การควบคุมการแสดงออกของ IL-6 โดยวิถีสัญญาณ Notch มิวรีนในแมโครฟาจ

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REGULATION OF IL-6 EXPRESSION BY NOTCH SIGNALING IN MURINE MACROPHAGES

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Notch รีเซบเตอร์และ ลิแกนด์เป็นกลุ่มโปรตีนบนเยื่อหุ้มเซลล์ที่เกี่ยวข้องกับการส่งสัญญาณของวิถีสัญญาณ Notch อันตรกิริยาระหว่าง Notch รีเซบเตอร์กับลิแกนด์เหนี่ยวนำให้มีการตัดโปรตีน Notch รีเซบเตอร์โดยแกมมาซีครีเตสและนำไปสู่การส่งสัญญาณ วิถีสัญญาณ Notch มีบทบาทสำคัญต่อกระบวนการพัฒนาการของสิ่งมีชีวิตหลายเซลล์ รวมทั้งเซลล์ของระบบภูมิคุ้มกัน ในการส่งสัญญาณนั้น Notch รีเซบเตอร์จะจับกับโปรตีน CSLในนิวเคลียสและกระตุ้นการถอดรหัสของจีนเป้าหมาย ในแมโครฟาจซึ่งเป็นเซลล์ในระบบภูมิคุ้มกันที่มีมาแต่กำเนิด วิถีสัญญาณ Notch มีบทบาทในการควบคุมการนำเสนอแอนติเจนต่อทีเซลล์และการตอบสนองที่เกี่ยวข้องกับการอักเสบ เช่น การหลั่งสารไซโตไคน์ต่างๆ IL-6 เป็นไซโตไคน์ที่หลั่งจากเซลล์หลายชนิดรวมทั้งในแมโครฟาจ IL-6 เป็นไซโตไคน์ที่มีบทบาทหลากหลาย เช่น ควบคุมระบบภูมิคุ้มกันและการอักเสบ จากรายงานก่อนหน้านี้และในการสืบค้นเบื้องต้นพบว่ามีตำแหน่งคอนเซนซัสบนโปรโมเตอร์ของ *IL-6* ที่ CSL รับรู้ และ CSL มีบทบาทในการกดการถอดรหัสของจีน *IL-6* แต่บทบาทของวิถีสัญญาณ Notch ในการควบคุมการแสดงออกของ IL-6 ในแมโครฟาจยังไม่มีการรายงานมาก่อน งานวิจัยนี้จึงศึกษาบทบาทของวิถีสัญญาณ Notch ในการควบคุมการแสดงออกของ IL-6 ในมิวรีนแมโครฟาจ จากการศึกษาพบว่าแมโครฟาจที่ถูกกระตุ้นโดยไลโปพอลิแซคคาไรด์และอินเตอร์เฟียรอนแกมมา มีการแสดงออกของจีน *IL-6* ขนานไปกับการเพิ่มขึ้นของ Notch1 เมื่อมีการกดวิถีสัญญาณ Notch โดยใช้ IL-CHO ซึ่งเป็นสารกดการทำงานของแกมมาซีครีเทส สามารถลดการแสดงออกของ IL-6 เมื่อเพิ่มสัญญาณของ Notch โดยการทรานสเฟคชันในเซลล์ไลน์ RAW264.7 พบว่าการแสดงออกของ IL-6 เพิ่มขึ้น เมื่อพิสูจน์ด้วยเทคนิค Chromatin Immunoprecipitation พบว่า anti RNA Polymerase II ซึ่งเป็นชุดตัวควบคุม จับบริเวณโปรโมเตอร์ของ *IL-6* ได้ ในขณะที่ไม่สามารถสรุปได้ว่า Notch รีเซบเตอร์จับกับบริเวณโปรโมเตอร์ของ *IL-6* เนื่องจากมีการจับแบบไม่จำเพาะของตัวควบคุม isotype ด้วย ผลที่ได้แสดงว่าวิถีสัญญาณ Notch มีบทบาทสำคัญในการควบคุมการแสดงออกของ IL-6 ในแมโครฟาจ ซึ่งอาจนำไปสู่การพัฒนายาที่ใช้ในการรักษาโรคที่เกิดจากความผิดปกติของการหลั่ง IL-6 ผ่านทางวิถีสัญญาณ Notch ต่อไปได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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WIPAWEE WONGCHANA: REGULATION OF IL-6 EXPRESSOIN BY NOTCH SIGNALING IN MURINE MACROPHAGES. THESIS ADVISOR: ASSISTANT PROFESSOR TANAPAT PALAGA, Ph.D., 85 pp.

Notch receptors and ligands are families of proteins involved in signal transduction of Notch signaling pathway. Interaction between Notch ligands and their receptors triggers cleavage of Notch receptor by gamma secretase which leads to signal transduction. Notch signaling plays important roles in development of metazoan cells, including immune cells. For signaling transduction, Notch receptors bind to CSL, a DNA-protein, in the nucleus and induce expression of target genes. In macrophages which are innate immune cells, Notch signaling regulates antigen presentation capacity and inflammatory responses such as secretion of pro-inflammatory cytokines including IL-6. IL-6 is a multifunctional cytokine secreted by various cell types, including macrophages. IL-6 functions in immune regulation and inflammation. Previous studies and our preliminary search identified the consensus CSL binding site in the promoter region of *IL-6* and CSL was shown to negatively regulate *IL-6* expression, but the role of Notch signaling in IL-6 expression in macrophages remained unresolved. Therefore, this study aimed at investigating the role of Notch signaling in regulation of IL-6 expression in murine macrophages. Our results showed that activated macrophages with LPS and interferon gamma induced *IL-6* expression in correlation with the upregulation of Notch1 expression. Inhibition of the Notch signaling in macrophages using IL-CHO, a gamma secretase inhibitor, significantly decreased IL-6 expression. Macrophages transiently transfected with truncated constitutively active Notch1 resulted in dramatic increase in both *IL-6* mRNA and secreted IL-6. When chromatin immunoprecipitation assay was used to prove whether Notch1 binds to the promoter of *IL-6*, only RNA polymerase II was found to bind to such promoter. The result for Notch1 binding was inconclusive because of the non-specific binding. Taken together, the results obtained in this study demonstrated that Notch signaling pathway plays an important role in IL-6 expression in macrophages. This finding may lead to a drug design targeting diseases with aberrant IL-6 secretion through Notch signaling.

Field of Study :Biotechnology Student's Signature .....

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ABBREVIATIONS

1	%	Percentage
2	/	Per
3	:	Ratio
4	+ve	Positive control
5	×	Fold
6	®	Registration
7	°C	Degree Celsius
8	µg	Microgram
9	µl	Micro liter
10	µM	Micro molar
11	µm	Micrometer
12	∞	Infinity
13	A	Absorbance
14	Ab	Antibody
15	ALPs	Autoimmune lymphoproliferative syndrome
16	AML	Acute myeloid leukemia
17	ANK	Ankyrin repeats
18	APP	Amyloid β-protein precursor
19	BCDF	B cell differentiation factor
20	bHLH	Basic helix-loop-helix
21	B-ML	B cell malignant lymphoma
22	BMMϕ	Bone marrow derived macrophage
23	bp	Base pair
24	BTD	Beta-trefoil domain

25	C/EBP	CAAT enhancer-binding protein
26	CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
27	CD	Clustter of differentiation
28	cDNA	Complementary DNA
29	ChIP	Chromatin immunoprecipitation
30	CIR	CBF1 interacting co-repressor
31	cM	Centimorgan
32	CO_2	Carbon dioxide
33	CRD	Cysteine-rich region domain
34	CSL	CBF-1/RBPJk in mammals, Su(H) in *Drosophila*, Lag-1 in *C. elegans*
35	CTD	C terminal domain
36	DC	Dendritic cells
37	DNA	Deoxyribonucleic acid
38	DNT	Double negative T cell
39	dNTP	DATP, dCTP, dGTP and dTTP
40	EGF-LR	Epidermal growth factor-like repeats
41	ELISA	Enzyme-linked immunosorbent assay
42	EMT	Epithelial-to-mesenchymal transition
43	g (centrifugation speed)	Gravity
44	GM-CSF	Granulocyte macrophages colony-stimulating factor
45	GSI	Gamma secretase inhibitor
46	HATs	Histone actyltransferases
47	HDACs	Histone deacetylase

48	*Hes1*	Hairy/Enhancer of Split1
49	HPLC	High performance liquid chromatography
50	hr	Hour
51	HRP	Horse radish peroxidase
52	IFNγ	Interferon gamma
53	Ig	Immunoglobulin
54	IL	Interleukin
55	kDa	Kilo Dalton
56	LB	Lauria bertani
57	LNR	LIN12/Notch repeats
58	LPS	Lipopolysaccharide
59	m	Murine
60	mA	Milliampere
61	MAML	Mastermind-like
62	mg	Milligram
63	min	Minute
64	ml	Milliliter
65	mM	Millimolar
66	N CoR	Nuclear receptor co repressor
67	N^{EC}	Extracellular domain of Notch
68	ng	Nanogram
69	N^{IC}	Intracellular domain of Notch
70	NLS	Nuclear localization signal
71	nm	Nanometer
72	No.	Number
73	NTD	N terminal domain

74	N^{TM}	Transmembrane domain of Notch
75	OD	Optical density
76	PAGE	Polyacrylamide gel electrophoresis
77	PAMPs	Pathogen-associated molecular patterns
78	PBS	Phosphate buffer saline
79	PBST	Phosphate buffer saline – Tween
80	pcDNA3	Plasmid cDNA3
81	pcDNA3NIC	Plasmid cDNA3 with intracellular Notch1
82	PCR	Polymerase chain reaction
83	PEST	Proline-glutamate serine-threonine-rich
84	PRRs	Pattern-recognition receptors
85	psi	Pound per square inch
86	PVDF	Polyvinylidine fluoride
87	r	Recombinant
88	RA	Rheumatoid arthritis
89	RAM	RAM32 domain
90	RB	Retinoblastoma
91	RNA	Ribonucleic acid
92	rpm	Round per minute
93	RT	Reverse transcription
94	SD	Standard deviation
95	SDS	Sodium dodecyl sulfate
96	SLE	Systemic lupus erythematosus
97	TACE	Tumor necrosis factor a converting enzyme
98	TAD	Transcriptional activation domain
99	T-ALL	T-cell acute lymphoblastic leukemia

100	Th	T helper cell
101	TLRs	Toll-like receptors
102	TNF	Tumor necrosis factor
103	™	Trade mark
104	U	Unit
105	v/v	Volume by volume
106	-ve	Negative control
107	w	Weight
108	α	Alpha
109	β	Beta
110	γ	Gamma

CHAPTER I

BACKGROUND

Notch receptors and ligands are transmembrane proteins in which Notch signaling pathway is involved. The binding of Notch ligands and their receptors leads to activation of the Notch signaling that regulates cell fate decision, cell proliferation and apoptosis during development. Once activated, Notch receptors bind to a DNA binding protein, CSL, and localize to the regulatory region in the promoters of the target genes. CSL binds to DNA at the consensus sequences as 5'(C/T)(A/G)TG(A/G/T)GA(A/G/T)3'. In the absence of intracellular domain of Notch (N^{IC}) CSL acts as a transcription repressor, while in the presence of N^{IC}, transcriptional co-repressors bound to CSL are replaced by N^{IC} and other co-activators are recruited, which in turn, switch on the transcription of the Notch target genes. IL-6 is multifunctional cytokines involved in immune regulation, inflammation and oncogenesis. Dyregulation of IL-6 expression is the cause of diseases such as autoimmune disease and cancers. IL-6 is produced by various types of cells such as macrophages, T cells, B cells and certain kinds of tumor cells. In innate immune cells, macrophages, the Notch signaling pathway functions in regulating inflammatory responses and antigen presentation capacity. The regulation of IL-6 expression depends on several transcription factors such as AP-1, C/EBP, IL6-κB, NF-κB and CSL, where binding sites of these transcription factors are found in the promoter region of IL-6.

Although CSL was indentified as regulator of IL-6, the relationship between Notch signaling and the expression of IL-6 in macrophages has not yet to be elucidated. Therefore, this study aimed at investigating the regulation of IL-6 expression in macrophages through the Notch signaling pathway.

Objective

To investigate the role of Notch signaling pathway in direct regulation of IL-6 expression in macrophages

CHAPTER II

LITERATURE REVIEWS

2.1 Notch signaling pathway

2.1.1 Notch receptors and ligands

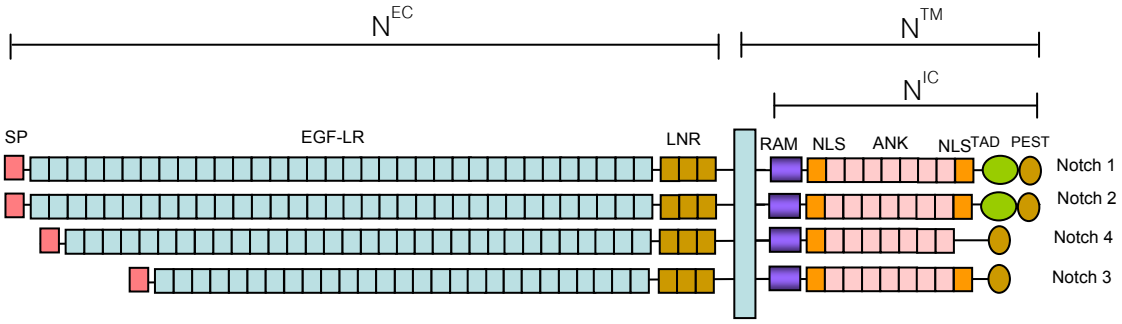
The Notch signaling pathway is highly conserved in all metazoan cells. In mammals, Notch receptors and ligands are type I membrane proteins divided into five Notch ligands (Delta-like 1, 3, and 4, Jagged 1 and 2) and four Notch receptors (Notch 1 - 4) (Kovall, 2007).

Notch receptors are single-pass transmembrane proteins, prior to the enzymatic cleavage within the trans-golgi by furin-like convertase during transportation to cell surface. This process results into two parts of the proteins: extracellular domain of Notch (N^{EC}) and transmembrane domain of Notch (N^{TM}) including intracellular domain of Notch (N^{IC}). Fringe glycosyltransferase in Golgi body modified N^{EC} by adding N-acetyl glucosamine (O-linked fucose residues) to epidermal growth factor-like repeats (EGF-LR) which plays an important roles in determining the binding affinity to ligands (Radtke *et al.*, 2005).

The schematic structure of Notch proteins are demonstrated in Figure 2.1. N^{EC} contains tandem EGF-LR, some of which are responsible for ligand-binding and LIN12/Notch repeats (LNR) which functions in preventing the binding of receptors and ligands in non-stimulated condition. N^{IC} composes of RAM32 domain (RAM) and ankyrin repeats (ANK) which are both required for signal transduction, two nuclear localization signal (NLS) motifs, and a C-terminal proline-glutamate serine-threonine-rich (PEST) domain which regulates N^{IC} stability. In addition, Notch1 and 2 contain a C-terminal transcriptional activation domain (TAD), while this domain is absence in Notch 3 and 4 (Allman *et al.*, 2002). Similar to Notch receptors, Notch ligands are composed of EGF-LR and a unique N-

terminal DSL domain. The cysteine-rich region domain (CRD), however, can be found in only Jagged 1 and 2, not in Delta like 1-3.

A



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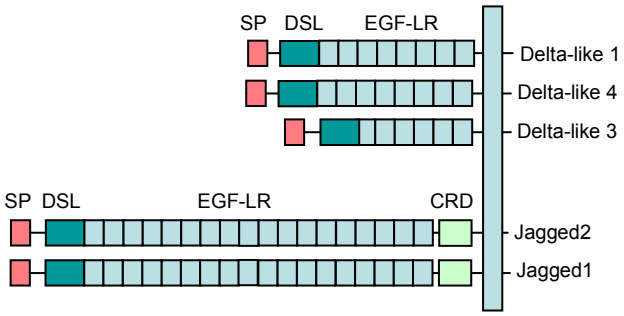


Figure 2.1 Structure of Notch receptors and Notch ligands.

(A) Notch receptor, Notch 1-4, N^{EC} contains 29-34 EGF-LR and 3 LNR. N^{IC} consists of RAM32 domain, ANK domain, NLS and PEST domain. Notch1 and 2 contain TAD domain, while is absence in Notch 3 and 4. (B) Notch ligands are composed of EGF-LR, DSL and CRD domain which can be found in only Jagged 1 and 2, not in Delta like 1,2 and 3. (Allman *et al.*, 2002).

2.1.2 Notch signaling pathway and its target genes

The interaction between Notch ligands and their receptors contributes to the releasing of N^{IC} via at least two proteolytic cleavages (Figure 2.2), *i.e.* tumor necrosis factor α converting enzyme (TACE) at N^{EC} and γ-secretase to release cleave N^{IC} (Shih Ie *et al.*, 2007) . The free N^{IC} translocates into nucleus where the RAM23 and ANK domains on N^{IC} directly bind to CSL and replace transcription co repressors complex (Kovall, 2007) . The RAM23 domain is responsible for the CSL binding, while the ANK domain is postulated to function in protein-protein interaction with CSL and other peptides (Allman *et al.*, 2002) . The arrival of N^{IC} converts this complex to be a transcription factor by recruiting transcriptional co-activator to the Notch/CSL complex (Lai, 2002). CSL/Notch/Co-activator, in turn, functions in regulating the expression of Notch target gene.

Notch signaling has been regulated or modified by various proteins such as Numb which prevents nuclear translocation of N^{IC} by promoting degradation of Notch1 (Mcgill *et al.*, 2003) , Itch which binds to the N terminal domain of N^{IC} and promotes ubiquitination of Notch (Qiu *et al.*, 2000) , and Mint/SHARP which competes with N^{IC} for binding to CSL (Radtke *et al.*, 2005) . These proteins function as the negative regulators of Notch signaling pathway, whereas Deltex is described as a positive regulator of Notch signaling (Yamamoto *et al.*, 2001).

The well known Notch target genes are member of basic helix-loop-helix (bHLH) family of transcriptional repressor such as hairy/enhancer of split (HES1-HES7) and HES related (HEY1-2 and HEYL) gene. HES and HEY proteins have been reported to function as the inhibitor of expression of other Notch signaling pathway, suggesting a feedback regulatory mechanism (Fischer *et al.*, 2007). Other reported Notch target genes are *cylcin D1* which is involved in the control of the G1/S transition during cell cycles (Ronchini *et al.*, 2001), *pre TCRα* (Maillard *et al.*, 2003), *NF-κB2* (Osborne *et al.*, 1999), and, *Deltex1* (Yamamoto *et al.*, 2001).

