การใช้เทคนิค RNAi เพื่อศึกษาบทบาทของจีน *Notch1* ในแมคโครฟาจ

นางสาวฐิติพร ภัทรกานต์กุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

USING RNAi TECHNIQUE TO INVESTIGATE THE ROLES OF *NOTCH1* GENE IN MACROPHAGES

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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 2009

Thesis Title	USING RNAi TECHNIQUE TO INVESTIGATE THE ROLES OF *NOTCH1* GENE IN MACROPHAGES
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Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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ฐิติพร ภัทรกานต์กุล : การใช้เทคนิค RNAi เพื่อศึกษาบทบาทของจีน *Notch1* ในแมคโครฟาจ. (USING RNAi TECHNIQUE TO INVESTIGATE THE ROLES OF *NOTCH1* GENE IN MACROPHAGES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. ธนาภัทร ปาลกะ, 94 หน้า

Notch เป็นจีนที่ประมวลรหัสของโปรตีนตัวรับ (receptor) บนผิวเยื่อหุ้มเซลล์ *Notch* ในสัตว์มีกระดูกสันหลังมี 4 ชนิด ได้แก่ *Notch1, 2, 3* และ *4* และมีลิแกนด์ 5 ชนิด ได้แก่ *Jagged1-2* และลิแกนด์ *Delta-like-1, 3* และ *4* วิถีสัญญาณ Notch เริ่มขึ้นเมื่อมีอันตรกิริยาระหว่างโปรตีนตัวรับกับลิแกนด์ วิถีสัญญาณ Notch มีบทบาทสำคัญในพัฒนาการของเซลล์หลากชนิด เช่น การคงสภาพเซลล์ต้นกำเนิด (stem cell) กำหนดชะตาของเซลล์ในระหว่างการพัฒนา การชักนำการเปลี่ยนสภาพ (differentiation) ในขั้นปลายของเซลล์บางชนิดและยังเกี่ยวข้องกับการเกิดมะเร็งด้วย แมคโครฟาจ (macrophage) เป็นเซลล์เม็ดเลือดขาวที่มีบทบาททั้งในภูมิคุ้นกันโดยกำเนิด (innate immunity) และภูมิคุ้นกันแบบจำเพาะ (acquired immunity) รายงานก่อนหน้านี้พบว่ามีการแสดงออกของโปรตีนตัวรับ Notch1 ในแมคโครฟาจที่ได้รับการกระตุ้นโดยไลโปพอลิแซคคาไรด์ (LPS) และอินเตอร์เฟียรอนแกมมา (IFNγ) ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาบทบาทของ Notch1 ในแมคโครฟาจโดยใช้เทคนิค RNAi เพื่อลดการแสดงออกของ Notch1 แบบจำเพาะในเซลล์ไลน์แมคโครฟาจ RAW264.7 ผลจากการลดการแสดงออกของ Notch1 ทำให้มีการเปลี่ยนแปลงทางสัณฐานวิทยาของแมคโครฟาจ มีการเพิ่มขึ้นของการผลิตโมเลกุลที่เกี่ยวข้องกับการอักเสบ ได้แก่ ไนตริกออกไซด์ (nitric oxide) การแสดงออกของโมเลกุล MHC class II บนผิวเซลล์แมคโครฟาจ และการแสดงออกของไซโตไคน์ (cytokines) ได้แก่ *IL-10 IL-12p40* และ *TNFα* รวมทั้งการแสดงออกของจีนเป้าหมายของวิถีสัญญาณ Notch ได้แก่ *Deltex1* ก่อนได้รับการกระตุ้นโดย LPS/IFNγ และลดลงในช่วงระยะเวลากระตุ้นซึ่งแตกต่างจากเซลล์ชุดควบคุมอย่างมีนัยสำคัญ แมคโครฟาจที่มีการลดการแสดงออกของ *Notch1* มีประสิทธิภาพในการฆ่าแบคทีเรียทั้งแกรมบวกและแกรมลบที่ดีขึ้น สอดคล้องกับการเพิ่มขึ้นของไนตริกออกไซด์และอีเฟคเตอร์โมเลกุลอื่นๆ ในแมคโครฟาจ ดังนั้น วิถีสัญญาณที่ส่งผ่าน Notch1 จึงมีบทบาทสำคัญในการควบคุมการทำงานของแมคโครฟาจ และ Notch1 จึงอาจใช้เป็นโมเลกุลเป้าหมายในการควบคุมการทำงานของแมคโครฟาจได้

ภาควิชาจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2552

4972286023 : MAJOR INDUSTRIAL MICROBIOLOGY

KEYWORDS : MACROPHAGE / NOTCH SIGNALING / RNAi

THITIPORN PATTARAKANKUL: USING RNAi TECHNIQUE TO INVESTIGATE THE ROLES OF *NOTCH1* GENE IN MACROPHAGES. THESIS ADVISOR: ASST. PROF. TANAPAT PALAGA, Ph.D., 94 pp.

Notch gene encodes a protein family of conserved transmembrane receptors expressed on a cell surface. There are four Notch genes, *i.e. Notch1*, *2*, *3* and *4* in veterbrates. Five ligands have been identified, *i.e.* Jagged-1 and-2 and Dll-1, 3 and 4. Notch signaling is initiated with the interaction of receptor and ligand. Notch signaling is involved in maintenance of stem cells, binary cell-fate decisions, induction of terminal differentiation and also in tumorigenesis. Macrophages are white blood cells performing important roles in both innate and acquired immunity. Previously, it was reported that Notch receptors are expressed in lipopolysaccharide (LPS)/interferon gamma (IFNγ) - activated macrophages. To investigate the role of Notch1 in macrophages in this study, RNAi technique was applied to specifically silence *Notch1* expression in RAW264.7 macrophage-like cell line. Silencing of *Notch1* resulted in cellular morphological alteration, and increased pro-inflammatory molecule production such as the nitric oxide and MHC class II and cytokines expression including *IL-10, IL-12p40* and *TNFα.* We unexpectedly found that one of the target genes of Notch signaling *Deltex1* was significantly up-regulated in the absence of LPS / IFNγ stimulation. Knock down Notch1 in macrophages increased efficacy in bactericidal activities against both gram negative and positive bacteria. Therefore, Notch1 is involved in effector functions of macrophages during both innate and acquired immune responses. Hence, Notch1 might be the therapeutic target for manipulating macrophage functions.

Department Microbiology Student's Signature .....

Field of Study Industrial Microbiology Advisor's Signature .....

Academic year 2009

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and profound gratitude to my advisor, Assistant Professor Dr. Tanapat Palaga for skillful supervision, excellent guidance and charitable encouragement throughout my thesis work.

I am also grateful to Associate Professor Dr. Suthep Thaniyavarn and Assistant Professor Dr. Wanchai Assavalapsakul for serving as the chairman and the committee. My gratitude is extended to the external committee, Dr. Choowong Auesukaree for his kind suggestions and valuable comments.

I would like to thank Max-Plank Institute for Infection Biology, Germany for *Escherichia coli* DH5α and Associate Professor Dr. Sirirat Rengpipat for *Staphylococcus aureus* ATCC 25923. Special thanks to Mr. Noppadol Sa-Ard-Iam for his great help, valuable advice and suggestion in FACS analysis. I also appreciate the supports from the Department of Microbiology, Faculty of Science, Chulalongkorn University for providing research facilities. I would like to extend my sincere appreciation for all my teachers, my friends and especially my 403 immunology laboratory members for their advices, assistant, kindness and friendship.

Finally, I would like to dedicate this successful to my beloved father, mother and my lovely family who encourage and love me always.

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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.	x	Fold
10.	∞	Infinity
11.	A	Absorbance
12.	Ab	Antibody
13.	bp	Base pair
14.	cDNA	Complementary DNA
15.	CFU	Colony forming unit
16.	CO_2	Carbon dioxide
17.	DNA	Deoxyribonucleic acid
18.	dNTP	dATP, dCTP, dGTP and dTTP

19.	ELISA	Enzyme-linked immunosorbent assay
20.	g (centrifugation speed)	Gravity
21.	GSI	Gamma secretase inhibitor
22.	hr	Hour
23.	*Hes1*	Hairy/Enhancer of Split1
24.	HRP	Horse radish peroxidase
25.	IFNγ	Interferon gamma
26.	IL	Interleukin
27.	*iNos*	Inducible nitric oxide synthase
28.	kDa	Kilo Dalton
29.	LB	Lauria bertani
30.	LPS	Lipopolysaccharide
31.	m	Murine
32.	mA	Milliampere
33.	mg	Milligram
34.	MHC class II	Major histocompatibility complex class II
35.	ml	Milliliter
36.	mM	Millimolar
37.	nm	Nanometer
38.	No.	Number

39.	OD	Optical density
40.	PAGE	Polyacrylamide gel electrophoresis
41.	PBS	Phosphate buffer saline
42.	PBST	Phosphate buffer saline – Tween
43.	pBAsi mU6 Neo DNA	Plasmid BAsi mouse U6 Neo DNA
44.	pShLuc	Plasmid encode short hairpin luciferase
45.	pShNotch1	Plasmid encode short hairpin Notch1
46.	PCR	Polymerase chain reaction
47.	psi	Pound per square inch
48.	PVDF	Polyvinylidine fluoride
49.	r	Recombinant
50.	RNA	Ribonucleic acid
51.	RNAi	RNA interference
52.	rpm	Round per minute
53.	RT	Reverse transcription
54.	SDS	Sodium dodecyl sulfate
55.	siRNA	Short interfering RNA
56.	T_0	Time 0 h
57.	T_{24}	Time 24 h
58.	*TNFα*	Tumor necrosis factor alpha

59.	U	Unit
60.	v	Volume
61.	-ve	Negative control
62.	w	Weight
63.	α	Alpha
64.	β	Beta
65.	γ	Gamma

CHAPTER I

BACKGROUND

Notch gene was first identified in *Drosophila melanogaster* with the notches at the end of their wing blades by Thomas Hunt Morgan and colleagues in 1917 (Radtke and Raj, 2003). *Notch* encodes a family of conserved transmembrane proteins and the Notch signaling pathway is highly conserved throughout the animal kingdom (Allenspach *et al.*, 2002). The molecules in the Notch signaling pathway include Notch receptors and theirs ligands and the cytoplasmic and nuclear proteins such as numb, CSL/RBP-Jκ and mastermind-like protein. Both the Notch receptors and their ligands contain the primarily epidermal growth factor (EGF) –like repeats in their ectodomain. The interaction between Notch receptor and the ligand leads to two enzymatic cleavage events in the Notch receptor molecule. These steps liberate the active intracellular domain of Notch (ICN). ICN translocates to the nucleus and binds to the DNA-binding protein called CSL (CBF1, Su (H) and LAG-1). The co-activators are recruited to this complex for target gene transcription such as *Hairy/Enhancer of Split* (*HES*) and *Hairy-related transcription-factor (HRT)* gene (Davis and Turner, 2001).

The functions of Notch signaling pathway includes maintenance of stem cells (Varnum-Finney *et al.*, 1998), participation in cell-fate decision during development and induction of cellular terminal differentiation (Radtke and Raj, 2003).

Macrophages are white blood cells playing essential roles both in innate and adaptive immunity. The first event in the innate resistance to infection is the activation of macrophages. Macrophages phagocytose pathogens, leading to activation of macrophages which produce a series of pro-inflammatory cytokines such as interleukin (IL)-1, IL-12, tumor necrosis factor-α (TNF-α) and interferon (IFN)-γ (Trinchieri, 1997).

Monsalve and colleagues (2006) reported that different members of the Notch receptors and ligands are expressed in macrophages and that Notch1 and Jagged-1 are up-regulated in activated macrophages (Monsalve *et al.*, 2006). They also showed that Delta-like 4 triggered the proteolysis of the Notch receptor and activation of the signaling pathway in macrophages. Fung and colleagues (2007) found that during human macrophage activation and differentiation, the expression of Notch3 is selectively increased (Fung *et al.*, 2007). In addition, inhibition of Notch signaling pathway using pharmacological inhibitor resulted in a profound negative impact on effector functions of macrophages (Palaga *et al.*, 2008). The details of the roles of each Notch receptor in macrophage activation, however, have not been well characterized.

RNA interference (RNAi) is the phenomenon which induces silencing of specific genes and can be observed in plants, animals and many fungi (Matzke and Birchler, 2005). The intermediate RNA of RNAi is short interfering RNA (siRNA) with the length of

approximately 21-25 nucleotides (nt). This technique is highly valuable in studying gene functions in mammals where targeted deletion or site specific recombination are rare events (McManus and Sharp, 2002).

In this study, the role Notch1 plays in mature macrophages was studied using RNAi to specifically silence *Notch1* expression and the phenotype of Notch1 knockdown macrophages were characterized.

CHAPTER II

LITERATURE REVIEWS

2.1 Notch Signaling Pathway

2..1.1 History of the Notch Signaling Pathway (Chiba, 2006)

Notch signaling pathway was first discovered in 1917 by Thomas Hunt Morgan, in the phenotype of *Drosophila melanogaster* with a notch in the wings. In the 1930s, this phenotype implied that the gene(s) coding for the protein was involved in the process of cell fate decision and neuronal tissue differentiation. It became clear later that *Notch* gene encodes a transmembrane protein expressing on the cell surface and the protein functions as a receptor. In neuronal differentiation, the interaction between Notch receptor and its ligand shift the signal receiving cells to adopt a non-neuronal cells fate by inhibition of signaling pathway leading to neuronal differentiation. This phenomenon is called “lateral specification” which is important for normal embryonic development and can be observed during development of other tissues.

2.1.2 Notch Receptors and Notch Ligands

Notch signaling pathway is highly conserved and plays an important role in the specification of cell fate during development. The family of Notch receptors and ligands in mammals consists of four transmembrane proteins, *i.e.* Notch1, 2, 3 and 4 and five

ligands composes of Jagged1, Jagged2, Delta-like1, Delta-like3 and Delta-like4 (Zeng *et al*., 2005).

The overall Notch receptor exists on the cell surface as heterodimer between the extracellular and intracellular domain. Notch extracellular domain consists of multiple epidermal growth factors (EGF)-like repeats. Notch1 and Notch2 have 36 repeats while Notch3 and Notch4 each have 34 and 29 repeats, respectively. The EGF-like repeats 11 and 12 are crucial for ligand binding. The Lin-Notch repeats (LNR) has inhibitory role against the cleavage by enzymes upon ligand engagement. Notch intracellular domain contains a RAM domain which is shown to associate with CSL protein, and the six ankyrin (ANK) repeats which are responsible for protein-protein interaction. The C-terminal PEST domain negatively regulates the half-life of the Notch proteins (Chiba, 2006) (Figure 2.1A).

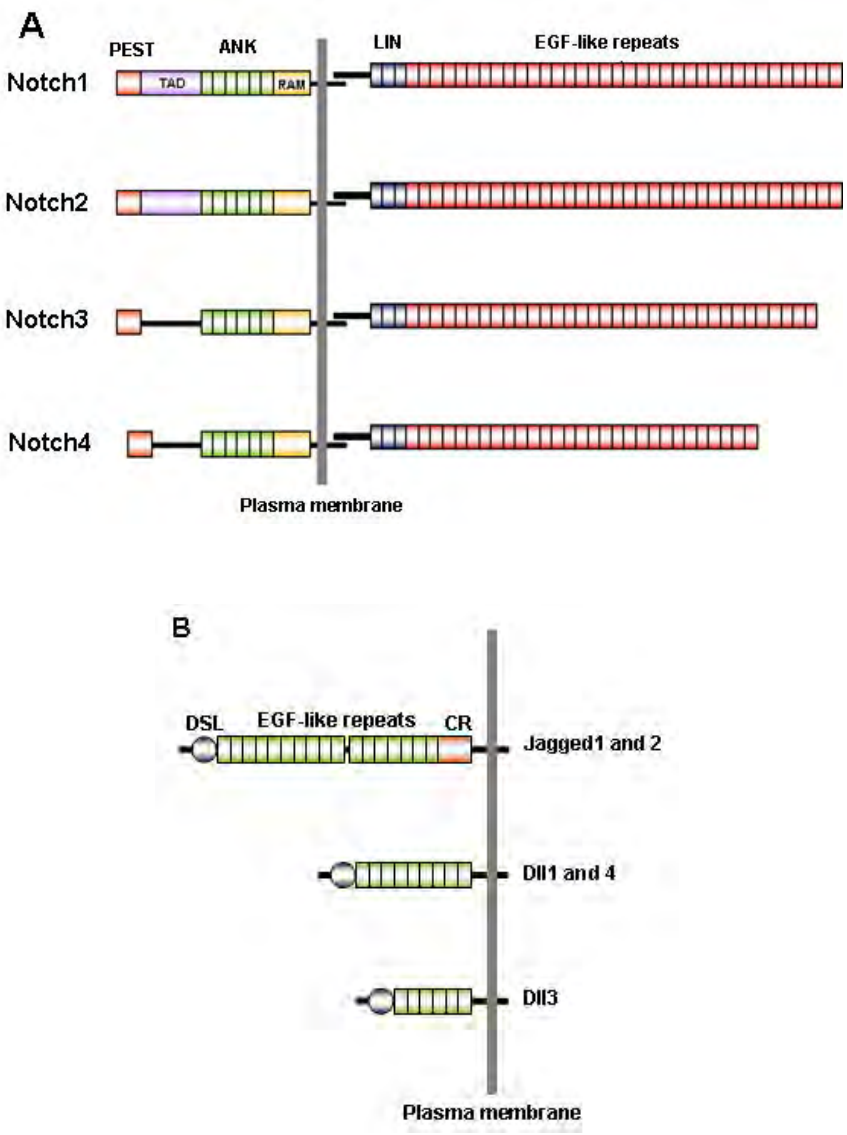


Figure 2.1 Schematic representation of the structure of Notch receptors and Notch ligands. A) Notch1, 2, 3 and 4 are depicted. The extracellular domains comprise of multiple EGF-like repeats and three LIN12/Notch repeats. Intracellular Notch has a RAM domain, six ankyrin repeats, transactivation domain or TAD (in Notch1 and 2) and PEST sequence. B) Notch ligands, Jagged1, 2, Dll1, 4 and 3, are shown. The extracellular domain of each ligand contains a DSL motif, multiple EGF-like repeats and cysteine-rich (CR) (found only in Jagged1 and 2).

