วิถีสัญญาณ Notch ควบคุมการตายแบบ apoptosis ในแมคโครฟาจที่ติดเชื้อมัยโคแบคทีเรีย

นางสาว ศิริลักษณ์ รัตนบรรยงค์

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NOTCH SIGNALING REGULATES APOPTOSIS IN MACROPHAGES INFECTED WITH MYCOBACTERIA.

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ศิริลักษณ์ รัตนบรรยงค์: วิถีสัญญาณ Notch ควบคุมการตายแบบ apoptosis ในแมคโครฟาจที่ติดเชื้อมัยโคแบคทีเรีย (Notch signaling regulates apoptosis in macrophages infected with mycobavteria.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. ธนาภัทร ปาลกะ, 91 หน้า.

การตายแบบ apoptosis มีบทบาทสำคัญในการตอบสนองทางระบบภูมิคุ้มกันในแมคโครฟาจที่ติดเชื้อ โดยการศึกษาก่อนหน้านี้ พบว่า Mcl-1 ซึ่งเป็นโปรตีนที่ทำหน้าที่ต้านการตายแบบ apoptosis มีการแสดงออกเพิ่มสูงขึ้นในมอโนไซท์และแมคโครฟาจที่ติดเชื้อ *Mycobacterium tuberculosis* (Mtb) สายพันธุ์ก่อโรครุนแรง ที่เป็นสาเหตุของโรควัณโรค Myeloid cell leukemia-1 (Mcl-1) เป็นโปรตีนที่เอื้อต่อการอยู่รอดของเซลล์ ซึ่งจัดอยู่ในกลุ่มโปรตีน Bcl-2 กลไกในการควบคุมการแสดงออกของ Mcl-1 ในแมคโครฟาจนั้นยังไม่รู้อย่างชัดเจน ในการศึกษาครั้งนี้ พบว่า Mcl-1 มีการแสดงออกเพิ่มสูงขึ้นใน bone marrow derived macrophage (BMMϕ) และ RAW264.7 ซึ่งเป็น macrophage like cell line ที่ถูกกระตุ้นด้วย Mtb-derived purified protein derivative (PPD) หรือที่ติดเชื้อด้วย *Mycobacterium bovis* BCG (*M. bovis* BCG) วิถีสัญญาณ Notch เป็นวิถีสัญญาณที่มีการอนุรักษ์ไว้สูง สามารถพบได้ในสิ่งมีชีวิตหลากชนิด มีหน้าที่ควบคุมการเพิ่มจำนวน การแปรสภาพเพื่อทำหน้าที่เฉพาะ รวมถึงการตายแบบ apoptosis และจากการศึกษาก่อนหน้านี้ได้มีรายงานว่า วิถีสัญญาณ Notch ถูกกระตุ้นในแมคโครฟาจที่ติดเชื้อ *M. bovis* BCG ในการศึกษาครั้งนี้ พบว่า แมคโครฟาจที่ถูกกระตุ้นด้วย PPD หรือติดเชื้อ *M. bovis* BCG มีการแสดงออกของ Mcl-1 และ Notch1 ที่มีความสัมพันธ์กันอย่างชัดเจน และยังพบอีกว่า ในแมคโครฟาจที่ถูกกระตุ้นด้วย PPD ตรวจพบ cleaved Notch1 ซึ่งเป็นตัวบ่งชี้ว่าวิถีสัญญาณ Notch ถูกกระตุ้น จากการให้ยากดแกมมาซีครีเตส (γ-secretase inhibitor; GSI) ซึ่งมีฤทธิ์ยับยั้งการทำงานของ Notch แบบจำเพาะ หรือการลดการแสดงออกของ Notch1 โดยวิธี knockdown ส่งผลทำให้การแสดงออกของ Mcl-1 ลดลง และเพิ่มการตายแบบ apoptosis ในแมคโครฟาจที่ติดเชื้อ และเนื่องจากการวิเคราะห์บริเวณโปรโมเตอร์ของ *mcl-1* พบว่ามีบริเวณจับที่จำเพาะของ CSL ซึ่งเป็น DNA binding protein และทำหน้าที่เป็นโปรตีนคู่จับกับ Notch จึงใช้วิธี chromatin immunoprecipitation (ChIP) assay ในการพิสูจน์ว่า จับกับโปรโมเตอร์ของ *mcl-1* โดยตรงหรือไม่ ผลจาก ChIP assay พบว่า แมคโครฟาจที่ถูกกระตุ้นด้วย PPD จะส่งผลชักนำให้บริเวณที่อยู่ด้านในเซลล์ของ Notch เคลื่อนที่ไปจับบนบริเวณโปรโมเตอร์ของ *mcl-1* ผลการศึกษาทั้งหมดทำให้สามารถบอกได้ว่า ในแมคโครฟาจที่ถูกกระตุ้นด้วย PPD หรือติดเชื้อ *M. bovis* BCG จะมีการแสดงออกของ Mcl-1 เพิ่มขึ้นโดยถูกควบคุมผ่านทางวิถีสัญญาณ Notch โดยตรง บทบาทใหม่ของ Notch1 ในการควบคุมการแสดงออกของ Mcl-1 ในการต้านทานต่อเชื้อแบคทีเรียนี้อาจนำไปสู่วิธีการใหม่ในการควบคุมการตอบสนองระบบภูมิคุ้มกันของเจ้าบ้านต่อโรควัณโรคได้

สาขาวิชา จุลชีววิทยาทางการแพทย์.....ลายมือชื่อนิสิต .....

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Apoptosis of macrophages infected with pathogens is considered to be one of the important host immune responses. Previously, Mcl-1 expression was found to be up-regulated in monocytes/macrophages during infection with virulent *Mycobacterium tuberculosis* (Mtb), a causative agent of tuberculosis. Myeloid cell leukemia-1 (Mcl-1) is a pro-survival member of the Bcl-2 protein family. The regulatory mechanism of Mcl-1 expression in macrophages is not well characterized. In this study, upregulation of Mcl-1 in bone marrow derived macrophages (BMMϕ) and macrophage-like cell line RAW264.7 were observed when cells were treated with Mtb-derived purified protein derivatives (PPD) or infected with *Mycobacterium bovis* BCG (*M. bovis* BCG). The Notch signaling pathway is a highly conserved cell signaling pathway present in most multicellular organisms. Notch signaling modulates numerous cellular functions such as proliferation, adhesion, angiogenesis and apoptosis. Recent studies reported that Notch signaling is activated in macrophages during infection with *M. bovis* BCG. We found a well correlation between Mcl-1 and Notch1 expression in PPD-activated or *M. bovis* BCG-infected macrophages. In addition, cleaved Notch1, an indicator of activated Notch signaling pathway, was detected upon PPD treatment of macrophages, suggesting that Notch signaling is activated. Treatment with γ-secretase inhibitor (GSI), a specific inhibitor of Notch signaling, or knockdown of Notch1 resulted in decreased expression of Mcl-1 and increased apoptosis of infected macrophages. Since a conserved potential binding site for CSL, a DNA binding protein and interacting partner with Notch, was identified in the promoter region of *mcl-1*, chromatin immunoprecipitation (ChIP) assay was used to determine whether Notch1 directly binds to *mcl-1* promoter. ChIP assay clearly demonstrated that stimulation with PPD recruited intracellular Notch to the *mcl-1* promoter. Taken together, these results suggested that Notch signaling directly regulates Mcl-1 expression in macrophages upon treatment with PPD or infection with *M. bovis* BCG. This new function of Notch1 in regulating Mcl-1 expression in antibacterial immunity may open a new strategy to manipulate host immune response against tuberculosis.

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ABBREVIATIONS

1.	%	Percentage
2.	°C	Degree Celsius
3.	μg	Microgram
4.	μm	Micrometer
5.	μM	Micromolar
6.	/	Per
7.	:	Ratio
8.	Ab	Antibody
9.	BMMϕ	Bone marrow derived macrophage
10.	bp	Base pair
11.	cDNA	Complementary DNA
12.	CFU	Colony forming unit
13.	CO_2	Carbon dioxide
14.	dATP	Deoxyadenosine triphosphate
15.	dCTP	Deoxycytosine triphosphate
16.	dGTP	Deoxyguanosine triphosphate
17.	DNA	Deoxyribonucleic acid
18.	dNTP	dATP, dCTP, dGTP and dTTP
19.	dTTP	Deoxythymidine triphosphate
20.	ELISA	Enzyme-linked immunosorbent assay
21.	g (centrifugation speed)	Gravity
22.	GSI	Gamma secretase inhibitor
23.	hr	Hour
24.	HPLC	High performance liquid chromatography
25.	HRP	Horse radish peroxidase

26.	IgG	Immunoglobulin G
27.	IL	Interleukin
28.	kDa	Kilo Dalton
29.	LB	Lauria bertani
30.	m	Murine
31.	mA	Milliampere
32.	mg	Milligram
33.	ml	Milliliter
34.	mM	Millimolar
35.	Mϕ	Macrophage
36.	nm	Nanometer
37.	No.	Number
38.	OD	Optical density
39.	PAGE	Polyacrylamide gel electrophoresis
40.	PBS	Phosphate buffer saline
41.	PBST	Phosphate buffer saline – Tween
42.	PCR	Polymerase chain reaction
43.	pShNotch1	Plasmid short hairpin loop for Notch1
44.	pShLuc	Plasmid short hairpin loop for Luciferase
45.	psi	Pound per square inch
46.	PVDF	Polyvinylidine fluoride
47.	RNA	Ribonucleic acid
48.	rpm	Round per minute
49.	RT	Reverse transcription
50.	SDS	Sodium dodecyl sulfate
51.	U	Unit
52.	v	Volume

53.	w	Weight
54.	α	Alpha
55.	β	Beta
56.	γ	Gamma

CHAPTER I

BACKGROUND

New cases of tuberculosis in 2007 were estimated to be 9.3 million and approximately 1.3 million patients die each year (Donald and van Helden, 2009). The causative agent of tuberculosis is an intracellular bacteria *Mycobacterium tuberculosis* (Mtb). Spread of tuberculosis has been increased by susceptibility of individuals infected with HIV and exacerbated due to the development of drug-resistant strains of Mtb. Mtb infects alveolar macrophages and uses them as a site for replication. The virulence of the mycobacteria depends on their ability to replicate in macrophages. Mtb uses immature phagosomes to replicate and cause pathological condition in human (Dao *et al.*, 2004). Intracellular survival of Mtb is possibly due in part to their interference with phagosomal acidification and fusion with lysosome (Sturgill-Koszycki *et al.*, 1994; Clemens and Horwitz, 1995). Macrophage apoptosis is a common host defense strategy against intracellular pathogens, including viruses, protozoa and bacteria (Spira *et al.*, 2003). Macrophages undergoing apoptosis kill intracellular mycobacteria and this process plays an important role in stimulation of the adaptive immune response (Fairbairn, 2004).

Apoptosis, a genetically controlled cell death program, is important for the development and cell homeostasis in multicellular organisms. Apoptosis can be triggered by either the intrinsic pathway or extrinsic pathway (Reed, 2000). Intrinsic pathway involves mitochondrial release of cytochrome *c* and activation of caspase 9 (Reed, 2000; Opferman and Korsmeyer, 2003). Extrinsic pathway involves stimulation of death receptors expressed on the cell surface and activation of caspase 8 (Reed, 2000; Opferman and Korsmeyer, 2003). Non-virulent mycobacteria, such as *Mycobacterium bovis* Bacille Calmette Guérin (*M. bovis* BCG), and the avirulent Mtb H37Ra, induce more macrophage apoptosis, compared with the

virulent Mtb H37Rv (Keane *et al.*, 2000). The mechanisms of preventing macrophage apoptosis by virulent mycobacteria have been indentified, including (i) through initiation of TLR-2-dependent activation of the NF-κB cell survival pathway (Gao and Kwaik, 2000); (ii) increasing the production of soluble TNF receptor2, which neutralizes the pro-apoptotic activity of TNF-α (Balcewicz-Sablinska *et al.*, 1998; Kornfeld *et al.*, 1999); (iii) inactivation of pro-apoptotic protein, such as Bad (Maiti *et al.*, 2001); and (iv) up-regulation of anti-apoptotic Mcl-1 protein (Sly *et al.*, 2003). Interestingly, increasing macrophage apoptosis by inhibiting Mcl-1 activity leads to decrease in mycobacterial survival (Sly *et al.*, 2003).

