *21*, 145-150.

Stout, R. D., and Suttles, J. (2004). Functional plasticity of macrophages: reversible adaptation to changing microenvironments. J Leukoc Biol *76*, 509-513.

Suwanjunee, S., Wongchana, W., and Palaga, T. (2008) Inhibition of gamma-secretase affects proliferation of leukemia and hepatoma cell lines through Notch signaling. Anticancer Drugs (in press)

Tada, Y., Asahina, A., Nakamura, K., Tomura, M., Fujiwara, H., and Tamaki, K. (2000). Granulocyte/macrophage colony-stimulating factor inhibits IL-12 production of mouse Langerhans cells. J Immunol *164*, 5113-5119.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. Immunity *11*, 443-451.

Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T., and Honjo, T. (1995). Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). Curr Biol *5*, 1416-1423.

Tian, G., Ghanekar, S. V., Aharony, D., Shenvi, A. B., Jacobs, R. T., Liu, X., and Greenberg, B. D. (2003). The mechanism of gamma-secretase: multiple inhibitor binding sites for transition state analogs and small molecule inhibitors. J Biol Chem *278*, 28968-28975.

Tohda, S., Kogoshi, H., Murakami, N., Sakano, S., and Nara, N. (2005). Diverse effects of the Notch ligands Jagged1 and Delta1 on the growth and differentiation of primary acute myeloblastic leukemia cells. Exp Hematol *33*, 558-563.

Utaisincharoen, P., Anuntagool, N., Chaisuriya, P., Pichyangkul, S., and Sirisinha, S. (2002). CpG ODN activates NO and iNOS production in mouse macrophage cell line (RAW 264.7). Clin Exp Immunol *128*, 467-473.

Van Ginderachter, J.A., Movahedi, K., Hassanzadeh Ghassabeh, G., Meerschaut, S., Beschin, A., Raes, G., and De Baetselier, P. (2006). Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. Immunobiology *211*, 487-501.

Weijzen, S., Velders, M. P., Elmishad, A. G., Bacon, P. E., Panella, J. R., Nickoloff, B. J., Miele, L., and Kast, W. M. (2002). The Notch ligand Jagged-1 is able to induce maturation of monocyte-derived human dendritic cells. J Immunol *169*, 4273-4278.

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000). MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. Nat Genet *26*, 484-489.

Wu, W., Mosteller, R. D., and Broek, D. (2004). Sphingosine kinase protects lipopolysaccharide-activated macrophages from apoptosis. Mol Cell Biol *24*, 7359-7369.

Yamada, T., Yamazaki, H., Yamane, T., Yoshino, M., Okuyama, H., Tsuneto, M., Kurino, T., Hayashi, S., and Sakano, S. (2003). Regulation of osteoclast development by Notch signaling directed to osteoclast precursors and through stromal cells. Blood *101*, 2227-2234.

Yamaguchi, E., Chiba, S., Kumano, K., Kunisato, A., Takahashi, T., Takahashi, T., and Hirai, H. (2002). Expression of Notch ligands, Jagged1, 2 and Delta1 in antigen presenting cells in mice. Immunol Lett *81*, 59-64.

Yamamoto, N., Yamamoto, S., Inagaki, F., Kawaichi, M., Fukamizu, A., Kishi, N., Matsuno, K., Nakamura, K., Weinmaster, G., Okano, H., and Nakafuku, M. (2001). Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. J Biol Chem *276*, 45031-45040.

Yamamura, K., Ohishi, K., Katayama, N., Kato, K., Shibasaki, T., Sugimoto, Y., Miyata, E., Shiku, H., Masuya, M., Nishioka, J., *et al.* (2007). Notch ligand Delta-1 differentially modulates the effects of gp130 activation on interleukin-6 receptor alpha-positive and -negative human hematopoietic progenitors. Cancer Sci *98*, 1597-1603.

Zhang, X., Edwards, J. P., and Mosser, D. M. (2006). Dynamic and transient remodeling of the macrophage IL-10 promoter during transcription. J Immunol *177*, 1282-1288.