Notch ligands are transmembrane proteins that contain an N-terminal Delta, Serrate and LAG-2 (DSL) domain that is required for Notch receptor interactions (Figure 2.1B). The extracellular domains of Notch ligands consist of multiple EGF-like repeats. Notch ligands are divided into two classes which is Delta or Delta-like (Dll) and Serrate-type ligand (referred to as Jagged in mammals), depending on the presence or absence of a cysteine rich (CR) repeat (Bray, 2006).

2.1.3 Activation of the Notch Signaling Pathway (Radtke and Raj, 2003; Bray, 2006)

Notch proteins are synthesized as single precursor protein in the endoplasmic reticulum. During this process, the Notch precursor becomes *O*-fucosylated by interaction with *O*-fucosyl transferase (O-Fut) and is translocated into the Golgi apparatus. In the Golgi, it is cleaved by Furin-like convertase to generate the extracellular and the intracellular plus the transmembrane domains. The extracellular domain is further glycosylated with *O*-Fut and glycosyltransferases such as Fringe before being transported to the cell surface. The glycosylation of Notch receptors appears to play a regulatory role in determining ligand specificity. At the cell surface, Notch receptors interact with their ligands triggers a cascade of two successive proteolytic processing of Notch. The first cleavage is mediated by an enzyme TACE (tumor-necrosis factor-α-converting enzyme/metalloproteinase), and the second cleavage is mediated by the γ-secretase complex (containing multiple subunits including presenilins). Notch intracellular domain (NIC) is liberated from the plasma

membrane by γ-secretase proteolysis and translocated to the nucleus. The released NIC binds to the transcription factor CSL/RBPJκ (Nagel *et al.*, 2005) which, in turn, displaces transcriptional co-repressors (CoR) and recruits transcriptional co-activators, including Mastermind-like protein (MAML) and CREB binding protein (CBP)/p300 (Koch and Radtke, 2007). This process leads to the transcription of downstream target genes (Figure 2.2).

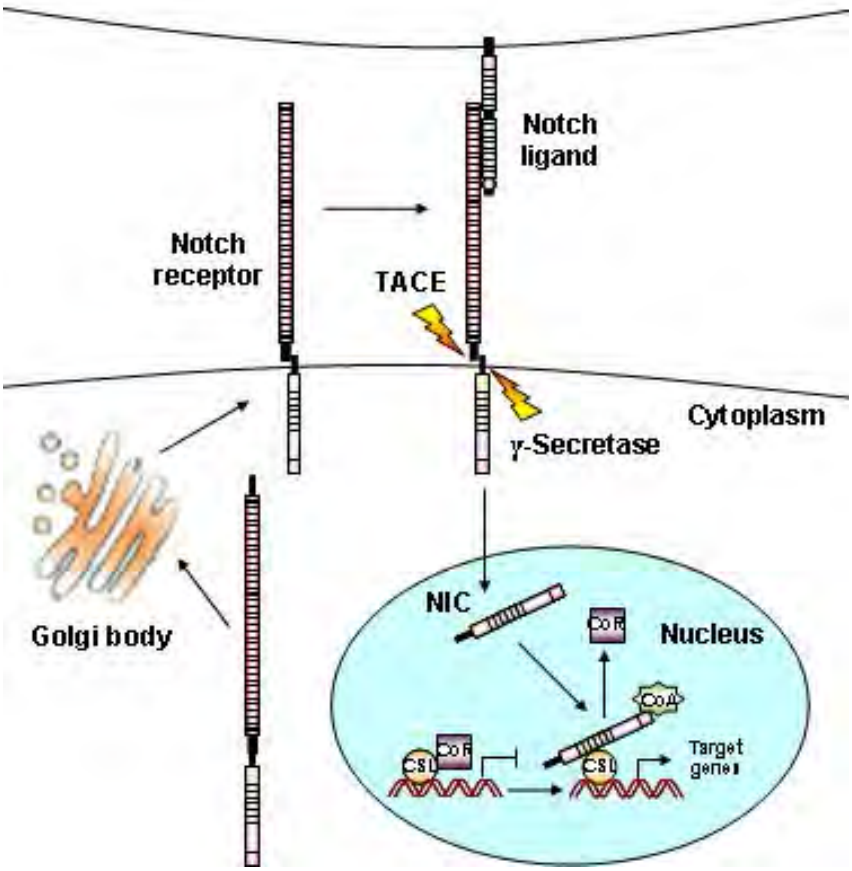


Figure 2.2 The Notch signaling pathway. Notch protein precursor is cleaved in the Golgi body and the heterodimer complex is translocated to the cell surface. Interaction between Notch receptor and its ligands is followed by several enzymatic cleavages. The first cleavage is mediated by an enzyme TACE and the last step is mediated by the γ-secretase complex. The resulted Notch intracellular domain is translocated to the nucleus and displaces co-repressor (CoR) for binding with CSL/RBP-Jk on promoter of the target gene leading to transcription of Notch target genes.

2.1.4 Target Genes of the Notch Signaling Pathway

One of the best characterized target genes of Notch signaling pathway is a group of a basic-helix-loop-helix (bHLH) protein family which is a type of transcriptional regulators. These proteins act as negatively repressors of downstream target genes such as the tissue-specific transcription factors. In most cases, but not always, *Hes1* and *Hes5* encode proteins that belong to the HES (Hairly/Enhancer of Split) protein family. Their expressions are up-regulated by NIC and are essentials for prevention of differentiation in neural precursor cells in mouse embryos (Iso *et al*., 2003). *Hes1* is the target gene of its own repression by a feedback regulatory mechanism. *Mash1* is a proneural gene and also a potential target gene of *Hes1*. *Hes1* acts as a negative regulator of neurogenesis by directly repressing *Mash1* gene (Iso *et al*., 2003). Recently, HERP has been isolated as a new bHLH family and the amino acid sequence in the basic domain of HERP1 is highly similar to HES1, indicating that HERP family is closely related to the HES family. It has been found that HERP1 negatively regulates its own gene expression (Iso *et al*., 2003). *Hey* gene is a member of the HERP family. Hey1, Hey2 and HeyL are expressed in a dynamic pattern in multiple tissues of the mouse embryo (Fischer *et al*., 2004). *Hey1* and *Hey2* were identified as crucial genes for Notch signaling pathway during vascular development in mammals (Fischer *et al*., 2004). HES1, HERP1 and HERP2 are primary target of Notch signaling pathway (Iso *et al*., 2003). In addition, *Deltex* is another reliable target gene of the Notch pathway. It is postulated to function as a negative regulator of Notch activity by antagonizing the

interaction between Notch and Suppressor of Hairless in *Drosophila* (Matsuno *et al*., 2002; Blacklow, 2005).

2.1.5 Biological Functions of the Notch Signaling Pathway

2.1.5.1 Notch Signaling Pathway in Hematopoietic System

The cell fate decisions of precursors in a wide range of the animal developments are known to be mediated by Notch signaling. Notch1, Notch2 and Notch3 are expressed in immature as well as mature blood cells and lymphocytes, suggesting crucial roles during development of hematopoietic system (Radtke *et al*., 2004).

Notch2 is expressed at higher level than Notch1 in hematopoietic stem cells (HSCs). Varnum-Finney and colleagues (1998) reported that in the stem cell compartment, the individual members of Notch family have non-overlapping distinct roles. They found that the survival of HSCs from mouse and human were increased when they are incubated with the Notch ligand, Jagged1. In human HSCs, a similar result was obtained when HSCs are exposed to human Dll1 and Dll4 (Varnum-Finney *et al*., 1998). In addition, Duncan and colleagues (2005) found that in HSCs, activation of the Notch signaling pathway inhibits HSC differentiation (Duncan *et al*., 2005).

The cytokine induced differentiation in myeloid progenitor cell lines was inhibited when intracellular Notch1 was overexpressed, suggesting that the Notch signaling pathway controls an early myeloid development (Milner *et al*., 1996). Schroeder and colleagues (2000) found that myeloid differentiation is promoted by the Notch signaling pathway (Schroeder and Just, 2000) while myeloid linage differentiation from progenitor

cells was inhibited by Notch signaling (Suzuki and Chiba, 2005). Monocytes express high levels of both Notch1 and Notch2 (Ohishi *et al.*, 2000). Stier and colleagues (2002) found that Notch signaling promoted the numbers of renewal stem cells and the differentiation of lymphoid lineage cells in murine bone marrow cells (Stier *et al.*, 2002). In an *in vitro* assay, Jagged1 was used to stimulate human HSCs, resulting in enhancement of HSC proliferation but no differentiation was observed. For an *in vivo* system, when HSCs were treated with Jagged1, repopulation of the lymphoid lineage cells and differentiating myeloid lineage cells were observed (Karanu *et al.*, 2000).

The high levels of Notch1 expression were reported in monocyte/macrophage precursors and Notch1 and Notch4 were expressed in high levels in maturing macrophages (Singh *et al.*, 2000). Mast cells and megakaryocytes both expressed high levels of Jagged1 in terminally maturing state. Singh and colleagues (2000) suggested that the distinct pattern of corresponding proteins could control cell fate decisions and the communication of lineage compartments during hematopoietic development (Singh *et al.*, 2000).

2.1.5.2 Notch Signaling in B Cell Lineage

Mature B cells express Notch2 (Saito *et al.*, 2003). Deletion or inactivation of CSL/RBP-Jk in B cells resulted in normal development of B cells in the bone marrow. Therefore, CSL-dependent Notch signaling is not essential for early B cell development (Tanigaki *et al.*, 2002).

During B cell maturation, expression of Notch2 is increased and most noticeable in splenic B cells, indicating that Notch2 may be necessary for the development of marginal zone B cells (MZB) in the spleen. Dll1 is shown to be a non-redundant ligand for Notch2 during MZB cell development (Hozumi *et al*., 2003). Inactivation of *Notch1* in the bone marrow precursor cells in a mouse model resulted in development of B cells in stead of T cells in the thymus. These results indicated that the absence of Notch1 in lymphoid precursors leads to developing B cells at the cost of T cell linage choice (Wilson *et al*., 2001).

2.1.5.3 Notch Signaling in T Cell Lineage

Several groups of researches have investigated the role of Notch signaling in T cell development. Notch receptors such as Notch1, 2 and 3 and Notch ligands, including Jagged1 and 2, have been shown to be expressed in thymic stromal cells, thymic epithelium cells and thymocytes (Robey, 1999; Deftos and Bevan, 2000)

Targeted deletion of *Notch1* in hematopoietic cells resulted in the absence of T cell development with significant increasing B cells in thymus (Radtke *et al*., 1999). In contrast, intracellular Notch1, which is a constitutively active form of Notch receptor, in the bone marrows led to development of CD4 and CD8 double-positive T cells and suppression of B cell development in the bone marrow. These results indicated that Notch1 and its mediated signaling pathway are crucial for T cell specification during T cell versus B cell decision (Hozumi *et al*., 2004).

The expression of several Notch receptors in peripheral $CD4^+$ and $CD8^+$ T cells were reported and inactivation of Notch signaling by the pharmacological inhibitors led to decreased in T cell proliferation and IFNγ production in both CD4+ and CD8+ T cells after stimulation with T cell receptor *in vitro* (Palaga *et al*., 2003). When purified $CD4^+$ T cells were co-cultured with a Dll1-Fc fusion protein, it led to increased IFNγ production. Taken together, these results indicated that the interaction of Dll1 and Notch3 could direct $CD4^+$ T cells towards IFNγ -secreted T_H1-cell fate (Maekawa *et al*., 2003).

When $CD4^+$ T cells were co-cultured with Jagged1 expressing antigen presenting cells (APCs), a T_H2-type cells were obtained which produced mainly IL-4 and IL-5 (Amsen *et al*., 2004). In murine $CD4^+$ T cells lacking CSL/RBP-Jκ, it was shown that IL-4 production was decreased (Tanaka *et al*., 2006). This result indicated that the function of RBP-Jκ is upstream of IL-4 and RBP-Jκ-dependent pathways might lead to IL-4 expression and secretion (Tanaka *et al*., 2006).

When $CD4^+$ T cells were transfected with plasmid encoding Dll1, it was found that upon stimulation through CD3 and CD28, the population of human $CD4^+CD25^+$ regulatory T cell appeared (Ng *et al*., 2001). In addition, when APCs with Jagged1 overexpression were co-cultured with naive $CD4^+$ T cells, it led to cells with the regulatory T cell phenotypes (Hoyne *et al*., 2000). Consistent with this finding, the expression of activated Notch3 in transgenic mice was shown to increase the percentages of $CD4^+CD25^+$ regulatory T cells (Anastasi *et al*., 2003).

2.2 Macrophages

Macrophages are mononuclear phagocytes of the white blood cells. Monocytes are the blood borne precursors of macrophages. Both monocytes and macrophages are present in the blood but only macrophages settle in the tissues and become resident macrophages.

2.2.1 Biological Functions of Macrophages

During infection, macrophages are attracted to the site of pathogen invasion, recognize and bind to pathogens. This step is followed by ingestion or phagocytosis and degradation of the pathogens within macrophages. The major biological functions of macrophages are as follows.

2.2.1.1 Phagocytosis

Polymorphonuclears (PMNs), monocytes and macrophages are the professional phagocytes (Rabinovitch, 1995). Phagocytosis is the main process for removing the pathogens from the body and there are several steps for this process. Phagocytosis is the uptake of any particles with the size over 0.5 μm (Aderem and Underhill, 1999). During infection, macrophages first move towards the infected pathogens. Following the recognition step, the microbes are attached to the phagocytic cell surface and the endocytosis takes place, which leads to the formation of phagosome inside the phagocytes. This phagosome later fuse with lysozyme and the microorganisms inside phagolysosome are killed. In addition, macrophages secrete a variety of crucial chemical mediator molecules such as radicals of oxygen species and nitric oxide and

cytokines that affect the behavior of the phagocytes and other cells involved in an immune response (Rabinovitch, 1995). There are some distinctive ligands on apoptotic cells which cannot be found on viable healthy cells. These ligands are recognized by macrophages in order to phagocytose the apoptotic cells for removal (Aderem and Underhill, 1999).

2.2.1.2 Antigen Presentation (Lydyard *et al*., 2004)

Antigen presentation is the process which peptide antigens are degraded and presented to T lymphocytes. The fragment of peptide antigens form a complex with the major histocompatibility complex (MHC) molecules on the cell surface of macrophages and other professional antigen presenting cells for T cell recognitions. MHC is the major gene locus critical in regulating T cell response. MHC is consisted of six major loci and is highly polymorphic. There are two classes of MHC molecules involved in antigen presentation, MHC class I and II. The peptide binding domain of MHC class I and class II can accommodate peptides with the length in ranging 8-10 and 10-20 amino acid residues, respectively.

Cells expressed MHC class II molecules, including B cells, dendritic cells and macrophages, plays an essential role in activation of $CD4^+$ helper T cells. The expressions of MHC class I and/or II molecules were regulated mainly by cytokines and activating stimuli.

2.2.2 Antigen Recognition and Activation of Macrophages

2.2.2.1 Toll-like Receptors (TLRs) (Lydyard *et al*., 2004)

TLRs are the transmembrane protein receptor and were first identified in the fruit fly *Drosophila* as a molecule that required during embryogenesis. It was later observed that Toll loss of function mutations were highly receptive to fungi and Gram-positive bacteria infections. These observations indicated that this molecule might be involved in immune defense of insect. Mammalian homologs of fruit fly TLRs were later identified and found to be conserved in protozoan. Recently, more than ten of the members were identified in mammals and form a large receptor protein family (Akira, 2003).

TLRs or Toll proteins are members of the protein family that have the domains of extracellular leucine-rich repeat and cytoplasmic domain. This protein structure mediates ligand recognition and signal activation of variety effector genes. The different TLRs proteins recognize pattern of molecular structure in different pathogens and can discriminate different pathogen groups (Table 2.1).

The different TLRs distinguish between the major molecular markers of the pathogens, such as peptidoglycan, lipopolysaccharide (Gram-negative bacteria), teichoic acids (Gram-positive bacteria), glucan and arabinomannans (yeast and fungi). Most of the TLRs are expressed on the cell surface of phagocytic cells and other cell types such as epithelial cells.

Table 2.1 TLRs and Ligands

Toll-like receptor	Identified Ligand	Pathogen
TLR1	Lipopeptides	Gram-negative bacteria Mycobacteria
TLR2	Lipoteichoic acid Lipoarabinomannan Zymosan	Gram-positive bacteria Mycobacteria Fungi
TLR3	dsRNA	Viruses
TLR4	LPS	Gram-negative bacteria
TLR5	Flagellin	Bacteria
TLR6	di-acyl lipopeptides	Mycobacteria
TLR9	CpG-DNA	Bacteria

2.2.2.2 Lipopolysaccharide (LPS)

LPS is the major component of cell wall found mainly in Gram-negative bacteria, functioning in protecting the chemical attack. It is a prototypical endotoxin which can induce innate immune response in animals. LPS binds to CD14, TLR4 and MD2 receptors that leads to the production of pro-inflammatory cytokines in macrophages and other innate immune cells (Lydyard *et al*., 2004)

The components of LPS include lipid A, core oligosaccharide and O-antigen. Lipid A consists of phosphate, glucosamine, disaccharides and multiple fatty acids. Lipid A is the major motif for toxicity in Gram-negative bacteria. The fragments that contain lipid A lead to several diseases (Lydyard *et al*., 2004). Core oligosaccharides are next to the lipid A and contain heptose and 3-deoxy-D-mannooctulosonic Acid (Hershberger and Binkley, 1968). The third component, O-antigen, is attached to the core oligosaccharide and in each strains of bacteria, the O components is vary. LPS are B cell mitogens and a powerful stimulus for macrophages (Lydyard *et al*., 2004).