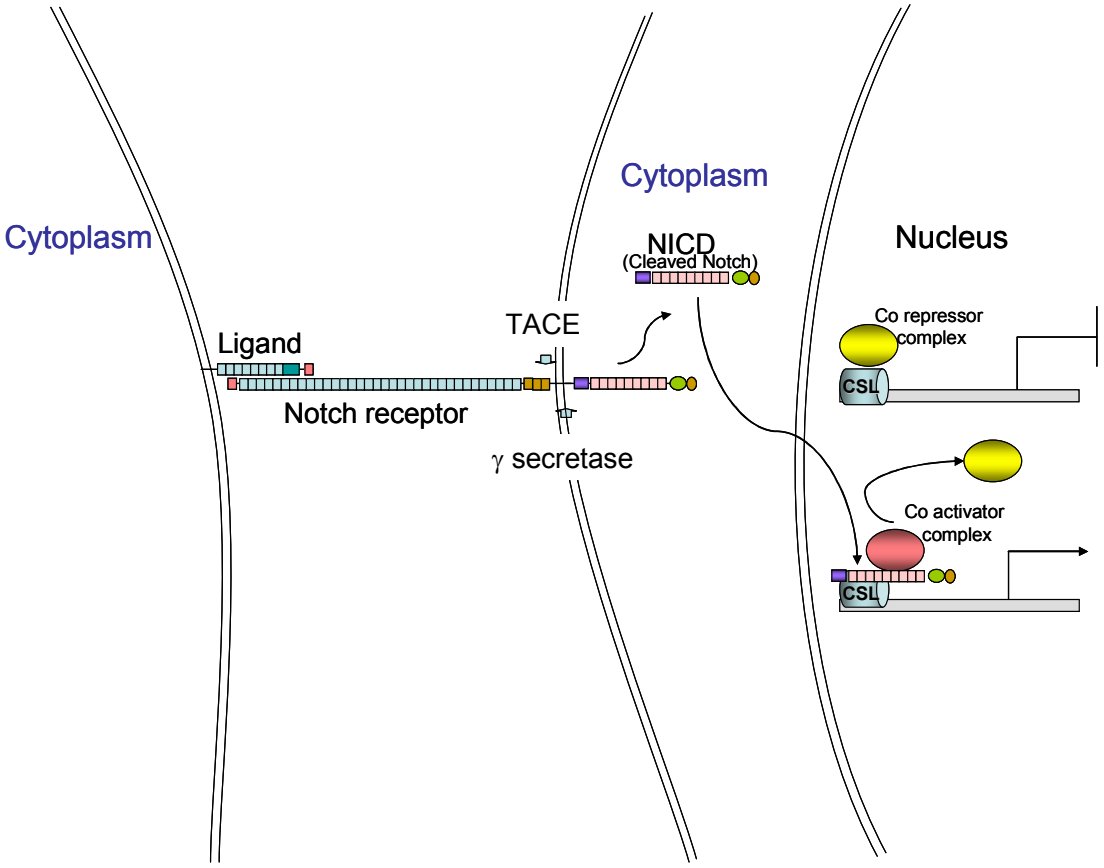


Figure 2.2 Notch signaling pathway

Interaction between Notch ligand and receptor initiated proteolytic cleavages by TACE and γ secretase led to Notch signaling pathway. Cleaved Notch, then, translocated into the nucleus and displaced Co repressors and formed complex with CSL. This complex recruited Co activator and MAML to the complex to turn on transcription of Notch inducible genes.

2.1.3 Biological functions of Notch signaling

Binding of Notch ligands and their receptors initiates Notch signaling which functions in regulating cell fate decisions during metazoan development. Notch signaling also influences cellular development, differentiation, proliferation and apoptosis in various cell types. This signaling pathway, thus, plays crucial roles in several developmental processes such as hematopoiesis, myogenesis and neurogenesis (Kojika *et al.*, 2001).

In hematopoiesis, it is clear that Notch signaling plays an important role in regulation of lymphoid development and functions such as development of thymocytes, differentiation of peripheral T cells and B cell (Tanigaki *et al.*, 2007). During development, signaling by Notch 1 inhibits B cell development but permits T cell development. Furthermore, Notch signaling also induces self-renewal of lymphoid repopulating cell (Ohishi *et al.*, 2003) and has been demonstrated to be involved in terminal differentiation of mature B cell to Ig secreting LPS-inducing splenic B cell (Santos *et al.*, 2007).

In myeloid precursor, Notch signaling has been reported to play several key functions such as inhibition of myeloid differentiation (Ohishi *et al.*, 2003) and regulation of dendritic cells (DC) differentiation and function. Targeted deletion of *Notch1* in embryonic stem cell leads to the reduction of DCs population (Cheng *et al.*, 2008).

In non-hematopoietic cell, Notch signaling is essential for an appropriate balance of chondrogenic proliferation and differentiation at initial stage of somite compartmentalization and skeleton development (Mead *et al.*, 2009). This signaling also functions in promoting self-renewal of mammary stem cells (Dontu *et al.*, 2004). During neural development, the maintenance of stem cell features of neuroprogenitor in human embryonic stem cell required Notch signaling (Woo *et al.*, 2009). Furthermore, signaling via Notch1 is also important for cardiac development by promoting Epithelial-to-mesenchymal transition (EMT) (Timmerman *et al.*, 2004). Several evidences indicate that some genetic disorders are the results of mutations in the Notch signaling, highlighting the importance of the Notch signaling in

development and maintenance of cardiovascular system, such as in Alagille syndrome and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Gridley, 2003).

2.1.4 Notch signaling and diseases

Aberrant activation of Notch signaling leads to alteration in cell properties, which may result in ontogenesis (Shih Ie *et al.*, 2007). Notch signaling promotes the growth of a wide range of tumors (Grego-Bessa *et al.*, 2004 and Garber, 2007). Activating mutations in *Notch1* are found in almost 50 % of T-cell acute lymphoblastic leukemia (T-ALL), suggesting a tumor promoting effect of Notch signaling (Weng *et al.*, 2004). Notch signaling has also been linked to autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) (Teachey *et al.*, 2008) and multiple sclerosis, based on its functions in regulating activation and differentiation of T cell (Talora *et al.*, 2008). As Notch signaling is involved in promoting EMT in heart development (Grego-Bessa *et al.*, 2004), mutation in Notch receptors are linked to cardiovascular disorder (Rusanescu *et al.*, 2008).

Mutations in genes involved in the Notch pathway contribute to inherited human diseases such as Alagille syndrome which is characterized by developmental abnormalities of liver, heart, eye, etc. The positional cloning evidences reveal that almost 70% of the patients harbor the point mutations in *Jagged1* gene. CADASIL is an autosomal dominant vascular disorder caused by *Notch3* mutations (Gridley, 2003).

2.1.5 Notch signaling and γ-secretase inhibitor (GSI)

Upon interaction between Notch ligands and receptors, Notch receptors are enzymatic cleaved; by TACE and γ-secretase. This cleavage results in releasing N^{IC}, a truncated Notch receptor, which plays crucial functions in regulating expression of the target genes. γ-

secretase is the protease complex which involves in the proteolytic cleavages of type I integral membrane proteins. Substrates of γ-secretase are identified such as amyloid β-protein precursor (APP) and the Notch receptors. This enzyme comprises of four different transmembrane proteins including catalytic subunit (presenilin-1 or presenilin-2), nicastrin, Aph-1 and Pen-2. Presenilin is an aspartyl protease which mediates transmembrane proteolytic processes, meanwhile, nicastrin contributes to substrate recognition by interacting with the N terminal region of substrates (Wolfe, 2006).

Similar to the releasing process of N^{IC} by γ-secretase, when APP is cleaved, the product Aβ-peptide, is released which contributes to neurotoxic protein aggregates that is linked to Alzheimer's disease.

In the past ten years, γ-secretase inhibitors (GSI) have been generated for blocking the generation of Aβ- peptide. Since Notch and APP shares the same enzymes for cleavage, GSI has also been applied for prevention of Notch signaling activation.

Using IL-CHO, a peptidometic GSI, resulted in inhibition of Notch1 expression in splenocytes, and partially blocked CD4 and CD8 T cell proliferation (Palaga *et al.*, 2003). Moreover, in human macrophages, two different GSIs inhibited *HES1, HEY1, IL2RA* and *IL7R* expression, all of which are Notch inducible genes, in TLR-induced condition (Hu *et al.*, 2008). Furthermore, Notch1 expression is dramatically reduced in IL-CHO treated bone marrow derived macrophages activated by LPS and IFNγ (Palaga *et al.*, 2008).

Recently, several types of GSI have been tested for suppression of tumors since there are evidences linking Notch signaling with tumorigensis. The findings demonstrated that GSI suppressed *in vitro* growth of some B cell malignant lymphoma (B-ML) cells line and acute myeloid leukemia (AML) cell line (Kogoshi *et al.*, 2007). Moreover, a phase I clinical trial for blocking of Notch signaling has been developed and used in T-ALL patients (Shih Ie *et al.*, 2007).

In autoimmune lymphoproliferative syndrome (ALPS) and SLE, abnormal induction of double negative T cell (DNT) population during T cell lineages development and hyperactivation of T cells resulted in autoimmune symptoms. Treatment with GSI, N-S-phenyl-glycine-t-butyl ester (DAPT), leads to decreasing in all disease parameters and the levels of DNT cell blood circulation (Teachey *et al.*, 2008).

2.2 DNA binding protein, CSL

CSL [CBF-1/RBPJκ in mammals, Su(H) in *Drosophila*, Lag-1 in *C. elegans*] is the DNA-binding protein which interacts with Notch receptor in the nucleus and is responsible for regulation of transcription of canonical target genes of Notch signalling pathway. CSL is a well conserved protein which shows 69% and 72% identity between *Drosophila* and mouse, and between *Drosophila* and human, respectively (Pursglove *et al.*, 2005). CSL composes of three structure domains: C terminal domain (CTD), beta-trefoil domain (BTD) and N terminal (NTD). CTD and NTD function in DNA binding, when it recognizes the consensus sequences of 5'(C/T)(A/G)TG(A/G/T)GA(A/G/T) 3' (Chung *et al.*, 1994; Tun *et al.*, 1994 and Pursglove *et al.*, 2005). N^{IC}, CSL and other complex are captured in the conserved hydrophobic pocket on BTD (Kovall *et al.*, 2004). CSL in mammals localizes in the nucleus while *Drosophila* CSL locates in cytoplasm until it binds to N^{IC}, and together they translocate to the nucleus. In mammals, CSL localizes to the nucleus due to its interactions with the SMRT/SKIP co-repressor complex, in which SMRT plays crucial role in inducing translocation of CSL to nucleus (Pursglove *et al.*, 2005).

2.2.1 CSL and Notch complex in nucleus

In the absence of N^{IC}, CSL acts as transcriptional repressor by directly binding to silencing mediator of retinoid and thyroid receptors (SMRT)/nuclear receptor co repressor (N

CoR), CBF1 interacting co-repressor (CIR) , and adaptor protein called SKIP. This multi-protein complex recruits histone deacetylase (HDACs) to the binding site and converts the local chromatin into a transcriptional repression form. In contrast, in a presence of N^{IC}, co-repressors forming complex on CSL protein are replaced by N^{IC}. Histone actyltransferases (HATs), a Mastermind-like co-activator (MAML) and other co-activators are recruited to the complex (Kovall, 2007). ANK domain and C-terminal transcriptional activation domain of N^{IC} functions in engaging additional co-activators, which contribute to the strong activation of target gene transcription (Allman *et al.*, 2002).

Previously, analysis of interaction among NIC, CSL and MAML shows that the stability for binding of this complex to DNA is determined by N^{IC} containing complexes can (1) bind cooperatively to DNA with properly arranged paired site or (2) interact with other associated factors (Krejci *et al.*, 2007).

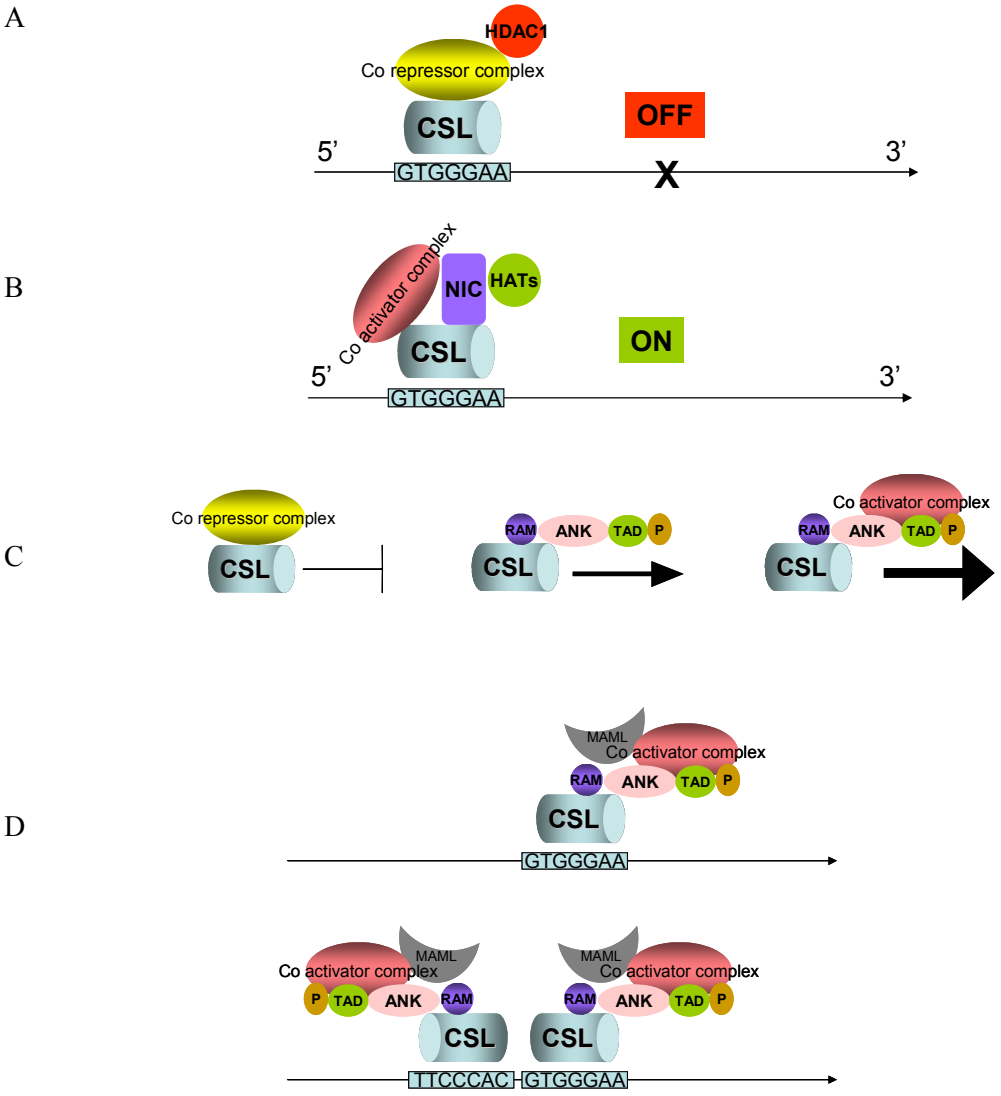


Figure 2.3 Schematic Representations of Events in Nucleus in the Absence or Presence of Notch Signaling

(A) CSL and co repressor complex repress target gene transcription (B) CSL and co activator complex induce target gene transcription (C) ANK domain and C-terminal transcriptional activation domain of N^{IC} plays functions in recruitment of additional co-activators which lead to the strong activation of transcription (Allman *et al.*, 2002). (D) The stability of CSL/Notch/DNA is determined by N^{IC} containing complexes can (1) bind cooperatively to DNA with properly arranged paired site or (2) interact with other associated factors (Krejci *et al.*, 2007).

2.3 Biology of Macrophages

Macrophages are white blood cells playing the essential roles in immune system by secreting pro-inflammatory cytokines and killing pathogens. After hematopoiesis in the bone marrow, promonocytes, which differentiate from granulocyte-monocyte progenitors, leave the specific area in the bone marrow and differentiate into mature monocytes. In blood circulation, inexperienced monocytes expose to soluble factors influence their biological functions and characters. After 8 h of circulation in the blood, these monocytes increase in size and migrate to different tissues, depending on the availability of chemokines or other specific homing factor. In particular tissue niche, these monocytes differentiate to become mature macrophages, which play an important role during innate immune response when they are activated by pathogens and cytokines such as IFNγ. Macrophages also function as antigen presenting cells to present peptide antigens to T cell and trigger acquired immune responses. Microenvironment in different tissues also affects biological functions of macrophages (Stout *et al.*, 2004).

Currently, macrophages are divided into at least three distinct subpopulations, based on stimuli, gene expression patterns and biological functions. The classically activated macrophages are macrophages which are activated by IFNγ and either exogenous TNF or TNF itself, resulting in higher production of TNF and IL-12, production of IL-1 and IL-6 and microbicidal chemical intermediates such as nitric oxide. This type of macrophages is proposed to be responsible for Th1 type immune response. Another type of macrophages is called the alternatively activated macrophages. They are induced by IL-4 or glucocorticiods which contributes to the immunosuppressive functions and are involved in tissue repairing. Type II activated macrophages are activated by stimuli similar to classical activating macrophages in the presence of antigen-antibody immune complex. This type was shown to be anti- inflammatory and induce Th2 type immune response according to the production of high amounts of IL-10 and affecting T cells to produce high level of IL-4 (Mosser, 2003).

2.4 Toll- like receptors (TLRs) and macrophages

The innate immune system is the first line of defense against microbial infection. Innate immune response is initiated by engagement of recognizing pathogen-associated molecular patterns (PAMPs) with specific pattern-recognition receptors (PRRs). One of PRRs which plays critical roles in initiation of acute inflammatory response is Toll-like receptors (TLRs). Currently, there are more than 10 members of the TLRs identified in mammals. Each one recognizes different PAPMs derived from foreign microbes and virus. For example; TLR2 recognizes peptidoglycan which is found mainly in Gram positive bacteria, while TLR4 is responsible for recognition of lipopolysaccharide (LPS) which has been conserved in Gram negative bacteria and TLR7 and 8 recognize RNA form RNA virus (Pasare *et al.*, 2004). The recognition via TLRs contributes to activation of NF-κB signaling pathway through MyD88 dependent and independent pathways. This evidence involves in beginning of innate immune response activation results in production of various types of cytokine, chemokine and expression of co-stimulatory molecules which plays crucial roles in adaptive immune response (Medzhitov *et al.*, 2000). In macrophages, signaling through TLRs improves phagocytosis and killing activities. Activation of macrophages with TLR ligands leads to production of inflammatory cytokines such as TNF and IL-1 (Medzhitov, 2007).

2.5 Notch signaling and macrophages

Expression of Notch receptors and ligands has been reported in macrophage. Notch signaling regulates specific gene expression patterns induced by different activation signals that may modulate the function of mature macrophages (Monsalve *et al.*, 2006 and Palaga *et al.*, 2008). In addition, involvement of Notch signaling in regulation of biological activities of macrophages by LPS plus IFNγ activation was observed (Palaga *et al.*, 2008).