APPENDIX

APPENDIX

1. Complete RPMI 1640 100 ml

RPMI 1640	90%
FBS	10%
Penicillin	100 U/ml
Streptomycin	0.4 mg/ml
Sodium pyruvate	1%
HEPES	1%

2. Freezing media 10 ml

Complete RPMI 1640	90%
DMSO	10%

3. DEPC water for RNA work 100 ml

HPLC water (100 ml) was added into a clean bottle, and 10 µl of DEPC (0.01% v/v) was added into the water. Then, the bottle was swirled and incubated overnight at room temperature. Next, the DEPC water was autoclaved at 121˚C and pressure 15 psi for 15 minutes.

4. FBS inactivation

Before using FBS, FBS must be inactivated at 56˚C for 30 minutes using water bath.

5. 50×TAE buffer for agarose gel electrophoresis 200 ml

Trisma base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA	20 ml

Adjusted pH to 8.0 and volume to 200 ml using deionized water

autoclaved at 121˚C and pressure 15 psi for 15 minutes

5.1 2% agarose gel preparation

Agarose gel	2%
1×TAE	20 ml

5.2 running buffer for agarose gel electrophoresis

50×TAE was diluted to final concentration 0.5× in 400 ml of deionized water.

6. Lauria-Bertani (LB) broth and agar plate 1000 ml

6.1 LB broth preparation

Bacto tryptone	10 g
Yeast extracts	5 g
NaCl	10 g

Adjusted pH to 7.4 and volume to 1000 ml using deionized water

6.2 LB agar preparation

After LB broth preparation, 1.5% of agar was added to the broth. Next, the LB broth and agar were autoclaved at 121˚C and pressure 15 psi for 15 minutes.

6.3 LB agar containing 50 µg/ml ampicillin

After sterilization, the LB agar was warmed to approximately 50˚C using water bath. Next, 50 µg/ml ampicillin was added to the warmed agar, and the agar was mixed well and poured plate.

7. SDS-polyacrylamide gel preparation

7.1 8% separating gel 8 ml

Sterile water	4.236 ml
40% Acrylamide and Bis-acrylamide solution	1.6 ml
1.5 M Tris-HCl pH 8.8	2 ml
10% SDS	0.08 ml
10% APS	0.08 ml
TEMED	0.004 ml

7.2 5% stacking gel 2 ml

Sterile water	1.204 ml
40% Acrylamide and Bis-acrylamide solution	0.25 ml
1 M Tris-HCl pH 6.8	0.504 ml
10% SDS	0.02 ml
10% APS	0.02 ml
TEMED	0.002 ml

8. 2×Laemmli buffer (SDS-dye) 10 ml

1 M Tris-HCl pH 6.8	1 ml (final concentration 100 mM)
10% SDS	4 ml (4% v/v)
99.5% glycerol	2.01 ml (20% v/v)
HPLC water	2.989 ml
Bromphenol blue	0.001 g

9. Reagent for protein extraction

9.1 Buffer A (stored at 4˚C)

10 mM EGTA	1 ml
10 mM DTT	1 ml
500 mM Tris-HCl pH 7.2	1 ml
1.4 M KCl	1 ml
25 mM $MgCl_2$	1 ml
Sterile water	5 ml
Protease Inhibitor Cocktail Tablets	1 tablet

9.2 Buffer B (stored at 4˚C)

Buffer A	990 µl
Nonidet P-40	10 µl

10. PBST (washing buffer for Western blot)

1×PBS	500 ml
Tween20	0.05%

11. Blocking solution for Western blot

PBST	200 ml
Non-fat dry milk	3%

12. 1×PBS pH 7.4

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Deionized water	1000 ml

autoclaved at 121˚C and pressure 15 psi for 15 minutes

13. 5×running buffer for Western blot

Trisma base	15.1 g
Glycine	94 g
SDS	5 g
Deionized water	1000 ml

14. Transfer buffer for Western blot

Trisma base	5.08 g
Glycine	2.9 g
SDS	0.37 g
Deionized water	800 ml
Absolute methanol	200 ml

15. 5×TBE buffer (for 5% acrylamide gel)

Trisma base	54 g
Boric acid	27.5 g
0.5 M EDTA pH 8.0	20 ml
Deionized water	to 1000 ml

autoclaved at 121˚C and pressure 15 psi for 15 minutes

16. 5% acrylamide gel 10 ml

Sterile water	6.23 ml
40% Acrylamide and Bis-acrylamide solution	1.7 ml
5×TBE buffer	2 ml
10% APS	0.07 ml
TEMED	0.0035 ml

17. Running buffer for 5% acrylamide gel

1×TBE buffer was prepared by diluting 5×TBE buffer to total volume 500 ml. The gel was run using Protein III system apparatus set at 50 volt for 2 h.