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

IFNγ are type II IFN which is expressed by several cell types in response to bacterial and viral infection. IFNγ is the pro-inflammatory cytokine that mediates the immune response against bacterial and viral infection. This cytokine is crucial for limiting infection during the development of specific humoral and cell mediated immune response.

IFNγ is the primarily cytokine in the adaptive immune system. It is essential for not only for viral infection but also necessary for the regulation of cell mediated immune response (Trinchieri, 1997). IFNγ perform significant role in induction of Th1-type immune response. It is also important for stimulation of macrophage, enhancing intracellular bactericidal activity of macrophage.

IL-12 induction is induced when animals are administrated by LPS. This can lead to IFNγ overexpression and lethality shock (Wysocka *et al*., 1995).

2.3 Notch Signaling Pathway and Macrophages

Notch ligands, Jagged1 and Dll1, but not Jagged2, were detected in bone marrow derived macrophages. Jagged1 in these cells was up-regulated with the additions of hematopoietic growth factor, suggesting that there is an involvement of macrophages and their growth factors in hematopoietic cell through Jagged1 production (Nomaguchi *et al.*, 2001)

Expression of Dll4 was increased in exposure to pro-inflammatory stimuli such as LPS in macrophages. Co-incubation of macrophages with cells expressing Dll4 activated Notch signaling pathway and the transcription of pro-inflammatory gene, including *iNOS*, mitogen-activated protein kinase, NF-κB and also Dll4 expression (Fung *et al.*, 2007).

RAW264.7 macrophage-like cell line expresses Notch1, 2 and 4 as well as Jagged1 and 2. When these cells were treated with LPS and/or IFNγ, it led to up-regulation of Notch1 and Jagged1. When a truncated inctracellular Notch1 domain was overexpressed in RAW264.7 cells, the levels of MHC class II was up-regulated, but *iNOS* expression and nitric oxide production decreased (Monsalve *et al.*, 2006).

The studies on the involvement of Notch signaling so far have been focused on developmental aspects of the cells and in effector functions of T and B lymphocytes. From the series of these works, it becomes clear that Notch signaling is involved in lymphocyte developments, maturations, activation and transformation (Fung *et al.*, 2007). On the other hand, the Notch signaling also affects the myeloid differentiation,

but the role of this signaling in effector phase of macrophages remain unclear, especially the contribution of individual Notch receptor during immune response of macrophages have not been investigated in details.

2.4 RNA interference (RNAi)

To study gene function, a forward genetic approach that generates the mutants and studies the phenotype of the mutants was highly utilized but it was not suitable for organisms with large scale genome. In stead of the forward genetic approach, the reverse genetic approach is the most effective to find out the functions of gene but the methods to study reverse genetics are time and money consuming. Antisense methods including antisense oligonucleotide and ribozyme techniques have advantages in reverse genetics but they have the limitation. Thus, a newly discovered phenomenon called RNAi is the efficient technology to knock down specific genes and study the gene function (McManus and Sharp, 2002).

2.4.1 Discovery of RNAi

Guo and Kemphues reported that both injected sense and antisense RNAs resulted in the phenocopies (Guo and Kemphues, 1995). This was later proved by Andrew Fire and Craig Mello in *Caenorhabditis elegans* that injection with sense and antisense RNAs produced the specific knock down phenotype (Fire *et al*., 1998). This technology using double stranded RNA (dsRNA) silencing for knock down a specific genes called RNA interference or RNAi. Consequently, in *Drosophila melanogaster*,

plant and undifferentiated mammalian cells the similar phenomenon were observed and widely used to specifically silent expression of genes (Marques and Williams, 2005).

2.4.2 Mechanism of RNAi

RNAi initiated by dsRNA is cleaved by an RNaseIII enzyme called Dicer into 21-26 nucleotides in length of small RNAs (Carmell and Hannon, 2004). Subsequently, multi-subunit enzyme complex, including the RNA-induced silencing complex (RISC) and Argonaute2 (AGO2), binds to siRNA and the sense strand is degraded (Figure 2.3). This reaction resulted in formation of the activated complex with an antisense strand of siRNA as a guide (Matranga *et al*., 2005). This activated AGO2-RISC complex attempts and binds to target mRNA strand with the complementary sequence. The specifically recognized mRNAs are degraded and expression of the target genes is inhibited (de Fougerolles *et al*., 2007).

Due to the high specificity of the process in gene silencing, the RNAi technique can be used for development of the new class of drugs. This development can be achieved by utilizing siRNA to interfere the genes that promoting diseases.

In the infection of murine lung with respiratory syncytial virus (RSV) and parainfluenza virus (PIV), the siRNA therapy by inhalation of the delivery naked siRNA and RNAi mediated the silencing of viral proteins (Bitko *et al*., 2005). In addition, *in vivo*, cholesterol conjugated with naked siRNA has been used successfully to increase efficacy of gene knock down (Soutschek *et al*., 2004).

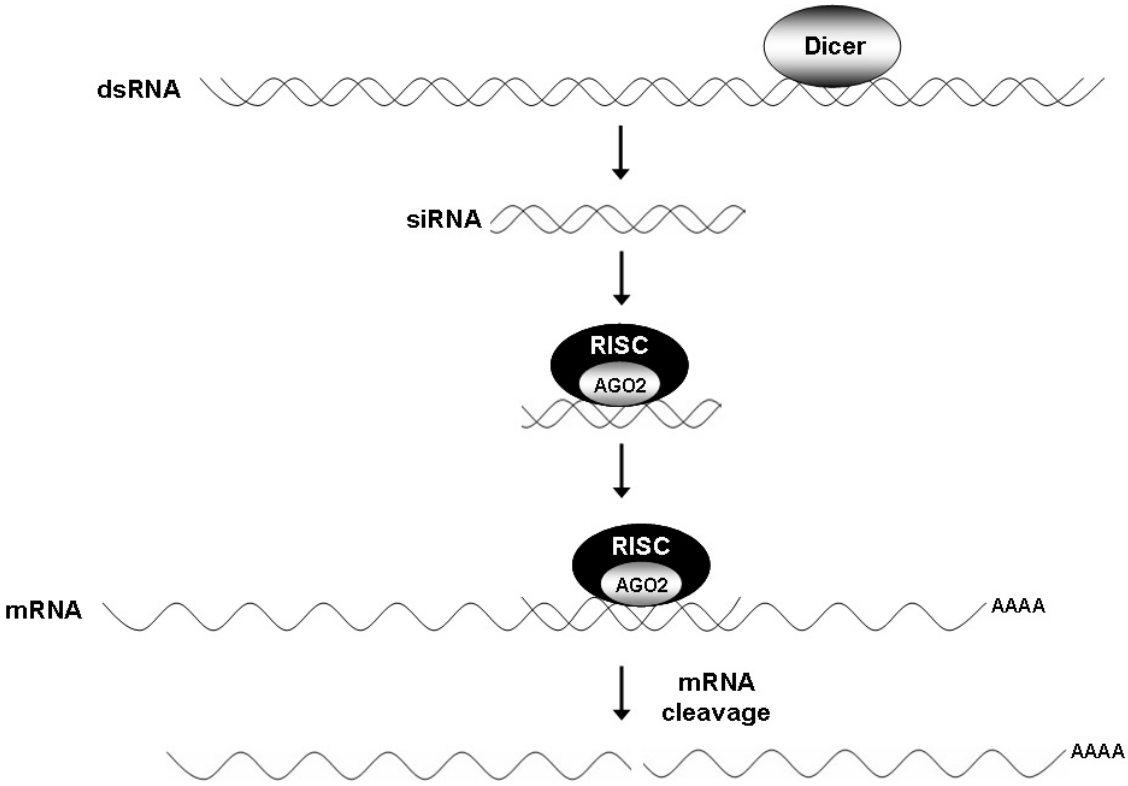


Figure 2.3 Mechanism of RNAi by small RNAs. siRNA begins with processing of long dsRNAs (21-26 nt) by Dicer enzyme. Argonaute (AGO2) and the RNAi-induced silencing complex (RISC) bind the siRNA and antisense strand of siRNA is cleaved by AGO2. This reaction is followed by the antisense strand of siRNA leading this complex to complementary target mRNA strand. This specific mRNA is degraded after this recognition and the expression of gene is silenced (de Fougerolles *et al*., 2007).

Objectives

Notch activations have pleiotropic effects upon development of many cell types, including macrophages. The effects of Notch signaling pathway on effector functions, however, remains unclear. In addition, the contribution of individual Notch receptor has not been investigated in details in macrophages. As described previously, RNAi is the useful approach for suppressing expression of specific gene. Therefore, the aims of this investigation are as follows:

1) To construct plasmid for silencing of *Notch1* expression in RAW264.7 macrophage cell line

2) To characterize the phenotypes of Notch1 silenced macrophages

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

1.	-20°C Freezer model MDF-U332	Sanyo, Japan
2.	-70°C Deep Freezer model ULT1780	Forma Scientific, USA
3.	37°C Incubator	Memmert, Germany
4.	4°C Refrigerator	Mitsubishi Electric, Japan
5.	5% CO_2 Incubator model 311	Thermo Electron Corporation, USA
6.	500 ml Bottle Top Vacuum Filter Necks 0.2 µm	Corning Incorporation, USA
7.	Autoclave model MLS 3020	Sanyo, Japan
8.	Balance	Mettler Toledo, Switzerland
9.	Bench-Top Centrifuge model Stratagene	Profuge, USA
10.	BioTrace Polyvinylidine Fluoride (PVDF) Membrane	PALL Life Science, USA
11.	Cell Culture Dish 35 mm	Corning Incorporation, USA
12.	Centrifuge : Rotafix32	Hettich, Germany
13.	Centrifuge Tube 15 and 50 ml	Corning Incorporation, USA

14.	Cryogenic Vial 2.0 ml	Corning Incorporation, USA
15.	Cuvette 0.4 cm	Bio-Rad, USA
16.	CX2 Inverted Microscope	Olympus, USA
17.	Flow Cytometer : BD FACSCalibur™	BD Bioscience, Canada
18.	Gel Documentation and Quantity One 4.4.1	Bio-Rad, USA
19.	Haemocytometer	Boeco, Germany
20.	High Performance Chemiluminescence Film: Amersham Hyperfilm™ ECL	Amersham Bioscience, England
21.	Hot Air Oven model D06063	Memmert, Germany
22.	Hypercassette™	Amersham Bioscience, England
23.	IX71 Inverted Microscope	Olympus, USA
24.	Laminar Flow Cabinet model H1	Lab Service LTD part, Thailand
25.	Liquid Nitrogen Tank 34 HC Taylor Wharton Cryogenic	Harsco Corporation, USA
26.	Magnetic Stirrer	Clifton, USA
27.	Microcentrifuge tube 1.5 ml	Axygen Scientific, USA
28.	Micropipette P2, P20, P100 and P1000	Gilson, France
29.	Microplate Reader : Elx 800	Bio-Tek Instrument, Canada
30.	Mini Gel Electrophoresis Unit for DNA, RNA and Proteins Mupid-2 Advance	Cosmo Bio, Japan
31.	MJ Mini™ Thermal Cycler	Bio-Rad, USA

32.	PCR tube 200 µl	Corning Incorporation, USA
33.	Petri Dish	Hycon, Germany
34.	pH-meter model S20-K	Mettler Toledo, Switzerland
35.	Pipette Aid	Drummond, USA
36.	Power Supply	Bio-Rad, USA
37.	Protein III System for SDS-PAGE	Bio-Rad, USA
38.	Qubit® fluorometer	Invitrogen, England
39.	Refrigerated Centrifuge model 1920	Kubota, Japan
40.	RNase-free Tip 2, 100 and 1000 µl	Corning Incorporation, USA
41.	Semi-dry Electrophoretic Transfer Cell	Bio-Rad, USA
42.	Spectrophotometer model Lambda 25	Perkin-Elmer, USA
43.	Sterile Aerosol Pipette Tips P2, P40 and P200	Labcon, USA
44.	Syringe 1 ml	Nitro, Japan
45.	Syringe Filter 0.2 µm	PALL Life Science, USA
46.	Thermal Cycler model TC-96/G/H (b)	Bioer, Japan
47.	Thermomixer Compact	Eppendorf, Germany
48.	Tissue Culture Plate 12, 24 and 96 well	Corning Incorporation, USA
49.	Ultra-Pure Water Purification System	Elga, England
50.	Vortex mixer model G560E	Scientific Industries, USA
51.	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.	1 Kb DNA ladder	Fermentas, Canada
5.	Absolute ethanol	Merck, Germany
6.	Absolute methanol	Merck, Germany
7.	Acrylamide and Bis-acrylamide solution	Bio-Rad, USA
8.	Agar	
9.	Agarose Gel	Research Organics, USA
10.	Ammonium persulfate	Bio Basic Inc., Canada
11.	Ampicillin	Bio Basic Inc., Canada
12.	Anti-Mouse I-Ab MHC biotin conjugate	Caltag Laboratories, USA
13.	Bactotryptone	Becton, Dickinson and company, France
14.	*Bam* HI	Fermentas, Canada
15.	BCA (bicinchoninic acid)™ protein assay	PIERCE, USA
16.	Bovine Serum Albumin (BSA)	Sigma Aldrich, USA
17.	Bromophenol blue	Sigma Aldrich, USA
18.	Chloroform	Lab-Scan, Ireland
19.	Diethylpyrocarbonate (DEPC)	Sigma Aldrich, USA

20.	Dimethylsulfoxide (DMSO)	Sigma Aldrich, USA
21.	Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany
22.	Dithiothreitol (DTT)	USB Corporation, USA
23.	Donkey anti-rabbit IgG-HRP	GE Healthcare, USA
24.	Endotoxin-free water	Sigma Aldrich, USA
25.	Ethylenediaminetetraacetic acid (EDTA)	Merck, Germany
26.	*Escherichia coli* DH5α	Max-Plank Institute for Infection Biology, Germany
27.	Ethidium Bromide	Sigma Aldrich, USA
28.	Fetal Bovine Serum (FBS)	Hyclone, England
29.	Film Developer and Fixer	J. Nasen Co.,Ltd., Thailand
30.	FuGene HD Tranfection Reagent	Roche, Germany
31.	Glycerol	Cario ERBA, France
32.	HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid	Hyclone, England
33.	*Hin* dIII	Fermentas, Canada
34.	HPLC grade water	Merck, Germany
35.	Hydrochloric acid (HCl)	Merck, Germany
36.	Hydrogen peroxide (H_2O_2)	Merck, Germany
37.	Hypure™ Molecular Biology Grade Water	Hyclone, England
38.	Isopropanol	Merck, Germany

39.	Lipopolysaccharide from *E. coli* serotype O26:B6	Sigma Aldrich, USA
40.	Luminol	Fluka, Czech Republic
41.	M-MuLV Reverse Transcriptase	Fermentas, Canada
42.	Maxima^{TM} SYBR Green qPCR master mix	Fermentas, Canada
43.	Mouse anti-actin antibody	Chemicon International, USA
44.	NED (N-1-naphthylethylenediamine dihydrochloride)	Sigma Aldrich, USA
45.	Nonidet P-40	Bio Basic Inc., Canada
46.	Opti-MEM	Invitrogen, England
47.	Paraformaldehyde	Sigma Aldrich, USA
48.	p-coumaric acid	Sigma Aldrich, USA
49.	Penicillin	General Drugs House Co., Ltd., Thailand
50.	Plasmid pBAsi mouseU6 Neo	Takara, Japan
51.	Potassium chloride (KCl)	Merck, Germany
52.	Potassium di-hidrogen phosphate (KH_2PO_4)	Merck, Germany
53.	Prestained molecular weight marker	Fermentas, Canada
54.	Protease Inhibitor Cocktail Tablets	Roche, Germany
55.	QIAGEN Plasmid midi kit	Qiagen, Germany
56.	QIAprep Spin Miniprep Kit	Qiagen, Germany

57.	QIAquick Gel Extraction Kit	Qiagen, Germany
58.	Quant-iT™ RNA Assay Kit	Invitrogen, England
59.	Rabbit anti-Notch1 antibody	Santa Cruz Biotechnology, USA
60.	Random Hexamer Primer	Qiagen, Germany
61.	Recombinant mouse interferon-γ	R&D systems, Inc., USA
62.	Ribolock™ Ribonuclease Inhibitor	Fermentas, Canada
63.	Dulbecco's modified minimum essential medium (DMEM)	Hyclone, England
64.	Skim milk	Becton Dickinson and company, France
65.	Sodium dodecyl sulfate (SDS)	Amersham Bioscience, England
66.	Sheep anti-mouse IgG-HRP	Amersham Bioscience, England
67.	Sodium azide (NaN_3)	Merck, Germany
68.	Sodium bicarbornate ($NaHCO_3$)	Sigma Aldrich, USA
69.	Sodium chloride (NaCl)	Merck, Germany
70.	Sodium hydrogen carbonate ($NaHCO_3$)	Sigma Aldrich, USA
71.	Sodium nitrite ($NaNO_2$)	Carlo Erba, Italy
72.	Sodium pyruvate	Hyclone, England
73.	*Staphylococcus aureus* ATCC 25923	ATCC, USA
74.	Streptavidin PE	BioLegend, USA
75.	Streptomycin	M & H Manufacturing Co., Ltd.,

		Thailand
76.	Sulfanilamide ($C_6H_8N_2O_2S$)	BDH Chemicals Ltd., England
77.	T4 DNA ligase	Fermentas, Canada
78.	*Taq* DNA Polymerase	Fermentas, Canada
79.	TEMED (N, N, N', N'-Tetramethyl ethylenediamide)	Bio Basic Inc., Canada
80.	TNFα ELISA kit	eBioscience, England
81.	Trisma Base (tris hydroxymethyl aminomethane, $CH_4H_{11}NO_3$)	Sigma Aldrich, USA
82.	Triton X-100	Amersham Bioscience, England
83.	TRI®zol reagent	Invitrogen, England
84.	Trypan Blue Solution	Hyclone, England
85.	Tween 20	Research Organics, USA
86.	Yeast Extract	Bio Springer, France