There are several reports on the role of Notch signaling in macrophages. Macrophages from various tissues in mice express Notch ligands (Jagged1, 2 and Delta1) (Yamaguchi *et al.*, 2002). Furthermore, expression of Notch receptors and ligands participate in the differentiation of monocytes which are precursors of macrophages, dendritic cells and osteoclasts. For example in presence of GM-CSF, Delta-1 inhibits differentiation of monocytes into macrophages (Ohishi *et al.*, 2003). Activation of macrophages by TLR stimulation such as LPS involved the Notch signaling through NF-kB activation, lead to IL-10, IL-6 and TNF-α expression (Palaga *et al.*, 2008). Likewise, macrophages which are exposed to LPS, mLDL and IL-1β expressed mRNA and protein of Delta-Like 4. Moreover, co incubation of macrophages with Delta- like 4 triggers activation of the Notch signaling and increases pro-inflammatory gene transcription (Fung *et al.*, 2007). These observations imply a role of Notch signaling in regulating cytokine production in macrophages.

2.6 Interleukin 6 (IL-6) and its functions

IL-6 was initially identified as an antigen-nonspecific B cell differentiation factor (BCDF) which stimulated B-lymphoblast stoid cell line (CESS) cells, and B-CLL cells to produce immunoglobulin. This factor was called BSF-2. In 1986, Hirano et al., successfully cloned cDNA encoding this peptides and it was called IL-6 (Kishimoto, 2005). The findings reported by other groups identified the identical molecules in many cells with various names such as interferon-β2 and 26-kDa protein in fibroblasts, plasmacytoma growth factor which was produced by mineral oil induced granulomas in murine macrophages and hepatocyte stimulating factor in liver cells (Gauldie *et al.*, 1987 and Castell *et al.*, 1988). Although this molecule was found in many types of cells and showed several different biological functions, they are all identical and now called IL-6. IL-6 is a multifunctional cytokine which is involved in immune regulation, hematopoiesis, inflammation and oncogenesis.

IL-6 is mainly produced by T cells, B-cells, macrophages, fibroblasts, synovial cells, endothelial cells, glia cells, keratinocytes and several types of tumor cells as depicted in Figure 2.5 (Naka *et al.*, 2002). Dyregulation of IL-6 expression is one of the causes of various diseases such as autoimmune disease and cancers. In tumors, high level expression of IL-6 has been detected in multiple epithelial tumors such as breast (Sansone *et al.*, 2007 and lung cancer (Gao *et al.*, 2007).

IL-6 is one of the most important cytokines in the acute inflammatory response in acute phase and other systemic response. It plays crucial roles in the pathophysiology of rheumatoid arthritis (RA) where expression of IL-6 was observed in fibroblast-like synoviocytes from joint tissues from RA patients (Rosenbaum *et al.*, 1992). IL-6 is also reported as an early marker for fat embolism which occurs when fat and marrow content (contain 92% fat) from bone enter the blood circulation, trap in the capillaries of the lungs, and activate an inflammatory response leading to the leakage of proteins in to alveoli (Yoga *et al.*, 2009). In bone remodelling, IL-6 has been shown in various cells lineages such as osteoblasts, osteocytes and osteoclasts to be involved in bone formation and resorption. The results demonstrate that IL-6 in osteoblastic cells, and stromal cells, but not osteoclastic cells is involved in bone pathophysiology by direct activation of bone resorption and osteoclastogenesis (Kwan Tat *et al.*, 2004). Moreover, during differentitation of helper T cells, IL-6 combined with TGF-β directly regulate the differentiation of IL-17 producing T cells from naïve T cell (Kimura *et al.*, 2007)

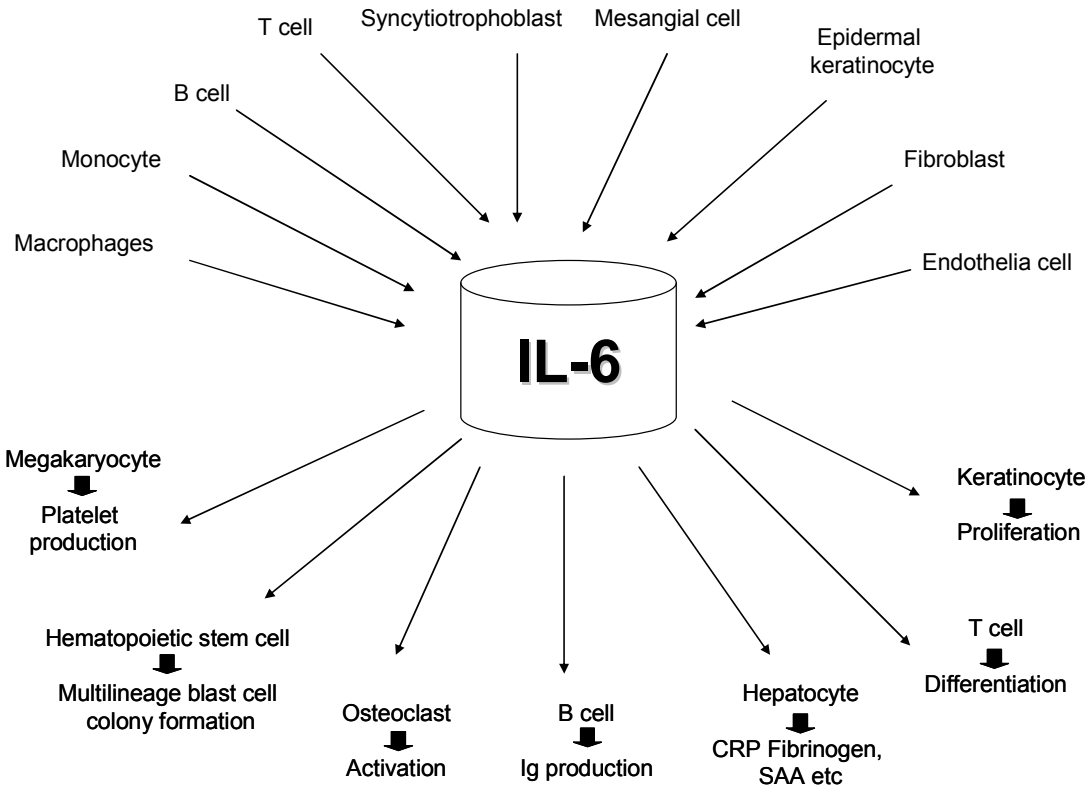


Figure 2.4 Various cellular sources of IL-6

IL-6 is produced from many cell types and is involved in various activities of cells (Naka *et al.*, 2002) .

2.6.1 Regulation of IL-6 expression

Signal transduction by IL-6 depends on IL-6 receptor composed of two subunits, *i.e.* IL-6 receptor (IL-6R) and gp130. The signal transduction is mediated by JAK-STAT pathway and Ras mitogen-activated protein kinase pathway via gp130 (Kishimoto, 2005).

The induction of IL-6 expression was mediated by several factors such as IL-1 as shown in stimulated synoviocytes taken from RA patient (Rosenbaum *et al.*, 1992), RNA and DNA virus infection, bacterial endotoxin, TNF and LPS (Hong *et al.*, 2007). In human adipose tissues, LPS activates adipocytes through TLR4 to induce IL-6 production (Hoch *et al.*, 2008).

At the molecular level, the regulation of IL-6 expression depends on various transcription factors such as AP-1, CAAT enhancer-binding protein (C/EBP), NF-κB and CSL. The consensus binding sites of these transcription factors are found in the promoter region of IL-6 (Kishimoto, 2005; Vales *et al.*, 2002; Palmieri *et al.*, 1999; Hong *et al.*, 2007 and Miyazawa *et al.*, 1998). Moreover, there is the report on the differences in LPS stimulated IL-6 production according to promoter haplotype and differential nuclear protein binding in the region of -174 Transcription factor motif of IL-6 promoter in *ex vivo* leukocytes (Rivera-Chavez *et al.*, 2003).

In case of IL-1β-induced IL6 expression in fibroblast-like synoviocytes, the report demonstrated that the regulation of IL-6 expression is under the control of C/EBPβ, NF-κB and CSL (Miyazawa *et al.*, 1998). In contrast, p53 which is nuclear tumor suppressor protein and retinoblastoma (RB) are reported to function as transcriptional repressors of *IL-6* expression during differentiation and tumorigenesis in p53 and RB transfected HeLa cell line (Santhanam *et al.*, 1991).

In addition, there are several reports show the involvement of CSL in regulation of *IL-6* gene. CSL binding site on IL-6 promoter is identified as IL-6κB which locates at -73 to -

60 and contains both putative NF-κB (-73 to -64) and CSL (-67 to -60) binding elements (Palmieri *et al.*, 1999 and Miyazawa *et al.*, 1998). Miyazawa *et al*., further showed that cooperation of NF-κB and CSL binding elements on IL-6 promoter plays an essential role in efficient transcriptional induction of *IL-6* gene in response to IL-1β stimulation in human rheumatoid fibroblast-like synoviocytes (Miyazawa *et al.*, 1998).

There are at least two reports, however, show that CSL overlapping the IL-6κB site is a negative regulator. In mouse fibrosarcoma cell (L929SA), CSL binds to κB site of IL-6 promoter, control the translocation of NF-κB to nucleus after activation and repress transcription of *IL-6* in the absence of NF-κB protein (Plaisance *et al.*, 1997). Moreover, over expression of CSL becomes a negative regulator of IL-6 expression in various human cell lines (Palmieri *et al.*, 1999). Palmieri et al. showed that HeLa cell line co-transfected with expression vector containing CSL decreases luciferase activity which was promoted with promoter in wide type. The result indicates that CSL is an inhibitor of *IL-6* gene transcription.

2.7 Notch signaling and IL-6 expression

There are several reports on the associated roles of Notch signaling and IL-6 expression. For examples; Increasing in secreted IL-6 from tumorigenic conversion of mammary stem cells causes the upregulation of the Notch-3 ligand (Jagged-1) which results in promotion of malignant features itself (Sansone *et al.*, 2007). In murine stromal cell line (SC9-19) which highly expressed Delta like-4, it had been shown that IL-6 was the most effective inducer of Delta like-4 protein expression via STAT3 activation (Suzuki *et al.*, 2006) and γ-secretase inhibitor decrease level of *IL-6* mRNA expression in LPS plus IFNγ stimulated macrophages (Palaga *et al.*, 2008).

Although CSL was previously proposed as a regulator of IL-6, the relationship between Notch signaling and IL-6 expression in innate immune cells has not been extensively investigated. Therefore, the previous findings lead us to purpose investigating the direct role of Notch signaling in regulation of IL-6 expression in macrophages. The results obtained from this study may shed a new light into the complex regulatory net work of IL-6 gene where Notch plays an essential role and lead to novel drug development for therapeutics of IL-6 related diseases.

CHAPTER III

MATERIALS AND METHODS

3.1 Cell line, primary cells and media

RAW 264.7 cell line (ATCC TIB-71) and bone marrow derived macrophages (BMMϕ) from female C57BL/6J mice (National Laboratory Animal Center, Mahidol University, Salaya, Thailand) were used in this study. All procedures involving laboratory animals were conducted according to the guidelines issued by Chulalongkorn University. The animal protocols used in this study have been approved by IACUC of Faculty of Science (Animal Protocol Rev. No. 0823001). RAW 264.7 and BMMϕ are maintained in DMEM media (Hyclone, England) with supplement of 10% FBS (v/v) (Hyclone, UK), 100 U/ml penicillin (General Drugs House Co., Ltd., Thailand), 0.4 mg/ml streptomycin (M & H Manufacturing Co., Ltd., Thailand), 1% sodium pyruvate (Hyclone, UK) and 1% HEPES (Hyclone, England) at 37°C and incubated in humidified 5% CO_2 incubator (Thermo Electron Corporation, USA).

3.2 Cell Preparation

3.2.1 Cell preparation

RAW 264.7 cell line was removed from non-treated culture dish (Hycon, Germany) using 1x cold PBS (Appendix A). Cell suspension was centrifuged at 1000 rpm for 5 min (Profuge, USA). The culture supernatant was discarded and cells were re-suspended in DMEM complete media. BMMϕ were prepared from femoral bone marrows of C57BL/6J

mice as described previously (Palaga et al., 2008). Briefly, cells were eluted from bone cavity and cultured in DMEM media containing 20% L929-conditioned media (v/v) and 5% horse serum (Hyclone, UK) for 10 days. Media were added every 3 days and cells were harvested using 1x cold PBS. Cell suspension was centrifuged at 1000 rpm for 5 min. Culture supernatant was discarded and cells were re-suspended in DMEM complete media. Viable cells were counted using trypan blue dye (Hyclone, England). Cells were diluted to appropriate concentrations as indicated and plated in the tissue culture plates for the experiments.

3.2.2 Cell preservation for storage

After centrifugation, RAW 264.7 cell line were resuspended in 1 ml of ice cold freezing media composed of 10% DMSO (v/v) (Sigma Aldrich, USA) in DMEM complete media, and stored in cryogenic vial (Corning Incorporation, USA). The cells were immediately stored at -70°C for at least overnight. For long term storage, cells were stored in Liquid Nitrogen Tank 34 HC Taylor Wharton Cryogenic (Harsco Corporation, USA). For BMMϕ, cells were re suspended in 500 µl of 80% DMEM media supplemented with 20% FBS and 500 µl of 60% DMEM media supplemented with 20% FBS and 20% DMSO. Cells were stored in cryogenic vial and kept at -70°C.

3.2.3 Thawing cells for use

Stored RAW 264.7 cell line was thawed in 37°C water bath (Memmert, Germany). Cells were washed in 9 ml serum-free media and centrifuged at 1000 rpm for 5 min. Supernatant was removed and 7 ml of DMEM complete media was added. Cells were plated in non-tissue culture treated plates and cultured until use for experiments. For frozen BMMϕ,

after thawing and centrifugation, culture supernatants were removed and 8 ml of DMEM media supplemented with 20% L929-conditioned media and 5% horse serum was added. Cells were plated on non tissue culture treated plates for at least 3 days before use.

3.3 Western blot

3.3.1 Protein extraction and quantitation

Cells treated as indicated were subjected to cell lysis and proteins were extracted as described by Palaga *et al*. (2003). In brief, culture supernatant was carefully removed from the plate, and cells were washed with 1 ml of 1x cold PBS and 250 µl of Buffer A (Appendix A). This procedure was followed by adding 30-40 µl of Buffer B (Appendix A) used for cell lysis. After mixing by micropipette (Gilson, France), cell lysates were transferred to 1.5 ml microcentrifuge tubes (Axygen Scientific, USA) and centrifuged at 5000 rpm for 5 min at room temperature. Supernatant were kept on ice for further analysis.

Protein concentrations were measured using BCA (bicinchoninic acid) ^{TM} protein assay (PIERCE, USA), according to manufacturer's instruction. The working reagent composed of reagent A and reagent B mixed at ratio 50: 1 was prepared and BSA (1mg/ml) was used as protein standard. BSA was diluted in sterile deionized water at 0, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml in 96-well microtiter plate (Corning Incorporation, USA). The samples were diluted at 1:10 (1 µl of sample in 9 µl of sterile deionized water) and 200 µl of working reagents was added to each well. The plates were incubated for 30 min at 37°C. After incubation, absorbance at 540 nm (A_{540} nm) was measured using microplate reader Elx 800 (Bio-Tek instrument, Canada).

3.3.2 SDS-polyacryamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels were prepared following the formulas shown in Appendix A. Fifteen to thirty µg of protein sample and 2×Laemmli buffer (Appendix A) were mixed in an equal volume in 1.5 ml microcentrifuge tube. The samples were heat at 100°C for 5 min on Thermomixer Compact (Eppendorf, Germany). Heated samples and prestained molecular weight markers (Fermentas, Canada) were loaded to the gels. The samples were separated at 100 volt for at least 120 min in Western blot running buffer (Appendix A) using Protein III system (BioRad, USA).

3.3.3 Protein transfer

After separation process, the stacking gel was removed and size of the separating gel was measured. The gels were equilibrated in transfer buffer (Appendix A) for 5 min. Six pieces of Whatman filter paper and a PVDF membrane (GE Healthcare, USA) were prepared. PVDF membranes were next soaked in absolute methanol (Merck, Germany) and rinsed with deionized water 2 times and immersed in transfer buffer. Gels, PVDF membrane and filter papers were placed in a semi-dry transfer Trans-Blot® SD (BioRad, USA) apparatus and air bubbles were eliminated by gently rolling a glass test tube on the top of stacks of filter paper. The semi-dry transfer was carried out under the following condition; current at 80 or 130 mA for 90 min for one gel or two gels, respectively.

3.3.4 Antibody probing

The PVDF membranes after protein transfer were blocked in blocking solution (Appendix A) twice for 5 min each on Labnet Rocker 25 (Labnet International Inc, USA). After blocking, the PVDF membranes were probed with 1:2,000 dilution of rabbit anti Notch1

(Santa Cruz Biotechnology, USA), 1:1,000 dilution of rabbit anti Cleaved Notch1 (Cell Signal Technology, USA) and 1:10,000 dilution of mouse anti β-actin (Chemicon International, USA) which are primary antibody at 4°C refrigerator overnight. The probed membranes were further incubated on a rocker for 1 hr at room temperature. The primary antibody solution was discarded, and the membrane was washed with PBST (Appendix A) for 5 min 2 times and 15 min 2 times. After washing, PBST was discarded, and 1:4,000 dilution of donkey anti rabbit and 1:5,000 of sheep anti mouse which are secondary antibody against mouse or rabbit immunoglobulins conjugated with horse-radish peroxidase (HRP) (Amersham Biosciences, UK) were added. The PVDF membranes were incubated for 1 hr with rocking before washing with PBST as follow described above.

3.3.5 Signal detection by chemiluminescence and autoradiography

The substrates were prepared using the formula shown in Appendix A. Briefly, solution A was mixed with solution B. PBST was removed from the container carrying the PVDF membranes The mixture of solution A and B was poured directly on the membranes and incubated for 1 min. The membranes were wrapped in the plastic wrap and placed in Hypercassette (Amersham Biosciences, UK) to expose to High Performance Chemilumunescence Film: Amersham Hyperfilm^{TM} ECL (Amersham Biosciences, UK) in the dark. Exposure time for Notch1/cleaved Notch1 and β-actin (as loading control) was 5 min and 10 sec, respectively. Exposed film was developed for 5 second in X-ray film developer, washed with tap water, fixed for 3 min in the fixer and finally washed with tap water.

3.4 RNA extraction

Cells were treated as indicated and culture supernatants were removed or collected for other experiments. One ml of TriZol reagent (Invitrogen, UK) was added directly to cells

and the mixture was incubated for 5 min at room temperature. TriZol reagents containing RNA were transferred to 1.5 ml microcentrifuge tubes and 0.2 ml of chloroform (Lab-Scan, Ireland) was added. All tubes were vigorously mixed by hands for 15 second and incubated at room temperature for 3 min. The samples were centrifuged using Refrigerated Centrifuge model 1920 (Kubota, Japan) at 12000xg for 15 min at 4°C. Only colorless aqueous phase was carefully transferred to fresh tubes. RNA was precipitated by gently mixing with 0.5 ml of isopropanol (Merck, Germany). The samples were incubated at room temperature for 10 min and centrifuged at 12000xg for 10 min at 4°C. The RNA pellets on the bottom side of each tube were visible at this stage. The supernatants were rinsed and the RNA pellets were washed once with 1 ml of ice cold 75% ethanol (Appendix A) in 0.01% DEPC water (Appendix A). The samples were mixed by Vortex mixer model G560E (Scientific Industries, USA) and centrifuged at 7500xg for 5 min at 4°C. RNA pellets were dried for 5-10 min, dissolved in 20 µl of 0.01% DEPC water, and incubated for 10 min at 55°C. RNA samples were kept at –70°C until use for further experiments.