18. Sulfanilamide solution (for Griess reaction) 50 ml (stored at 4˚C)

Sulfanilamide 1% (w/v) was dissolved in 5% phosphoric acid, and total volume was adjusted to 50 ml by deionized water.

19. NED solution (for Griess reaction) 50 ml (stored at 4˚C)

NED 0.1% (w/v) was dissolved in 50 ml deionized water.

20. Standard nitrite

Sodium nitrite 0.1 M was prepared as a stock solution. For working solution, 100 μM was prepared by diluting the stock solution in complete RPMI 1640 to 1 ml total volume.

21. ECL substrate of HRP

Coumaric acid (90mM) was dissolved in DMSO in total volume 10 ml. Then, the solution aliquots were kept at –20˚C.

Luminol (250 mM) was also dissolved in DMSO in total volume 10 ml. Then, the solution aliquots were kept at –20˚C.

21.1 Solution A

100 mM Tris-HCl pH 8.5 (stored at 4˚C)	4 ml
90 mM coumaric acid	17.6 μl
250 mM luminol	40 μl

21.2 Solution B

100 mM Tris-HCl pH 8.5 (stored at 4˚C)	4 ml
30% H_2O_2	2.4 µl

22. Bacterial glycerol stock

An inoculum preparation, a bacterial colony on agar plate was picked and cultured in 2 ml of LB broth overnight. Then, bacterial culture (250 µl) was taken to 25 ml of LB broth overnight. The bacterial culture (400 µl) was transferred to a new microcentrifuge tube, and glycerol was added to 20% final concentration. The aliquots were kept at –80˚C. To measure the CFU of stock culture, the aliquot was diluted and determined the CFU by spread plate.

23. Film developer and fixer

Film developer and fixer were diluted in tap water at dilution 1 : 4 in total volume 500 ml.

24. Ampicillin, penicillin, streptomycin and G418 solution

Ampicillin, streptomycin and G418 solution were prepared at final concentration 50 mg/ml, and penicillin was prepared at final concentration 10^6 U/ml by diluting in sterile deionized water. Then, the solutions were filtered by using 0.22 µm syringe filter. The solution aliquots were kept at –20˚C.

25. 4% paraformaldehyde

Paraformaldehyde (4g) was dissolved in 100 ml of PBS. Then, 1 N NaOH was added for 2 – 3 drops. The solution was heated at 65°C in a chemical hood. Then, the solution was cooled to room temperature and, the pH was adjusted to 7.4.

Biography

Chayanit Buranaruk was born in Bangkok, Thailand on July 22, 1983. After graduated with the second honor of Bachelor's degree of Science from the Department of Microbiology, Faculty of Science at Chulalongkorn University in 2004, she subsequently enrolled in the Master Program of Science in Industrial Microbiology at Chulalongkorn University in 2005.

Academic presentation

Buranaruk, C. and Palaga, T. Notch signaling regulates effector functions in macrophages. The 23th National Congress on Allergy and Immunology, 29-30 March 2007, Radisson Hotel, Bangkok Thailand. p.28

Buranaruk, C. and Palaga, T. Notch signaling regulates effector functions in macrophages. The 12th Biological Sciences Graduate Congress 2007, 17-19 December 2007, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. p.173

Submitted paper

Palaga, T., Buranaruk, C., Rengpipat, S., Fauq, A. H., Golde, T. E., Kaufmann, S. H., and Osborne, B. A. (2008). Notch signaling is activated by TLR stimulation and regulates macrophage functions. Eur J Immunol *38*, 174-183.