3.2 Oligonucleotide Primers

Table 3.1 Oligonucleotide primers used in experiments

Primer	Gene Bank Association Number	Sequence (5' to 3')	References
Murine *Notch1* forward	AF508809	GACAACTCCTACCTCTGCTTATGCC	(Palaga *et al*., 2008)
Murine *Notch1* reverse		TTACTGTTGCACTCGTTGACCTCG	
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)

Primer	Gene Bank Association Number	Sequence (5' to 3')	References
Murine *Notch4* reverse	NM_010929	ACACAGTCATCTGGGTTCATCATCTCAC	(Palaga *et al.*, 2008)
Murine *Deltex1* forward	NM_008052	GTAAGGCTTCAAGGGGTCGCT	(Palaga *et al.*, 2008)
Murine *Deltex1* reverse		CTCAGCTTGATGCGTGTATAGCC	
β-actin forward	NM_001101	ACCAACTGGGACGACATGGAGAA	(Palaga *et al.*, 2008)
β-actin reverse		GTGGTGGTGAAGCTGTAGCC	
IL-10 forward	NM_010548	TCAAACAAAGGACCAGCTGGACAACAT ACTGC	(Palaga *et al.*, 2008)
IL-10 reverse		CTGTCTAGGTCCTGGAGTCCAGCAGAC TCAA	
IL-12p40 forward	S82420S3	AACCTCACCTGTGACACGCC	(Tada *et al.*, 2000)
IL-12p40 reverse		CAAGTCCATGTTTCTTTGCACC	

Primer	Gene Bank Association Number	Sequence (5' to 3')	References
*TNF*α forward	NM_013693	CCTGTAGCCCACGTCGTAGC	(Lee *et al.*, 2007)
*TNF*α reverse		TTGACCTCAGCGCTGAGTTG	
iNOS forward	NM_010927	CCCTTCCGAAGTTTCTGGCAGCAGC	(Lee *et al.*, 2007)
iNOS reverse		GGCTGTCAGAGCCTCGTGGCTTTGG	

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 Plasmid Construction

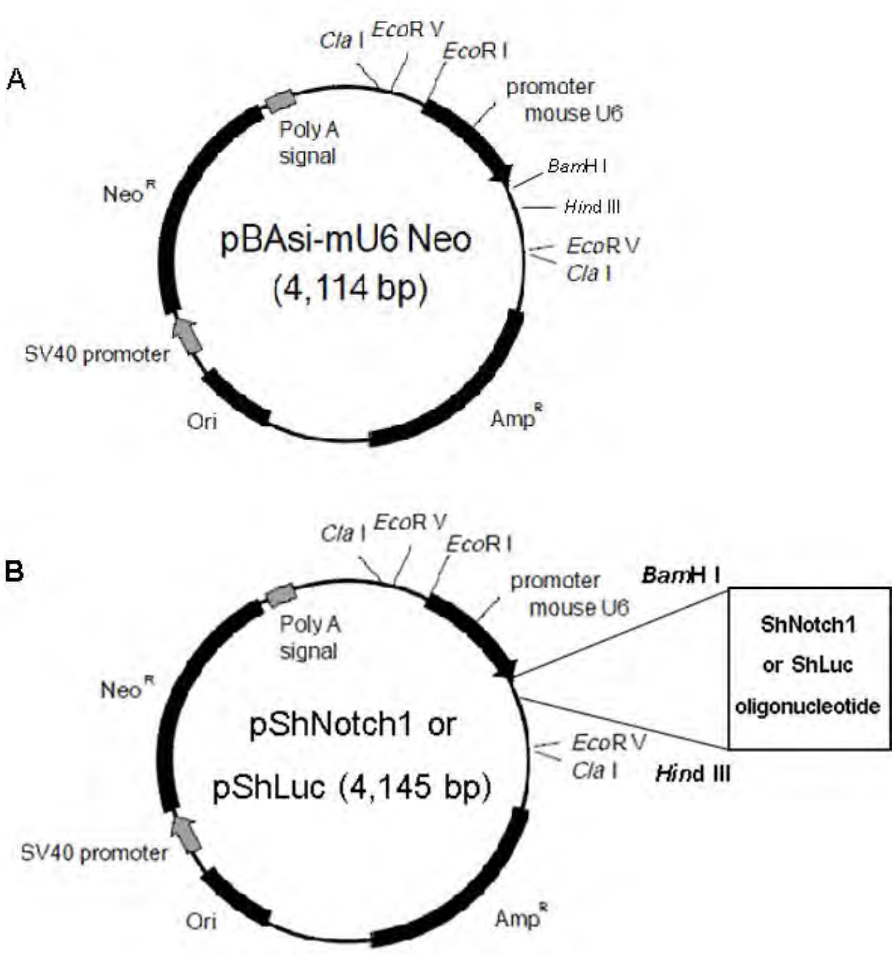


Figure 3.1 Map of restriction enzyme recognition sites of plasmids used in this study

A. Restriction map of pBAsi-mU6 Neo

B. Restriction map of pShNotch1 or pShLuc

3.4.1 Design of shRNA and plasmid construction

The target region for Notch1 silencing was identified and selected using free software available online at www.ambion.com/techlib/misc/siRNA_finder.html (Gen Bank Accession No. NM_008714.2 for murine Notch1). The sequence corresponded to bases

1529 - 1547. To generate an insert for plasmid, the appropriate restriction enzyme sites *Bam*H I (pink underlines) and *Hin*d III (blue underlines) was added to the end of the oligonucleotides targeting the region of *Notch1* (green underlines) (Figure 3.2). The hairpin loop sequence (yellow underlines) was added in between the two targeting nucleotides. Sense strand (top strand) and antisense strand (bottom strand) of oligonucleotides (1st BASE) were annealed to generate the duplex for using as an insert for plasmid construction by mixing and heating equal amount of each strand. After the reaction was cooled off slowly (95°C for 5 min, 85°C for 4 min, 75°C for 4 min, 65°C for 4 min, 55°C for 4 min, 45°C for 4 min, 35°C for 5 min, 25°C for 5 min, and 4°C), the mixtures were used as an insert for plasmid ligation.

Top strand : 5'-GATCCGGTGTATACTGTGAAATCAGTGTGCTGTCCTGATTTCACAGTATACACCTTTTTTA-3'

Bottom strand : 3'-GCCACATATGACACTTTAGTCACACGACAGGACTAAAGTGTCATATGTGGAAAAAATTCGA-5'

Figure 3.2 DNA sequences of ShNotch1 oligonucleotide insert

The control silencing target (grey area) was part of luciferase gene (Accession No. NM M63501.1) and used the unchanged free online software. The sequence corresponded to bases 423 - 441. For contructing the control silencing insert, similar protocol was used as ShNotch1 and the obtained insert was referred to as ShLuc.

Top strand : 5'-GATCCAGCAATAGTTCACGCTGAAGTGTGCTGTCCTTCAGCGTGAACTATTGCTTTTTTTA-3'

Bottom strand : 3'-GTCGTTATCAAGTGCGACTTCACACGACAGGAAGTCGCACTTGATAACGAAAAAAATTCGA-5'

Figure 3.3 DNA sequences of ShLuc oligonucleotide insert

The plasmid was prepared by treatment of pBASi-mU6 Neo with restriction enzymes, *Bam*H I and *Hin*d III and purification using Qiaquick Gel Extraction Kit according to the manufacturer's instruction.

For ligating plasmid and insert, the molar ratio of the duplex oligonucleotides used for ligation and the vector was 1:5 as calculated by the following equation.

insert [ng] = [100 ng x insert size (bp) x molar ratio] / vector size (bp)

The plasmid and insert were annealed in 1X annealing buffer using T4 DNA ligase at 16°C for overnight.

ShNotch1 and ShLuc were ligated into the pBAsi-mU6 Neo which were double digested with *Bam*H I and *Hin*d III. The ligation mixtures were transformed into competent *E. coli* DH5α cells by the following protocol. For transformation, approximately 100 ng of plasmids were added to 50 µl of the competent cells and the mixtures were incubated on ice for 30 min. Following the incubation, competent cells were treated by heat shock at 42°C for 90 sec followed by 4°C for 2 min. After that, cells were inoculated in 1 ml LB broth and incubated at 37°C for 1 hr. Fifty µl of bacterial culture was spread on LB agar plate containing 50 µg/ml ampicillin and the plates were incubated at 37°C for 16 -18 hr. The plasmids obtained from colonies

appeared on LB agar containing ampicillin was isolated and referred as pShNotch1 and pShLuc, respectively.

3.4.2 Plasmid Isolation

The colony of transformants was picked and inoculated in 2 ml LB broth containing 50 µg/ml of ampicillin. The bacteria cultures were incubated by shaking at 37°C for 16-18 hr. The bacterial cultures were harvested by centrifugation at 13,000 rpm for 1 min and the supernatant was discarded. Cell pellet was subjected to plasmid isolation by QIAprep Spin Miniprep according to the manufacturer's instructions. The obtained plasmids were store at -20°C until use.

3.4.3 Plasmid quantitative assay

The purified plasmid at -20°C was thawed on ice and diluted in sterile deionized water at 50 fold dilution. Next, the diluted plasmid was measured by the optical density at 260 nm and 280 nm. The concentration of plasmids was calculated using the following the equation (Ausubel *et al*., 1999):

Plasmid concentration (µg/ml) = OD_{260} x 50 x dilution factor

The ratio of OD_{260}/ OD_{280} indicates the purity of plasmid which the suitable ratio is 1.8 - 2.0.

3.5 Cell line and media

RAW264.7 macrophage-like cell line (ATCC TIB-71) was obtained from American Type Culture Collection (USA) and maintained in DMEM containing 10% v/v

FBS, 100 U/ml penicillin, 0.4 mg/ml streptomycin, 1% sodium pyruvate and 1% HEPES and incubated at 37°C in 5% CO_2 incubator.

3.5.1 Maintaining RAW264.7 Cell Line

RAW264.7 cell line in non-tissue culture treated dish was softly rinsed with repetitive pipetting in PBS and discarded it. RAW264.7 was harvested in PBS by centrifugation at 1,000 rpm for 5 min. After that, PBS was removed and cell pellet was resuspended in DMEM complete media. Trypan blue dye exclusion assay was used to determine cell viability. The following equation was used to calculate viable cell numbers.

Viable cells (cells/ml) = Number of counted cell in 16-large squares x 2 x 10^4

3.5.2 Cell storage

Cells were harvested as described above and cell pellet was resuspended in cold freezing media (DMEM complete media containing 10%DMSO) and aliquoted in the cryogenic tubes. The cryogenic tubes were kept on ice and stored at -80°C immediately for at least overnight. For long term storage, the tubes were stored in liquid nitrogen.

3.5.3 Thawing Cells

RAW 264.7 cells in cryogenic tubes were immediately thawed in water bath at 37°C. RAW 264.7 cells were gently transferred into 9 ml of serum free DMEM media and harvested by centrifugation at 1,000 rpm for 5 min. Supernatant was discarded and the cell pellets were resuspended in pre-warm DMEM complete media.

3.6 Transfection of RAW264.7 Cells

RAW 264.7 cells were seeded at 7.5 x 10^4cells/well in 24-well tissue culture treated plate in complete DMEM media and incubated at 37°C in 5% CO_2 overnight. To generate plasmid complexes for transfection, plasmids (0.5 µg each) obtained above were mixed with 1 µl of FuGENE HD transfection reagent at the ratio of 4:2. These complexes were prepared in serum free Opti-MEM (in 25 µl total volume), shortly mixed and incubated for 15 min at room temperature. This step was followed by addition of the complexes to RAW264.7 cell line. After swirling the plate to mix plasmid complexes and cells thoroughly, the plate was incubated for 30 hr in CO_2 incubator. After this incubation period, cell lysates were harvested and subjected to Western blot to measure the efficiency of Notch1 silencing.

3.7 Western blot

3.7.1 Cell lysate extraction and quantitative analysis of protein

RAW 264.7 cell lines were plated at 7.5 x 10^4 cells in 24-well tissue culture plate for overnight. Cells were transiently transfected with pShNotch1 or pShLuc as described in method 3.5 to obtain RAW264.7pShNotch1 or RAW264.7pShLuc, respectively. Thirty hrs after tranfection, these transfected cells were activated by the combination of 10 ng/ml IFNγ and 100 ng/ml LPS for 18 hr. Supernatant was discarded and cells were rinsed with 1 ml cold PBS, followed by rinsing with 250 µl buffer A (Appendix). After discarding buffer A, protein was extracted using 30 µl buffer B (Appendix) by repetitive

pipetting and cell debris was removed by centrifugation at 5000 rpm for 3 min at room temperature. The supernatant was transferred to the new 1.5 ml microcentrifuge tubes and stored at -80°C until use.

Protein concentrations were measured using BCA protein assay kit, according to the manufacturer's recommendation in 96-well microplate format. BCA protein assay reagents were made up by mixing Reagent A and Reagent B at the ratio of 50:1. Bovine serum albumin (BSA) was used as a standard protein at concentrations of 0, 31.25, 62.5, 125, 250, 500 and 1,000 µg/ml. Samples were diluted in sterile deionized water at the dilution of 1:10 in 96-well microtiter plate. Two hundreds µl of the working BCA reagent was measured at 540 nm by microplate reader (Bio-Tek Instrument, Canada)

3.7.2 Western Blot

The formula for preparing SDS-polyacrylamide gel (SDS-PAGE) was described in Appendix. Size of SDS-polyacrylamide gel was 0.15x8.4x8.4 cm^3 and 20 µg of protein samples (total volume 25 µl) were mixed with 25 µl 2X Laemmli buffer (Appendix). The mixture of protein samples were denatured by heating at 100°C for 5 min. These denatured protein samples and prestained molecular weight markers were loaded in 8% SDS-polyacrylamide gel. Protein samples were separated in running buffer (Appendix) using Protein III system (Bio-Rad), at 100 volts for 90 min. SDS-PAGE contained separated protein was subjected to protein transfer on PVDF membrane in semi-dry transfer apparatus (Bio-Rad) in transfer buffer (Appendix) at 70 mA for 90 min.

After blotting proteins onto PVDF membrane, the membrane was incubated twice in the blocking solution (Appendix) for 5 min each. This was followed by incubation with 4 ml of specific primary antibody in blocking solution (at the working dilution as described in table 3.2) at 4°C overnight. The probed membrane was further incubated on rocking platform for 1 hr at room temperature. After that, primary antibody was discarded and washed in PBST (Appendix) as followed: 5 min twice and 15 min twice. After discarding PBST, 4 ml of diluted secondary antibody conjugated with horseradish peroxidase (HRP) was added and incubated for 1 hr on rocking platform. After incubation, membrane was washed in PBST for 5 min twice, 15 min twice.

Chemiluminescence detection method was used in this study. The substrate of HRP was prepared that consist of solution A and solution B (Appendix). These solutions were mixed and incubated with membrane for 1 min. The membranes were wrapped in plastic wrap and exposed to an X-ray film in the autoradiography cassette. For detecting the signals, X-ray films was exposed to the blot in the dark room. The exposed film was developed in the developer for 30 sec, washed in tap water, fixed in the fixer for 3 min and washed in tap water again. This film was air dried at the room temperature.

3.8 Determination of nitric oxide

RAW264.7, RAW264.7pShNotch1 or RAW264.7pShLuc were prepared as described above and activated with 10 ng/ml IFNγ and 100 ng/ml LPS for 18 hr. The supernatant was collected and kept on ice. To measure nitric oxide produced by

activated macrophages in the form of nitrite, Griess's reaction was performed (Guevara *et al*., 1998). Briefly, standard nitrite solution was prepared by diluting 0.1 M nitrite standard at 1:1000 dilution in DMEM complete media. Nitrite standard was diluted by 6-serial 2-fold dilutions (50 µl/well) in triplicate of 96-well plate (to prepare nitrite at 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0 µM). The collected samples (50 µl) were added to the same plate in duplicate. After adding sulfanilamide solution (50 µl) to all wells, the plate was incubated for 5-10 min at room temperature in dark. After that, NED solution (Appendix) was added to all wells and incubated in dark at room temperature for another 5-10 min. After incubation, the absorbance at 540 nm was measured using microplate reader (Bio-Tek Instrument, Canada).

3.9 Assay for bactericidal activities by total plate count technique

RAW264.7pShNotch1 or pShLuc were prepared as described above in 12-well plate. Bacterial glycerol stocks *E. coli* (strain DH5α) and *S. aureus* (ATCC 25923) were thawed and LB broth contained glycerol was eliminated. After that, bacterial pellet was resuspended in DMEM media without antibiotic at the dilution which gave the multiplicity of infection (MOI) of 1:10 (macrophage:bacteria). After removing the culture supernatant from RAW264.7, the diluted bacteria pellet in DMEM media without antibiotic was added to cells for 1 hr. After infection, the supernatant was discarded and macrophages were rinsed twice with 1 ml of PBS. Complete DMEM media (containing antibiotics) were added to all wells and incubated for 30 min. The culture supernatant was discarded (for

T_0) and macrophage was rinsed with twice 1 ml of PBS. After that, macrophages were lysed using 200 µl of 0.1% triton X-100 in cold PBS and incubated for 5 min. The numbers of intracellular bacteria at T_0 were determined using total plate count technique in duplicate. After 24 hr of infection, CFUs of intracellular bacteria were measured as described with T_{24}. Bactericidal activity was calculated by the following formula:

$$\% \text{ Bactericidal activity} = (\text{Sample } T_0 \text{ or } T_{24} - \text{Control } T_0) \times 100 / \text{Control } T_0$$

3.10 Determination of MHC class II expression by flow cytometry

RAW264.7pShNotch1 or pShLuc were generated as described above. After 24 hr of transiently transfection, cells were activated with 10 ng/ml IFNγ and 100 ng/ml LPS for 24 hr. The culture supernatant was discarded and cells were rinsed by 1 ml PBS. Cells were harvested by cell scrapers in 1 ml of cold PBS using centrifugation at 8,000 rpm for 5 min at 4°C and resuspended in 100 µl of staining buffer (Appendix). Anti-Mouse I-Ab MHC biotin conjugate (0.5 µg per sample) was added to samples where it was not added to the control sample and incubated on ice for 20 min. Cells were centrifuged at 8,000 rpm for 5 min at 4°C and supernatant was discarded. Cell pellets were resuspended in 100 µl of staining buffer containing 0.5 µg of streptavidin-PE and incubated on ice for 20 min in dark. After incubation, cells were washed twice with staining buffer by centrifugation at 8,000 rpm for 5 min at 4°C. Following the staining and washing, stained cells were fixed with 500 µl of 4% paraformaldehyde for 20 min in dark and resuspended in cold PBS before subjecting to flow cytometric analysis (FACS

Calibur). The results were acquired and analyzed using CellQuest software (BD Becton Dickinson).