3.4.1 Quantitation of RNA using spectrophotometer

RNA was diluted to 50 to 100-fold dilution in 0.01% DEPC-treated water. The diluted RNA was subjected to absorbance measurement at 260 and 280 nm in the spectrophotometer. An OD_{260} corresponds to a concentration of 40 µg/ml single stranded RNA. Therefore, the concentration of RNA was calculated in µg/ml by using the following equation.

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

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

3.4.2 Quantitation of RNA using Quanti iT Assays (Invitrogen, UK)

Measuring amount of RNA using Quanti iT were performed according to the manufacturer's instruction. Briefly, Quanti-iT reagent and Quanti-iT buffer (Invitrogen, UK) were calculated and prepared to be Quanti-iT working solution. Ten µl of RNA standards composing of 0 ng/µl of RNA and 10 ng/µl of RNA were mixed with 190 µl of working solution. RNA samples was diluted to 10-fold dilution in Hypure® water PCR grade (Hyclone, England) and 2 µl of diluted RNA was mixed with 198 µl of working dilution. Calibrations of RNA standard were performed by Quanti-iT and concentrations of RNA samples were measured. The concentrations of RNA were calculated in µg/ml by using the following equation.

$$\text{RNA } (\mu g/ml) = \text{measured concentration} \times \text{dilution factor}$$

3.5 cDNA synthesis by reverse transcriptase

Five hundred ng to 1 µg of obtained total RNA was used to generate cDNA. Total RNA was mixed with 0.2 µg of random hexamer (Qiagen, Germany), and the volume was adjusted to 12.5 µl by 0.01% DEPC treated water. The RNA mixture was heated at 65°C for 5 min and placed on ice for 5 min. Then, 1×Reverse transcriptase buffer (Fermentus, Canada), 1 mM dNTP mix (Fermentus, Canada) and 20 U of RNase Inhibitor (Fermentus, Canada) were added in the mixture and followed by incubation at room temperature for 5 min. Reverse transcriptase (Fermentus, Canada) was added to final amount of 200 U per reaction, and the reaction was manipulated on Bioer Life Express (Bioer technology, China) at 25°C for 10 min, 42°C for 60 min, 70°C for 10 min and 25°C for infinity. The cDNA was stored at -20°C until use.

3.6 Polymerase chain reaction (PCR)

The components of PCR reactions were as follows: 1xTag buffer (Fermentus, Canada), 0.64 mM dNTP mix (Fermentus, Canada), 2mM $MgCl_2$ (Fermentus, Canada) ,0.2 µM forward and reverse primers, 25 U of Tag polymerase and Hypure® water PCR grade adjusted to volume 20- 22.5 µl per reaction. Obtained cDNAs were used 2.5- 5 µl as templates to amplify, murine *IL-6* and murine *IL-6* promoter. *β-actin* was used as loading control. RT-PCR without reverse transcriptase was used as negative control. The forward and reverse primers used for PCR amplification are as follows: murine *IL-6* (5'-CATGTTCTCTGGGAAATCGTGG-3' and 5'-AACGCAC TAGGT TTGCCGAGTA-3'); β-actin (5'-ACCAACTGGGACGACATGGAGAA-3' and 5'-GTGGTGGTGAAGCTGTAGC C-3') and murine *IL-6* promoter (5'-TCGATGCTAAACGACGTCAC-3' and 5'-TCAATTC CAGAAACCGCTATG-3'). The PCR reactions were carried out using Bioer Life Express® by condition as follows; 94°C 5 min, 94°C for 1 min, 52°C (murine *IL-6*) or 55°C (β-actin) or 57°C (murine *IL-6* promoter) for 1 min, 72°C for 1 min and 72°C for 10 min. PCR were amplified for 30-32 cycles. The PCR products were analyzed on 2% agarose gel by Mini Gel Electrophoresis Unit for DNA, RNA and Proteins Mupid-2 Advance (Cosmo Bio, Japan). The amplified DNA bands were detected after staining with ethidium bromide using Gel Documentation and Quantity one 4.4.1 (BioRad, USA).

3.7 Semi-Quantitative Polymerase chain reaction (qPCR)

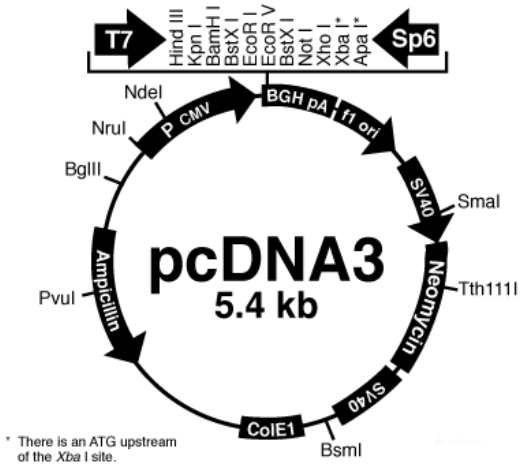
The qPCR amplification was performed with 1x Maxima™ SYBR Green/ROX qPCR Master Mix, with 0.3 µM forward and reverse primer, RNase Free water and 2 µl obtained cDNAs according to the manufacturer's protocol (Fermentus, Canada). The forward and reverse primers used for qPCR amplification are as follows: murine *IL-6* (5'-CTCTGGGA

AATCGTGGAAATG-3' and 5'-AAGTGCATCATCGTTGTTCATACA-3') and β-actin (5'-ACCAACTGGGACGACATGGAG AA-3' and 5'- GTGGTGGTGAAGCTGTAGCC-3'). *β-actin* was used as a Reference gene. Reaction without cDNA was used as negative control. The qPCR was carried out using MJ Mini personal Thermal cycler (Biorad, USA) by condition as follows 95°C for 10 min, 95°C for 5 min, 55°C (*β-actin)* for 30 sec or 57°C (murine *IL-6)* for 1min, 72°C for 1 min and 72°C for 10 min, follows by repeating for 40 cycles. The relative expression levels were calculated and analyzed by $2^{-\Delta\Delta CT}$. The PCR products were seldom analyzed on 2% agarose gel by electrophoresis and visualized after staining with ethidium bromide using the Gel Documentation System.

3.8 Overexpression of truncated Notch1 in RAW 264.7 by transient transfection

Overexpression of truncated Notch1 in RAW 264.7 was achieved by using plasmid pcDNA3 containing intracellular Notch1 (N^{IC}) corresponding to amino acid 1759 to 2556 and pcDNA3 empty plasmid as control vector. The plasmid constructs are shown in Figure 3.1. These two plasmids were kindly provided by Professor Barbara Osborne (University of Massachusetts at Amherst, USA). Transient transfection was done by FuGeneHD transfection reagent (Roche, Germany) according to the manufacturer's instruction. The procedures are as follows:

A



B

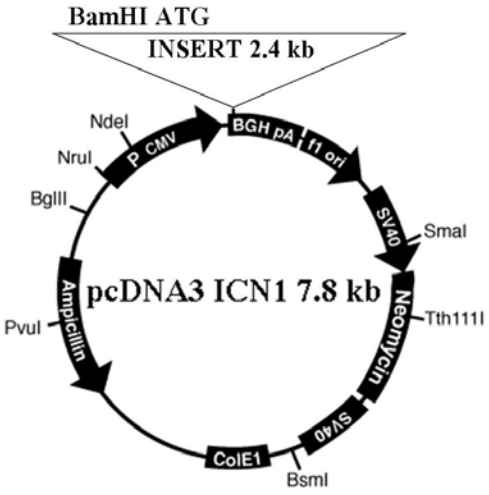


Figure 3.1 Plasmid maps of pcDNA3 and pcDNA3 ICN1 (pcDNA3NIC) used in this study

Restriction enzyme map of plasmid (A) pcDNA3 and (B) pcDNA3NIC containing ICN1 corresponding to the region between amino acid 1759 to 2556 under the control of cytomegalovirus (CMV) promoter

3.8.1 Plasmid preparation

Plasmids were transformed into competent *E. coli* DH5α by heat-shock technique. Briefly, fifty µl of competent from -80°C was thawed on ice. One µl of plasmid was added to competent cells and the plasmid-competent cell mixture was incubated on ice for 30 min. This mix was heated shock at 42°C for 90 sec and immediately placed on ice for 2 min. After the heat shock process, cells were transferred to 1 ml of LB broth (Appendix A) and incubated at 37°C for 1 hr by shaking at 200 rpm. After that, fifty to one hundred µl of LB broth containing the cells was plated on LB agar (Appendix A) plates containing 50 µg/ml of ampicillin. The plate was incubated at 37°C for 16-24 hr and the colony was picked for plasmid preparation.

3.8.2 Plasmid isolation

A few colonies were picked up and cultured for 16-18 hr at 37°C in 2 ml LB broth containing 50 µg/ml of ampicillin. One ml of the culture was sterilely moved to 1.5 ml microcentrifuge tube, and cells were pelleted by centrifugation at 13,000 rpm for 1min at 4°C. The supernatant was discarded and the plasmid was extracted using QIA prep spin miniprep kit (Qiagen, Germany) according to the manufacturer's instructions. The obtained plasmid was eluted in 50 µl sterile HPLC water and stored at -20°C until use.

3.8.3 Plasmid quantitative assay

Plasmid stored at -20°C was diluted in sterile deionized water at 50 fold dilution. The absorbance was measured at wavelength 260 and 280 nm. One OD_{260} corresponds to a

concentration of 50 µg/ml plasmid DNA. Therefore, the concentration of plasmid was calculated by using the following equation.

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

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

3.8.4 Transient transfection using FuGene® HD transfection reagent

RAW 264.7 cell line (1.5×10^5 cell/ml) in 0.5 ml culture media was plated overnight in 24-well tissue culture treated plates. Preparing the FuGene® HD transfection reagent was conducted according to the manufacturer's instructions. Optimal FuGene® HD transfection reagent and plasmid ratio was chosen at 6:2. Briefly, FuGene® HD transfection reagent was brought to room temperature prior to use for 15 min. Volume of serum free media Opti-MEM (Invitrogen, UK) and plasmid were calculated to dilute the plasmids and they were prepared in a 1.5 ml microcentrifuge tube. To form the transfection complex, FuGene® HD transfection reagent was directly added to the diluted plasmids without being in contact with the wall of the plastic tubes and the mixtures were gently vortexed for 1-2 second. They were incubated at room temperature for 15 min and the complex mixture was added dropwise to cells. Finally, the plate was swirled and incubated for 12-48 hr. After this incubation, the culture supernatant was collected for measuring secreted IL-6, and cell lysates was analyzed by Western blot for Notch1 protein. Total RNA was isolated for qPCR to detect *IL-6* expression.

3.9 Inhibition of Notch signaling by IL-CHO

For inhibition of Notch signaling by GSI, IL-CHO (a kind gift of Professor Todd Golde, Mayo Clinic, FL, and USA), macrophages were pretreated by 25 μM or 50 μM of IL-CHO or vehicle control for 1 hr before Lipopolysaccharide from Salmonella (Sigma Aldrich, USA) and rIFNγ (R&D System, USA) activation. Total RNA was extracted by Trizol reagent and converted to cDNA as described above. The qPCR was carried out using the primer sets specific for *IL-6* and *β-actin* as listed above and the relative expression of *IL-6* were calculated using $2^{-\Delta\Delta CT}$ Expressions of Notch1 were confirmed by Western blot.

3.10 ELISA for IL-6

Secreted IL-6 produced by activated macrophage using LPS and IFNγ was measured by Ready-set-go! Mouse Interleukin 6 (IL-6) (eBioscience, UK). Culture supernatants were collected after treatment as indicated and kept at –70°C until use. ELISA was carried out according to the manufacture's instructions. Briefly, ELISA plate was coated with 100 μl/well of capture antibody in coating buffer and incubated for overnight at 4°C. Unbound antibodies were removed and the wells were washed with 250 μl/well of wash buffer for 5 times. During each washing step, 1 min soaking was performed to increase the effectiveness of the washing. Wells were blocked with 200 μl/well of 1x Assay Diluent and incubated for 1 hr at room temperature. Wells were aspirated and washed as described above. The samples which were diluted to 1:10 and standards, recombinant mouse IL-6 were prepared in 2-fold serial dilutions in new 1.5 ml micro centrifuge tube. One hundred μl of diluted samples and standards were added in the wells and the plates were incubated for 4 hr at room temperature. Following this step, wells were emptied and washed as detailed above. Biotinylated anti-mIL-6 antibody was used as a detection antibody and was added in each well at 100 μl and

incubated for 1 hr at room temperature. After that, the wells were washed as described above and avidin-HRP (100 µl/well) was added and the plates were incubated at room temperature for 30 min. Wells were washed for 7 times after this step. Substrate solution (TMB) (100 µl/well) was added and the plates were incubated for 15 min at room temperature. Finally, the reactions were stopped by addition of 50 µl of 2N H_2SO_4 and the absorption was read at 450 nm.

3.11 Chromatin Immunoprecipitation (ChIP) assay

RAW 264.7 cell line ($1x10^7$ cells/plate) was activated by LPS and IFNγ for 6 hr. EZ Magna ChIP™ Chromatin Immunoprecipitation Kit (Upstate, USA) was used according to the manufacture's protocol for ChIP assay. Briefly, 37% formaldehyde was used to cross link proteins and DNA molecules located in close proximity at the final concentration of 1%. To avoid over cross-linking, 1.25M Glycine at the final concentration of 0.125M, was added to quench formaldehyde. Subsequently, cells were subjected to lysis by treating with 500 µl of nuclear lysis buffer containing 2.5 µl of protease inhibitor cocktails II. Lysed cells (250 µl) were sonicated using UP 50 H Ultrasonic Processor (dr.hielscher GmbH, Germany) at 80% amplitude, 1 cycle, sonication period for 10 second and rest period for 10 second for 15 times to shear chromatins. Sonicated cells were centrifuges at 10,000xg at 4°C for 10 min to eliminate insoluble materials. Following the sonication, genomic DNA lengths was optimized to be in the range of 200 to 1,000 bp. Afterward, two hundred and fifty µl of sheared chromatin was moved to new 1.5 microcentrifuge tube in 50 µl aliquots. In each tube, four hundred and fifty µl of dilution buffer containing 2.25 µl of protease inhibitor cocktails II was added. After this step, one to ten µg of target antibody as follows; 2-4 µg of anti-Notch1 (Santa Cruz Biotechnology, USA), 1 µg of Normal Rabbit IgG, 1 µg of Normal Mouse IgG and 1 µg of RNA Poll II (Chemicon International, USA) and 20 µl protein G-conjugated magnetic beads was added and incubated at 4°C with Mini–rotator Bio RS-24 (Biosan,

Latvia) for 2 hr. Magnetic beads were separated by MiniMACS Separator (Miltenyi Biotec, Germany) and the supernatants were discarded. Beads were washed and during each washing step, the incubation with rotator for 5 min was carried out. This step was followed by reversing crosslinking of protein/DNA complex by adding 100 µl of ChIP Elution buffer containing 1 µl of proteinase K, and the mixture was incubated at 62°C for 2 hr with shaking, and further incubated at 95°C for 10 min. The mixture was left to cool down at room temperature. The supernatants were collected and purified using spin column or phenol-chloroform extraction and the presence of DNA fragments of the target promoter (IL-6) was detected by PCR with primers specific for the regions on promoter of *IL-6* with the putative CSL binding sites.

3.12 Statistical analysis

One-way ANOVA (F test) and multiple comparisons (LSD) were used to analysis the data. The values of $p<0.05$ were considered statistically significant.

3.13 Database search

NCBI sequence viewer v.2 was used to investigate nucleotide sequences of *IL-6* promoter and EBI ClustalW2 was used to find the consensus sequence alignment of human and murine IL-6. Mulan (Loots *et al.*, 2007) was used to identify the potential transcription factor binding sites evolutionarily conserved in human and murine species.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Identification of the consensus CSL binding sites in the promoter region of *IL-6*.

Previous studies demonstrated that the regulation of IL-6 expression was under the control of several transcription factors such as CEBPβ, ATF3, AP-1 and IL6-κB (Miyazawa *et al.*, 1998; Palmieri *et al.*, 1999; Vales *et al.*, 2002; Kishimoto, 2005 and Hong *et al.*, 2007). CSL was identified as one of the *IL-6* transcription regulators in the absence of Notch signaling in human and mouse (Plaisance *et al.*, 1997 and Palmieri *et al.*, 1999). However, there was no report on the investigation of the conserved CSL binding sites between both species. Therefore in this study, the consensus CSL binding sites in the promoter region of *IL-6* were re-evaluated in both human and mouse species. The results from the database searches indicated that there is at least one potential CSL binding site in both human and mouse.

In human, IL-*6* locus is located on chromosome 7 in the region of 7p21. NCBI sequence viewer v.2 was used to investigate the nucleotide sequences of *IL-6* promoter. Mulan database (Loots *et al.*, 2007) was used to identify the potential transcription factor binding sites evolutionarily conserved across human and mouse species. The results from Mulan database reported the potential CSL binding sites on human *IL-6* promoter which was shown in Figure 4.1.

CSL binding site on human *IL-6* promotor located in IL6-κB site (-77 to -59) where putative NF-κB binding site; 5'-GGGATTTTCC-3' (-73 to -64) overlapping with CSL consensus sequence; 5'-CATGGGAA-3' (-67 to -60) (Miyazawa *et al.*, 1998). This result was consistent with the previous report on CSL consensus binding site 5'-A(G/C)CGTGGGAA-3' by Tun *et al*. (1994) and Chung *et al.* (1994).

In mouse, *IL-6* gene is located on chromosome 5 in the region of B1; 5 17.0 cM. The results from Mulan database identified potential CSL binding sites on mouse *IL-6* promoter which was shown in Figure 4.2. Similar to human, mouse CSL binding site on *IL-6* promotor is located on IL6-κB site.

Subsequently, Mulan database was used to identify the potential transcription factor binding sites evolutionarily conserved across human and murine species. The nucleotide sequence approximately 1,243 base pairs in length upstream of the start codon (ATG) was determined. The results from the database searches indicated that CSL binding site was completely conserved in human and murine species (Reference: Request ID: mlr03202010113913320) (Figure 4.3). This result was consistent with previous reports which identified the same site as potential CSL binding sites (Miyazawa *et al.*, 1998; Tun *et al.*, 1998 and Chung *et al*., 1998).

In TNFα or IL-1β induced mouse fibrosarchoma cell (L929sA), CSL was reported as negative regulation of *IL-6* transcription by competitively binding to the IL6-κB site and repressing transcription of *IL-6* in the absence of NF-κB (Plaisance *et al.*, 1997). In contrast, in IL-1β stimulated human rheumatoid fibroblast-like synoviocytes, CSL was shown to function as positive regulator of *IL-6* transcription by deletion analysis (Miyazawa *et al.*, 1998). Although CSL regulated *IL-6* expression by various types of inducers and cells were investigated, the role of CSL in regulation of *IL-6* expression through Notch signaling in LPS induced macrophages was not investigated in previous studies. Therefore, we investigated whether Notch signaling regulates IL-6 expression in murine macrophages upon stimulation.