Mcl-1 (myeloid cell leukemia-1), an anti-apoptotic protein, is a key member of Bcl-2 protein family containing Bcl-2 homology domain. Mcl-1 is rapidly degraded in response to cell death signals and immediately re-induced by survival stimuli (Michels *et al.*, 2005). The regulation of *mcl-1* expression is controlled at transcriptional, post-transcriptional, and post-translational levels (Le Gouill *et al.*, 2004). At the transcriptional level, it is modulated by numerous extra-cellular stimuli including interleukins, growth factors, colony stimulating factors, interferon (IFN), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the protein kinase C activator (Domina *et al.*, 2000; Craig, 2002; Le Gouill *et al.*, 2004). The extra-cellular stimuli trigger *Mcl-1* expression via several signaling pathways such as JAK/STAT, MAPK, PI3K/AKT and MEK/ERK pathways (Wang *et al.*, 1999; Bingle *et al.*, 2000; Craig, 2002; Liu *et al.*, 2003; Le Gouill *et al.*, 2004). At the post-transcriptional level, *mcl-1* mRNA undergoes alternative splicing. The products of two splicing forms, Mcl-1L and Mcl-1S, form heterodimers and are capable of neutralizing each other. At the post-translational level, Mcl-1 is controlled either by caspase cleavage, phosphorylation or ubiquitination (Le Gouill *et al.*, 2004; Thomas *et al.*, 2010). Mcl-1 plays an important role in development and cellular differentiation, particularly within the hematopoietic cell lineage (Craig, 2002; Opferman *et al.*, 2005; Akala and Clarke, 2006). Mcl-1 inhibits apoptosis by maintaining the integrity of the mitochondrial membrane by forming protein complex with pro-apoptotic protein Bak or

Bax (Clohessy *et al.*, 2004; Opferman *et al.*, 2005; Willis *et al.*, 2005; Zhuang and Brady, 2006). Oishi *et al*. reported that Mcl-1 protein is up-regulated in neural precursor cells during development through Notch signaling (Oishi *et al.*, 2004; Yoshimatsu *et al.*, 2006).

The Notch signaling pathway is a highly conserved cell signaling pathway present in most multicellular organisms (Artavanis-Tsakonas *et al.*, 1999; Schweisguth, 2004). Notch signaling is initiated through ligand-receptor interaction, primarily by proteolytic cleavage of the receptor, a process that releases the intracellular domain of Notch (NIC). NIC translocates to nucleus and binds directly to the transcription factor CSL (Ohishi *et al.*, 2003). CSL protein binds to a consensus DNA sequence in the promoter region of Notch-inducible genes (Pursglove and Mackay, 2005). Notch signaling modulates numerous cellular functions such as proliferation, adhesion, angiogenesis and apoptosis. In myeloid lineage cells, Notch signaling promotes cell commitment to differentiation and modulates cell-fate decisions (Schroeder and Just, 2000; Schroeder *et al.*, 2003, Ohishi *et al.*, 2003). Our unpublished data and findings from other groups suggested that Notch signaling is activated in macrophages during infection with *M. bovis* BCG (Jayakumar *et al.*, 2008) (Palaga *et al.*, unpublished data).

Although Mcl-1 expression is regulated by many pathways such as MAPK, PI3K/AKT, and JAK/STAT pathways, it is unclear whether activation through Notch signaling pathway regulates Mcl-1 expression in macrophages infected with mycobacteria. In this study, we investigated whether PPD, Mtb-derived purified protein derivative, or *M. bovis* BCG infection upregulate Notch receptors and activates Notch signaling pathway, leading to the expression of Mcl-1 and inhibition of apoptosis.

Objective

To investigate whether treatment with PPD or infection with *M. bovis* BCG upregulate Notch receptors and activates Notch signaling pathway, leading to the expression of Mcl-1 and inhibition of apoptosis.

CHAPTER II

LITERATURE REVIEWS

2.1 Tuberculosis

2.1.1 The infection and recognition

New cases of tuberculosis in 2007 were estimated to be 9.3 million and approximately 1.3 million patients have died (Donald and van Helden, 2009). The causative agent of tuberculosis is *Mycobacterium tuberculosis* (Mtb). The main route of infection for the Mtb is the respiratory tract and the infections progress to the lung to establish an infection. Mtb is a facultative intracellular parasite infecting mainly alveolar macrophage. The parasitic relationship is made possible by the capacity of Mtb to block phagosome maturation subsequent to the entry into the host macrophages (Sturgill-Koszycki *et al.*, 1994; Clemens and Horwitz, 1995). When Mtb enters macrophages, it creates an environment that supports bacillary replication. Mtb was recognized by many different phagocytic receptors including complement receptor, scavenger receptor, and mannose receptor. For innate immune recognition, proteins in the family of TLRs play an important role in Mtb recognition. For example, TLR2 binds lipoarabinomannan, TLR2 and 6 bind a 19-kDa lipoprotein, TLR-4 binds an undefined heat-labile cell associated factor, and TLR-9 recognized Mtb DNA (Trinchieri and Sher, 2007). Spread of tuberculosis has been increased by susceptibility of individuals infected with HIV and exacerbated due to the development of drug-resistant strains of Mtb.

2.1.2 Pathogenesis of tuberculosis

The virulence of the mycobacteria depends on their ability to replicate in macrophages. The interaction between Mtb and host macrophages determines outcome of

disease. When active tuberculosis develops, disease localization, severity, and outcome are highly variable. The formation of characteristic multicellular structures within infected lung tissue is called granulomas. The granuloma is a dynamic unit, with macrophages dying and being replaced by newly activated monocytes. This structure consists of a core of macropahges surrounded by lymphocytes. Granulomatous lesions appear to be initiated by nonspecific inflammatory signals arising from the interaction of macrophages with microbial products. These lesions are essential for controlling and limiting both tissue damage and bacterial dissemination (Saunders and Cooper, 2000). Tuberculosis can manifest in other parts of organs besides lung tissue, but it ordinarily presents as pulmonary infection, ranging from mild penetration to chronic, and severe pathologic condition. The different manifestrations of infection with Mtb reflect the balance between the bacilli and host defense mechanisms.

Control of tuberculosis requires mounting of effective T-cell responses and the recruitment of inflammatory cells as shown in Figure 2.1. These recruited cells surround and contain infected macrophages and limit spread of the bacilli. The acquired immune response to Mtb infection can be divided into three overlapping phases; (1) initiation and development of specific cellular immunity (2) expression of protective immunity in the lung (3) maintenance of protective immunity (Saunders and Cooper, 2000; Jacobs *et al.*, 2007).

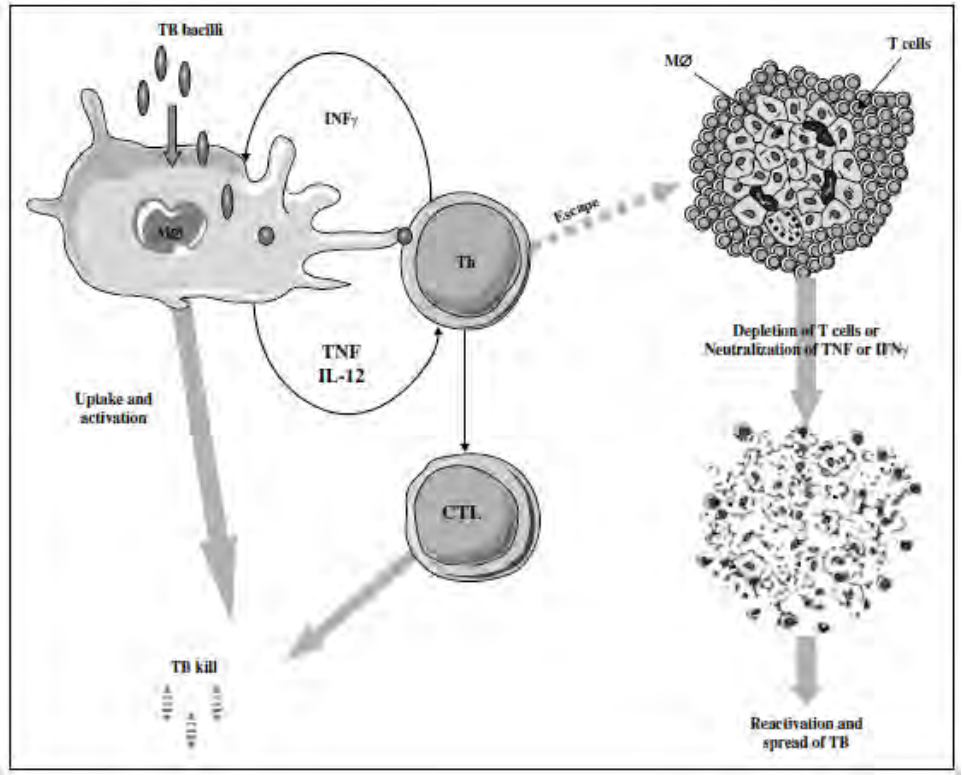

Figure 2.1 Macrophage and T cell activation, killing of Mtb and granuloma formation. Macrophages are activated by Mtb to produce cytokines leading to T cell activation. Activation of cells induces lymphocyte recruitment by chemokines, resulting in the formation of granulomas that contain the bacilli. Antibody neutralization of TNF or IFN-γ, or T cell depletion result in dissolution of the granuloma structure, rescuing of surviving bacilli with dissemination of infection (modified from Jacobs *et al.*, 2007).

2.1.3 Mtb-purified protein derivative (PPD)

PPD is prepared from autolysed cultures of Mtb taken after approximately 6 weeks of culture. PPD is used for intradermally injection (Mantoux test or prick test) or into the skin (Heal test) and for a positive delayed hypersensitivity reaction test, that demonstrates previous exposure of Mtb infection or BCG immunization (Lachmann, 1988). Mycobacterial PPD contains undefined molecules that act as ligands to stimulate innate immune responses. In addition, PPD can induce multiple cytokine responses such as IFN-γ, TNF-α and IL-1β, proinflammatory cytokines, or IL-10, antiinflammatory cytokine that regulate the adaptive

immune response (Weir *et al.*, 2004). Recently report suggested that TLR2 is an essential role for tuberculin PPD-induced macrophage to MAPK activation and proinflammatory cytokine expression (Yang *et al.*, 2008).

2.1.4 *Mycobacterium bovis* Bacille Calmette Guérin (*M. bovis* BCG)

M. bovis BCG is a live attenuated strain of *M. bovis*, a virulent tubercle bacillus very closely related to Mtb, and was originally derived from cattle with tuberculous mastitis (Imaeda *et al.*, 1985; Frothingham *et al.*, 1994). In an effort to control the threat of tuberculosis, attenuated BCG has been used as a live attenuated vaccine. Lagranderie reported that mice immunized orally or intragastrically with high doses of *M. bovis* BCG showed similar levels of protective immunity to mice immunized via the subcutaneous route and induced protection against intravenous challenge with Mtb (Lagranderie *et al.*, 2000).

BCG as a current TB vaccine is partially successful in protecting against TB. In neonates, BCG vaccination appears to reduce the possibility of severe early childhood TB, whereas efficacy against adult pulmonary TB is inconsistent (Barker *et al.*, 2009).

2.2 Macrophage apoptosis during mycobacterial infection

2.2.1 The apoptotic components and the pathways

Apoptosis, a genetically controlled cell death program, is important for the development and cell homeostasis in multicellular organisms. The characteristics of the apoptotic cell include chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebing, and cell shrinkage, and cell death without lysis or damage to neighboring cells (Kerr, 1972). The vital components for apoptotic pathway are caspases, a group of cysteien proteases. Activation of caspases and subsequent apoptosis can be triggered by at least two distrinct pathways. First, the extrinsic pathway is triggered by ligation and oligodimerization of tumor necrotic receptor (TNFR) family cell surface receptors such as

TNFR1 and Fas by their related ligands tumor necrosis factor (TNF)-α and Fas ligand (FasL) (Chen *et al.*, 2002). When death signal is induced via TNFR, it induces activation of the procaspase-8, the initiator caspase, that triggers a cascade of downstream caspases by proteolytic cleavage of their precursor and terminates the activation of effector caspases such as caspase-3, 6 and 7, resulting in apoptosis of cells (Taylor *et al.*, 2008).