3.11 RT-PCR

3.11.1 RNA extraction using TRI®zol reagent

7.5 x 10^4 cells of RAW264.7pShNotch1 or pShLuc were generated as described above. After transfection (30, 36, and 40 hr), transfected cells were treated with 10 ng/ml IFNγ and 100 ng/ml LPS for 6, 12 and 18 hr. All samples were collected at 48 hr after transfection. After activation, culture supernatant was discarded and 1 ml of TRI®zol reagent was added directly to cells in each well for 5 min. The TRI®zol reagent was pipetted several times and transferred to new sterile microcentrifuge tubes. Chloroform (0.2 ml) was added to these solutions. After vigorously shaking by hands for 15 sec, the samples were incubated for 2-3 min at room temperature and separated by centrifugation at 12,000xg for 15 min at 2-8°C. After phase separation, a colorless upper aqueous phase was transferred to the new tubes. Isopropanol (0.5 ml) was added and gently mixed for RNA precipitation. The samples were let sit at room temperature for 10 min and centrifuged at 12,000xg for 10 min at 2-8°C. A gel-like pellet of RNA precipitate appeared on the side and bottom of the tube. Following the removing of the supernatant, RNA pellet was washed with 1 ml of cold 75% ethanol in 0.01% DEPC-treated water. The RNA solution was mixed by vortexing and centrifuged at 7,500xg for 5 min at 2-8°C. This pellet was air dried for 5-10 min and dissolved in 20 µl

of 0.01% DEPC-treated water. To completely dissolve RNA, the solution was incubated at 60°C for 10 min and stored at -70°C. To measure amount of RNA in samples, the using Qubit® fluorometer and Quant-iT™ RNA Assay Kit (Invitrogen) were used according to manufacturer's recommendation.

3.11.2 cDNA synthesis by reverse transcription

Total RNA (0.5 μg) was mixed with 0.2 μg of random hexamer and the final volume was adjusted with 0.01% DEPC-treated water to 12.5 μl. After that this RNA mixture was gently mixed and briefly centrifuged. The mixture was incubated at 65°C in Thermal Cycler (Bioer, Japan) for 5 min, chilled on ice for 5 min and briefly centrifuged. The reverse transcriptase buffer (5X), 10 mM dNTP mix and RNase inhibitor were added to the RNA mixture at the final concentration 1x, 1 mM and 20 U, respectively. Finally, 200 U of reverse transcriptase was added to the sample. Following this step, the samples were gently mixed and briefly centrifuged. The cDNA synthesis was carried out by incubation at 25°C for 10 min, 42°C for 60 min, 70°C for 10 min. cDNA generated in this manner was stored at -80°C until use for PCR.

3.12 Polymerase chain reaction (PCR)

Synthesized cDNA was used as templates for amplification using primers specific for *Notch1-4, IL-10, IL-12p40, iNOS, TNFα, Deltex1* and *β-actin*.

3.12.1 Conventional PCR

The components of PCR reaction mixture are shown in Table 3.3

Table 3.3 Components of PCR Reaction Mixture

Component	Final concentration
10x *Taq* buffer	1x
10 mM dNTP mix	0.64 mM
25 mM $MgCl_2$	2 mM
10 mM forward primer	0.2 µM
10 mM reverse primer	0.2 µM
Taq polymerase	25 U
Hypure™ Molecular Biology Grade Water	To total volume 25 µl
cDNA	2 µl
Total volume	25 µl

Primer sets specific for *β-actin* was used to amplify a house keeping gene as a loading control. Negative control was carried out using RT-PCR reaction without reverse transcriptase and total RNA from mouse thymus was used as positive control for all genes.

PCR condition:

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

Annealing temperatures, number of cycles and sizes of PCR products for each gene are shown in Table 3.4.

Table 3.4 Annealing temperatures, number of cycles and expected sizes of PCR products for each gene in this study

Gene	Annealing temperature (°C)	number of cycles performed	Expected size of PCR product (bp)
Murine *Notch1*	60	30	305
Murine *Notch2*	55	30	522
Murine *Notch3*	55	30	467
Murine *Notch4*	60	30	239
β-actin	55	30	380

The PCR reaction was carried out using Thermal Cycler (Bioer, Japan). PCR products were analyzed on 2% agarose gel by electrophoresis and the gels were stained with ethidium bromide for visualization after destaining in deionized water with Gel Documentation System (Bio-Rad).

3.12.2 Quantitative Real-time PCR

The components of quantitative real-time PCR reaction mixture are shown in Table 3.5.

Table 3.5 Components of quantitative real-time PCR reaction mixture

Component	Final concentration
2x SYBR Green master mix (Maxima™ SYBR Green qPCR master mix)	1x
10 mM forward primer	0.3 mM
10 mM reverse primer	0.3 mM
RNase-free water	Total volume 20 µl
cDNA	1 µl
Total volume	20 µl

β-actin gene was used for normalization to calculate expression level.

The thermal cycling profile was as the followings:

Hot start	95°C	15 min	
Denaturation	95°C	30 sec	40 cycles
Annealing	X°C	30 sec	
Extension	72°C	30 sec	
Fluorescent dye SYBR Green detection			
Melting curve analysis from 65°C to 95°C			

Table 3.6 Annealing temperatures and expected sizes of real-time PCR products

Gene	Annealing temperature (°C)	Expected size of PCR product (bp)
Murine *IL-10*	60	305
Murine *IL-12p40*	55	522
Murine *iNOS*	55	467
Murine *TNFα*	60	239
Deltex1	57	366
β-actin	55	380

Real-time PCR was carried out in MJ Mini™ Thermal Cycler (Bio-Rad, USA). The threshold cycle (C_T) was used as the levels of mRNA expression and the $2^{-\Delta\Delta CT}$ was calculated for relative quantification using Gene Expression Macro™ Version 1.1 software (Bio-Rad, USA).

3.13 Statistical analysis

To calculate the statistical differences between control and samples, Student's independent-samples T test was used (SPSS 16.0). The values of $p<0.05$ were considered statistically significant.

CHAPTER IV

RESULTS

4.1 Specific silencing of Notch1 by pShNotch1 in RAW264.7

The constructed plasmid pShNotch1 containing an insert targeting Notch1 and the control plasmid with luciferase targeted insert were subjected for oligonucleotide sequencing to confirm the plasmids (Appendix). These two plasmids were used to transiently transfected RAW264.7 and the expression of Notch1 was followed at 0, 6, 12, 24 and 30 hr (Figure 4A). From this result, the optimal duration to get the maximum silencing was found to be 12-30 hr. To compare the expression of Notch1 in untransfected, pShLuc or pShNotch1 transfected RAW264.7 cells, cells were stimulated with 100 ng/ml LPS and 10 ng/ml IFNγ for 18 hr after 30 hr of transfection. As shown in Figure 4B, expression of Notch1 was significantly reduced, in comparison to those of control cells. In fact, Notch1 was almost undetectable in our system.

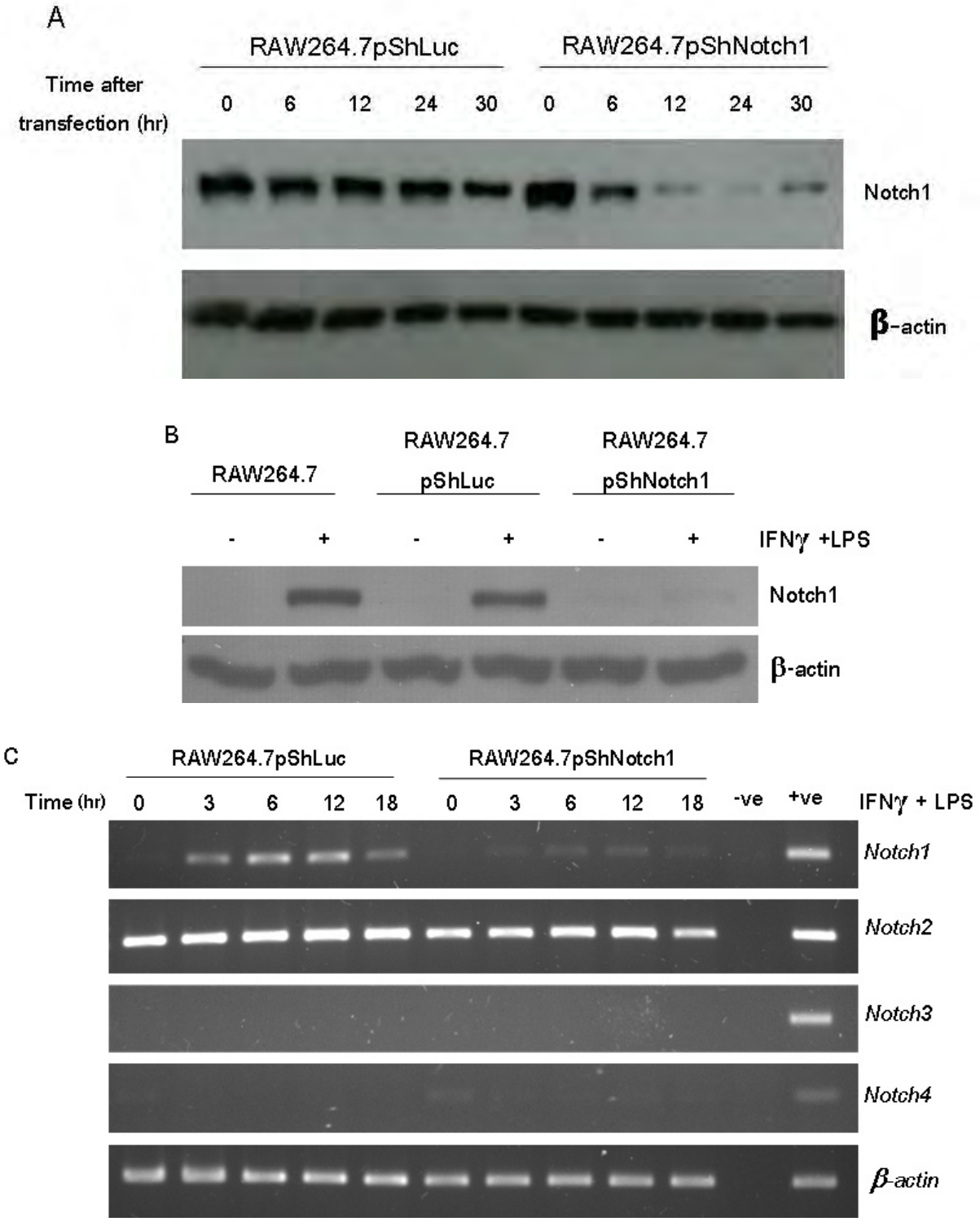


Figure 4.1 Specific silencing of Notch1 by pShNotch1 in RAW264.7. Kinetics of Notch1 silencing in RAW264.7 cells as determined by Western blot (A). Comparison of Notch1 expression in untransfected, pShLuc and pShNotch1 transfected RAW264.7 cells was

investigated by Western blot (B) and the specificity of *Notch1* knock down was tested by RT-PCR (C).

After confirming the silencing effect of pShNotch1 RAW264.7 cells hence, RT-PCR was utilized to test the specificity of *Notch1* silencing. The reduction of *Notch1* expression in stimulated RAW264.7pShNotch1 cells was observed from 0 hr until 18 hr, consistent with the results obtained from Western blot (Figure 4.1A and B), whereas there was no detectable effect on *Notch1* expression in stimulated RAW264.7pShLuc cells. Expression of others Notch family members, *Notch2*, *Notch3* and *Notch4* were not significantly different in both RAW264.7pShNotch1 and RAW264.7pShLuc cells in all time points (Figure 4.1C). These results indicated that plasmid shNotch1 was effective and specific in silencing Notch1 expression in RAW264.7 cells.

4.2 Effects of Notch1 silencing in RAW264.7 cells

To investigate the effect of Notch1 silencing in RAW264.7 cell line, the following phenotypes were examined.

4.2.1 Morphology of RAW264.7 cells with Notch1 knockdown

In RAW264.7 cells with knockdown Notch1, the alteration in the cellular morphology was observed which was similar to those of stimulated RAW264.7 cells (Figure 4.2 B and E). Cells appeared as flat and showed multiple projections from the cell body. In addition, vacuoles in cytoplasm were also observed in RAW264.7 with pShNotch1. After 18 hr of activation with LPS and IFNγ, RAW264.7pShNotch1 cells

showed a typical morphology as activated macrophages, such as flat on the tissue plate, large vacuoles in cytoplasms. At this time point, there was no drastic difference between RAW264.7pShNotch1 and the control (Figure 4.2E and F).

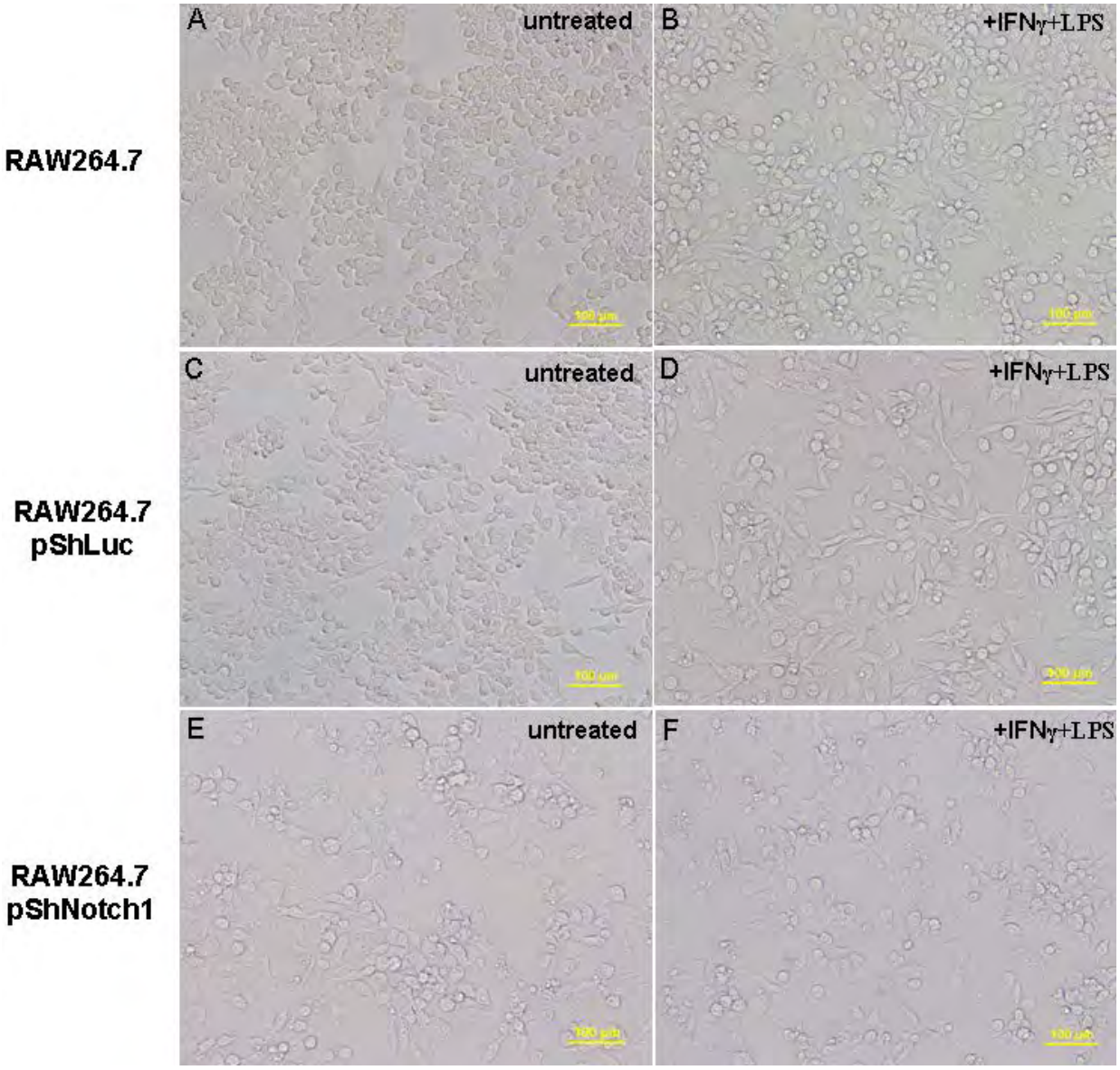


Figure 4.2 Changes in morphology of RAW264.7 cells with silencing Notch1. In A, C and F were untreated as B, D and F were stimulated with 100 ng/ml LPS and 10 ng/ml IFNγ for 18 hr.