>gi|25990180|gb|AY170325.1| Homo sapiens interleukin 6 (IL6) gene, promoter and partial cds

GGATCCTCCTGCAAGAGACACCATCCTGAGGAAGAGGGCTTCTGAACCAGCTTGACCCAATAAGAAATTC
TTGGGTGCCGACGCGGAAGCAGATTCAGAGCCTAGAGCCGTGCCTGCGTCCGTAGTTTCCTTCTAGCTTC
TTTTGATTTCAAATCAAGACTTACAGGGAGAGGGAGCGATAAACACAAACTCTGCAAGATGCCACAAGGT
CCTCCTTTGACATCCCCAACAAAGAGGTGAGTAGTATTCTCCCCCTTTCTGCCCTGAACCAAGTGGGCTT
CAGTAATTTCAGGGCTCCAGGAGACCTGGGGCCCATGCAGGTGCCCCAGTGAAACAGTGGTGAAGAGACT
CAGTGGCAATGGGGAGAGCACTGGCAGCACAAGGCAAACCTCTGGCACAGAGAGCAAAGTCCTCACTGGG
AGGATTCCCAAGGGGTCACTTGGGAGAGGGCAGGGCAGCAGCCAACCTCCTCTAAGTGGGCTGAAGCAGG
TGAAGAAAGTGGCAGAAGCCACGCGGTGGCAAAAAGGAGTCACACACTCCACCTGGAGACGCCTTGAAGT
AACTGCACGAAATTTGAGGGTGGCCAGGCAGTTCTACAACAGCCCCTCACAGGGAGAGCCAGAACACAGA
AGAACTCAGATGACTGGTAGTATTACCTTCTTCATAATCCCAGGCTTGGGGGGCTGCGATGGAGTCAGAG
GAAACTCAGTTCAGAACATCTTTGGTTTTTACAAATACAAATTAACTGGAACGCTAAATTCTAGCCTGTT
AATCTGGTCACTGAAAAAAAAAATTTTTTTTTTCAAAAAACATAGCTTTAGCTTATTTTTTTCTCTTTG
TAAAACTTCGTGCATGACTTCAGCTTTACTCTTTGTCAAGACATGCCAAAGTGCTGAGTCACTAATAAAA
GAAAAAAAGAAAGTAAAGGAAGAGTGGTTCTGCTTCTTAGCGCTAGCCTCAATGACGACCTAAGCTGCAC
TTTTCCCCCTAGTTGTGTCTTGCGATGCTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAATC
AGCCCCACCCGCTCTGGCCCCACCCTCACCCTCCAACAAAGATTTATCAAATGTGGGATTTTCCCATGAG
TCTCAATATTAGAGTCTCAACCCCCAATAAATATAGGACTGGAGATGTCTGAGGCTCATTCTGCCCTCGA
GCCCACCGGGAACGAAAGAGAAGCTCTATCTCCCCTCCAGGAGCCCAGCTATGAACTCCTTCTCCACAAG
TAAGTGCAGGAAATCC

Figure 4.1 Nucleotide sequences of human *IL-6* promoter region and partial coding sequences (ACCESSION: AY170325)

The area which is highlighted in green indicates the potential CSL binding sites in human *IL-6* promoter which was identified by Mulan database search. The translational starting site is highlighted in red and 5' UTR (Transcription initiation) is in pink.

>gi|198369|gb|M20572.1|MUSIL6A Mouse interleukin 6 (IL-6) gene, complete cds

GGATCCTGAGAGTGTGTTTTGTAAATGGTTTTGGATTTTATGTACAGAGCCTACTTTCAAGCCTGGAATC
ATTCTGAATGCTAGCTAGATATCTGGAGACAGGTGGACAGAAAACCAGGAACTAGTCTGAAAAAGAAACT
AACCAAAGGGAAGAAGTCTGTTTAAGTTTGACCCAGCCTAGAAGACTTGAGCATTGGAGGGGTTATTCAG
AGTGAGACGTACCACCTTCAGATTCAAATCCTGTCATCCAGTAGAAGGGAGCTTCAAACACAAGCTAGCT
AAGATACAATGAGGTCCTTCTTCGATATCTTTATCTTCCATATACCATGAATCAAAGAAACTTCAACAAC
ATGAGGACTGCAACAGACCTTCAAGCCTCCTTGCATGACCTGGAAATTGGGTGTTTTGGGGTGTCGGGCA
GCAGCAGTGGGATCAGCACTAACAGATAAGGGCAACTCTCACAGAGACTAAAGGTCTTAACTAAGAAGAT
AGCCAAGAGACCACTGGGGAGAATGCAGAGAATAGGCTTGGACTTGGAAGCCAAGATTGCTTGACAACAG
ACAGAAGATATTTCTGTACTTCACCCACTTTACCCACCTGGCAACTCCTGGAAACAACTGCACAAAATTT
GGAGGTGAACAAACCATTAGAAACAACTGGTCCTGACAAGACACAGGAAAAACAAGCAATATGCAACATT
ACTGTCTGTTGTCCAGGTTGGGTGCTGGGGGTGGGAGAGGGAGTGTGTGTCTTTGTATGATCTGAAAAAA
CTCAGGTCAGAACATCTGTAGATCCTTACAGACATACAAAAGAATCCTAGCCTCTTATTCATGTGTGTGT
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATGTGTGTGTCGTCTGTCATGCGCGCGTGCCT
GCGTTTAAATAACATCAGCTTTACGTTCTCTTTCTCCTTATAAAACATTGTGAATTTCAGTTTTCTTTCC
CATCAAGACATGCTCAAGTGCTGAGTCACTTTTAAAGAAAAAAAGAAGAGTGCTCATGCTTCTTAGGGCT
AGCCTCAAGGATGACTTAAGCACACTTTTCCCTTCCTAGTTGTGATTCTTTCGATGCTAAACGACGTCAC
ATTGTGCAATCTTAATAAGGTTTCCAATCAGCCCCACCCACTCTGGCCCCACCCCCACCCTCCAACAAAG
ATTTTTATCAAATGTGGGATTTTCCCATGAGTCTCAAAATTAGAGAGTTGACTCCTAATAAATATGAGAC
TGGGGATGTCTGTAGCTCATTCTGCTCTGGAGCCCACCAAGAACGATAGTCAATTCCAGAAACCGCTATG

Figure 4.2 Nucleotide sequences of murine *IL-6* gene and partial promoter which has been reported in NCBI (ACCESSION: M20572.1)

The area which is highlighted in green indicates the potential CSL binding sites in mouse *IL-6* promoter. The translational starting site is highlighted in red and 5' UTR (Transcription initiation) is in pink.

A

Multi-species RBPJK binding sites have blue background:

Positions in the base sequence:
1110 1120 V$RBPJK 01
1111 1118 V$RBPJK Q4

Mulan tba-based alignment

******************* ORTHOLOGOUS BLOCKS *******************

:::::: block no. **1**, score=30837.0, strand:+, base position:**9-1208**

```
MurineIL6       CTGAAAAAGAAACTAACCAAAGGGAAGAAG--TCTGTTTAAGTTTGACCCAGCCTAGAAG
HumanIL6        CTGCAAGAGACACCATCCTGAGGAAGAGGGCTTCTGAACCAGCTTGACCCAATAAGAAAT 68
                *** ** *** ** * **  *** *    *  ****    ** ********      **

MurineIL6       ACTTGAGCATTGGAGGGGT----TATTCAGAGT---GAGACGTACCA-------------
HumanIL6        TCTTGGGTGCCGACGCGGAAGCAGATTCAGAGCCTAGAGCCGTGCCTGCGTCCGTAGTTT 128
                 **** *    *  * **      ********    *** *** **

MurineIL6       -----------CCTTCAGATTCAAATCCTGTCATCCAGTAGAAGGGAGCTTCAAACACAA
HumanIL6        CCTTCTAGCTTCTTTTGATTTCAAATCAAGACTTACAGGGAGAGGGAGCGATAAACACAA 188
                          * **    ********  * * * ***    *******   ********

MurineIL6       GCTAGCTAAGATACAATGAGGTCCTTCTTCGATATC----------------------TT
HumanIL6        ACTCTGCAAGATGCCACAAGGTCCTCCTTTGACATCCCCAACAAAGAGGTGAGTAGTATT 248
                 **    ***** * *  ******* *** ** ***                       **

MurineIL6       TATCTTCCATATACCATGAATCAAAGAAACTTCAACAACATGAGGACTGCAACAGACCTT
HumanIL6        CTCCCCCTTTCTGCCCTGAACCAAGTGGGCTTCAGTAATTTCAGGGCTCCAGGAGACCT- 307
                   *  *  * * ** **** ***     *****  **  * *** ** **  ******

MurineIL6       CAAGCCTCCTTGCA--TGACCTGGAAATTGGGTGTTTTGGGGTGTCGGGCAGCAGCAGTG
HumanIL6        --GGGGCCCATGCAGGTGCCCCAGTGAAACAGTGGTGAAGAGACTC----AGTGGCAATG 361
                   *   ** ****  ** **  *  *    *** *   * *  **    **  *** **

MurineIL6       GGATCAGCACTAACAGATAAGGGCAACTCTC----ACAGAGACTAAAGGTCTTAACTAAG
HumanIL6        GGGAGAGCACTGGCAGCACAAGGCAAACCTCTGGCACAGAGAGCAAAG-TCCTCACTGGG 420
                **   ******  ***   * *****  ***    *******  **** ** * ***  *

MurineIL6       AAGATAGCCAAGAGACCACTGGGGAGAATGCAGAG-------------------AATAGG
HumanIL6        AGGATTCCCAAGGGGTCACTTGGGAGAGGGCAGGGCAGCAGCCAACCTCCTCTAAGTGGG 480
                * ***  ***** *  **** ******  **** *                   * * **

MurineIL6       CT--------TGGACTTGGAAGCCAAGATTGCTTGACAACAGACAGAAGATATTTCTGTA
HumanIL6        CTGAAGCAGGTGAAGAAAGTGGCAGAAGCCACGCGGTGGCAAAAAGGAG----------- 529
                **        ** *    *  **  *     *  *    ** * ** **

MurineIL6       CTTCACCCACTTTACCCACCTGGCAACTCCTGGAAACAACTGCACAAAATTTGGAGGTGA
HumanIL6        --TCACACACT----CCACCTGGAGACGCCTTGAAGTAACTGCACGAAATTTGAGGGTGG 583
                  **** ****    ********  ** *** ***  ******** *******  ****

MurineIL6       ACAAACCATTAGAAACAACTGGTCCTGACAAG----------ACACAGGAAAAA-CAAGC
HumanIL6        CCAGGCAGTTCTA--CAACAGCCCCTCACAGGGAGAGCCAGAACACAGAAGAACTCAGAT 641
                 **  *  **  *  **** *  *** *** *          ****** * **  **

MurineIL6       AATATGCAACATTACTGTCTGTTGT----CCAGGTTGGGTGCTGGGGGTGGGAGAGGGAG
HumanIL6        GACTGGTAGTATTACCTTCT-TCATAATCCCAGGCT------TGGGGGGCTGCGATGGAG 694
                 *   * *  *****  *** *  *    ***** *      ******   * ** ****

MurineIL6       TGTGTGTCTTTGTATGATCTGAAAAAACTCAGGTCAGAACATCTGTAGATCCTTACAGAC
HumanIL6        T-----------------CAGAGGAAACTCAGTTCAGAACATCTTTGG-TTTTTACAAAT 736
                *                 * **  ******** ********** * * *  ***** *

MurineIL6       ATACAAAAG-----------AATCCTAGCCTCTTATTCATGTGTGTGTGTGTGTGTGTGT
HumanIL6        ACAAATTAACTGGAACGCTAAATTCTAGCCTGTTAATCTGGTCACTGAAAAAAAAAATTT 796
                * * *  *            *** ******* *** **  **   **          * *

MurineIL6       GTGTGTGTGTGTGTGTGTGTGTGTATGTGTGTGTCGTCTGTCATGCGCGCGTGCCTGC
HumanIL6        TTTTTT---------------------------------------------------- 802
                 * * *
```

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MurineIL6       GTTTAAATAACATCAGCTTTA---CGTTCTCTTTCTCCTTATAAAACATTGTGAAT---T
HumanIL6        --TCAAAAAACAT-AGCTTTAGCTTATTTTTTTTCTCTTTGTAAAACTTCGTGCATGACT 859
                  * *** ***** *******     ** * ****** ** ****** * *** **   *

MurineIL6       TCAGTTTTCTTTCCCATCAAGACATGCTCAAGTGCTGAGTCACTTTTAAAGAAAAAAA--
HumanIL6        TCAGCTTTACTCTTTGTCAAGACATGCCAAAGTGCTGAGTCACTAATAAAAGAAAAAAAG 919
                **** ***  *     ***********  ***************  ****  ******

MurineIL6       ---------GAAGAGTGCTCATGCTTCTTAGGGCTAGCCTCAAGGATGACTTAAGC-ACA
HumanIL6        AAAGTAAAGGAAGAGTGGTTCTGCTTCTTAGCGCTAGCCTCAATGACGACCTAAGCTGCA 979
                         ******** *  ********** *********** ** *** *****  **

MurineIL6       CTTTTCCCTTCCTAGTTGTGATTCTTTCGATGCTAAACGACGTCACATTGTGCAATCTTA
HumanIL6        CTTTTCCC--CCTAGTTGTG--TCTTGCGATGCTAAAGGACGTCACATTGCACAATCTTA 1035
                ********  **********  **** ********** ************  ********

MurineIL6       ATAAGGTTTCCAATCAGCCCCACCCACTCTGGCCCCACCCCCACCCTCCAACAAAGATTT
HumanIL6        ATAAGGTTTCCAATCAGCCCCACCCGCTCTGGCCCCACCCTCACCCTCCAACAAAGA--T 1093
                ************************ ************* ***************  *

MurineIL6       TTATCAAATGTGGGATTTTCCCATGAGTCTCAAAATTAGAGAGTTGACTCCTAATAAATA
HumanIL6        TTATCAAATGTGGGATTTTCCCATGAGTCTCAATATTAGAGTCTCAACCCCCAATAAATA 1153
                ********************************* *******  *  ** ** ********