The second pathway called an intrinsic pathway is induced by intracellular stresses, including DNA damages, nutrient deprivation and oxidative stress. These stimuli promote mitochondrial outer membrane permeability, permitting cytochrome *c* release. In cytosol, cytochrome *c* is associated with procaspase-9 and apoptosis protease and apoptosis protease activating factor-1 (Apaf1) to form a signaling complex called the proteasome (Riedl and Salvesen, 2007). Activated caspase-9 promotes the downstream activation of effector caspases and initiation of apoptosis.

Mitochondrial permeability is controlled by the integration of pro-apoptotic and anti-apoptotic actions of the Bcl-2 protein family (Xu and Shi, 2007; Youle and Strasser, 2008). Pro-apoptotic Bax and Bak form pores in the mitochondrial outer membrane, permitting cytochrome *c* release. The activation of pro-apoptotic proteins were inhibited by anti-apoptotic protein such as Bcl-2, Bcl-$_{XL}$ and Mcl-1. An upstream pro-apoptotic Bcl-2 family protein called Bid is activated by enzymatic cleavage to form truncated protein (tBid), which orchestrates the activities of Bax and Bak to promote cytochorme *c* release. Cleavage of Bid can be mediated by many proteases and is a characteristic of most intrinsic pathway. The cross-talk between the extrinsic and intrinsic pathway is mediated by caspase-8 cleavage which results in activation of Bid as shown in Figure 2.2.

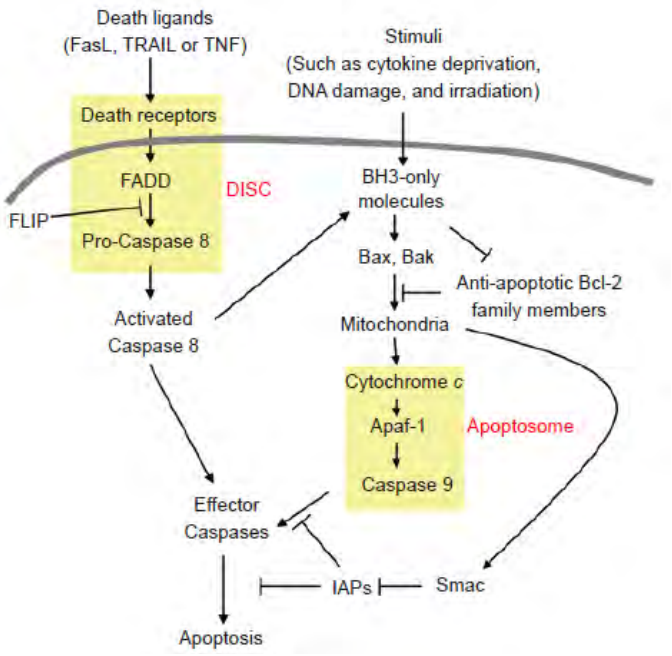

Figure 2.2 Apoptosis pathways. On the left, the extrinsic pathway is shown, and on the right the intrinsic pathway is shown. These pathways converge at the activation of caspases. The cross-talk between the extrinsic and intrinsic pathway is permitted by caspase-8 cleavage and this results in activation of BH3-only molecules such as Bid (modified from Xu and Shi, 2007)

2.2.2 Apoptosis and intracellular pathogens

Macrophage apoptosis is a common host defense strategy against intracellular pathogens, including viruses, protozoa and bacteria (Spira *et al.*, 2003). Apoptosis of macrophages plays critical roles in modulating the pathogenesis of a variety of infectious diseases. In contrast, apoptosis of macrophages is associated with disease promotion, including the elimination of critical host defense cells, invasion of epithelial barriers and spreading of infection by the delivery of pathogens to naïve host phagocytes engulfing apoptotic bodies. Intracellular bacterial pathogens induce and/or block apoptosis, which benefit their survival. Induction of host macrophage apoptosis eliminates a potential site for bacterial replication (Fairbairn, 2004). Virulent Mtb strains inhibit macrophage apoptosis by various mechanisms such as interfering with TNF-α signaling and by upregulating the

expression of *mcl-1*, an anti-apoptotic gene, which enhances intracellular bacterial replication (Balcewicz-Sablinska *et al.*, 1998; Sly *et al.*, 2003; Spira *et al.*, 2003).

2.2.3 Benefits of macrophage apoptosis to the host

Macrophages undergoing apoptosis kill intracellular mycobacteria, whereas those undergoing necrosis do not. This has been demonstrated using a number of apoptotic stimuli such as Fas ligand, TNF-α, ATP and the mycobacterial 19 kDa lipoprotein (Oddo *et al.*, 1998). Apoptosis of macrophages is postulated as a host strategy to prevent the progression of infection by causing death of the intracellular bacteria (Molloy *et al.*, 1994). The induction of apoptosis in infected monocytes/macrophages limits pathogen growth and retains bacilli in apoptotic bodies (Monack *et al.*, 1996; Ojcius *et al.*, 1998). Macrophages undergoing apoptosis play an important role in stimulation of the adaptive immune response by these presentation of bacterial antigen by dendritic cells to $CD8^+$ T cell (Fairbairn, 2004). In tuberculosis, macrophage apoptosis occurs within the granuloma that appears to benefit host immunity (Fairbairn, 2004). Winau *et al.* (2004) suggested that intracellular bacteria infected-macrophage apoptosis was a beneficial gateway to promote protective immunity (Winau *et al.*, 2004). Upon mycobacterial infection, antigen presenting cells (APC) including macrophage and dendritic cells were apoptosis and release apoptotic bodies. Apoptotic vesicles represent an autonomous immunological body when they carry the antigen and presenting molecule to T cell for effective T cell activation (Schaible *et al.*, 2003; Winau *et al.*, 2006).

2.3 Myeloid cell leukemia 1 (Mcl-1)

2.3.1 Discovery and structure

Mcl-1 is an anti-apoptotic member of the Bcl-2 protein family. The family members are identified by having conserved regions termed Bcl-2 homology (BH) domain at one or more regions (Mark F van Delft, 2006). *Mcl-1* was first identified as an early gene expressed

in human myeloid leukemia cell line ML-1 when treatment with phorbol 12-myristate 13-acetate (PMA) (Kozopas *et al.*, 1993).

Mcl-1 is highly expressed in hematopoietic stem cells while the expression level decreased in differentiated progenitor populations, including the myeloid and the lymphoid progenitors (Opferman *et al.*, 2005). In early monocyte differentiation, Mcl-1 is expressed and it helps sustain cell viability in immature myeloid cells (Zhou *et al.*, 1998). Mcl-1 is expressed in multiple cell lineages and essential for their survival and development. For example, Mcl-1 is important for the development and maintenance of B and T lymphocytes. During early lymphocyte differentiation, Mcl-1 plays a pivotal role in pro-B-cell and double-negative T-cell development (Opferman *et al.*, 2003). Mcl-1 is also essential for macrophage survival, which is mediated through STAT3 and Akt-1 pathway (Liu *et al.*, 2003). Hikita *et al.* reported that in adult liver, Mcl-1 is important for inhibition of cell apoptosis and essential for normal liver development (Hikita *et al.*, 2009).

Mcl-1 contains three putative BH domains which mediate protein-protein interactions among members of Bcl-2 family which is important for apoptosis regulation (Michels *et al.*, 2005). The C-terminal region of Mcl-1 contains transmembrane (TM) domain that promotes the localization to various intracellular membranes, most especially the outer mitochondrial membrane (Yang *et al.*, 1995). The N-terminal region of Mcl-1 contains two PEST sequences, weak and strong PEST, which is rich in proline, glutamic acid, serine and threonine. PEST sequence is possibly responsible for the short half-life of this protein, and as shown in Figure 2.3 (Wang *et al.*, 1999; Bingle *et al.*, 2000; Michels *et al.*, 2005). At the N-terminus of Mcl-1, it contains a large numbers of modifiable amino acid residues, and therefore appears to function as a regulatory region (Thomas *et al.*, 2010).

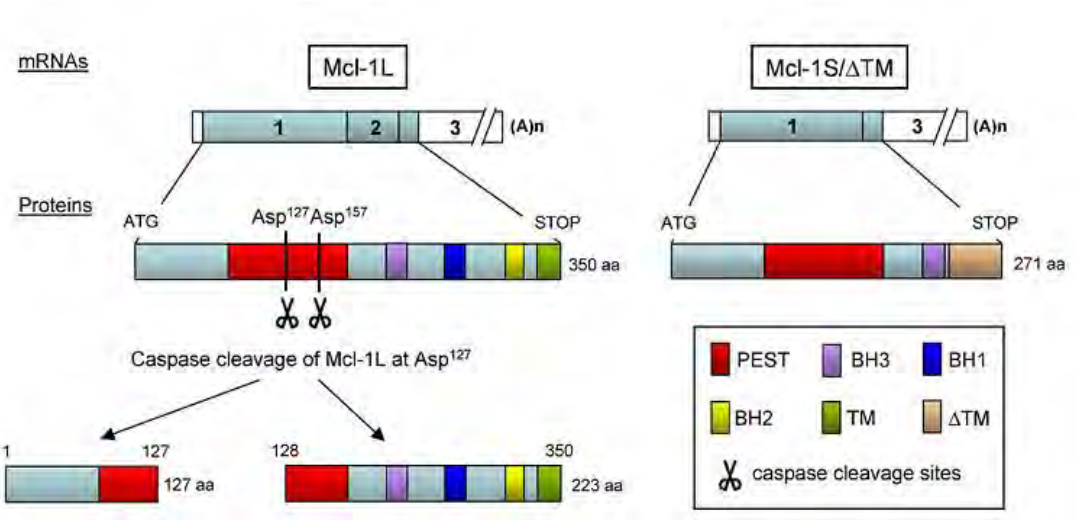

Figure 2.3 Transcription and translation of Mcl-1. A schematic representation showed the alternative splicing which gives rise to two distinct mRNAs. These 2 mRNAs encode Mcl-1L and Mcl-1S isoforms. The PEST, BH and TM domains are indicated, along with the caspase cleavage sites. The product of Mcl-1 that is cleaved by caspase at Asp^{127} residue is shown (modified from Michels *et al.*, 2005).

2.3.2 Expression and regulation of Mcl-1

Mcl-1 expression is a growth-factor dependent in various situations. Expression of *mcl-1* was regulated at transcriptional, post-transcriptional and post-translational levels. At the transcriptional level, *mcl-1* is induced by numerous extra-cellular stimuli, including interleukins (IL-3, IL-6, IL-15), growth factors (VEGF, EGF), colony stimulating factors (G-CSF, GM-CSF, SCF), IFN, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator (Le Gouill *et al.*, 2004). The extracellular stimuli trigger Mcl-1 expression via several cytoplasmic signaling pathways such as JAK/STAT, MAPK, PI3K/AKT and MEK/ERK pathways (Wang *et al.*, 1999; Bingle *et al.*, 2000; Liu *et al.*, 2003; Le Gouill *et al.*, 2004). The promoter region of Mcl-1 contains a putative transcription factor binding sites to upregulate Mcl-1 expression such as cAMP response element binding protein (CREB), PU.1, STAT3, SP1 and HIF-1 α (Townsend *et al.*, 1999; Wang *et al.*, 1999; Epling-Burnette *et al.*,

2001; Wang *et al.*, 2003; Liu *et al.*, 2006). Mcl-1 expression is downregulated by binding of the E2F-1 transcription factor to its promoter (Croxton *et al.*, 2002).

At the post-transcriptional level, *mcl-1* mRNA undergoes alternative splicing which gives rise to two distinct *mcl-1* mRNAs encoding the Mcl-1L and Mcl-1S isoforms, which contains or lacks exon 2, respectively (Bingle *et al.*, 2000; Akgul, 2009). Mcl-1S, a shorter form, lacks BH domains 1, 2 and the TM domain. The two splicing forms, Mcl-1L and Mcl-1S, form heterodimers and are capable of neutralizing each other. Another shorter form called Mcl-1ES, a splicing variant, has also been identified which lacks a portion of exon 1, removing 53 amino acids from the PEST region, but retains all three BH domains and the TM domain (Kim *et al.*, 2009).