4.2.2 Nitric oxide production in Notch1 silencing RAW264.7 cells

In order to investigate the effects of Notch1 silencing on inflammatory responses in macrophages, the phenotypes associated with inflammation were examined. Nitric oxide is one of the chemical meditaors macrophages produced with anti-microbial activity during activation. RAW264.7 transfected with control pShLuc vector or pShNotch1 was stimulated with LPS and IFNγ and culture supernatants were harvested and subjected to Griess's reaction for determining the amount of nitric oxide produced and secreted. As shown in Figure 4.3A, we unexpected found that RAW264.7pShNotch1 produced significant amount of nitric oxide in unstimulated condition, while control RAW264.7 or RAW264.7pShLuc produced background level of nitric oxide. Upon activation with 100 ng/ml LPS and 10 ng/ml IFNγ for 18 hr, RAW264.7 and RAW264.7pShLuc secreted significantly increased amount of nitric oxide, while Notch1 silencing RAW264.7 produced significantly less nitric oxide that the control cells (Figure 4.3A). This result correlated well with *iNOS* expression which was measured by quantitative real-time PCR (Figure 4.3B). RAW264.7pShNotch1 expressed significantly higher *iNOS* expression than RAW264.7pShLuc in unstimulated state, while the level remained almost at the same level even after stimulation for 18 hr. In contrast, the level of *iNOS* expression of both control groups increased significantly after stimulation. Taken together, these results indicated that signaling via Notch1 negatively influenced nitric oxide production via iNOS expression in RAW264.7 cells.

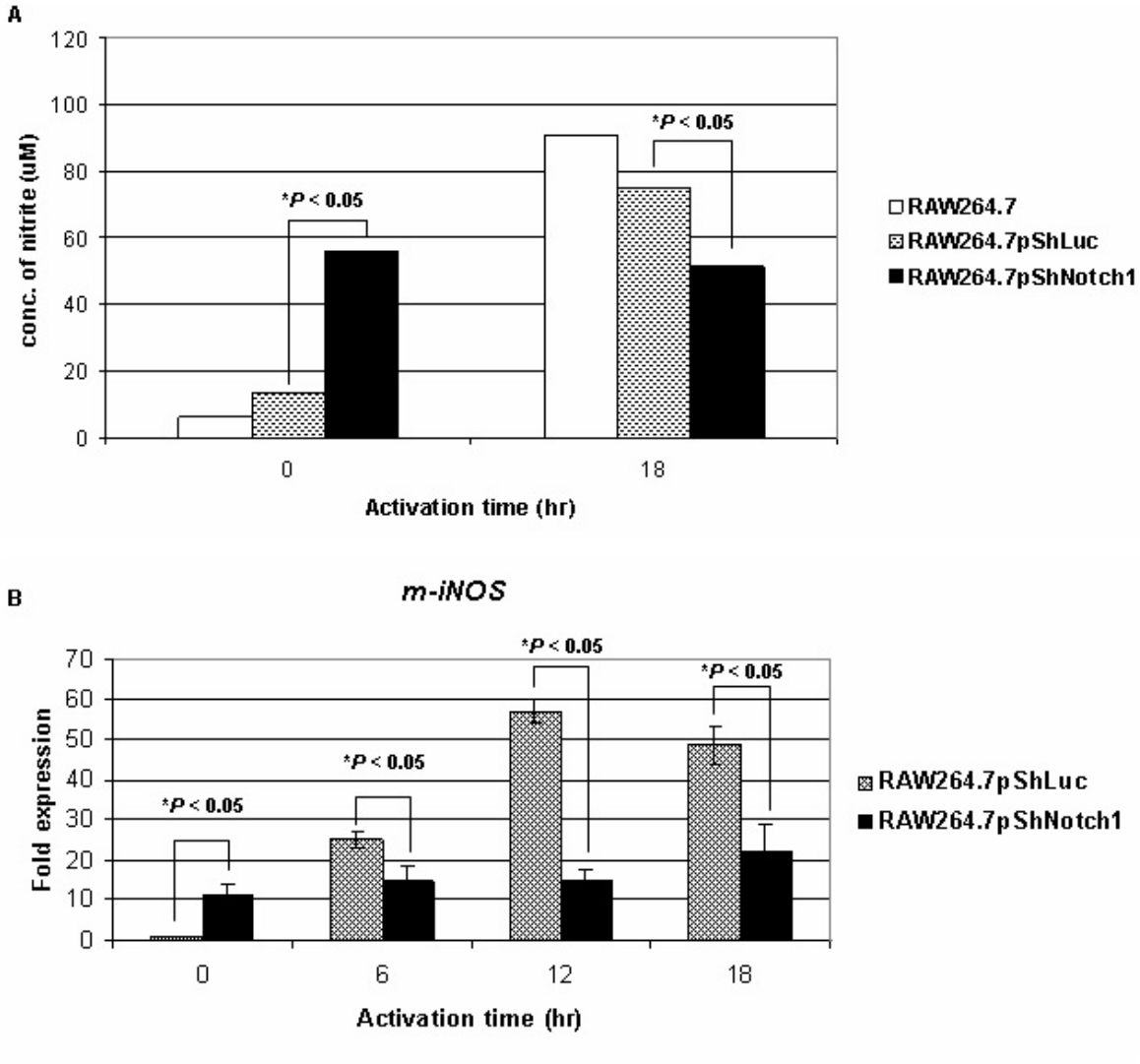


Figure 4.3 Nitric oxide production in RAW264.7 stimulated with LPS and IFNγ for 18 hr was measured by Griess's reaction (A). *iNOS* expressions in RAW264.7 stimulated at 0, 6, 12 and 18 hr were examined by quantitative real-time PCR. Stimulation was achieved by using 100 ng/ml LPS and 10 ng/ml IFNγ (B). β-actin was used to normalize the expression level and the expression of *iNOS* in unstimulated RAW264.7 was set as 1. The results are shown as mean ± S.D. of triplicate. The results represented two independent experiments.

4.2.3 TNFα production in Notch1 silencing RAW264.7 cells

TNFα is the pro-inflammatory cytokine produced during early phase of infection by innate immune cells. To investigate whether silencing Notch1 affected the production of this cytokine, TNFα production in the culture supernatant of RAW264.7pShNotch1 and RAW264.7pShLuc were determined by ELISA. To stimulate RAW264.7 cells, LPS (100 ng/ml) and IFNγ (10 ng/ml IFNγ) were used and the culture supernatant of RAW264.7pShNotch1 and RAW264.7pShLuc were harvested at 6 and 24 hr post stimulation. Secretion of TNFα from unstimulated RAW264.7pShNotch1 were significantly higher that that from unstimulated RAW264.7pShLuc, while No significant difference, however, was observed in TNFα of stimulated RAW264.7pShNotch1 and RAW264.7pShLuc in 6 and 24 hr of activation times (Figure 4.4A). These results correlated well with *TNFα* mRNA expression as measured by quantitative real-time PCR method (Figure 4.4B). *TNFα* expression in RAW264.7pShNotch1 was higher than that in RAW264.7pShLuc at 0 hr of activation time while no significant difference was found at others times after stimulation.

4.2.4 Bactericidal activity of Notch1 silencing RAW264.7 cells

To further characterize the phenotypes of macrophages with silencing Notch1, the bactericidal activity was measured using *E. coli* and *S. aureus* as model. In *E. coli* infected RAW264.7pShNotch1, the bactericidal activity at day 0 was demonstrated while this activity was slightly increased at day 1 of infection when compared to RAW264.7pShLuc (Figure 4.5A). Similarly, the bactericidal activity against *S. aureus* -

infected RAW264.7pShNotch1 cells was higher than infected RAW264.7pShLuc at day 0 and day 1 of infection (Figure 4.5B). This result together with nitric oxide and TNFα production suggested that Notch1 silencing in macrophages resulted in the spontaneous activation phenotypes.

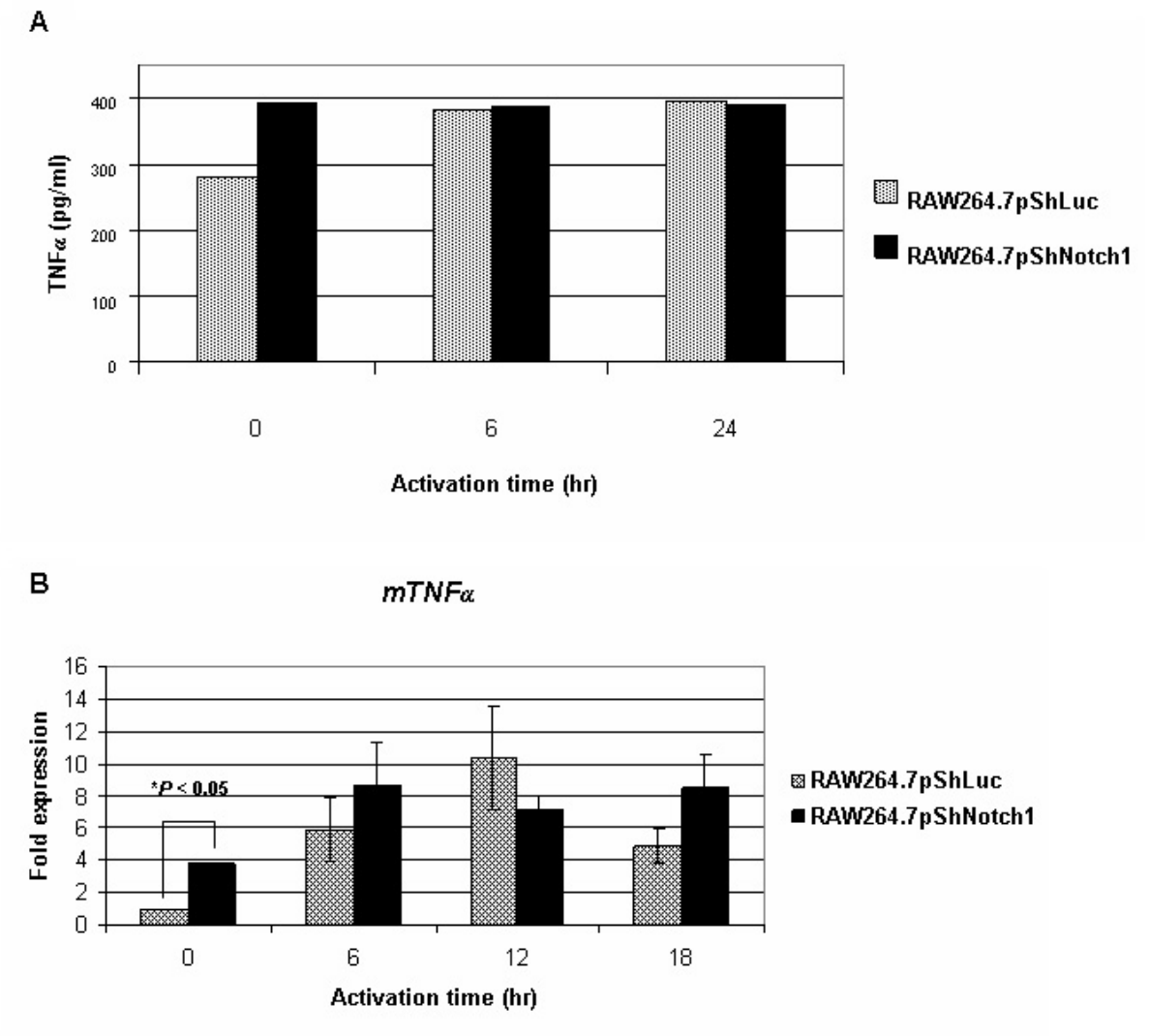


Figure 4.4 Effects of Notch1 silencing in RAW264.7 cells on TNFα secretion and TNFα mRNA expression. TNFα in the culture supernatant at 0, 6 and 24 hr after stimulation were measured by ELISA (A) and mRNA expression of *TNFα* was examined with quantitative real-time PCR (B). Stimulation was achieved by using 100 ng/ml LPS and 10 ng/ml IFNγ. β-actin was used to normalize the expression level and the expression of *iNOS* in unstimulated RAW264.7 was set as 1. The results are shown as mean± S.D. of triplicate. The results represented two independent experiments.

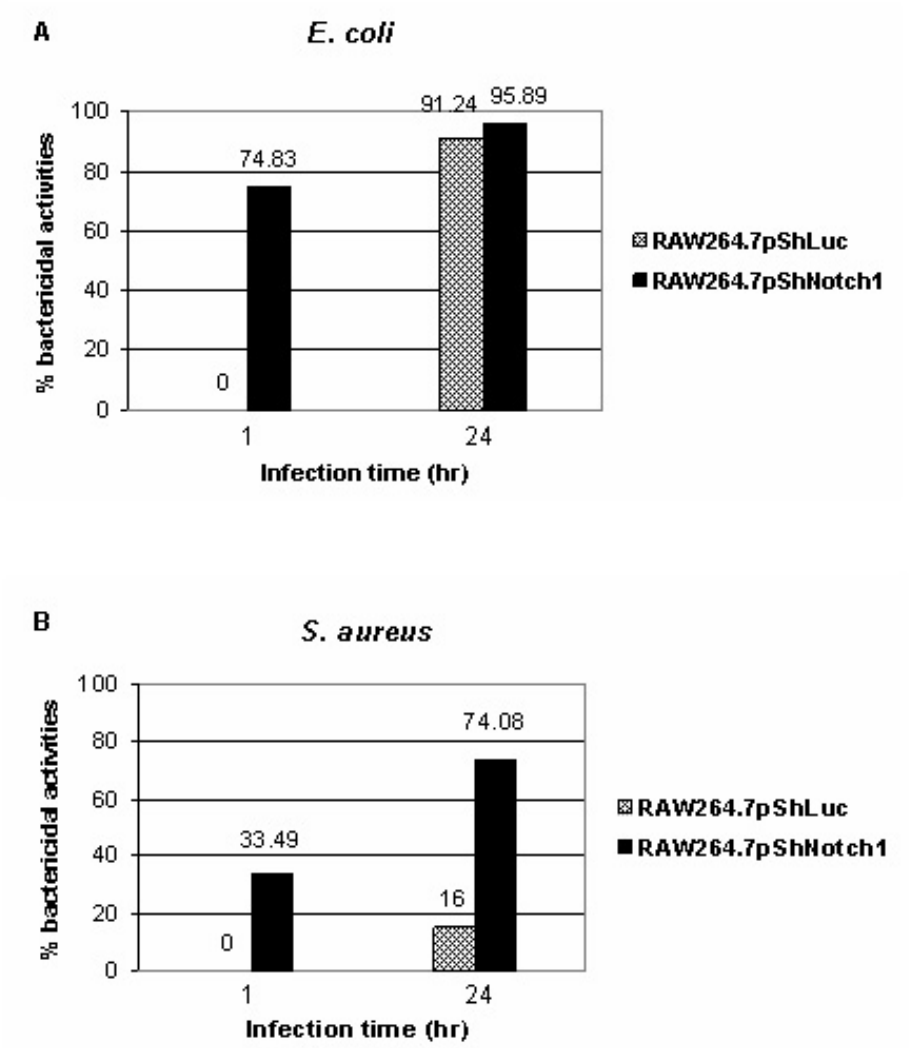


Figure 4.5 Bactericidal activities of Notch1 silencing RAW264.7 cells. RAW264.7pShLuc and RAW264.7pShNotch1 were infected with *E. coli* (A) or *S. aureus* (B) at MOI of 10:1 (bacteria:macrophage) for 1 hr. After infection, infected macrophages were washed and replenished with antibiotics containing media. After 24 hr of incubation, RAW264.7pShLuc and RAW264.7pShNotch1 were lysed to release intracellular bacteria. Viable bacteria were determined by total plate count technique. Percent of bacteria killing was calculated by

$$\% \text{ Bactericidal activity} = (\text{Sample } T_0 \text{ or } T_{24} - \text{Control } T_0) \times 100 / \text{Control } T_0$$

4.2.5 mRNA expressions of cytokines in Notch1 silencing RAW264.7 cells

In order to explored the roles of Notch1 in cytokine expression in macrophages, expression of *IL-10* and *IL-12p40* in RAW264.7pShLuc and RAW264.7pShNotch1 were measured after being stimulated with 100 ng/ml LPS and 10 ng/ml IFNγ for 0, 6, 12 and 18 hr. Quantitative real-time PCR was used to measure cytokine gene expressions. In comparison with RAW264.7pShLuc, the level of *IL-10* mRNA in activated RAW264.7pShNotch1 was significantly higher at 0 and 6 hr after activation, whereas there were no significant differences at later time points (Figure 4.6A). In addition, RAW264.7pShNotch1 expressed more *IL-12p40* than RAW264.7pShLuc at 6 hr after stimulation, while there was no difference between RAW264.7pShNotch1 and the control at others time point (Figure 4.6B).