MurineIL6       TGAGACTGGGGATGTCTGTAGCTCATTCTGCTCTGGAGCCCACCAAGAACGATAG
HumanIL6        TAGGACTGGAGATGTCTGAGGCTCATTCTGCCCTCGAGCCCACCGGGAACGAAAG 1208
                *  ****** ********  *********** ** *********  ****** **
```

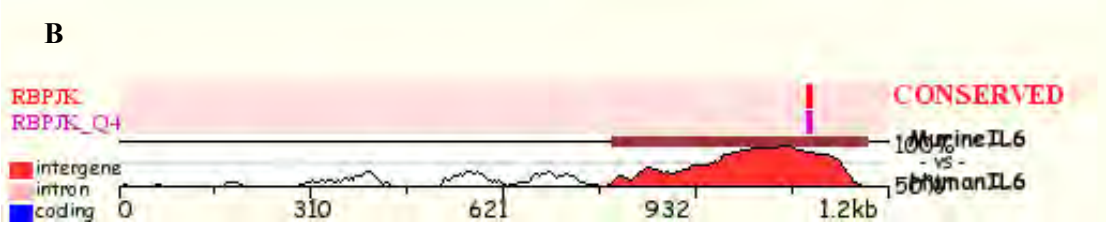


Figure 4.3 Promoter Sequence alignment of human and mouse IL-6 (continued)

(A) Alignment of human and mouse IL-6 promoters was conducted by Mulan. The region highlighted in green is the identified conserved sequences of CSL binding site.

(B) Dynamically overlay of Transcription factor binding site predicted with conserved profiles. The figure indicated that CSL binding site (RBPJκ) is conserved in human and mouse promoter.

4.2 Expression of Notch1 and cleaved Notch1 in macrophages activated by LPS or LPS plus IFNγ

It has been previously suggested that the activation of macrophages with LPS and IFNγ led to up-regulation of Notch1 which is mediated by TLR signaling pathway in MyD88 dependent manner (Palaga *et al.*, 2008). To investigate whether Notch signaling plays a role in IL-6 expression in activated macrophages, the expression profile of Notch1 and activated form of Notch1 (cleaved Notch1) was first examined in macrophages. BMMϕ and RAW264.7 cell line were activated by LPS (100 ng/ml) or LPS (100 ng/ml) plus IFNγ (10 ng/ml) for 3, 6, 12 and 24 hr. Expressions of Notch1 and cleaved Notch1 were determined by Western blot. As shown in Figure 4.4 and 4.5A, stimulation of BMMϕ with LPS or LPS plus IFNγ triggered Notch1 expression and the appearance of cleaved Notch1 in both BMMϕ and RAW 264.7 cell line. These results were consistent with previous report (Palaga *et al.*, 2008). However, Notch1 and cleaved Notch1 protein were detected in non-stimulated BMMϕ, suggesting that there was basal activation of Notch signaling in resting BMMϕ consistent with the report by Hu *et al*. (2008) which studied human primary macrophages. Interestingly, in BMMϕ, using LPS or LPS plus IFNγ, no significant differences in Notch1 expression and the appearance of cleaved Notch1 were detected, whereas stimulation of RAW 264.7 cell line with LPS plus IFNγ induced much greater expression of Notch1 than LPS or IFNγ (Figure 4.5 B) alone. The result suggested that primary macrophages and RAW264.7 cell line had some differences in signaling pathway leading to Notch1 upregulation. Furthermore, cleaved Notch1 was more prominent in stimulated BMMϕ than in stimulated RAW264.7 cell line. In RAW 264.7 cell line, LPS induced expression of Notch 1 was found to be started to up regulate at 3 hr of activation and increasingly higher until 24 hr of activation while the expression of cleaved Notch1 was found to be at the lowest level at 12 hr of activation. Using LPS plus IFNγ to activate RAW 264.7 cell line led to the highest expression of Notch1 at 6 hr while expression

of cleaved Notch1 was the highest at 3 hr and then declined until 24 hr after activation. BMMϕ stimulated with LPS showed the lowest expression of cleaved Notch1 at 3 hr and the level increased until it reached 24 hr of activation. For activation by LPS plus IFNγ in BMMϕ, the expression of Notch 1 was detected but no differences were found at the indicated times. For cleaved Notch 1, the expression appeared the lowest at 24 hr of activation.

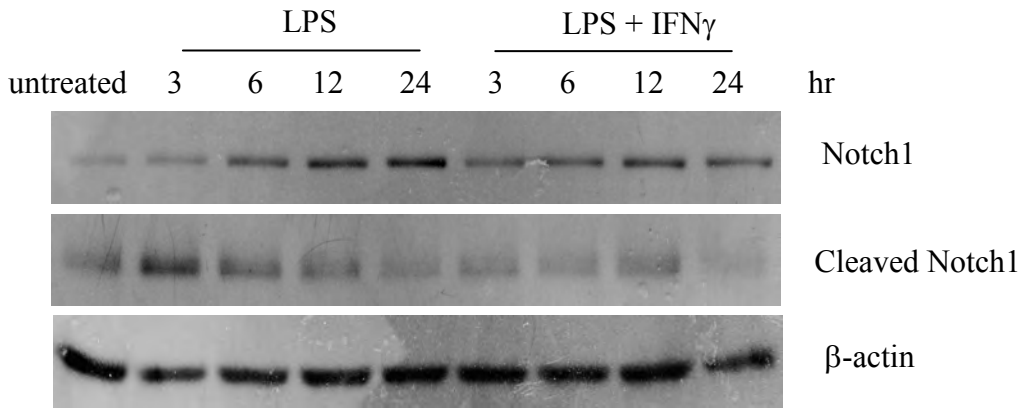
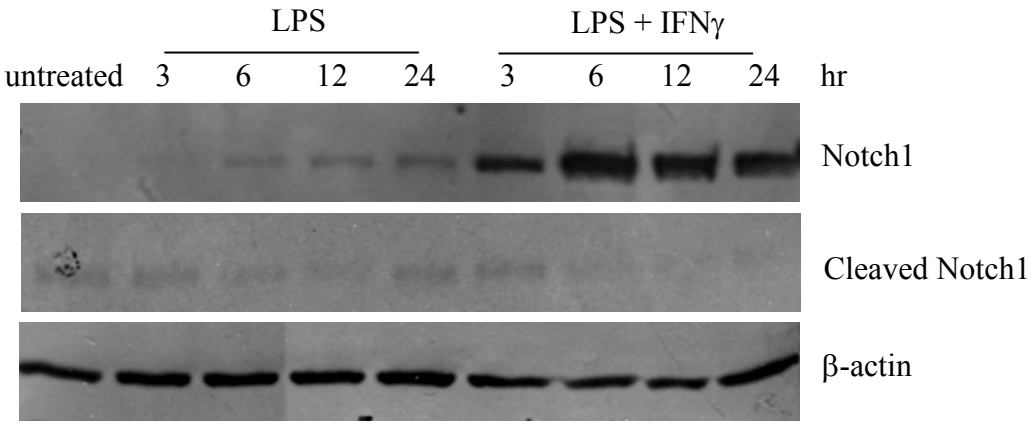


Figure 4.4 Notch 1 and cleaved Notch1 in LPS or LPS plus IFNγ stimulated BMMϕ

BMMϕ were stimulated with LPS (100 ng/ml) and LPS (100 ng/ml) + IFNγ (10 ng/ml) for 3, 6, 12 and 24 hr and the expression of Notch1, cleaved Notch1 and β actin were measured by Western blot.

A



B

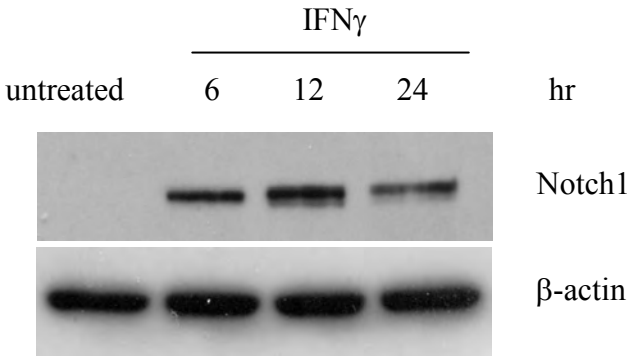


Figure 4.5 Notch 1 and cleaved Notch1 in LPS, IFNγ and LPS plus IFNγ stimulated RAW264.7

(A) RAW 264.7 were stimulated with LPS (100 ng/ml) and LPS (100 ng/ml) + IFNγ (10 ng/ml) for 3, 6, 12 and 24 hr and the expression of Notch1, cleaved Notch1 and β-actin were measured by Western blot. (B) RAW 264.7 were stimulated with IFNγ (10 ng/ml) for 6, 12 and 24 hr and the expression of Notch1 and β-actin were measured by Western blot.

4.3 Expression of *IL-6* in LPS or LPS+IFNγ activated macrophages

Since activation with LPS plus IFNγ slightly increased Notch1 and cleaved Notch1 expression, we aimed at examining the expression profile of IL-6, in comparison to the expression profile of Notch1. Previous data revealed that the expression level of Notch1 and cleaved Notch1 were not different in LPS or LPS plus IFNγ activated BMMϕ. Macrophages were able to be activated by LPS, IFNγ and LPS plus IFNγ and these activations up-regulated expression of Notch1 in a similar pattern (Palaga, *et al*., 2008). Therefore, we decided to investigate *IL-6* expression only in LPS plus IFNγ activated BMMϕ by qRT-PCR.

Expression of *IL-6* mRNA significantly increased at 3 hr after stimulation and reached the highest level at 6 hr after stimulation (Figure 4.6). The expression gradually decreased after this time point but was still detectable at 24 hr post stimulation. This *IL-6* expression profile correlated well with the profile of Notch1 and cleaved Notch1 (Figure 4.4), which was consistent with previous report (Palaga *et al.*, 2008).

Because the difference in Notch1 and cleaved Notch1 expression in RAW 264.7 cell line when activated by LPS or LPS plus IFNγ were detected (Figure 4.5), we decided to examine *IL-6* mRNA expression in both conditions in RAW264.7 cell line. The results was shown in Figure 4.7, which demonstrated that higher expression of *IL-6* mRNA was found in activation condition of LPS plus IFNγ than when using LPS alone (Figure 4.7). These results also correlated well with the expression profile of Notch1 and cleaved Notch1 in RAW264.7 (Figure 4.5). Taken together, the expression of both Notch1 and *IL-6* mRNA were highest at 6 hr after activation by LPS plus IFNγ in both primary macrophages and RAW264.7 cell line. Therefore, this time point and stimulation condition were chosen for further study.

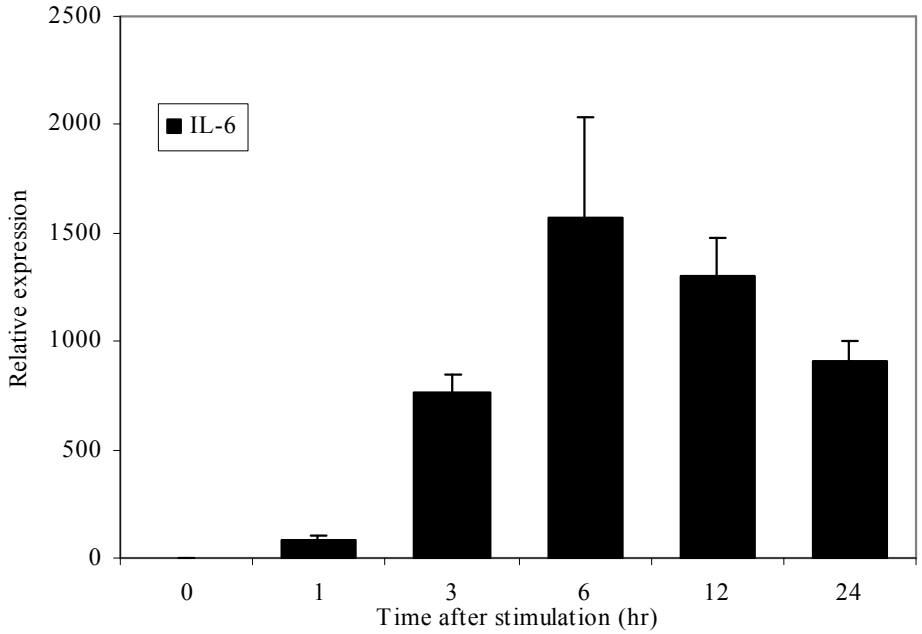


Figure 4.6 Expression profile of *IL-6* mRNA in BMMϕ stimulated with LPS and IFNγ

BMMϕ were stimulated with LPS (100 ng/ml) + IFNγ (10 ng/ml) for 1, 3, 6, 12, 24 hr. mRNA were measured by semi-quantitative realtime RT-PCR. The relative expression was calculated by normalizing to β-actin. The expression of *IL-6* mRNA in non-stimulated BMMϕ was set at 1. Data are shown as mean+SD of triplicate determinants and representative of duplicated independent experiments.

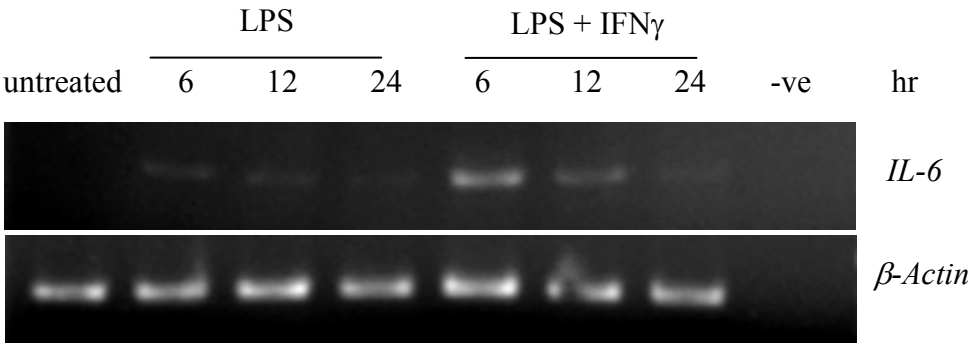


Figure 4.7 Expression profile of *IL-6* mRNA in RAW264.7 cell line stimulated with LPS or LPS and IFNγ

RAW 264.7 were stimulated with LPS (100 ng/ml) and LPS (100 ng/ml) + IFNγ (10 ng/ml) for 6, 12 and 24 hr. Untreated was non-stimulated macrophages. The expression of *IL-6* was examined by RT-PCR.

4.4 Expression Profile of Notch1 and Cleaved Notch1 in IL-CHO-treated macrophages

IL-CHO, a γ-secretase inhibitor, was used to inhibit cleavage of Notch receptors and the Notch signaling in macrophages. Palaga *et al.*, 2008 reported that 25 μM of IL-CHO inhibit both cleaved Notch1 and Notch1 in BMMϕ (Palaga *et al.*, 2008). Using other γ-secretase inhibitors such as Compound E or L685458 also resulted in suppression of cleaved Notch1 expression and TLR induced genes such as *HES1* and *HEY1* (Hu *et al.*, 2008) In this study, the effect of IL-CHO on expression of Notch1 and cleaved Notch1 was evaluated in BMMϕ. The effects of IL-CHO or vehicle control DMSO were first observed in the cellular morphology of BMMϕ (Figure 4.8). The results indicated that treatment with 25 μM of IL-CHO for 1 hr affected cellular morphology by decreasing adhesive properties of BMMϕ without increasing cell numbers and resulted in cells with more round shape (Figure 4.8C), while no obvious changes were detected in DMSO treated control (Figure 4.8A and B). Afterwards, the effect of IL-CHO on the expression of Notch1 and cleaved Notch1 was determined by Western blot. BMMϕ was pre-treated with IL-CHO or DMSO for 1 hr and was stimulated with LPS (100 ng/ml) plus IFNγ (10 ng/ml) for 6 hr. As shown in Figure 4.9 A, in non-stimulated cells, treatment with DMSO did not affect the expression level of Notch1 and cleaved Notch1, whereas treatment with IL-CHO at 25 μM completely eliminated the cleaved Notch1 expression (Figure 4.9A). Treating BMMϕ with 25 μM and 50 μM of IL-CHO in the presence of LPS plus IFNγ resulted in complete disappearance of cleaved Notch1 protein (Figure 4.9A and Figure 4.9B). On the other hand, the result of stimulation by LPS plus IFNγ in DMSO treated BMMϕ (Figure 4.9A) showed the expression of Notch 1 and cleaved Notch 1 which was similar to LPS plus IFNγ activated BMMϕ (Figure 4.4). These results were observed at both 6 and 24 hr after activation. Nevertheless, there was no difference in the level of total Notch1 in any treatments, implying that the level of Notch1 was not affected by inhibition of γ-secretase.

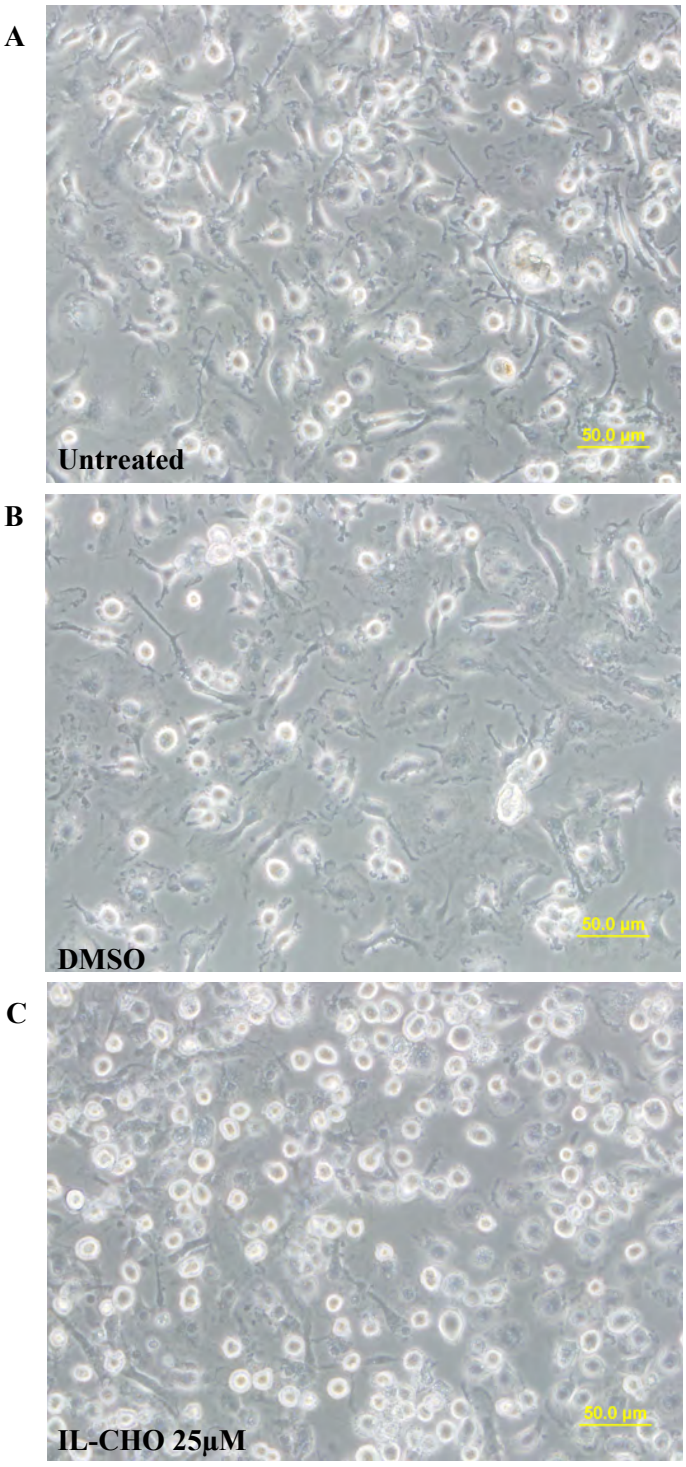


Figure 4.8 Morphology of BMMϕ upon treatment with IL-CHO

(A) BMMϕ, (B) BMMϕ treated by vehicle control (1%DMSO) for 1 hr without LPS+IFNγ activation, (C) BMMϕ treated by IL-CHO 25 µM for 1 hr without LPS+IFNγ activation. Images were captured under inverted microscope.

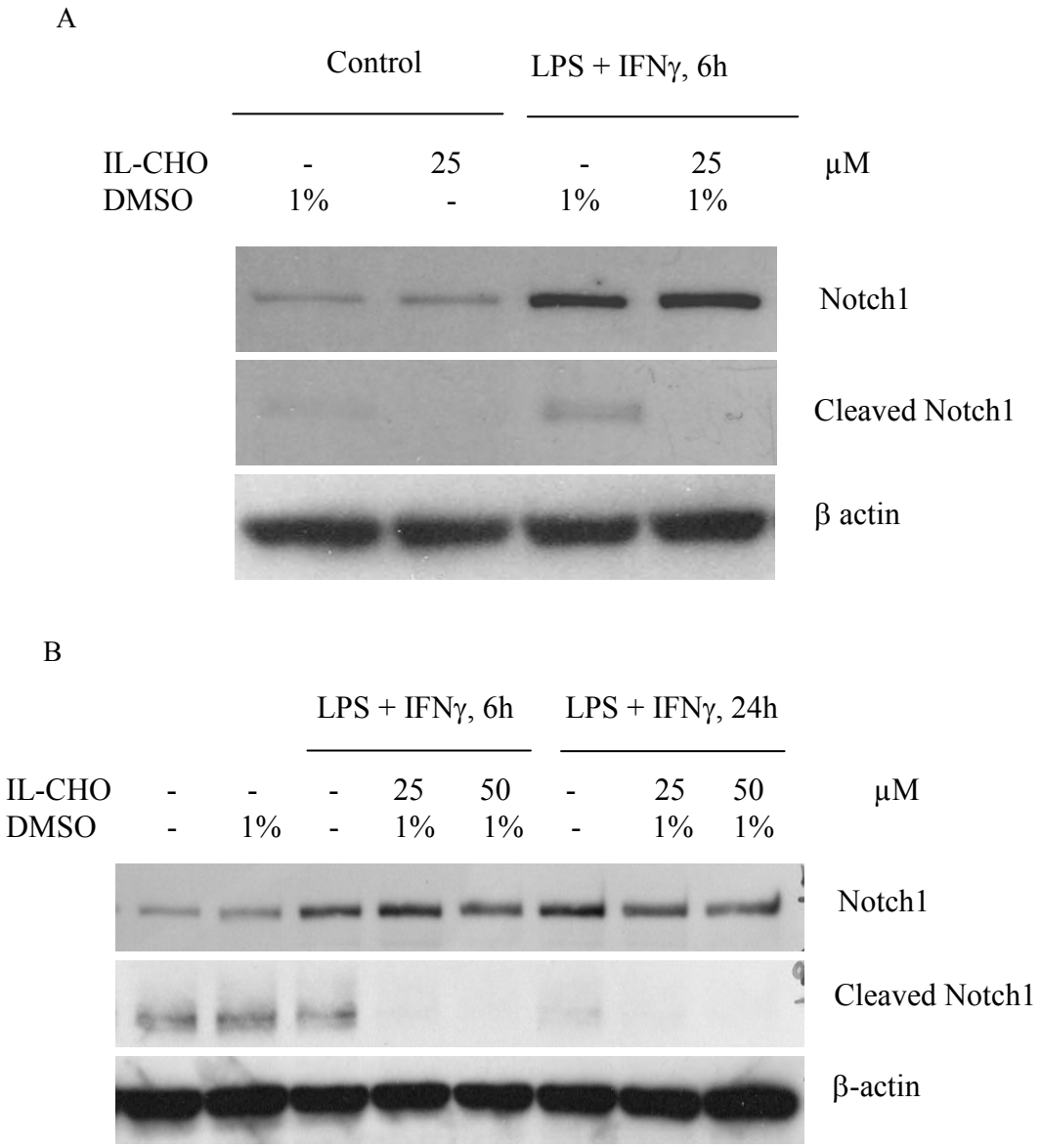


Figure 4.9 IL-CHO Treatment Decreased Cleaved Notch1 in BMMϕ

(A) BMMϕ were pre treated with 25 μM of IL-CHO and vehicle control for 1 hr and stimulated with LPS (100 ng/ml) + IFNγ (10 ng/ml) for 6 hr. Notch1, cleaved Notch1 and β-actin protein were measured by Western blot. Result indicated that IL-CHO reduced cleaved Notch1 in non-stimulated macrophages. (B) BMMϕ were pre treated with IL-CHO and vehicle control for 1 hr and stimulated with LPS (100 ng/ml) + IFNγ (10 ng/ml) for 6 and 24 hr. Notch1, cleaved Notch1 and β- actin protein were measured by Western blot.

4.5 Expression Profile of *IL-6* and secreted IL-6 in IL-CHO treated macrophages