At the post-translational level, Mcl-1 is controlled by caspase cleavage, phosphorylation or ubiquitination. Human Mcl-1 has two caspase cleavage sites locating at Asp^{127} and Asp^{157}, while mouse Mcl-1 has one caspase cleavage site locating at Asp^{108}. The N terminal region of Mcl-1 contains a large numbers of modifiable amino acid residues. In ML-1 leukemia cell, TPA activates Erk signaling and thus triggers Mcl-1 phosphorylation at Thr^{163}. This single Thr^{163} phosphorylation slows Mcl-1 turnover and promotes cell survival (Clohessy *et al.*, 2004). Thomas *et al*. reported that phosphorylation at Thr^{163} contributes to the stabilization and destabilization of Mcl-1, depending on the activity of other kinases. Phosphorylation Mcl-1 by GSK-3 at multiple residues modulates rapid degradation of Mcl-1. Erk-1 phosphorylates Mcl-1 at Thr^{163} residue and promotes Mcl-1 stabilization. Phosphorylation by JNK appears to be required at Thr^{163} for destabilizing phosphorylation of Ser^{159} and Ser^{155} by GSK-3 (Figure 2.4) (Thomas *et al.*, 2010).

The Mcl-1 protein level decreases quickly in response to signals that trigger cell death. Mcl-1 is degraded at an early stage of apoptosis. The degradation of Mcl-1 was first identified in HeLa, a cervical cancer cell line, exposed to UV irradiation via proteasome

pathway (Nijhawan *et al.*, 2003). The lysine residues are the targets for ubiquitination. Zhong *et al.* discovered a novel E3 ubiquitin-ligase responsible for the constitutive polyubiquitination of Mcl-1 and this is called Mcl-1 Ubiquitin Ligase E3 or MULE (Zhong *et al.*, 2005). Gelinas found that in healthy cells, Bak BH3 domain binds with high affinity to Mcl-1 in an inactive protein complex. Accordingly, Noxa, a pro-apoptotic BH3-only protein, replaces Bak from Mcl-1 and promotes degradation of Mcl-1 by the proteasome-dependent pathway (Gelinas and White, 2005).

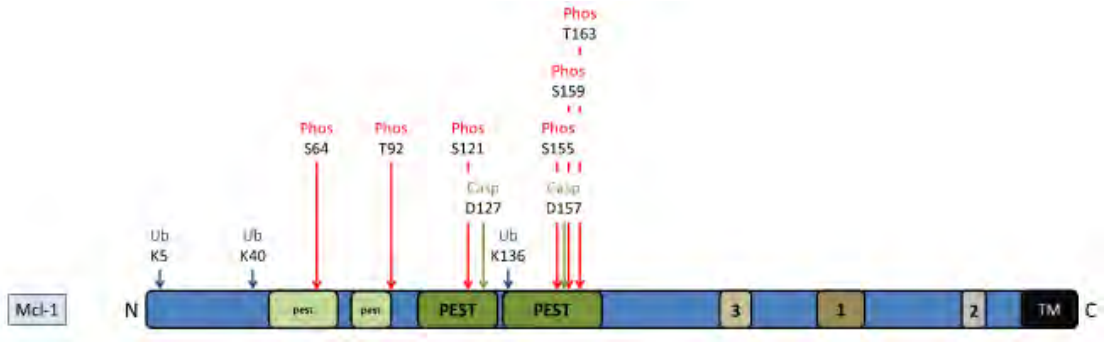

Figure 2.4 The molecular regulation of Mcl-1 protein. A schematic representation demonstrated the Mcl-1 protein with the functional regions and sites of post-translational modification. These include the transmembrane domain (TM), the Bcl-2 homology domains (numbered 1-3), two weak (lower case) and two strong (upper case) PEST sequences. Sites of post-translational modification are also shown, including ubiquitination (Ub), caspase cleavage (Jurisicova *et al.*), and phosphorylation (Phos) (modified from Thomas *et al.*, 2010).

2.3.3 Function of Mcl-1

Mcl-1 is widely expressed but has its own specific tissue distribution (Krajewski *et al.*, 1995; Yang *et al.*, 1995) along with its own specific physiological roles. Mcl-1 promotes cell viability during phenotypic transitions such as stimulation of proliferation or differentiation, responses to stress, or regulation of mature cell lifespan (Vrana *et al.*, 2006). Mcl-1 is essential for the survival of hematopoietic stem cell (Akala and Clarke, 2006). Mcl-1 plays a critical role in regulation of macrophage and neutrophil apoptosis (Edwards *et al.*, 2004; Steimer *et al.*, 2009). Reynolds *et al*. reported that Mcl-1 over-expression increased the

survival of CHO cells (Reynolds *et al.*, 1996). Oishi *et al*. reported that Bcl-2 and Mcl-1 promote neural precursor cell survival (Oishi *et al.*, 2004). Dysfunction of Mcl-1 regulation, expression, or degradation is studied in various diseases (Le Gouill *et al.*, 2004). For example, over-expression of Mcl-1 increased cell viability over the long term and therefore open the window for immortalization and additional genetic changes which lead to tumorigenesis (Akgul, 2009).

Mcl-1 promotes cell survival by suppression of cytochrome *c* release from mitochondria, probably via heterodimerisation and neutralization of pro-apoptotic Bcl-2 family protein (Michels *et al.*, 2005). Mcl-1 blocks the progression of apoptosis by binding and sequestering the pro-apoptotic proteins Bak and Bax, which are capable of forming pores in the mitochondrial membrane, allowing the release of cytochrome *c* into the cytoplasm to induce apoptosis (Thomas *et al.*, 2010).

Mcl-1 actively counteracts activity of pro-apoptotic BH3-only proteins in both intrinsic and extrinsic signaling pathways of apoptosis (Figure 2.5). Mcl-1 functions as an early critical negative regulator of apoptosis by directly interacting with BH3-only proteins as well as Bim, tBid, Noxa and Puma. This interaction inhibits the induction of cytochrome *c* release and activation of the mitochondrial apoptotic pathway. In addition, Mcl-1 is also able to interact with Bak and suppress its apoptotic activity. Therefore, Mcl-1 functions at multiple steps in the apoptotic pathways (Zhuang and Brady, 2006).

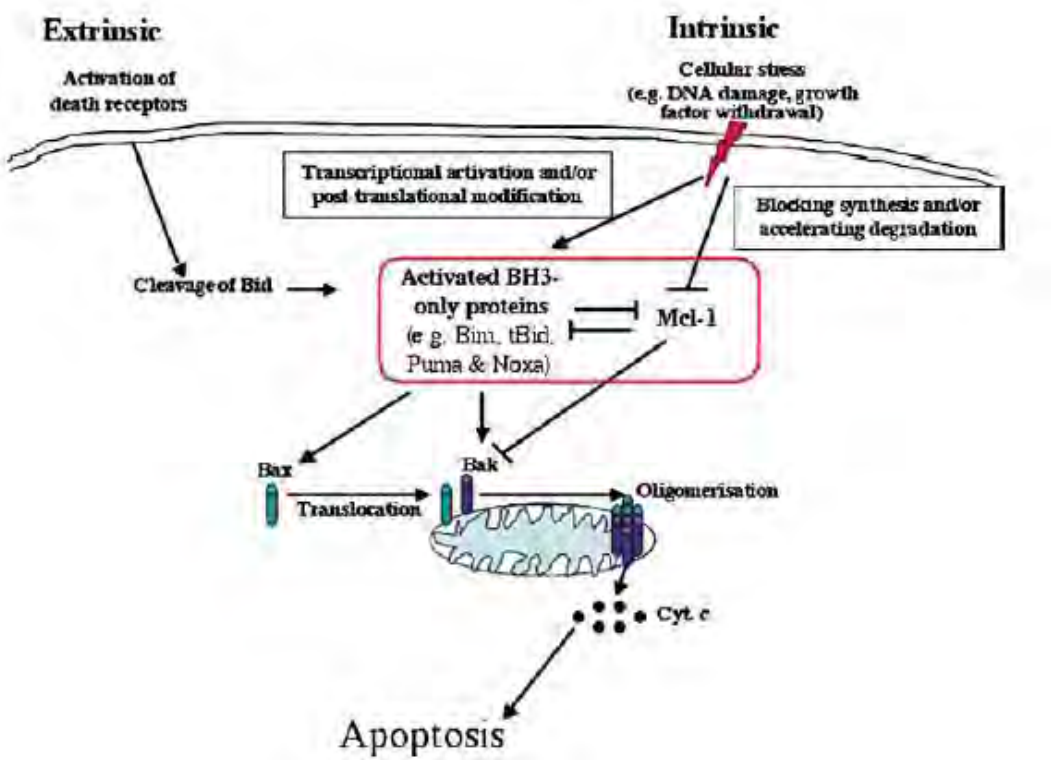

Figure 2.5 A schematic representation demonstrating Mcl-1 functions during apoptosis. Mcl-1 actively counteracts BH3-only proteins in both intrinsic and extrinsic signaling pathways of apoptosis (Zhuang and Brady, 2006)**.**

2.3.4 The role of Mcl-1 in infected cells

Recent studies reported that Mcl-1 has important role in many bacterial and viral-infected cells. As an example for bacterial infection, human neutrophils infected with *Trichomonas vaginalis* suppressed Mcl-1 expression and induced caspase-3 activation, thereby inducing neutrophil apoptosis (Kang *et al.*, 2006). Marriott *et al.* reported that during pneumococcal infection, survival of macrophages was regulated by Mcl-1 which promoted macrophage transition from resistance to susceptibility to apoptosis (Marriott *et al.*, 2005). Cappon *et al.* demonstrated that the gastric mucosa of *H. pylori*-infected neutrophils increased Mcl-1 protein expression which accumulated at 24 hr and remained high at 72 hr after infection (Cappon *et al.*, 2010). For *Chlamydia trachomatis* infection, Mcl-1 upregulation is primarily required to delay infected cell apoptosis (Rajalingam *et al.*, 2008).

Generally, apoptosis in viral-infected cells is control by the Bcl-2 protein family. When cells response to viral infection, Mcl-1 is upregulated resulting in delayed apoptosis. For example, in EBV-infected B lymphocytes, Mcl-l upregulation was induced by LMP1, an Epstein Barr Virus transforming protein, which may help to maintain Mcl-l expression and to delay cell death under conditions not promising to cell survival (Wang *et al.*, 1996). Recent study reported that the primary lung epithelial cells infected with RSV also showed strong induction of Mcl-1, which may account for the delayed RSV-infected cell apoptosis (Kotelkin *et al.*, 2003). In monocytes, cells infected with CMV upregulated Mcl-1 and Bcl-2 expression that play an essential role in the early and late phases of survival of CMV-infected monocytes (Chan *et al.*, 2010). Furthermore, over-expression of Mcl-1 protects against HCV core protein induced Huh7 cells apoptosis (Mohd-Ismail *et al.*, 2009).

2.3.5 Mcl-1 in mycobacterial infection

In mycobacterial infection, macrophages delay apoptosis by upregulated Mcl-1 and A1, an anti-apoptotic protein. This promotes mycobacterial survival and chronic intracellular persistence (Kausalya *et al.*, 2001; Sly *et al.*, 2003; Marriott and Dockrell, 2007). Recent study demonstrated that THP-1, a human monocytic cell line, infected with K-strain, an isolated clinical strain of Mtb in Korea, significantly upregulated anti-apoptotic genes such as *Bcl-2*, *Mcl-1, Bfl-1* and *Bcl-xL* expression (Sohn *et al.*, 2009).

2.4 Notch signaling pathway

2.4.1 Notch receptors and Notch ligands

Notch receptors and ligands are cell surface proteins expressed in various cell types. Notch signaling is essential for cell fate decisions in development of embryos and adults. It appears to function in mediating communication between cells. Notch signaling is highly conserved throughout the vertebrate and invertebrate species (Artavanis-Tsakonas *et al.*, 1999). The structures of Notch receptors and ligands are shown in Figure 2.6. Notch encodes

conserved transmembrane receptors in mammals which have four receptors (Notch1-Notch4). The extracellular domain of Notch (ECN) contains multiple EGF like repeats essential for ligand binding and three cysteine-rich Notch/LIN12 repeats (LIN) that prevent ligand-independent activation of the signaling pathway. The intracellular domain of Notch (ICN) contains several functional domains, which mediate transcriptional activity of Notch signaling. These include the membrane-proximal RBP-j-associated molecule (RAM) domain and ankyrin repeats (ANK) that interact with downstream effector proteins, two nuclear localization signal (NLS) motifs, and a C-terminal proline-glutamate serine-threonine-rich (PEST) domain which regulates protein stability.