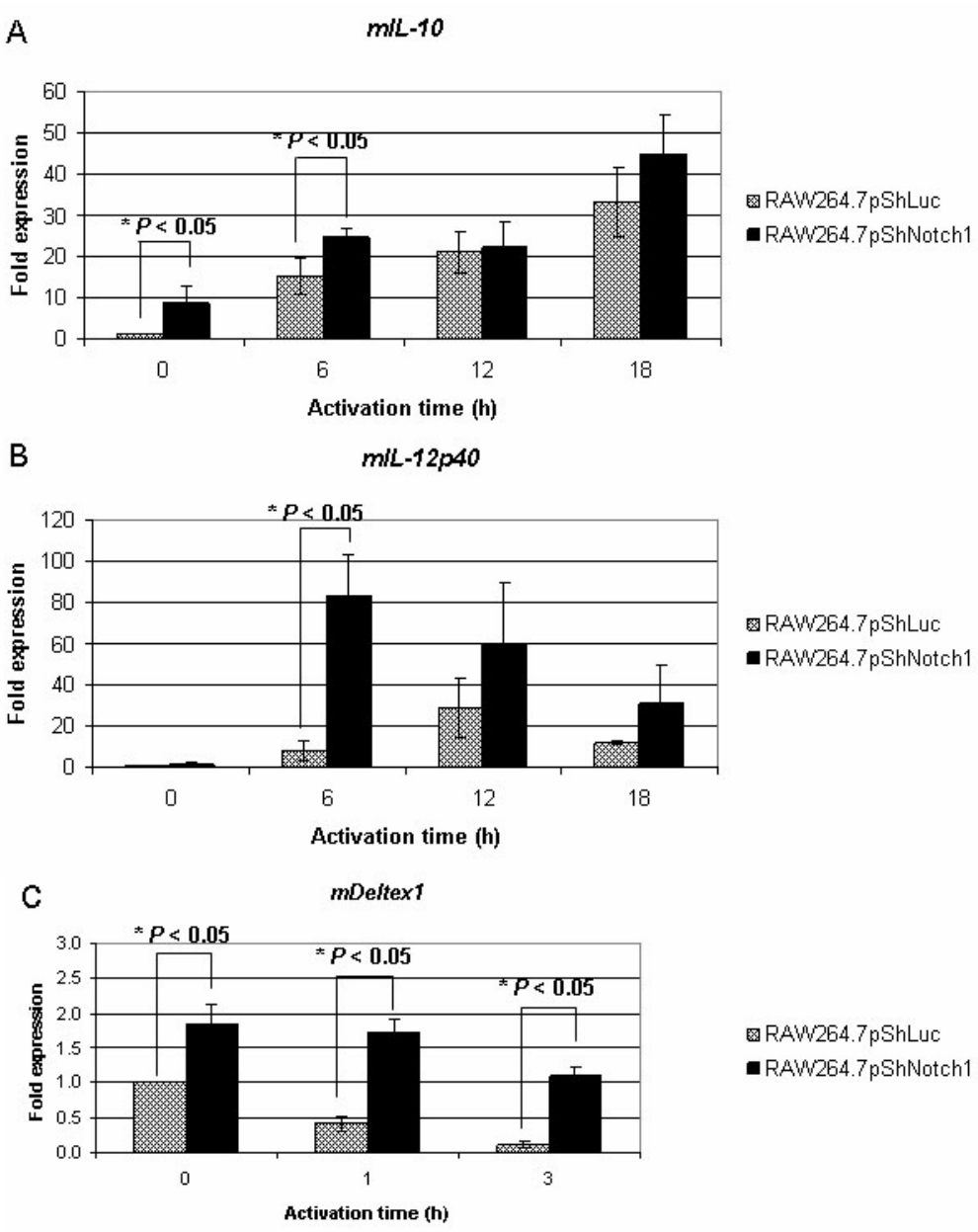


Figure 4.6 Effects of Notch1 silencing in RAW264.7 cells on cytokine genes and *Deltex1* expression. Quantitative real-time PCR method was utilized for this analysis. RAW264.7pShLuc and RAW264.7pShNotch1 stimulated with 100 ng/ml LPS and 10 ng/ml IFNγ for 0, 6, 12 and 18 hr for cytokine expressions including *IL-10* (A) and *IL-12p40* (B). *Deltex1*(C) as Notch target gene was expressed for 0, 1 and 3 hr of

activation time with 100 ng/ml LPS and 10 ng/ml IFNγ. The results are shown as mean ± S.D. of triplicate. The results represented two independent experiments.

4.2.6 MHC Class II expression in Notch1 silenced RAW264.7 cells

In order to examine the functions as antigen presenting cells of macrophages, flow cytometry was utilized to determined the amounts of MHC class II molecules expressed on the cell surface. Unstimulated RAW264.7pShNotch1 showed significantly higher MHC class II (77%) than those of unstimulated RAW264.7 (15%) or RAW264.7pShLuc cells (37%) (Figure 4.7A). After stimulation for 24 hr, it was found that RAW264.7, RAW264.7pShLuc and RAW264.7pShNotch1 similarly expressed MHC class II molecules at the same level but greater than unstimulated cells (Figure 4.7B).

4.2.7 Expression of *Deltex*, a Notch signaling target gene, in Notch1 silencing RAW264.7

Deltex is one of the most well studied target genes of the Notch signaling pathway. It is often used as a surrogate marker for detecting the activation status of Notch pathway. The effect of silencing *Notch1* on expression of *Deltex1* was investigated. The levels of mRNA of this gene was analysed by quantitative real-time PCR. Total RNA was isolated from RAW264.7pShLuc and RAW264.7pShNotch1 which had been stimulated with LPS (100 ng/mL) and IFNγ (10 ng/mL) for 0, 1 and 3 hr. As shown in Figure 4.6C, *Deltex1* expression in RAW264.7pShLuc was higher than that in RAW264.7pShNotch1. Regardless of the activation, RAW264.7pShNotch1 showed significantly higher *Deltex1* expression than activated RAW264.7pShLuc at 6 and 18 hr

of the activation time (data not shown). This is an unexpected finding since silencing Notch1 was expected to decrease expression of the target genes. Therefore, this result strongly suggested that Notch1 silencing leads to activation of the Notch signaling pathway, presumably via other remaining Notch receptors and the phenotypes observed so far may be the result of Notch activation.

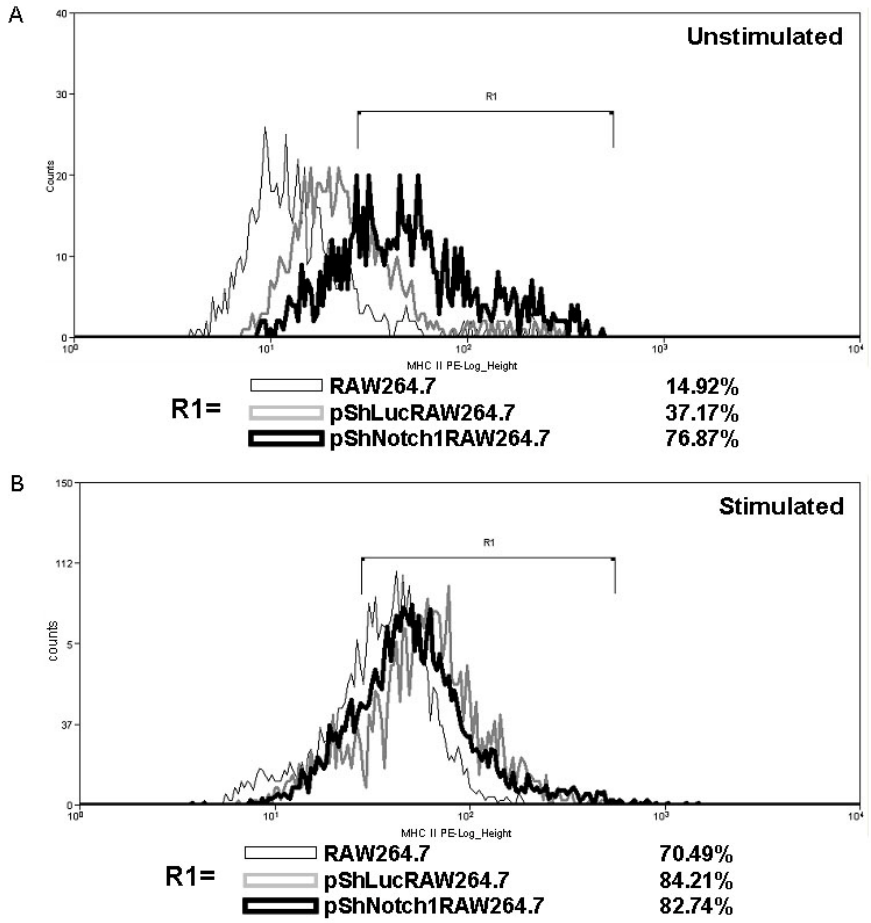


Figure 4.7 Effects of Notch1 silenced in RAW264.7 cells on MHC class II expression for antigen presentation. 100 ng/ml LPS and 10 ng/ml IFNγ were utilized to RAW264.7 cell activations for 0 and 24 hr. These effects were determined with flow cytometry technique and the obtained data were analysed by Summit 5.0 software.

CHAPTER V

DISCUSSIONS

Notch signaling pathway regulates developmental process and various effector phase of immune cells. For myeloid lineage cells, it was shown to affect the differentiation of myeloid cells (Carlesso *et al*., 1999), but the roles Notch signaling plays in macrophages remained largely uncleared. The link between Notch signaling and macrophage effector function may be useful in controlling inflammation and many others diseases (Aikawa and Libby, 2004; Hansson, 2005; Mehra *et al*., 2005). It was shown previously that *Notch 1, 2, 4* and *Hes-1* were observed in TLR-activated macrophages (Monsalve *et al*., 2006; Palaga *et al*., 2008). In this study, we aimed at investigating specifically the role of Notch1 in macrophages using RAW264.7cell line as a model.

During both innate and adaptive immune response, macrophages play crucial roles. Precursors of macrophages are produced in the bone marrow and, later, they are translocated via the blood to different tissues in the body, where they differentiate to mature resident macrophages (Monsalve *et al*., 2006). When macrophages were infected with pathogens, they confront the invading organisms and eliminate them by various measures. The response leads to inflammation where macrophages and other immune cells migrate to this site and eliminate the pathogens (Mosser, 2003).

There are 3 different types of enzyme nitric oxide synthase (NOS) based on the origins of expression, which is called endothelial NOS (eNOS), neuronal NOS (nNOS)

and cytokine-inducible NOS (iNOS), respectively. eNOS and nNOS are constitutively expressed at low level. When macrophages are activated by pro-inflammatory cytokines including IFNγ and TNFα and other agents such as TLR agonists, the expression of iNOS is induced. Induction of iNOS is regulated at the gene transcription level which result in increasing nitric oxide production (Garcia and Stein, 2006). Nitric oxide has strong anti-microbial activities and is used as a weapon to counter the invasion of pathogens. Mancineli and McKay found that low concentration (0 – 1.9 ppm) of nitric oxide were bacteriostatic in log-phase culture of *S. aureus* (Mancinelli and McKay, 1983). Bactericidal activities against *S. aureus* were found to be lower in infected RAW264.7pShLuc than infected RAW264.7pShNotch1. This may imply that Notch1 is involved in negative regulatuion of generation of others types of inflammatory mediators such as reactive oxygen intermediates and anti-microbial peptides. The level of bactericidal activity against *S. aureus* in infected RAW264.7pShLuc for 24 hr was lower than those of RAW264.7pShNotch1 at both 1 and 24 hr after infection. This can be explained by the fact that Notch1 reduction results in increased nitric oxide and *iNOS* expression (Figure 4.3 A and B).

TNFα is a pleiotropic cytokine produced during activation of the immune system by various cell types, including macrophages. In this study, RAW264.7pShNotch1 were found to express high levels of *TNFα* and secrete more TNFα. Previously, Palaga et al, reported that inhibition of Notch signaling resulted in decreased iNOS, TNFα expression and nitric oxide production, suggesting that Notch signaling is partially involved in

regulation of this pathway (Palaga *et al*., 2008). The result obtained in this study revealed a surprising function of Notch1 where silencing Notch1 resulted in higher iNOS expression and more nitric oxide even in the absence of any stimulation. Taken together with the results of iNOS and nitric oxide, it could be concluded that *Notch1* may be involved in the regulation of inflammation in macrophages. In addition, the results obtained here is also consistent with the ability of Notch1 knock down macrophages to kill bacteria better than the control.

In the innate resistance to intracellular infection macrophages phagocytose the pathogens and kill them inside the cells. Activated macrophages generate a series of pro-inflammatory cytokines and the inflammatory response (Trinchieri, 1997). A wide range of cytokines and reactive oxygen and nitrogen species are produced by macrophages during infection (Sasmono *et al*., 2003). IL-10 prevents macrophage activation and decrease inflammatory cytokine productions (Bogdan *et al*., 1991) where IL-12 is a pro-inflammatory cytokine and essential for efficient cell mediate immune response of Th1-type (Anderson and Mosser, 2002). TLR4 signaling initiated with LPS induces pro-inflammatory cytokines such as TNFα and IL-12p40 (Fujihara *et al*., 2003) Cytokine expressions in activated macrophages depend on TLR engagement with specific components of pathogens (Franchini *et al*., 2006). Notch signaling is shown previously to get activated via TLR stimulation through MyD88 (Palaga *et al*., 2008).

Thus, cytokine expressions in activated macrophages are possibly associated partially with the Notch signaling.

In this study, the level of *IL-10* expression in RAW264.7 transfected with pShNotch1 was slightly increased while the level of *IL-12p40* expression in activated RAW264.7pShNotch1 was also higher than the control. These results correlated well with the results of *iNOS* expression, nitric oxide production, *TNFα* expression and TNFα secretion, indicating that *Notch1* maybe participated in the mechanism of inflammation in macrophages. It was probably due to the up-regulation of nitric oxide and TNFα production and *IL-12p40* expression that the bactericidal activity of RAW264.7 with pShNotch1 was significantly higher than the control.

MHC class II molecules essential for antigen presentation and T cell activation are up-regulated on cell surface of LPS-treated RAW264.7 cells (Saxena *et al*., 2003). In this study, the amount of MHC class II expression in unstimulated RAW264.7pShNotch1 was significantly increased as high as that of the stimulated RAW264.7 cells. This result suggested that Notch1 possibly control the mechanism of MHC class II expression in macrophages.

From these results, it was found that unstimulated macrophages with silenced *Notch1* expressed in high levels of several cytokines including *iNOS*, *TNFα*, *IL-12p40* and a Notch signaling target gene, *Deltex1*. These expression patterns are similar to the patterns found in stimulated macrophages. Hence, when Notch1 is silenced, it is possible that other Notch receptors replace Notch1 and function to activate the

signaling cascade. The likely candidate is Notch2 receptor, since its expression can be clearly detected in macrophages. Taken together, we propose a model to explain and put into context the results obtained by this study and others (Figure 5.1). In macrophages with silenced Notch1, it is possible that expression of the Notch ligands is up-regulated. This up-regulation leads to initiation of Notch signaling via other Notch receptors, possibly Notch2. The activated Notch signaling, in turn, regulate pro-inflammatory response of macrophages. Thus, this model proposes that Notch1 acts as a negative regulator of Notch ligand expression. In the presence of Notch1, Notch ligands are kept in check and no overt Notch signaling is initiated. In the absence of Notch1, this negative control is removed and Notch ligands are expressed (de la Pompa *et al.*, 1997). When TLR is activated, repression of ligand expression is overrided and Notch signaling is activated with inflammatory phenotype of macrophages. When GSI is used, all Notch receptors are suppressed, and decreased inflammatory response was observed.

In conclusion, we reported that silencing Notch1 in macrophages resulted in a spontaneous activated phenotype in the absence of any stimulation. This novel finding may be utilized to control inflammation.

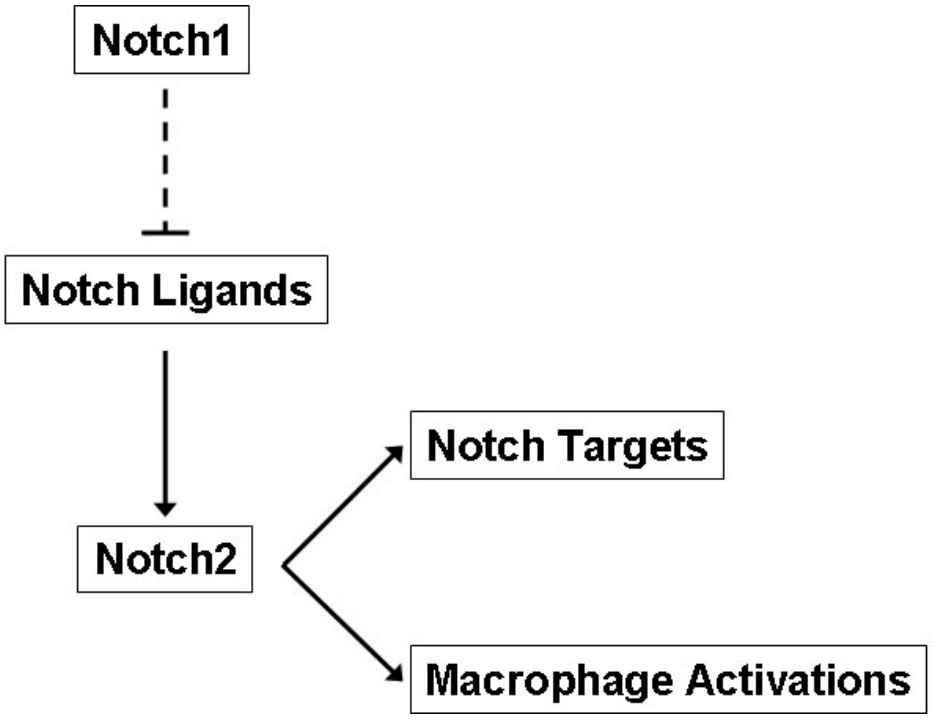


Figure 5.1 Model of the proposed mechanism of Notch activation in *Notch1* silencing macrophages. Broken line depicted step(s) needed further investigation.

CHAPTER VI

CONCLUSIONS

RNAi was used to specifically target Notch1 silencing in RAW264.7 macrophage cell line. Silencing Notch1 resulted in upregulation of mRNA for *iNos* and pro-inflammatory cytokines in the absence of any stimuli. Some of the products of these genes are confirmed to be increased. The gene expression profile was correlated well with the bactericidal activity of Notch1 knockdown macrophages. In contrast, upon activation using LPS and recombinant IFNγ, macrophages with silencing Notch1 did not upregulate any genes involved in inflammation. Taken together, these results demonstrate that Notch1 play a unique non-redundant function in macrophages.

Suggestions for the future works

1. Since Notch signaling is activated in Notch1 knockdown macrophages, the involvement of other Notch receptors should be studied by simultaneously silencing multiple receptors.
2. The signaling pathways regulating inflammatory responses are well documented in macrophages such as NF-κB and MAP kinase pathway. The status of such pathways should be studied in Notch1 knockdown macrophages.

REFERENCES

Aderem, A. and Underhill, D.M. 1999. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol **17**: 593-623.

Aikawa, M. and Libby, P. 2004. The vulnerable atherosclerotic plaque: pathogenesis and therapeutic approach. Cardiovasc Pathol **13**(3): 125-138.

Akira, S. 2003. Toll-like receptor signaling. J Biol Chem **278**(40): 38105-38108.

Allenspach, E.J., Maillard, I., Aster, J.C., and Pear, W.S. 2002. Notch signaling in cancer. Cancer Biol Ther **1**(5): 466-476.