Since treatment with IL-CHO inhibited Notch1 cleavage in macrophages both in resting and stimulated macrophages, the effect of such treatment was determined on the expression of *IL-6*. Previous studies showed that IL-CHO treatment led to decreased *IL-6* expression at 12 and 24 hr after stimulation (Palaga *et al.*, 2008). Total RNA was extracted from BMMϕ which were pre-treated with 25 μM of IL-CHO or DMSO for 1 hr and activated by LPS plus IFNγ for 6 hr, and the expression of *IL-6* was measured by qRT-PCR. As shown in Figure 4.10, the treatment with IL-CHO (25 μM) significantly decreased mRNA of *IL-6* upon activation of macrophages by LPS plus IFNγ, compared to DMSO treated control (Figure 4.10). Treatment with IL-CHO could not completely abrogate IL-6 expression, suggesting that the other signaling pathways were responsible for optimal *IL-6* expression in stimulated macrophages. The evidence was supported by Lityak et al., which reported that the kinetics of recruitment of transcription factors (NF-κB, ATF3 and C/EBPδ) on *IL-6* promoter were observed in LPS-TLR4 induced activation of macrophages (Litvak *et al.*, 2009). The report showed that C/EBPδ and NF-κB together stimulated transcription of *IL-6* while ATF3 functioned in inhibition of *IL-6* transcription. It was also reported that synergistic transcription factors such as C/EBPβ, NF-κB and CSL are involved in regulation of *IL-6* expression in IL-1β-activated human rheumatoid fibroblast-like synoviocytes. (Miyazawa *et al.*, 1998). These evidences indicated that the regulation of *IL-6* expression is controlled by the various types of transcription factors, including Notch/CSL.

Subsequently, in order to examine whether IL-CHO treatment affects on both transcription and translation of IL-6, secreted IL-6 were measured by ELISA. The culture supernatants were collected from BMMϕ pre-treated with IL-CHO or DMSO vehicle control and stimulated with LPS plus IFNγ. The results indicated that the level of secreted IL-6 significantly decreased upon IL-CHO treatment as compared with DMSO in stimulated

BMMϕ (Figure 4.11). Consistent with the results of mRNA expression, the level of IL-6 was not completely abrogated by IL-CHO treatment, suggesting that other signaling pathways play a role in regulating IL-6 production in macrophages.

In this study, both Notch1 and cleaved Notch1 protein were found to be expressed in non-stimulated BMMϕ (Figure 4.9), whereas expression of IL-6 was not detectable. Many researches reported that irregular expression of *IL-6* caused various diseases such as autoimmune and cancers, implying that the tight regulation of *IL-6* expression was crucial. We hypothesized that Notch signaling directly regulated *IL-6* expression; however, according to this experiment, the presence of Notch in resting macrophages did not result in IL-6 expression. In fact, in the absence of Notch, Plaisance *et al.*, 1997 reported in TNFα-induced mouse fibrosarcoma cells (L929sA) that CSL was able to sufficiently repress basal transcription of *IL-6* in the absence of NF-κB protein to prevent inflammatory stimulus (Plaisance *et al.*, 1997). Therefore, the result of this study implied that there might be some transcriptional repressors of *IL-6* which could prevent expression of *IL-6* before activation by stimuli.

Together with the results obtained from Western blot, these results indicated that Notch signaling pathway was a crucial, if not a sole, pathway which regulates IL-6 expression in macrophages.

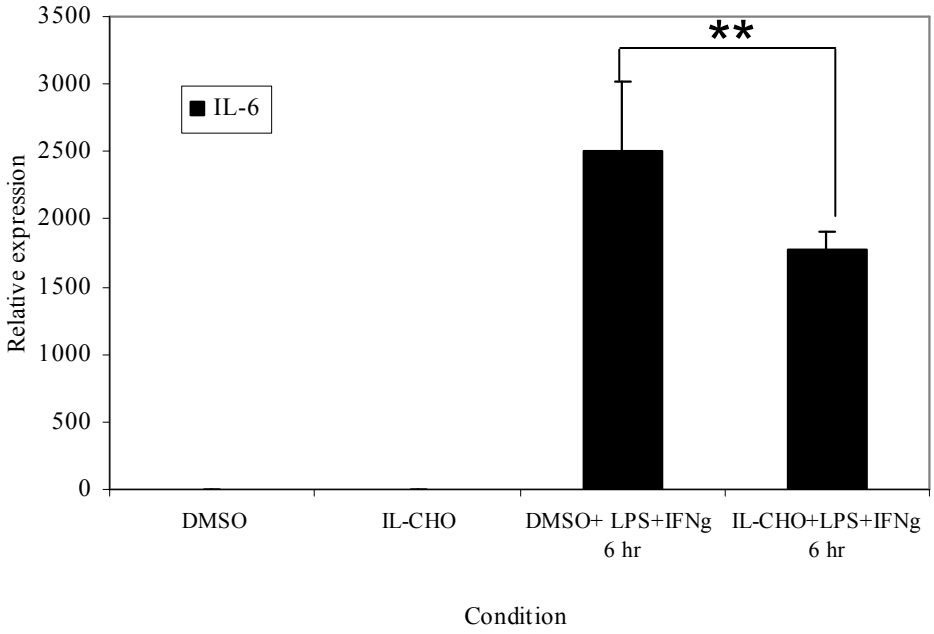


Figure 4.10 Expression of *IL-6* in macrophages upon IL-CHO treatment

BMMϕ were pre-treated with 25 μM of IL-CHO and vehicle control for 1 hr and stimulated with LPS (100 ng/ml) plus IFNγ (10 ng/ml) for 6 hr. The relative expression level of *IL-6* mRNA was measured by qPCR. Data are shown as mean+SD of triplicate determinants and representative of duplicated independent experiments. ** = significant at $p\ value < 0.05$

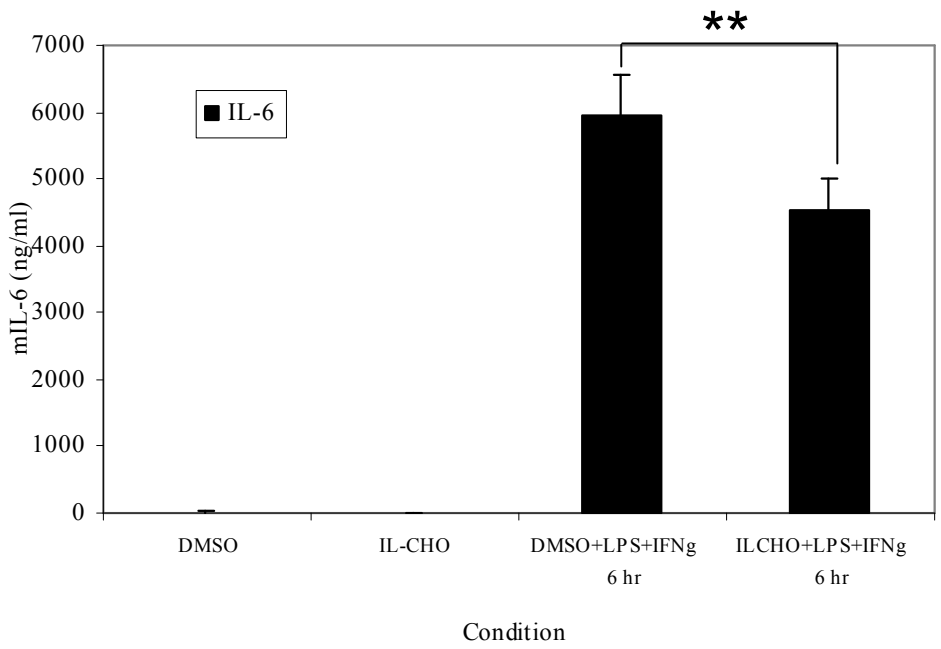


Figure 4.11 IL-6 Production in BMMϕ upon treatment with IL-CHO

BMMϕ were pre-treated with 25 μM of IL-CHO and vehicle control for 1 hr and stimulated with LPS (100 ng/ml) plus IFNγ (10 ng/ml) for 6 hr. Secreted IL-6 was determined by ELISA. Data are shown as mean+SD of triplicate determinants and representative of duplicated independent experiments. ** = significant at *p value* < 0.05

4.6 Over expression of truncated Notch1 in RAW264.7 cell line

The results obtained from this study indicated that inhibition of Notch signalling by gamma secretase treatment in activated macrophages resulted in decreasing expression of *IL-6* mRNA and protein production. In order to investigate the effect of hyperactivation of Notch signaling on *IL-6* expression, overexpression of truncated Notch1 was carried out in RAW264.7 cell line. RAW 264.7 cell line was transiently transfected with pcDNA3 harboring DNA inserts corresponding to intracellular domain of Notch 1(N^{IC}) from amino acids 1759 - 2556 (pcDNA3NIC) or pcDNA3 empty vector described in Chapter III. The truncated N^{IC} is constitutively active and does not require ligand binding to Notch receptor, which resulted in a strong activation of CBF1-dependent reporter gene in control or LPS plus IFNγ activated macrophages (Monsalve *et al*., 2006). The morphology of transiently tranfected RAW 264.7 cell line was first observed. The images showed that morphology pcDNA3NIC transfected RAW 264.7 appeared flat and had some extensions from cytoplasm (Figure 4.12). However, transient transfection at 48 hr resulted in a dramatic decrease in surface attaching properties on tissue culture treated plates, suggesting that transfection of pcDNA3 and pcDNA3NIC for 48 hr affected on cell morphology.

To confirm that pcDNA3NIC resulted in over expression of Notch1 in RAW264.7, total proteins were extracted after transient transfection for 12, 24 and 48 hr and subjected to analysis by Western blot. The results showed that Notch1 expression in RAW 264.7 transiently transfected with pcDNA3NIC was higher than the control vector transfected cells at both 12 and 24 hr of transfection (Figure 4.13). This result was consistent with Monsalve *et al.* which reported that pcDNA3NIC overexpressed HEK 293T cells by transient transfection expressed higher Notch1 protein compared with controls, whereas by stable transfection of this construct in RAW 264.7 cell line, Notch1 protein expressed lower than obtained from transient transfection (Monsalve *et al.*, 2006). Furthermore, pcDNA3NIC transiently transfected RAW 264.7 at 48 hr resulted in decreased expression of Notch1 protein, as

compared with those at 12 and 24 hr (Figure 4.13), suggesting that longer duration of tranfection may result in plasmid loss or toxicity to cells. Based on this result, the transfection at 12 hr time point was chosen as a condition for further study.

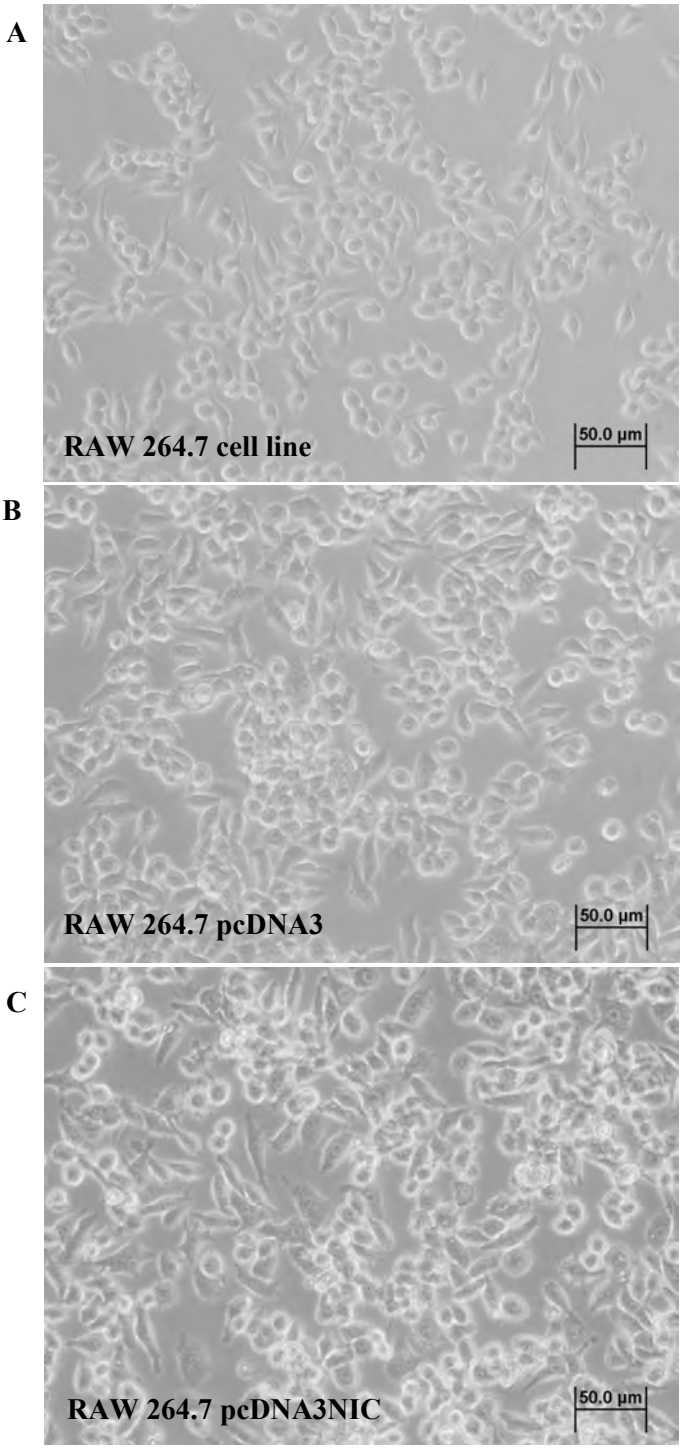


Figure 4.12 Morphology of RAW264.7 cell line transiently transfected with pcDNA3 and pcDNA3NIC

(A) Morphology of untransfected RAW264.7 (B) pcDNA3 empty vector transfected RAW264.7 (C) and pcDNA3NIC transfected RAW264.7 at 12 hr after transfection. The images were captured by using inverted microscope

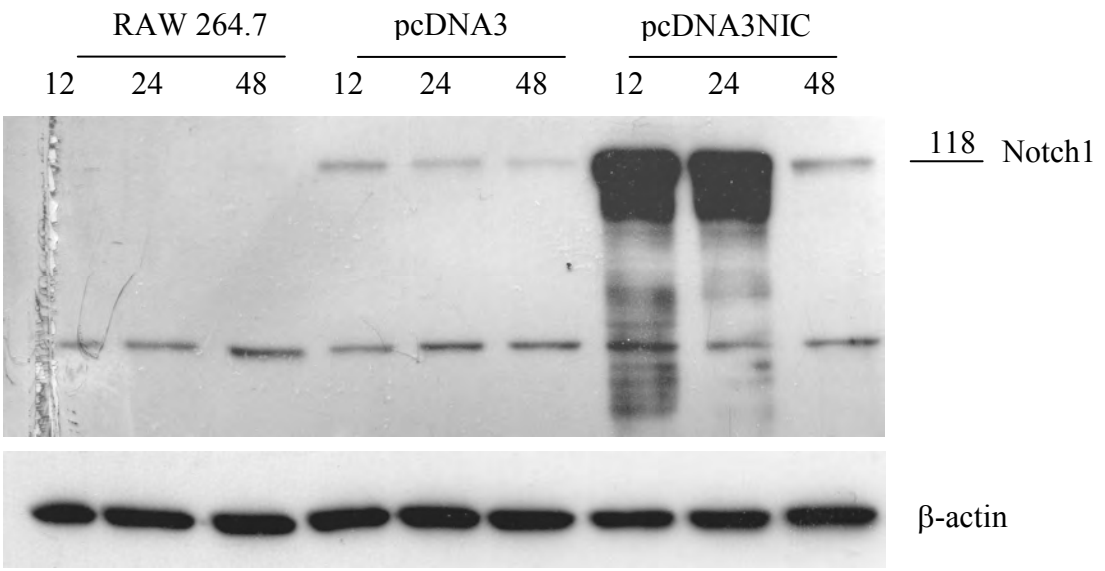


Figure 4.13 Overexpression of truncated Notch1 in transiently transfected RAW264.7 cell line

RAW264.7 cell line was transiently transfected with pcDNA3 and pcDNA3NIC for 12, 24 and 48 hr. Notch1 and β- actin (loading control) protein were analyzed by Western blot.

4.7 Expression of *IL-6* and secretion of IL-6 in Notch1 overexpressing macrophages

To determine whether increasing Notch signaling by transfection of truncated constitutively active Notch1 is associated with *IL-6* expression, mRNA was harvested from RAW264.7 transiently transfected with pcDNA3 or pcDNA3NIC for 12 hr, as described above and subjected to qPCR. As shown in Figure 4.14, the level of *IL-6* mRNA significantly increased in pcDNA3NIC transiently transfected RAW 264.7, in comparison with empty vector pcDNA3 transiently transfected RAW 264.7 (Figure 4.14).

To confirm that overexpression of constitutively active Notch1 affect both transcription and translation of IL-6, culture supernatants of transfected cells were collected to determine the amount of secreted IL-6 by ELISA. As shown in Figure 4.15. Increasing Notch signaling also resulted in significantly increased level of secreted IL-6 (Figure 4.15), even in the absence of other stimuli such as LPS and IFNγ.

These findings, together with the results obtained from IL-CHO treatment, strongly suggested that Notch signaling is positively involved in *IL-6* expression at the transcriptional level. In addition, the signaling itself was sufficient to induce IL-6 expression in macrophages in the absence of other stimuli.

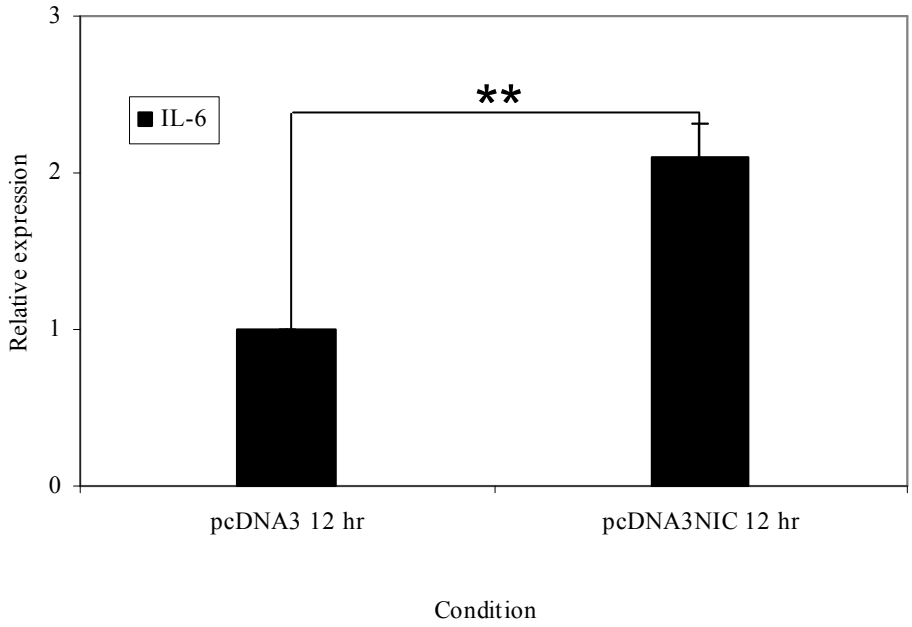


Figure 4.14 Relative expression of IL-6 in transiently transfected RAW264.7 cell line

RAW264.7 was transiently transfected with pcDNA3 and pcDNA3NIC for 12 hr. Total RNA was extracted and subjected to qPCR for *IL-6* mRNA expression. The relative expression of IL-6 was normalized to β−actin. Data are shown as mean+SD of triplicate determinants and representative of duplicated independent experiments. ** = significant at *p value* < 0.05

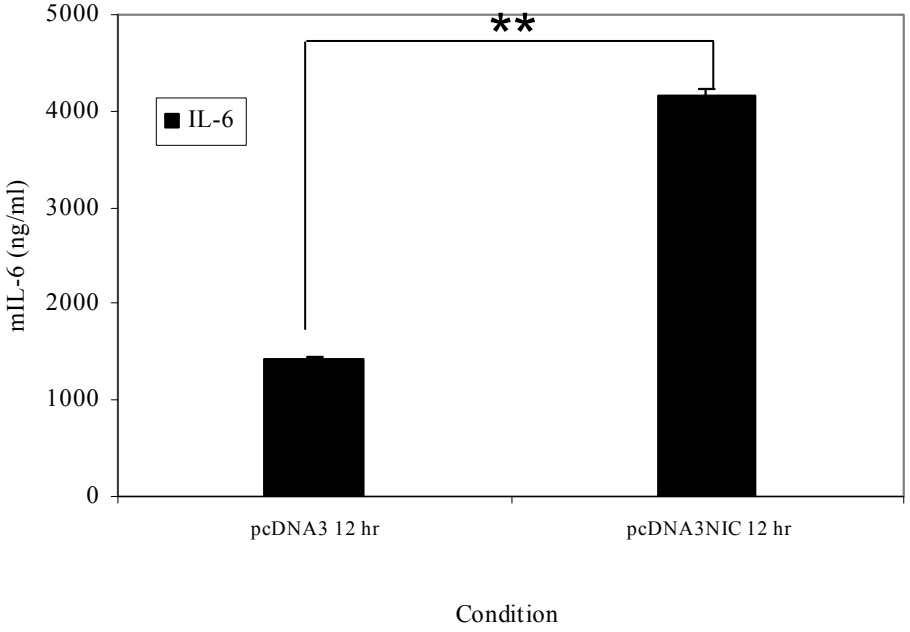


Figure 4.15 Production of IL-6 from transiently transfected RAW264.7 cell line

RAW264.7 was transiently transfected with pcDNA3 and pcDNA3NIC for 12 hr. Secreted IL-6 was determined by ELISA from harvested culture supernatant. Data are shown as mean+SD of triplicate determinants and representative of duplicated independent experiments. ** = significant at *p value* < 0.05.

4.8 Investigating the binding of Notch/CSL complex on *IL-6* promoter by ChIP assay

Since we and others identified the potential CSL binding sites in the promoter of *IL-6* and the results obtained in this study suggested that Notch signaling pathway positively regulates *IL-6* expression, we set to test whether Notch/CSL complex binds to this potential site in the promoter of *IL-6*. To determine whether Notch (in the complex with CSL) directly binds to *IL-6* promoter, ChIP was performed. In the study by Krejci *et al,* ChIP assay was used to study the dynamic binding of CSL/Notch on promoter of Notch target genes in *Drosophila* and the binding affinity in Notch-expressing *Drosophila* S2 cell line. They found that Su(H), a *Drosophila* homolog of CSL, binds to Notch target genes in the presence or absence of Notch signaling. After RAW 264.7 were activated by LPS plus IFNγ for 6 hr, cells were fixed and lysed and subsequently sheared by sonicator. Five µl of unsheared and sheared DNA were extracted to determine whether DNA has been sheared to suitable sizes (200 bp-1000 bp). The result was shown in figure 4.16 and this condition was used for the ChIP assay.