The mammalian Notch receptors are activated by five ligands; Jagged1, Jaged2, Delta like1, Delta like3 and Delta like4. Notch ligands are transmembrane proteins with EGF-like repeats and a unique N-terminal DSL domain (for Delta, Serrate and *C. elegans* homolog Lag2) and cysteine-rich region (CR) found in only Jagged 1 and 2 in the extracellular domain. The intracellular domains of the ligands are small (75-210 residues) and not highly conserved, but they are also important for signal initiation (Allman *et al.*, 2002).

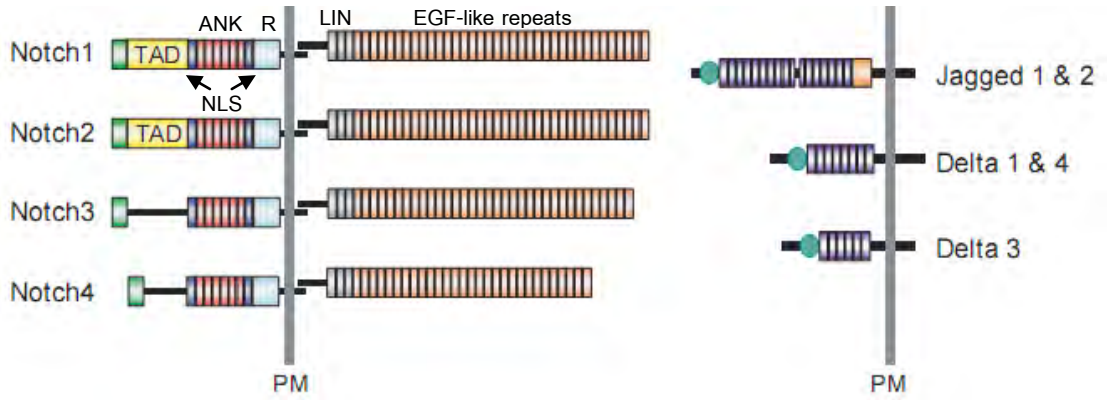

Figure 2.6 A schematic representation of protein structure of Notch receptors and ligands. For Notch receptor, the ECN of Notch 1-2 contain of 36 EGF-like repeats and 3 LIN12/Notch repeats. Notch 3-4 have 34 and 29 EGF-like repeats, respectively. ICN includes a RAM domain, 6 ANK repeats, 2 NLS and PEST domain. Notch ligands are consisted of DSL, EGF and CR (found in Jagged 1 and 2). PM indicates a plasma membrane (modified from Radtke *et al.*, 2004).

2.4.2 Activation of Notch signaling pathway

Notch signaling is initiated through ligand-receptor interaction that promotes two proteolytic cleavages of the receptor (Figure 2.7). The first cleavage is mediated by ADAM-family metalloproteases or TACE, and the second is mediated by γ-secretase, an enzyme complex containing at least presenilin, nicastrin, PEN2 and APH1 (Mumm and Kopan, 2000; Fortini, 2002; Selkoe and Kopan, 2003; Bray, 2006). Proteolytic cleavage releases the intracellular domain of Notch (ICN) which translocates to the nucleus. ICN co-operates with a DNA binding protein called CSL (CBF-1 for humans, Suppressor of hairless for *Drosophila* and Lag-1 for *C. elegans*; also known as RBP-J) and co-activator Mastermind like protein (MAML) to promote transcription of target genes. Target genes of Notch signaling pathway include basic helix-loop-helix transcription factors of the Hairy enhancer of split (Hes) family

such as *Hes1* and *Hes5* , *Deltex1* as well as *Hey1*, *Hey2* and *HeyL* (subfamily of *Hes*) (Deftos *et al.*, 2000; Davis and Turner, 2001; Iso *et al.*, 2003).

CSL is essential effector molecule of the Notch signaling pathway. It is a highly conserved protein, exhibiting 69% and 72% identity between *Drosophila* and mouse and *Drosophila* and human protein, respectively (Pursglove and Mackay, 2005). In the absence of Notch signaling, CSL represses the transcription of Notch target genes by binding to a consensus DNA motif at the promoter region of Notch-inducible genes and interacting with the co-repressor complex (Zhou and Hayward, 2001). CSL recognizes consensus sequences of (C/T)(A/G)TG(A/G/T)GA (A/G/T) in the promoter of target genes (Lai, 2002). When Notch has been activated through ligand-receptor interaction, leading to ICN translocates to the nucleus, ICN binds to CSL protein and recruits co-activator including MAML to form an transcriptional activation complex and induces target genes expression.

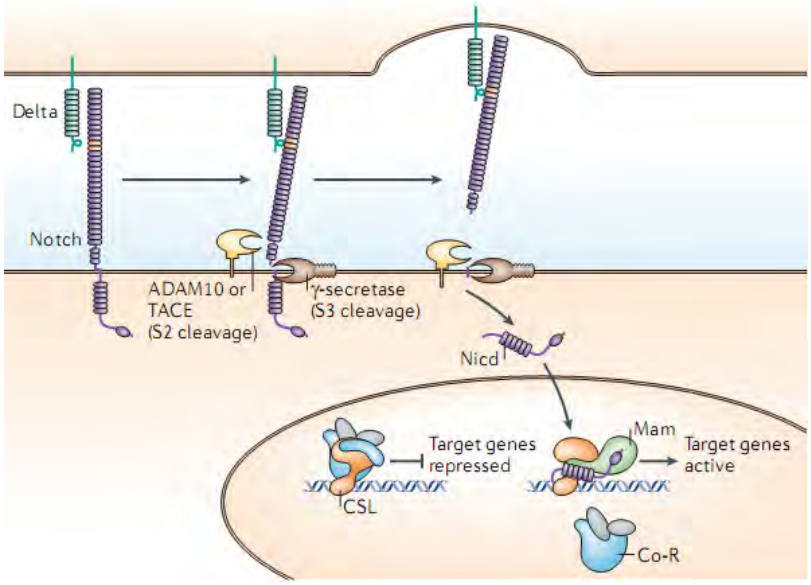

Figure 2.7 Notch signaling pathway. Notch ligand binds to the receptor, resulting in two proteolytic cleavages of the receptor. This proteolytic processing mediates the release of ICN and its translocation to the nuclease. ICN interacts with the DNA binding protein, CSL. The co-activator MAML and other transcription factor are recruited to the CSL complex, whereas co-repressors are release (modified from Bray, 2006).

2.4.3 Biological function of Notch signaling pathway

Notch signaling pathway plays an important role in the regulating of many biological functions such as proliferation, stem cell maintenance, differentiation during embryonic and adult development and apoptosis (Bray, 1998; Greenwald, 1998; Artavanis-Tsakonas *et al.*, 1999). For example, Notch signaling pathway suppresses differentiation of intestinal crypt progenitor cells in the gut (Fre *et al.*, 2005). In neuronal differentiation, activation of Notch signaling leads to suppression of neurogenesis and promotes survival of neural precursors by upregulating anti-apoptotic proteins Bcl-2 and Mcl-1 (Oishi *et al.*, 2004).

Notch signaling maintains stem cell numbers by regulating their self renewal and differentiation (Spradling *et al.*, 2008). Notch-mediated cellular interactions are essential for determining the numbers and lineages of hematopoietic precursor cells. In hematopoiesis, Notch signaling plays an important role in regulating cell fate decisions at various stages of hematopoietic development. Notch signaling inhibits differentiation of hematopoietic stem cells leading to increased self renewal (Suzuki and Chiba, 2005). Duncan *et al*. reported that the activation of Notch signaling in adult hematopoietic stem cells is reduced in differentiated cells (Duncan *et al.*, 2005).

2.4.4 Notch signaling in myeloid lineages

Recent study reported that Notch signaling via CSL clearly correlates with myeloid differentiation (Schroeder and Just, 2000). In monocytes, Notch signaling modulates cell fate decisions, e.g. monocytes incubating with GM-CSF and Delta-1 were suppressed from differentiation into macrophages. These cells underwent an early stage of dendritic cell differentiation. On the other hand, in the presence of GM-CSF and IL-4, monocytes were promoted to dendritic cells with activation of Notch signaling (Ohishi *et al.*, 2001). During erythroid development, Notch signaling regulates erythroid homeostasis by inducing apoptosis (Robert-Moreno *et al.*, 2007). In macrophages, Monsalve *et al*. reported that Notch signaling is involved in the modulation of macrophage function (Monsalve *et al.*, 2006). For

example, macrophages receive appropriate Notch-activating signals promoted their Ag-presenting capability and migrated to lymphoid organs to activate T cells (Monsalve *et al.*, 2006). In inflammatory diseases such as atherosclerosis, Notch signaling pathway plays an important role in macrophages, a key cell type in inflammation. When macrophage stimulated with LPS, Dll4, Notch ligand, was increase and bind to neighboring macrophages and trigger Notch signaling pathway to induce inflammation through activation of the MAPK, Akt, and NF-κB pathways (Fung *et al.*, 2007). Palaga *et al.* (2008) have recently demonstrated that stimulation of macrophages through the TLR signaling cascade triggered activation of Notch signaling pathway. This trigger is activation of NF-κB and turn regulated pro-inflammatory gene expression (Palaga *et al.*, 2008). In cancer, Notch signaling pathway plays critical roles in M1 versus M2 polarization of macrophages through SOCS3. M1 macrophages produce IL-12 to promote tumoricidal responses, whereas M2 macrophages produce IL10 and help tumor progression (Wang *et al.*, 2010). In osteogenesis, Notch signaling pathway might be regulation the balance between osteoclasts and osteoblasts in the bone. Osteoclast are derived from hematopoietic precursor cells belonging to the monocyte/macrophage lineage, Notch signaling pathway reduced the expression of c-Fms on the cell surface and inhibited terminal differentiation into osteoclasts but in osteoblast differentiation Notch signaling pathway was positively regulator (Yamada *et al.*, 2003).

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

Gamma-secretase, a large protease complex locating on the plasma membrane, is composed of a catalytic subunit (presenilin (ps) 1 or 2) and accessory subunit (presenilin enhancer-2 (pen-2), anterior pharynx-defective phenotype-1 (aph-1) and nicastrin. Presenilin is proposed to be the catalytic subunit containing separate binding and catalytic sites (Tian *et al.*, 2003).

Multiple substrates, such as amyloid β-protein, have been identified as endogenous substrate of γ-secretase (Seubert *et al.*, 1993). The amyloid β-protein (Aβ) deposited in brain tissue from patient of Alzheimer's disease (AD) is derived from the amyloid β-protein precursor (APP) by two proteolytic cleavages. An initial β-secretase cleavage at the N-terminus of Aβ sequence is followed by γ-secretase cleavage at the C-terminus of Aβ (McLendon *et al.*, 2000).

Inhibitors specific for γ-secretase have been developed for blocking the generation of Aβ peptide for Alzheimer's disease therapy. Because ICN is also cleaved by γ-secretase during Notch signaling activation, GSI can be used to suppress Notch receptor activation. Small peptide based (peptidomimetic) inhibitors were the first reported inhibitors of γ-secretase activity (Rochette and Murphy, 2002). Peptide aldehyde, such as z-IL-CHO (IC50 ~ 10 μM (McLendon *et al.*, 2000) was used in recent studies for inhibiting Notch signaling pathway. Blockade of Notch signaling using IL-CHO resulted in inhibition of TCR-induced Notch1 expression in splenocytes, and IL-CHO completely blocked CD4 and CD8 T cell proliferation (Palaga *et al.*, 2003). In addition, IL-CHO also blocked Notch1 expression in activated bone marrow macrophages. In the presence of IL-CHO, NO production was suppressed, but MHC class II was enhanced in activated bone marrow macrophages (Palaga *et al.*, 2008).

Treatment with dipeptide GSI N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-5-phenylglycine t-butyl ester (DAPT) resulted in inhibition in proliferation of Jurkat, a human acute T cell leukemia cell line and HepG2, a hepatocellular carcinoma cell line but did not affect apoptosis in both cell lines. By contrast, when ICN1 was over-expressed in both cell lines, GSI did not affect proliferation of both cell lines. Moreover, decreased expression of *Hes1* and increased expression of Notch1 in both cell lines were detected after treatment with DAPT for 4 days (Suwanjunee *et al.*, 2008).