Amsen, D., Blander, J.M., Lee, G.R., Tanigaki, K., Honjo, T., and Flavell, R.A. 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell **117**(4): 515-526.

Anastasi, E., Campese, A.F., Bellavia, D., Bulotta, A., Balestri, A., Pascucci, M., Checquolo, S., Gradini, R., Lendahl, U., Frati, L. et al. 2003. Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes. J Immunol **171**(9): 4504-4511.

Anderson, C.F. and Mosser, D.M. 2002. Cutting edge: biasing immune responses by directing antigen to macrophage Fc gamma receptors. J Immunol **168**(8): 3697-3701.

Ausubel, L.J., Bieganowska, K.D., and Hafler, D.A. 1999. Cross-reactivity of T-cell clones specific for altered peptide ligands of myelin basic protein. Cell Immunol **193**(1): 99-107.

Bitko, V., Musiyenko, A., Shulyayeva, O., and Barik, S. 2005. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med **11**(1): 50-55.

Blacklow, S.C. 2005. A new niche for Notch on Deltex? Structure **13**(11): 1579-1580.

Bogdan, C., Vodovotz, Y., and Nathan, C. 1991. Macrophage deactivation by interleukin 10. J Exp Med **174**(6): 1549-1555.

Bray, S.J. 2006. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol **7**(9): 678-689.

Carlesso, N., Aster, J.C., Sklar, J., and Scadden, D.T. 1999. Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. Blood **93**(3): 838-848.

Carmell, M.A. and Hannon, G.J. 2004. RNase III enzymes and the initiation of gene silencing. Nat Struct Mol Biol **11**(3): 214-218.

Chiba, S. 2006. Notch signaling in stem cell systems. Stem Cells **24**(11): 2437-2447.

Davis, R.L. and Turner, D.L. 2001. Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. Oncogene **20**(58): 8342-8357.

de Fougerolles, A., Vornlocher, H.P., Maraganore, J., and Lieberman, J. 2007. Interfering with disease: a progress report on siRNA-based therapeutics. Nat Rev Drug Discov **6**(6): 443-453.

de la Pompa, J.L., Wakeham, A., Correia, K.M., Samper, E., Brown, S., Aguilera, R.J., Nakano, T., Honjo, T., Mak, T.W., Rossant, J. et al. 1997. Conservation of the Notch signalling pathway in mammalian neurogenesis. Development **124**(6): 1139-1148.

Deftos, M.L. and Bevan, M.J. 2000. Notch signaling in T cell development. Curr Opin Immunol **12**(2): 166-172.

Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N. et al. 2005. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol **6**(3): 314-322.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **391**(6669): 806-811.

Fischer, A., Schumacher, N., Maier, M., Sendtner, M., and Gessler, M. 2004. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev **18**(8): 901-911.

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**(1-2): 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**(2): 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**(23): 2948-2956.

Garcia, X. and Stein, F. 2006. Nitric oxide. Semin Pediatr Infect Dis **17**(2): 55-57.

Guevara, I., Iwanejko, J., Dembinska-Kiec, A., Pankiewicz, J., Wanat, A., Anna, P., Golabek, I., Bartus, S., Malczewska-Malec, M., and Szczudlik, A. 1998. Determination of nitrite/nitrate in human biological material by the simple Griess reaction. Clin Chim Acta **274**(2): 177-188.

Guo, S. and Kemphues, K.J. 1995. par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81**(4): 611-620.

Hansson, G.K. 2005. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med **352**(16): 1685-1695.

Hershberger, C. and Binkley, S.B. 1968. Chemistry and metabolism of 3-deoxy-D-mannooctulosonic acid. I. Stereochemical determination. J Biol Chem **243**(7): 1578-1584.

Hoyne, G.F., Le Roux, I., Corsin-Jimenez, M., Tan, K., Dunne, J., Forsyth, L.M., Dallman, M.J., Owen, M.J., Ish-Horowicz, D., and Lamb, J.R. 2000. Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4(+) T cells. Int Immunol **12**(2): 177-185.

Hozumi, K., Abe, N., Chiba, S., Hirai, H., and Habu, S. 2003. Active form of Notch members can enforce T lymphopoiesis on lymphoid progenitors in the monolayer culture specific for B cell development. J Immunol **170**(10): 4973-4979.

Hozumi, K., Negishi, N., Suzuki, D., Abe, N., Sotomaru, Y., Tamaoki, N., Mailhos, C., Ish-Horowicz, D., Habu, S., and Owen, M.J. 2004. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. Nat Immunol **5**(6): 638-644.

Iso, T., Kedes, L., and Hamamori, Y. 2003. HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol **194**(3): 237-255.

Karanu, F.N., Murdoch, B., Gallacher, L., Wu, D.M., Koremoto, M., Sakano, S., and Bhatia, M. 2000. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. J Exp Med **192**(9): 1365-1372.

Koch, U. and Radtke, F. 2007. Notch and cancer: a double-edged sword. Cell Mol Life Sci **64**(21): 2746-2762.

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**(1): 180-188.

Maekawa, Y., Tsukumo, S., Chiba, S., Hirai, H., Hayashi, Y., Okada, H., Kishihara, K., and Yasutomo, K. 2003. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. Immunity **19**(4): 549-559.

Mancinelli, R.L. and McKay, C.P. 1983. Effects of nitric oxide and nitrogen dioxide on bacterial growth. Appl Environ Microbiol **46**(1): 198-202.

Marques, J.T. and Williams, B.R. 2005. Activation of the mammalian immune system by siRNAs. Nat Biotechnol **23**(11): 1399-1405.

Matranga, C., Tomari, Y., Shin, C., Bartel, D.P., and Zamore, P.D. 2005. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell **123**(4): 607-620.

Matsuno, K., Ito, M., Hori, K., Miyashita, F., Suzuki, S., Kishi, N., Artavanis-Tsakonas, S., and Okano, H. 2002. Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling. Development **129**(4): 1049-1059.

Matzke, M.A. and Birchler, J.A. 2005. RNAi-mediated pathways in the nucleus. Nat Rev Genet **6**(1): 24-35.

McManus, M.T. and Sharp, P.A. 2002. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet **3**(10): 737-747.

Mehra, V.C., Ramgolam, V.S., and Bender, J.R. 2005. Cytokines and cardiovascular disease. J Leukoc Biol **78**(4): 805-818.

Milner, L.A., Bigas, A., Kopan, R., Brashem-Stein, C., Bernstein, I.D., and Martin, D.I. 1996. Inhibition of granulocytic differentiation by mNotch1. Proc Natl Acad Sci U S A **93**(23): 13014-13019.

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**(9): 5362-5373.

Mosser, D.M. 2003. The many faces of macrophage activation. J Leukoc Biol **73**(2): 209-212.

Nagel, A.C., Krejci, A., Tenin, G., Bravo-Patino, A., Bray, S., Maier, D., and Preiss, A. 2005. Hairless-mediated repression of notch target genes requires the combined activity of Groucho and CtBP corepressors. Mol Cell Biol **25**(23): 10433-10441.

Ng, W.F., Duggan, P.J., Ponchel, F., Matarese, G., Lombardi, G., Edwards, A.D., Isaacs, J.D., and Lechler, R.I. 2001. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. Blood **98**(9): 2736-2744.

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**(7): 850-855.

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**(9): 2847-2854.

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**(1): 174-183.

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**(6): 3019-3024.

Rabinovitch, M. 1995. Professional and non-professional phagocytes: an introduction. Trends Cell Biol **5**(3): 85-87.

Radtke, F. and Raj, K. 2003. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer **3**(10): 756-767.

Radtke, F., Wilson, A., Mancini, S.J., and MacDonald, H.R. 2004. Notch regulation of lymphocyte development and function. Nat Immunol **5**(3): 247-253.

Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H.R., and Aguet, M. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity **10**(5): 547-558.

Robey, E. 1999. Regulation of T cell fate by Notch. Annu Rev Immunol **17**: 283-295.

Saito, T., Chiba, S., Ichikawa, M., Kunisato, A., Asai, T., Shimizu, K., Yamaguchi, T., Yamamoto, G., Seo, S., Kumano, K. et al. 2003. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity **18**(5): 675-685.

Sasmono, R.T., Oceandy, D., Pollard, J.W., Tong, W., Pavli, P., Wainwright, B.J., Ostrowski, M.C., Himes, S.R., and Hume, D.A. 2003. A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. Blood **101**(3): 1155-1163.

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**(1): 129-134.

Schroeder, T. and Just, U. 2000. Notch signalling via RBP-J promotes myeloid differentiation. EMBO J **19**(11): 2558-2568.

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**(5): 527-534.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J. et al. 2004. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature **432**(7014): 173-178.

Stier, S., Cheng, T., Dombkowski, D., Carlesso, N., and Scadden, D.T. 2002. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood **99**(7): 2369-2378.

Suzuki, T. and Chiba, S. 2005. Notch signaling in hematopoietic stem cells. Int J Hematol **82**(4): 285-294.

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**(10): 5113-5119.

Tanaka, S., Tsukada, J., Suzuki, W., Hayashi, K., Tanigaki, K., Tsuji, M., Inoue, H., Honjo, T., and Kubo, M. 2006. The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. Immunity **24**(6): 689-701.

Tanigaki, K., Han, H., Yamamoto, N., Tashiro, K., Ikegawa, M., Kuroda, K., Suzuki, A., Nakano, T., and Honjo, T. 2002. Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. Nat Immunol **3**(5): 443-450.

Trinchieri, G. 1997. Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma). Curr Opin Immunol **9**(1): 17-23.

Varnum-Finney, B., Purton, L.E., Yu, M., Brashem-Stein, C., Flowers, D., Staats, S., Moore, K.A., Le Roux, I., Mann, R., Gray, G. et al. 1998. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. Blood **91**(11): 4084-4091.

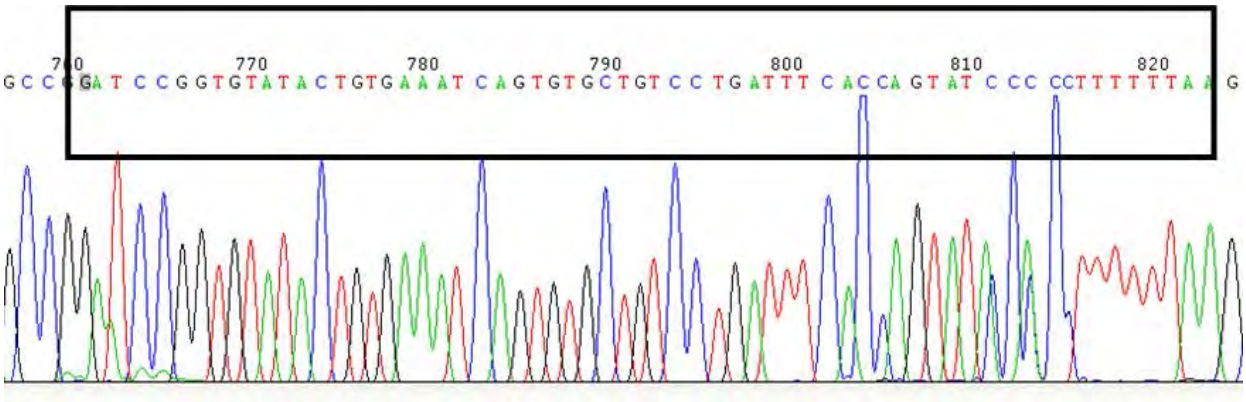
Wilson, A., MacDonald, H.R., and Radtke, F. 2001. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J Exp Med **194**(7): 1003-1012.

Wysocka, M., Kubin, M., Vieira, L.Q., Ozmen, L., Garotta, G., Scott, P., and Trinchieri, G. 1995. Interleukin-12 is required for interferon-gamma production and lethality in lipopolysaccharide-induced shock in mice. Eur J Immunol **25**(3): 672-676.

Zeng, Q., Li, S., Chepeha, D.B., Giordano, T.J., Li, J., Zhang, H., Polverini, P.J., Nor, J., Kitajewski, J., and Wang, C.Y. 2005. Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. Cancer Cell **8**(1): 13-23.

APPENDIX

A

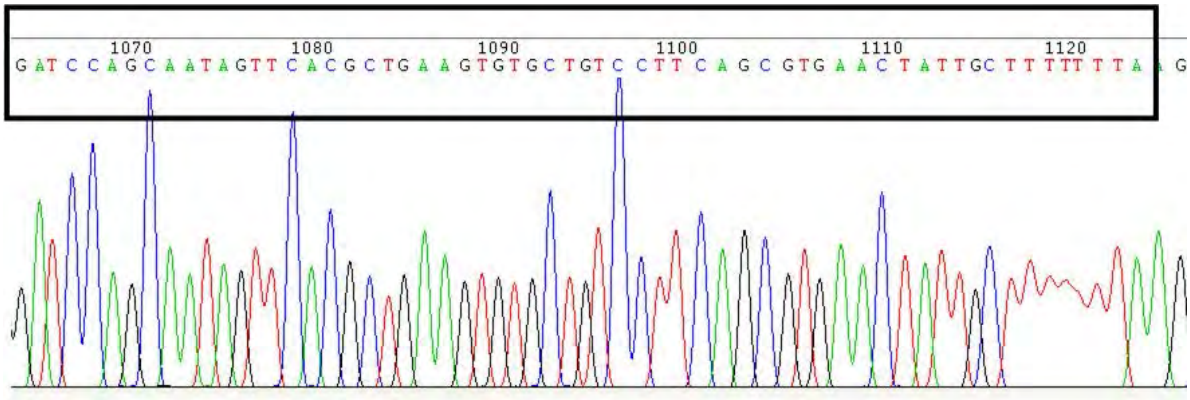


shNotch1 top strand (5’ to 3’)

5’- GATCCGGTGTATACTGTGAAATCAGTGTGCTGTCCTGATTTCACAGTATACACCTTT

TTTA-3’

B



shLuc top strand (5’ to 3’)

5’-GATCCAGCAATAGTTCACGCTGAAGTGTGCTGTCCTTCAGCGTGAACTATTGCTTTT

TTTA-3’

Figure 6.1 Chromatogram of nucleotide sequencing from 5’ to 3’ of pShNotch1 (figure 6.1 A) and pShLuc (figure 6.2B), respectively.

Complete DMEM 100 ml

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

Freezing media 10 ml

Complete DMEM	90%
DMSO	10%

10X annealing buffer

100 mM Tris-HCl pH 8.0

500 mM NaCl

50X Tris-acetic acid EDTA (TAE) buffer solution 200 ml

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

Adjust pH to 8.0 and make volume to 200 ml using deionized water. Then this solution was autoclaved at 121°C and pressure 15 psi for 15 min.

1X TAE for agarose gel preparation

50X TAE : deionized water = ratio 1 : 49

0.5X TAE for running buffer in agarose gel electrophoresis

50X TAE : deionized water = ratio 1 : 99

2% agarose gel preparation

Agarose	0.8 g
1X TAE	40 ml

1X PBS 1 L

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

In 800 ml of deionized water and adjust pH to 7.4. Then make volume to 1 L and sterile by autoclave at 121°C and pressure 15 psi for 15 min.

Reagents for protein extraction

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	1 tablet

Buffer B (stored at 4°C)

0.99 ml Buffer A + 0.01 ml Nonidet P-40

SDS polyacrylamide gel preparation

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

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

2X Laemmli buffer (SDS loading buffer 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

5X Running buffer 1 liter for western blot

Trisma base	15.1 g
Glycine	94 g
SDS	5 g

Adjust volume with deionized water to 1000 ml.

Transfer buffer 1 liter for western blot

Trisma base	5.08 g
Glycine	2.9 g
SDS	0.37 g

Adjust volume with deionized water to 800 ml.

This solution was added with absolute methanol 200 ml.

Blocking solution for western blot

PBST	100 ml
skim milk	3%

PBST (washing buffer for western blot)

1×PBS	500 ml
Tween20	0.05%

Substrate of HRP

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

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

Solution A

100 mM Tris-HCl pH 8.5 (stored at 4°C)	2.5 ml
90 mM coumaric acid	11 µl
250 mM luminol	25 µl

Solution B

100 mM Tris-HCl pH 8.5 (stored at 4°C)	2.5 ml
30% H_2O_2	1.5 µl

Components of Griess reaction

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.

NED solution (for) 50 ml (stored at 4°C)

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

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 DMEM complete media to 1 ml total volume.

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.

Film developer and fixer

Film developer and fixer were diluted in tap water at dilution 1 : 4 in total volume 100 ml.

Ampicillin, penicillin and streptomycin solution

Ampicillin and streptomycin 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.

4% paraformaldehyde

Paraformaldehyde (4 g) 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 chilled to room temperature and the pH was adjusted to 7.4.

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.

Luria-Bertani (LB) broth and agar 1000 ml

LB broth preparation

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Adjust pH to 7.4 and make a total of 1000 ml.

LB agar preparation

After prepare LB broth, add 1.5% of agar to broth. Autoclaved at 121°C pressure 15 psi for 15 minutes. Let agar cool about 55°C and then pour a thin layer of LB agar in petri dishes.

LB agar containing 50µg/ml ampicillin

After sterile LB broth and 1.5% agar with autoclaved at 121°C pressure 15 psi for 15 minutes. Then let LB media cool about 55°C and ampicillin was added to final concentration 50 µg/ml. After that this LB was poured in petri dishes.

Biography

NAME	Miss Thitiporn Pattarakankul
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ACADEMIC PRESENTATION

Pattarakankul, T. and Palaga, T. Silencing expression of *Notch1* in macrophages using RNAi. Development for sustainable livelihoods through Multidisciplinary Research, 28-29 July 2009, Sunee Grand Hotel, Ubonratchathani Thailand. P.447-453 (Poster Presentation)

Palaga, T., Boonyatecha, N. and Pattarakankul, T. The roles of Notch signaling in macrophage activation. The Notch Meeting 2009, 28 September-1 October 2009, Athens, Greece. (Poster Presentation)