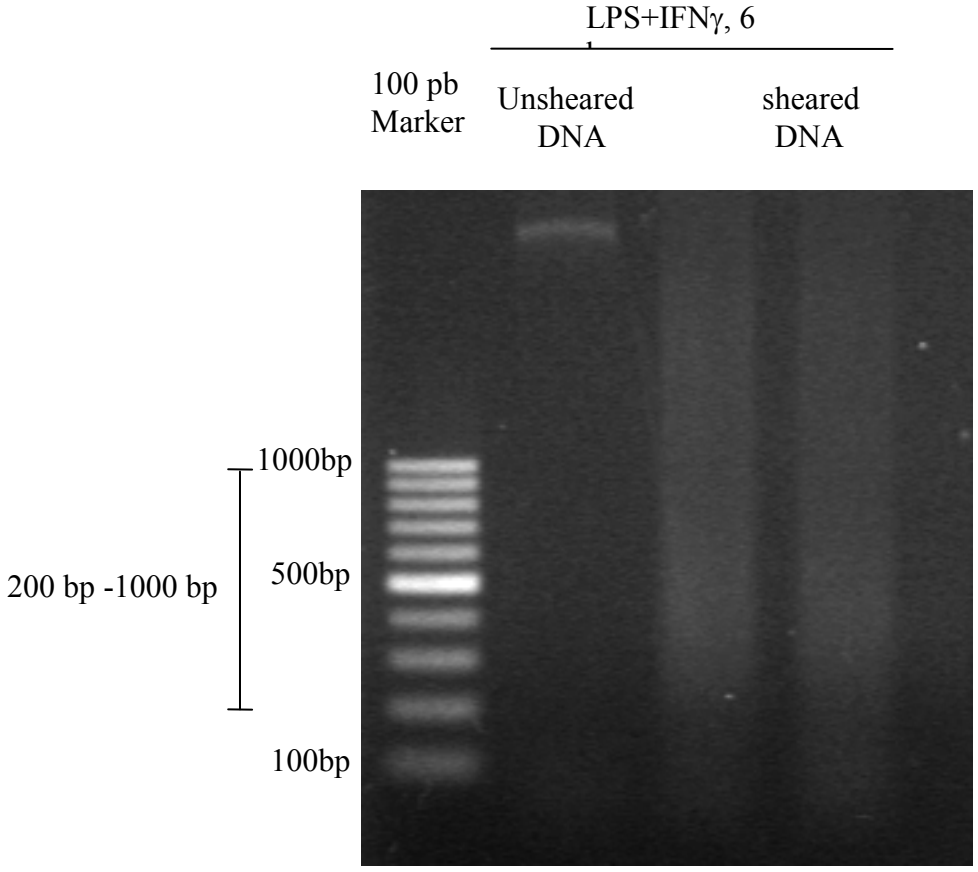


Figure 4.16 Shearing of genomic DNA for ChIP assay

Genomic DNA from RAW264.7 cell line stimulated with LPS and IFNγ were subjected to sonication for DNA shearing. After purification, 10 µl of unsheared and sheared DNA were loaded on 2% agarose gel. DNA was visualized by ethidium bromide staining.

Using the shearing condition described above, immunoprecipitation were carried out using anti-Notch1 antibody and rabbit isotype control. Anti-RNA Polymerase II and mouse isotype control were used to evaluate the performance of ChIP assay. Precipitated DNA was purified and analyzed by PCR using primers specific for the *IL-6* promoter in the regions where CSL binding site was found. PCR products were observed in 'Input' which was 2% of total DNA sample used for immunoprecipitation. Antibody against RNA polymerase was used as positive control for immunoprecipitation and the results indicated that RNA polymerase II bound directly to the IL-6 promoter of stimulated RAW264.7 cell line and validated the successful of ChIP assay for this study. For ChIP assay using anti-Notch1 antibody, the positive band was detected but the band at the similar size also appeared when normal rabbit IgG was used, suggesting that this result was a non-specific effect of the immunprecipitation. Therefore, it was not conclusive whether Notch1 binds directly to the *IL-6* promoter. Future investigation using Notch1 overexpressing cell line is needed to conclude the regulatory role of Notch signaling in *IL-6* expression.

The results obtained in this study demonstrated that Notch signaling pathway plays an important role in IL-6 expression in macrophages, adding another molecular mechanism in regulating IL-6 expression in macrophages. This is the first report on the positive role of Notch signaling in regulating IL-6. This finding may lead to a drug design targeting diseases with aberrant IL-6 production.

A

Primer-Murine

GCTTTACGTTCTCTTTCTCCTTATAAAACATTGTGAATTTCAGTTTTCTTTCCCATCAAGACATGCTCAA
GTGCTGAGTCACTTTTAAAGAAAAAAAGAAGAGTGCTCATGCTTCTTAGGGCTAGCCTCAAGGATGACTT
AAGCACACTTTTCCCTTCCTAGTTGTGATTCTT**TCGATGCTAAACGACGTCAC**ATTGTGCAATCTTAATA
AGGTTTCCAATCAGCCCCACCCACTCTGGCCCCACCCCCACCCTCCAACAAAGATTTTTATCAAATGTGG
GATTTTCCCATGAGTCTCAAAATTAGAGAGTTGACTCCTAATAAATATGAGACTGGGGATGTCTGTAGCT
CATTCTGCTCTGGAGCCCACCAAGAACGATAG**TCAATTCCAGAAACCGCTATG**

PCR product: 230

B

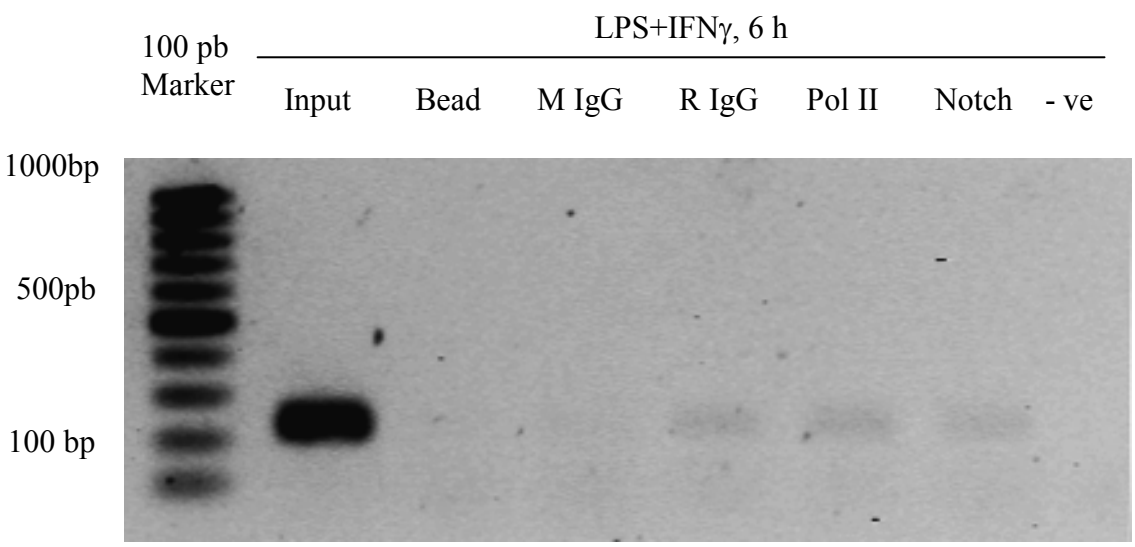


Figure 4.17 ChIP assay for IL-6 promoter using anti-Notch1 antibody

(A) Specific primers (underline) were designed cover CSL binding site (highlight in green) on IL-6 promoter by Primer3 program. The translational starting site is highlighted in red and 5' UTR (Transcription initiation) is in pink.

(B) PCR analysis of chromatin immunoprecipitation. DNA was precipitated by using Mouse IgG, Rabbit IgG, anti-RNA Polymerase II and anti-Notch1 antibody. ChIP was performed following to the Magna ChIP™ protocol. Ten µl of each DNA sample were loading in 2% agarose gel. Electrophoresis was carried out at 100 volt for 30 min and gel, then, was strained with ethidium bromide and detected in UV light.

CHAPTER V

CONCLUSION

(1) There was one potential CSL binding site on *IL-6* promoter of human and mouse at region approximately 1,243 base pairs upstream of the start codon (ATG) and was evolutionarily conserved in both species.

(2) Stimulation with LPS or LPS plus IFNγ triggered Notch1 and cleaved Notch 1 expression in BMMϕ and RAW 264.7 cell line. Notch1 and cleaved Notch1 protein were detected in non-stimulated BMMϕ, suggesting that there were basal activation of Notch signaling in BMMϕ.

(3) The expression of Notch1 and cleaved Notch1 related to the expression of *IL-6* mRNA after activation by LPS plus IFNγ in primary macrophages and RAW264.7 cell line.

(4) Treatment with 25 μM of IL-CHO affected cellular morphology and was sufficient to eliminate the cleaved Notch1 expression does not Notch1 in activated macrophages.

(5) The expression level of IL-6 was not completely abrogated by IL-CHO treatment, suggesting that other signaling pathways also play a role in regulating IL-6 expression in macrophages.

(6) Increasing of truncated Notch1 in RAW 264.7 by transient transfection at 12h involved in increasing of Notch1 protein expression and also increased *IL-6* mRNA and Secreted IL-6.

(7) ChIP assay was validated the success by using anti RNA Polymerase II and mouse isotype control as positive control of immunoprecipitation. RNA Polymerease II bound directly to *IL-6* promoter in activated macrophages whereas it was not conclusive

whether Notch1 bound directly to the IL-6 promoter according to non-specific effect of the immunprecipitation.

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APPENDIX

1. Completed DMEM 100 ml

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

2. Freezing media 10 ml

Completed DMEM	90%
DMSO	10%

3. FBS inactivation

Commercial FBS which were kept at -20°C was thawed at 4°C for overnight and inactivated at 56°C for 30 min. in water bath prior using.

4. Penicillin and streptomycin

Streptomycin was prepared at final concentration 50 mg/ml, and penicillin was prepared at final concentration 10^6 U/ml by diluting in sterile deionized water. The solutions were filtered by using 0.22 µm syringe filter and then aliquoted and kept at –20°C.

5. Buffer A for protein extraction

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

6. Buffer B for protein extraction

Buffer A	990 µl
Nonidet P-40	10 µl

7. 10% SDS-polyacrylamide 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

8. 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

9. 2×Laemmli buffer (SDS-dye) 10 ml

1 M Tris-HCl pH 6.8	1 ml
10% SDS	4 ml
99.5% glycerol	2.01 ml
HPLC water	2.989 ml
Bromphenol blue	0.001 g

10. 5×running buffer for Western blot (1000 ml)

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

11. Transfer buffer for Western blot

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

12. 1×PBS pH 7.4 1000 ml

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

Autoclaved at 121°C and pressure 15 psi for 15 min.

13. PBST (washing buffer for Western blot)

1×PBS	500 ml
Tween20	0.05%

14. Blocking solution for Western blot

PBST	200 ml
Non-fat dry milk	6 g

15. ECL substrate of HRP

90mM of Coumaric acid was dissolved in DMSO in total volume 10 ml, aliquoted and kept at –20°C.

250 mM of Luminol was also dissolved in DMSO in total volume 10 ml, aliquoted and kept at –20°C.

16. Solution A

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

17. Solution B

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

18. Film developer and fixer

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

19. 0.01% DEPC water for RNA 100 ml

One hundred ml of HPLC water was added into a clean bottle follow by 10 µl of DEPC (0.01% v/v). The bottle was swirled and incubated overnight at room temperature. Afterwards, DEPC water was sterile at 121°C, pressure 15 psi for 15 min.

20. 75% Ethanol in DEPC 100 ml

25 ml of 0.01% DEPC water was added in 75 ml of Ethonol and kept at –20°C.

21. 50xTAE buffer for agarose gel electrophoresis 200 ml

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

Adjusted pH to 8.0 and volume to 200 ml using deionized water autoclaved at 121°C and pressure 15 psi for 15 min.

22. Running buffer for agarose gel electrophoresis

50xTAE was diluted to final concentration 0.5x in 400 ml of deionized water.

23. Gel preparing buffer for agarose gel electrophoresis

50xTAE was diluted to final concentration 1x in 400 ml of deionized water.

24. 7. 2 % agarose gel preparation

Agarose gel	0.4 g
1×TAE	20 ml

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

LB broth preparation

Bacto tryptone	10 g
Yeast extracts	5 g
NaCl	10 g

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

LB agar preparation

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

LB agar containing 50 µg/ml ampicillin

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

26. 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 1.5 µl microcentrifuge tube, and glycerol was added to 20% final concentration. The aliquots were kept at –20°C. To measure the CFU of stock culture, the aliquot was diluted and determined the CFU by spread plate.

BIOGRAPHY

I earned my undergraduate degree of Science in Microbiology from Chulalongkorn University in 2002. After graduation, I immediately started working with Mars Cooperation, Effem Foods (Thailand) Ltd., as Quality System technician where I was responsible for setting the quality specification for dried and wet pet food in terms of food safety and hygiene. Three years after working with Effem Foods, I decided to join the Itex foods International Ltd to work as a Quality System Assistant. My responsibility was mainly to audit and provide the relevant recommendations to the food manufacturers about the risk assessment and conforming to the particular regulations such as GMP and HACCP. Furthermore, I gained new experiences about the extra strict requirements on food regulations for exporting foods to EU which is more subtle than Thai local regulations. After being in the role of quality system in total of 4 years, I began to reconsider about my career and my interest in basic biological sciences. Consequently, I decided to pursue the higher education in Biotechnology for M.S. degree at Chulalongkorn University and ultimately found immunology as my topic of interest.

In 2009, I was selected from American Association of Immunologist (AAI) and International Union Immunological Societies (IUIS) to attend the introductory course in immunology during 19-25 June at University of Pennsylvania, Philadelphia.

In 2010, I made the oral presentation in the topic of regulation of IL-6 expression by Notch signaling in macrophages at The 3rd Technology and Innovation for Sustainable Development International Conference at Nong Khai, Thailand.

PUBLICATION

Suwanjunee, S., **Wongchana, W.,** Palaga, T. (2008). Inhibition of gamma-secretase affects proliferation of leukemia and hepatoma cell lines through Notch signaling. Anti-Cancer Drugs. 19, 477-486.