2.4.6 Notch signaling in mycobacterial infection

Recent study demonstrated that macrophages infected with *M. bovis* BCG upregulated Notch1 expression and Notch-activated signal mediated induction of SOCS3, a critical negative regulator of cytokine signaling, for the suppression of a generalized inflammatory response and the persistence of mycobacteria within the host (Narayana and Balaji, 2008). Our unpublished data and findings from other groups suggested that Notch signaling is activated in macrophages during infection with *M. bovis* BCG (Narayana and Balaji, 2008) (Palaga *et al.*, unpublished data). In addition, Ito *et al.* reported that mycobacterial infection induce dll4, Notch ligand, expression on dendritic cell through TLR9 and plays an important role in promoting Th17 effector activity during a mycobacterial infection. Furthermore this activation is regulating the mycobacterial antigen–elicited granuloma formation in mice (Ito *et al.*, 2009).

CHAPTER III

MATERIALS AND METHODS

3.1 Cell line, primary cells and media

RAW264.7 cell line (ATCC TIB-71) and bone marrow derived macrophages (BMMϕ) from female C57BL/6 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). RAW264.7 and BMMϕ were maintained in DMEM (Hyclone, England) with supplement of 10% FBS (v/v) (Hyclone, UK), 100 U/ml penicillin (General Drugs House, Thailand), 0.4 mg/ml streptomycin (M & H Manufacturing, 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

RAW264.7 cell line was removed from non-treated culture dish (Hycon, Germany) using 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/6 mice as described previously (Palaga *et al.*, 2008). In brief, 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. Fresh media were added every 3 days and cells were harvested using cold PBS. Cell suspension was centrifuged at 1000 rpm for 5 min. Culture

supernatant was discarded and cells were resuspended in DMEM complete media. Viable cells were assessed by trypan blue dye exclusion method using a hemacytometer. The cell number was calculated according to the following formula;

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

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

3.2.2 Cell preservation for storage

After centrifugation, RAW264.7 cell line were resuspended in 1 ml of ice cold freezing media composed of 10% DMSO (v/v) (Sigma Aldrich, USA) in complete DMEM, and stored in cryogenic vial (Corning Incorporation, USA). Cells were immediately stored at -80°C for at least overnight. For long term storage, cells were stored in Liquid Nitrogen Tank model 34 HC Taylor Wharton Cryogenic (Harsco Corporation, USA). For BMMϕ, cells were resuspended in 500 µl of DMEM media supplemented with 20% FBS and 500 µl of DMEM media supplemented with 20% FBS and 20% DMSO. Cells were stored in cryogenic vial and kept at -80°C.

3.2.3 Thawing cells for use

Stored RAW264.7 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 complete DMEM was added. Cells were plated in non-tissue culture treated plate and cultured until use for experiments. For frozen BMMϕ, after thawing and centrifugation, culture supernatants were removed and 8 ml of DMEM supplemented with 20% L929-conditioned media and 5% horse serum. Cells were seeded on non tissue culture treated plates for at least 2 days before use.

3.3 Bacteria

Mycobacterium bovis Bacille Calmette Guérin (*M. bovis* BCG) (Copenhagen strain) were grown to log phase in Middlebrook 7H9 broth (Difco, USA) supplemented with 10% albumin-dextrose-catalase (OADC) (Difco, USA) and 0.05% Tween 80 (Research Organics, USA). To storage *M. bovis* BCG, bacteria were frozen in M7H9 broth supplemented with 15% glycerol at -80^{o}C. Representative samples were thawed and the CFUs per ml were counted by plating on Middlebrook 7H11 supplemented with10% OADC and 0.5% glycerol and incubating at 37^{o}C for 21 days.

3.4 Infection of macrophage with *M. bovis* BCG

Macrophages were seeded in 12-well flat-bottom tissue culture plates and allowed to adhere at 37^{o}C in a humidified, 5% CO_2 atmosphere for 18 hr. Bacteria were centrifuged at 9000 rpm for 10 min at 4^{o}C. Culture supernatant was discarded and bacteria were resuspended in DMEM complete media without antibiotics. To eliminate clumping, the *M. bovis* BCG was passed through a 25-gauge needle 10 times. Numbers of cells were counter in hemacytometer using the following formula;

$$\text{Bacteria number (cell/ml)} = \text{number of counted cell in 16-large squares} \times 2 \times 10^4$$

The infection was carried out at a multiplicity of infection (MOI) of 10:1 (10 bacilli to 1 cell) for 4hr, subsequently extracellular bacteria were removed by washing with phosphate buffered saline (PBS). Cells were incubated in complete media supplemented with 100 U/ml penicillin and 0.4 mg/ml streptomycin.

3.5 Western blot

3.5.1 Protein extraction and quantitation

RAW264.7 (1.5×10^5cells/ml in 0.5 ml culture) or BMMϕ ($3x10^5$cells/ml in 1ml culture) was plated on 24 or 12 wells tissue culture plate for overnight respectively. The next day cells were stimulated with 5µg/ml PPD (kindly provided by Dr. Thipjuta Bhantong, Queen Saovabha Memorial Institute) or *M. bovis* BCG MOI 10 for indicated time. Cells stimulated as indicated were subjected to cell lysis and proteins were extracted as described by Palaga *et al*. (2003). Briefly, culture supernatant was carefully removed from the plate, and cells were washed with 1 ml of cold PBS and 250 µl of cold Buffer A (Appendix). Afterward 30-40 µl of cold Buffer B (Appendix) was added 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. Supernatants 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. 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 with 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, plate was measurement at 540 nm (A_{540} nm) by using microplate reader Elx 800 (Bio-Tek instrument, Canada).

3.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Cell lysates were diluted by an equal volume of protein sample and 2×Laemmli buffer (Appendix) 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).

Boiled samples and prestained molecular weight markers (Fermentas, Canada) were loaded and separated under reducing conditions on 8% or 12% SDS-PAGE gels. The samples were separated at 100 volt for 100-150 min in Western blot running buffer (Appendix) using Protein III system (BioRad, USA).

After separation, the stacking gel was removed. The gels were equilibrated in transfer buffer (Appendix) for 5 min. PVDF membrane (GE Healthcare, USA) and 6 pieces of Whatman filter paper were prepared. PVDF membranes were soaked in absolute methanol (Merck, Germany) and rinsed twice with deionized water, afterward immersed in transfer buffer. Gels, PVDF membrane and filter papers were placed in a semi-dry transfer Trans-Blot® SD (BioRad, USA) apparatus. The proteins were transferred at 80 mAmp for 90 min.

3.5.3 Antibody probing

After proteins transferred onto polyvinylidene fluoride (PVDF) membranes (PALL), membrane was blocked twice at room temperature for 5 min with 3% non-fat skim milk in PBST each on Labnet Rocker 25 (Labnet International Inc, USA). Subsequently blots were reacted with the primary antibody for overnight at 4^{o}C. Blots were probe with rabbit anti-Notch1 Ab (Santa Cruz Biotechnology, USA) at 1:2000 dilution, rabbit anti-cleaved Notch1 Ab (Val 1774, Cell Signaling Technology; Boston, MA) at 1:1000 dilution , rabbit anti-Mcl-1 Ab (Santa Cruz Biotechnology, USA) at 1:1000 dilution or anti-β-actin mAb (Chemicon International, USA) at 1:5000 dilution. The primary antibody solution was removed, and the membrane was washed with PBST (Appendix) 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.

3.5.4 Signal detection by chemiluminescence and autoradiography

The substrates were prepared using the formula shown in Appendix. In brief, mixed solution A and solution B. PBST was removed and the mixture of solution A and B was poured directly on the membranes and incubated for 1 min by rocking. The membranes were wrapped in the plastic wrap and placed in Hypercassette (Amersham Biosciences, UK) to expose to High Performance Chemilumunescence Film (Amersham Biosciences, UK) in the dark. Exposure time for Notch1, cleaved Notch1, Mcl-1 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.

3.6 RNA extraction

BMMϕ ($3x10^5$cells/ml in 1ml culture) were plated in 12-well tissue culture plate overnight. Cells were stimulated with 5μg/ml PPD or *M. bovis* BCG MOI 10 for indicated time the next day. At indicated time, culture supernatants were removed and 1 ml of TriZol reagent (Invitrogen, UK) was added directly to cells. The mixture was incubated for 5 min at room temperature. TriZol reagents containing RNA were transferred to 1.5 ml microcentrifuge tubes and added with 0.2 ml of chloroform (Lab-Scan, Ireland). All tubes were vigorously mixed by hands for 15 sec 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 new tubes. For RNA precipitation, the aqueous phase was added with 0.5 ml of isopropanol (Merck, Germany) and gently mixing. The samples were incubated at room temperature for 10 min and centrifuged at 12000xg for 10 min at 4°C. The supernatants were removed and the RNA pellets were washed once with 1 ml of ice cold 75% ethanol (Appendix) in 0.01% DEPC water (Appendix). 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 air dried for

10 min, and resuspended in 15 µl of 0.01% DEPC water. After resuspension, RNA was incubated for 10 min at 60°C. RNA samples were kept at –80°C until use for further experiments.

3.6.1 Quantitation of RNA using Quant iT Assays (Invitrogen, UK)

Measurement total of RNA by using Quant-iT were performed according to the manufacturer's instruction. In brief, Quant-iT reagent and Quant-iT buffer (Invitrogen, UK) were prepared to working solution. For standard RNA, 10 µl of standard RNAs composing of 0ng/µl of RNA and 10ng/µl of RNA were mixed with 190 µl of working solution. RNA samples were diluted to 10-fold dilution in Hypure® water PCR grade (Hyclone, England). Two µl of diluted RNA was added into 198 µl of working dilution. Calibrations of standard RNA were performed by Quant-iT and concentrations of RNA samples were measured. The concentrations of RNA were calculated in µg/ml by using the following formula;

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

3.7 cDNA synthesis by reverse transcriptase

To generate cDNA, 0.5 µg of total RNA was used. 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 chilled on ice for 5 min. After that, 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 at 200 U per reaction, and the reaction was carried out in Bioer Life Express (Bioer technology, China) at 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min. The cDNA was stored at -20°C until use.

3.8 Polymerase chain reaction (PCR)

The components of PCR reactions were as follows; 1xTag buffer (Fermentus, Canada), 0.2mM dNTP mix (Fermentus, Canada), 1.5mM $MgCl_2$ (Fermentus, Canada) ,0.2μM forward and reverse primers, 0.1U of *Taq* polymerase and Hypure® water PCR grade adjusted to volume 18 μl per reaction. Two μl of DNAs were used as templates to amplify, mouse *mcl-1* promoter. The forward and reverse primers used for mouse *mcl-1* promoter are as follows; forward 5'-CCGGGCTGAGAGTTGTACC-3' reverse 5'-CGGAAGTCAGGAGT GAGGAA-3'. The PCR reactions were carried out using Bioer Life Express® by condition as follows; 94°C 5 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and 72°C for 10 min. PCR were amplified for 32-40 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 products were detected after staining with ethidium bromide using Gel Documentation and Quantity one 4.4.1 (BioRad, USA).

3.9 Semi-Quantitative Polymerase chain reaction (qPCR)

The qPCR amplification was performed with 1x Maxima™ SYBR Green/ROX qPCR Master Mix, with 0.3μM forward and reverse primer, RNase Free water and 2 μl obtained cDNAs according to the manufacturer's (Fermentus, Canada). The forward and reverse primers used for qPCR amplification are as follows: murine *mcl-1* (forward; 5'-GACCGGCTCCAAGGAC TC-3', reverse 5'-TGTCCAGTTTCCGGAGCAT-3') and β-actin (5'-ACCAACTGGGACGACATGGAGAA-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 in 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 58°C (murine *mcl-1)* for 30 sec, 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 $2^{-\Delta\Delta CT}$.

3.10 siRNA transfections

To generate Notch1 silencing RAW264.7 cell line, DNA fragments corresponding to nucleotide 1529-1538 of murine Notch1 gene and a hairpin loop sequence (5'-GATCCG GTGTATACTGTGAAATCAGTGTGCTGTCCTGATTTAACAGTATACACCTTTTTTA-3') were subcloned into pBAsi plasmid under mouse U6 promoter to drive expression of small hairpin RNA (Takara Bio, Japan) (Pattarakankul, 2009). The plasmids were transfected into RAW264.7 cell line using FuGeneHD transfection reagent according to the manufacturer's (Roche Molecular Biochemicals, Indianapolis, IN). Plasmid pBAsi with luciferase insert was used as vector control in all experiments (Palaga *et al.*, 2008). Protein lysates were subjected to Western blot using monoclonal anti-Notch1, and polyclonal anti-Mcl-1 antibodies.

3.11 Transformation of *E. coli* for 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 and added 1 µl of plasmid into competent cells. 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 chilled on ice for 2 min. After the heat shock process, cells were transferred to 1 ml of LB broth (Appendix) and incubated at 37°C for 1 hr by shaking at 200 rpm. Fifty to one hundred µl of LB broth containing the cells was plated on LB agar (Appendix) plates containing 50µg/ml of ampicillin. The plate was incubated at 37°C for 16-24 hr and the colony was picked for plasmid preparation.

3.12 Plasmid isolation

A few colonies were picked up and cultured for 6-8 hr at 37°C in 2 ml LB broth containing 50µg/ml of ampicillin. After that 100-200 µl of culture were inoculated in 100 ml LB broth containing 50µg/ml of ampicillin for 16-18 hr. Bacteria culture was centrifuged at 13,000 rpm for 15min at 4°C. The supernatant was discarded and the plasmid was extracted

using QIA prep spin maxiprep kit (Qiagen, Germany) according to the manufacturer's instruction. Obtained plasmid was eluted in 300 µl sterile water and stored at -20°C until use.

3.13 Transient transfection using FuGene® HD transfection reagent

RAW264.7 cell line (1.5×10^5cells/ml) in 0.5 ml culture media was plated overnight in 24-well tissue culture treated plates. To form the transfection complex, FuGene® HD transfection reagent was used. The transfection complex was prepared according to the manufacturer's instructions. Briefly, FuGene® HD transfection reagent was brought to room temperature prior to use for 15 min. Plasmids were diluted by using serum free media Opti-MEM (Invitrogen, UK). 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. The plate was swirled and incubated for 36 hr prior to PPD stimulation. After this incubation, cell lysates was analyzed by Western blot for expression of Notch1 and Mcl-1 protein.

3.14 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, USA), macrophages were pretreated with 25µM of IL-CHO or vehicle control DMSO for 1 hr before stimulated with PPD or *M. bovis* BCG infection. After incubation, cell lysates were analyzed by Western blot for expression of Notch1 and Mcl-1 protein. 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 murine *mcl-1* and *β-actin* as listed above and the relative expression of murine *mcl-1* were calculated using $2^{-\Delta\Delta CT}$.

3.15 Detection of apoptotic macrophages infected with *M. bovis* BCG

Analysis of macrophage apoptosis was performed using Cell Death Detection ELISA (Roche, Mannheim, Germany) according to the manufacturer's instructions and as previously described (Means *et al.*, 2001). Macrophages were plated at a density of 4×10^4cells/well in 96-well plates. Cells were infected with *M. bovis* BCG, in the absence or present of 25μM IL-CHO. Four hours later the medium was removed and replaced with fresh medium. After 5 days of infection, the supernatant was removed and the adherent macrophages were resuspended in 200 μl of lysis buffer, and the lysate was centrifuged at 20000 × g for 10 min. These culture supernatant contain mono- and oligonucleosomes released by apoptotic cells and an Ag-capture ELISA were used to measure the level of histone-associated DNA fragments present in the cell lysate. Sample values were subtracted with the background value (blank). The specific enrichment of mono- and oligonucleosomes released into cytoplasm was calculated from these values using from the following formula;

$$\text{Enrichment factor} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the negative control}}$$

3.16 Chromatin Immunoprecipitation (ChIP) assay

BMMϕ ($1x10^7$cells/plate) was activated by 5μg/ml PPD for 24 hr. To determine the promoter binding site, EZ Magna ChIP™ Chromatin Immunoprecipitation Kit (Upstate, USA) was used according to the manufacture's protocol. Briefly, 37% formaldehyde was used to cross link proteins and DNA molecules located in close proximity at the final concentration of 1% and 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 1x protease inhibitor cocktails II. Lysed cells (500 μl) were sonicated using UP 50 H Ultrasonic Processor (GmbH, Germany) at 100% amplitude, 1 cycle, sonication period for 10 second and rest period for 20 second for 20 times to shear chromatins. Sonicated cells were centrifuges at 12,000×g at 4°C for 10 min to eliminate

insoluble materials. Afterward 50 µl of sheared chromatin was moved to new 1.5 microcentrifuge tube. In each tube, added with 450µl of dilution buffer containing 1x protease inhibitor cocktails II. After this step, 2µg of anti-Notch1 antibody (Santa Cruz Biotechnology, USA) or 1µg of Normal Rabbit IgG (compare) or 1µg of Normal Mouse IgG (compare) or 1µg of RNA Poll II (Chemicon International, USA) was added. Next 20 µl protein G-conjugated magnetic beads was added into the mixture and incubated at 4°C with Mini–rotator Bio RS-24 (Biosan, Latvia) for overnight. 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. To reverse cross linking of protein/DNA complex, adding 100 µl of ChIP Elution buffer containing 1 µl of proteinase K, and incubated at 62°C for 2 hr with shaking. After that the mixture was incubated at 95°C for 10 min and left to cool down at room temperature. The supernatants were collected and subjected to purification using spin column. The region with potential CSL binding site in the mouse *mcl-1* promoter was detected by PCR and Real-time PCR.

3.17 Determination of CFUs from infected macrophages

Enumeration of CFUs were performed as described previously (Sly *et al.*, 2003). Bacilli were plated immediately after 4 hr of co-incubation with cells and washing (time 0) and at 2, 3 and 7 days after infection. Macrophage was lysed using 100 µl of 0.1% Triton X-100 in cold PBS and incubated for 5 min. Bacteria were serially diluted in Middlebrook 7H9 with OADC, and 100 µl of three dilutions were plated on Middlebrook 7H11 with OADC in duplicate. CFUs were counted after 14 days of incubation at 37°C and plates were maintained for 21 days to ensure that no additional CFUs appeared.

3.18 Database search

NCBI sequence viewer v.2 was used to investigate nucleotide sequences of *mcl-1* promoter and EBI ClustalW2 was used to find the consensus sequence alignment of human and murine *mcl-1*. Mulan (Loots and Ovcharenko, 2007) was used to identify the potential transcription factor binding sites evolutionarily conserved in human and murine species.

3.19 Statistical analysis

To calculate the statistical differences between control and samples, Student's paired independent t-test was used (SPSS 15.0). Values of $p < 0.05$ were considered significant.

CHAPTER IV

RESULTS

4.1 Treatment with PPD or *M. bovis* BCG infection upregulated Notch1 and Mcl-1 expression in BMMϕ

4.1.1 Expression of Notch1 and Mcl-1 in BMMϕ upon PPD stimulation

To follow the kinetics of Mcl-1 and Notch1 expression in activated BMMϕ at the protein level, Western blot using antibody specific for Notch1 or Mcl-1 were performed. BMMϕ were stimulated with PPD at indicated times and cell lysates were analyzed. As shown in Figure 4.1a, Mcl-1 was detected at a low level in unstimulated cells, and the level of expression increased and peaked at 24 hr after treatment. Similarly, Notch1 expression was readily detected at a low level in unstimulated macrophage but the expression level increased as the duration of treatment extended (Figure 4.1b). Expression profiles of Mcl-1 and Notch1 correlated well in PPD-stimulated macrophage, suggesting that both proteins may be involved in response to stimulation with PPD in macrophage.

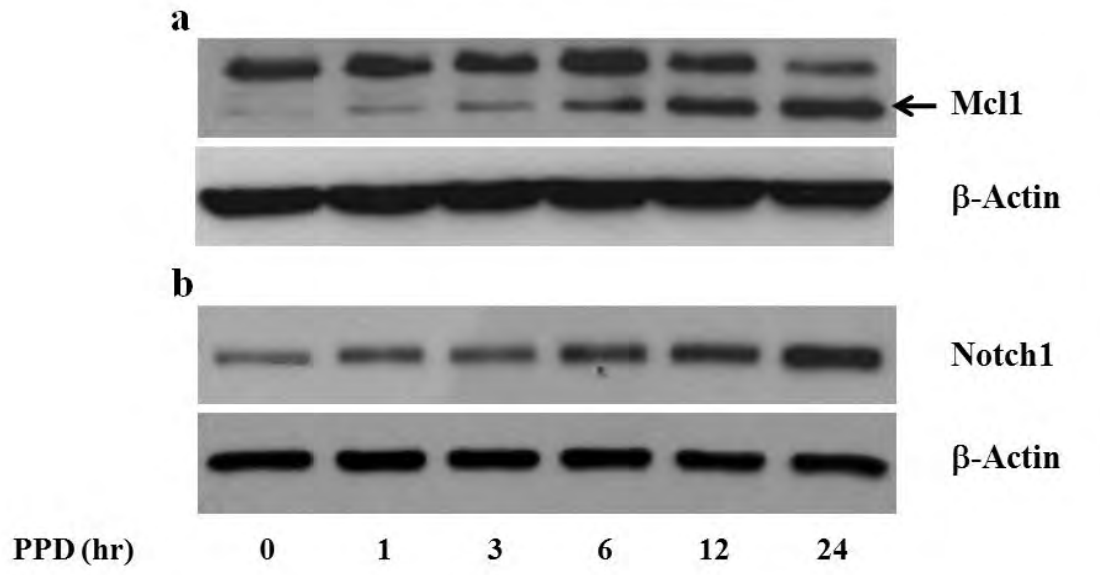

Figure 4.1 Treatment with PPD upregulated Mcl-1 and Notch1 protein expression in BMMϕ. BMMϕ were treated with 5μg/ml PPD at indicated times. Total cell lysates were prepared and analyzed for expression of Mcl-1 (a) or Notch1 (b) proteins by Western blot. β-actin was used as a loading control.

4.1.2 Upregulation of *mcl-1* in PPD-stimulated BMMϕ

To examine *mcl-1* mRNA expression in BMMϕ, semi-quantitative real-time RT-PCR was performed using specific primers for murine *mcl-1*. Expression of *mcl-1* mRNA was determined at 1, 6 and 24 hr after stimulation with PPD. Upregulation of *mcl-1* was detected as early as 1 hr upon treatment (Figure 4.2). This result indicated that PPD treatment upregulated *mcl-1* expression both at the transcriptional and translational level, but the level of protein and mRNA were not correlated, suggesting that post-transcriptional regulation may be involved.

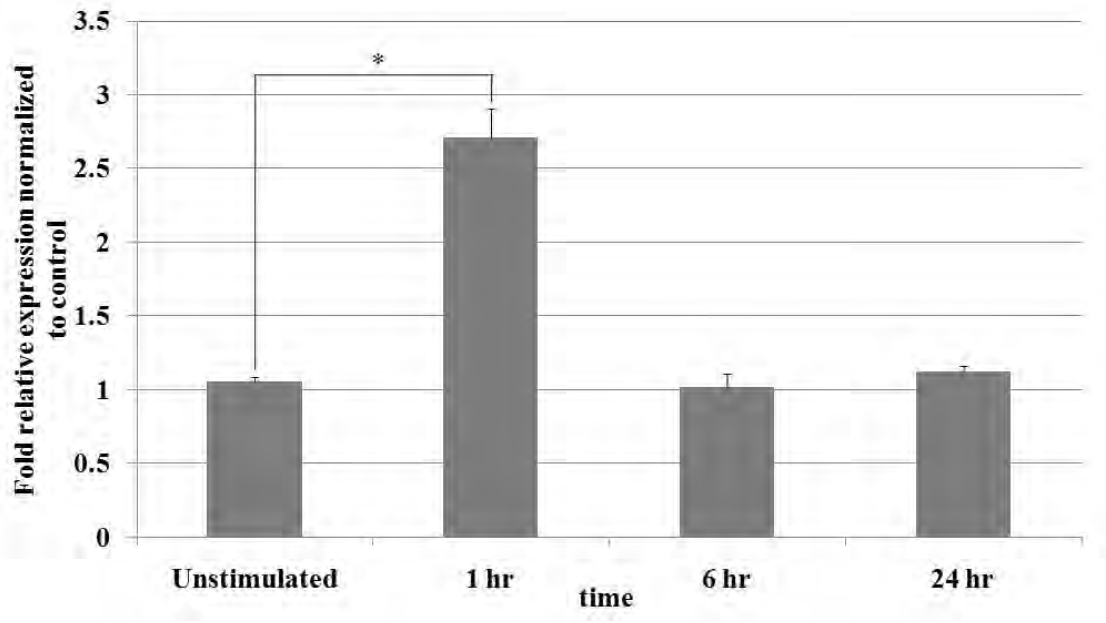

Figure 4.2 Treatment with PPD upregulated *mcl-1* mRNA in BMMϕ.

BMMϕ were treated with 5μg/ml PPD at indicated times. Total RNAs were analyzed for relative expression of *mcl-1* by semi quantitative real-time RT-PCR. β-actin was used as reference control**.** * indicated the statistic significance ($p < 0.05$) analyzed by student's t-test, as compared with unstimulated cells. The results represented two independent experiments, in triplicates.

4.1.3 Expression of Notch1 and Mcl-1 in BMMϕ infected with *M. bovis* BCG

To determine Notch1 and Mcl-1 expression in *M. bovis* BCG-infected BMMϕ, Western blot using specific antibodies against Notch1 and Mcl-1 were performed. BMMϕ were infected with *M. bovis* BCG for 4 hr (indicated as 0 hr post-infection), and extracellular bacteria were removed by washing. Infected cells were further incubated for indicated times. As shown in Figure 4.3a, BMMϕ infected with *M. bovis* BCG upregulated Mcl-1 expression at 0 hr and the level remained high until 24 hr after infection (Figure 4.3a). Similarly, Notch1 expression was readily detected at low level in unstimulated macrophage and the protein level increased all the durations of infection which peaked at 24 hr (Figure 4.3b). This result

indicated that infection with *M. bovis* BCG induced Notch1 and Mcl-1 in *M. bovis BCG*-infected BMMϕ.

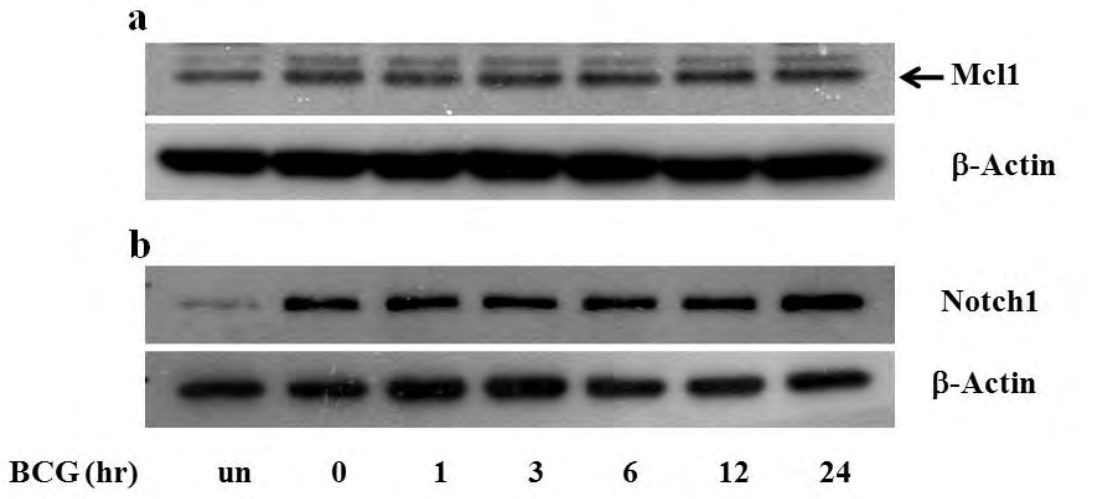

Figure 4.3 *M. bovis* BCG induced Mcl-1 and Notch1 in BMMϕ.

BMMϕ were infected with *M. bovis* BCG at an MOI of 10 for 4 hr. The extracellular bacteria were removed and infected cell were further incubated for indicated times. Cell lysates were analyzed for Mcl-1 (a) or Notch1 (b) by Western blot. β-actin was used as loading control.

4.1.4 Upregulation of *mcl-1* expression in BMMϕ upon *M. bovis* BCG infection

To examine the kinetics of *mcl-1* mRNA expression, semi-quantitative real-time RT-PCR was performed. *Mcl-1* mRNA was determined at 1, 6 and 24 hr after infection with *M. bovis* BCG. As shown in Figure 4.4, in BMMϕ infected with *M. bovis* BCG, *mcl-1* expression peaked at 6 hr after infection where the level dropped to basal level at 24 hr post infection. This result indicated that *mcl-1* expression was upregulated in *M. bovis* BCG infected-macrophages. In addition, the profile of mcl-1 expression in PPD treated and BCG infected macrophages were different, indicating that the signaling involved in responses to these stimuli may be differently regulated.

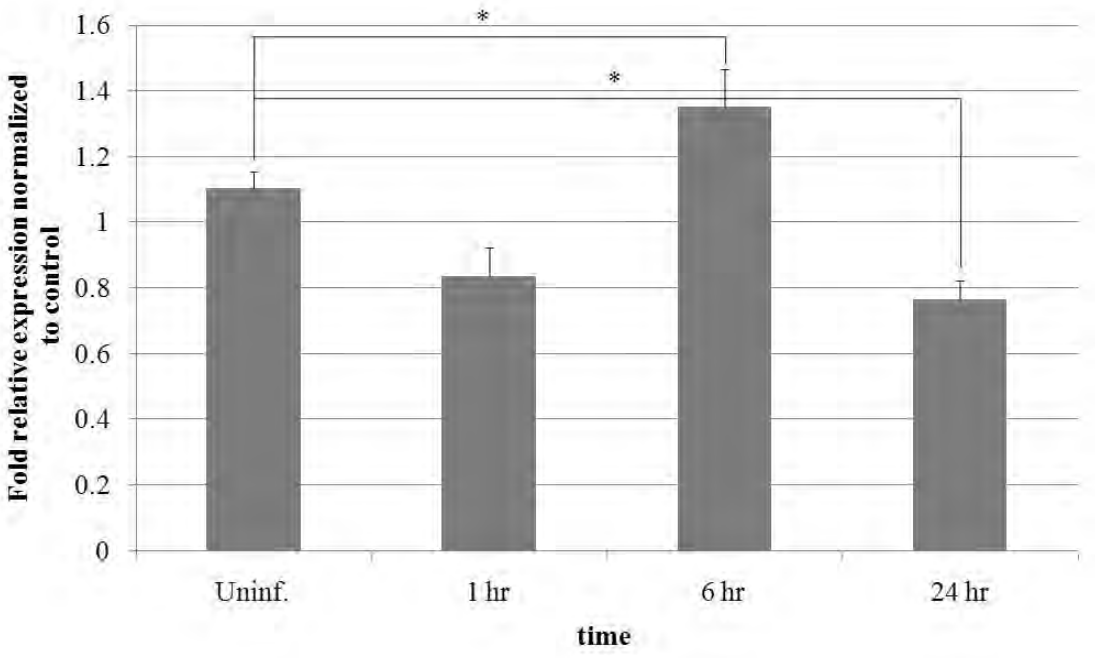

Figure 4.4 *M. bovis* BCG infection upregulated *mcl-1* expression in BMMϕ.

BMMϕ were infected with *M. bovis* BCG at an MOI of 10. Total RNA were isolated at indicated times and analyzed for *mcl-1* expression by semi quantitative real-time RT-PCR. β-actin was used as reference control**.** * indicated the statistic significance ($p < 0.05$) analyzed by student's t-test, as compared with unstimulated cells. The results represented two independent experiments, in triplicates.

4.2 Effect of γ-secretase inhibitor treatment on Notch1 and Mcl-1 expression in BMMϕ

4.2.1 Effects of GSI treatment on cell morphology of BMMϕ

To study the role of Notch signaling in macrophages, we used a pharmacological approach by using IL-CHO, a peptidomimetic inhibitor of γ-secretase (Palaga *et al.*, 2008). BMMϕ were pretreated with IL-CHO (25μM) or vehicle control (DMSO) before subjecting to PPD treatment or *M. bovis* BCG infection. As shown in Figure 4.5, DMSO treatment did not

affect morphology of cells but GSI treatment resulted in increasing in numbers of cells with round shapes.

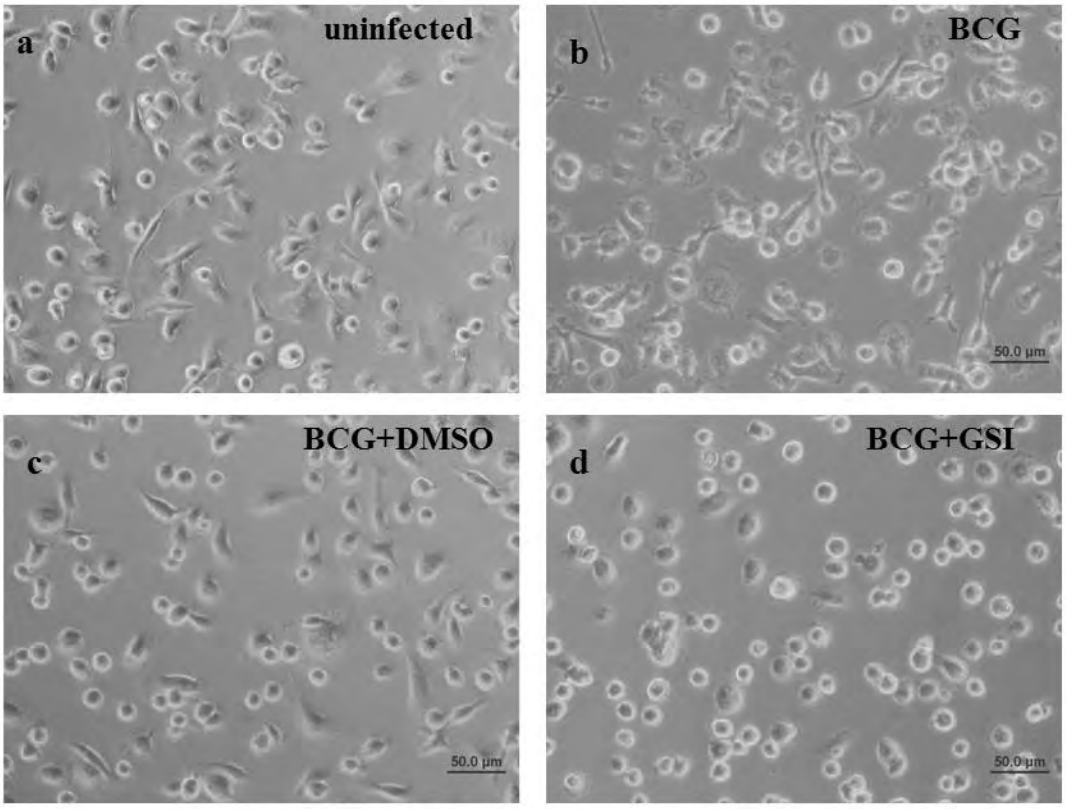

Figure 4.5 Effects of GSI treatment on morphology of BMMϕ.

BMMϕ were pretreated with 25µM IL-CHO or DMSO (vehicle control) for 1 hr and infected with *M. bovis* BCG at an MOI of 10 for 6 hr. The morphological changes of macrophages were observed under an inverted microscope. (a) Uninfected cells, (b) BMMϕ infected with *M. bovis* BCG for 6 hr, (c) BMMϕ pretreated with DMSO prior to *M. bovis* BCG infection and (d) BMMϕ pretreated with 25µM IL-CHO prior to *M. bovis* BCG infection.

4.2.2 Effect of GSI on Notch1 and Mcl-1 expression in PPD-activated BMMϕ

To study the effect of GSI treatment on Notch signaling in BMMϕ, cells were pretreated with 25μM IL-CHO or vehicle control DMSO for 1 hr prior to PPD stimulation for 24 hr. When pretreatment of BMMϕ with GSI, the level of cleaved Notch1, an active form of Notch1, was reduced in activated macrophages, compared to the mock treatment (Figure 4.6a). This result suggested that GSI treatment suppressed cleavage of Notch1. Furthermore, GSI treatment resulted in reducing level of total Notch1 expression at 24 hr after PPD stimulation (Figure 4.6b).

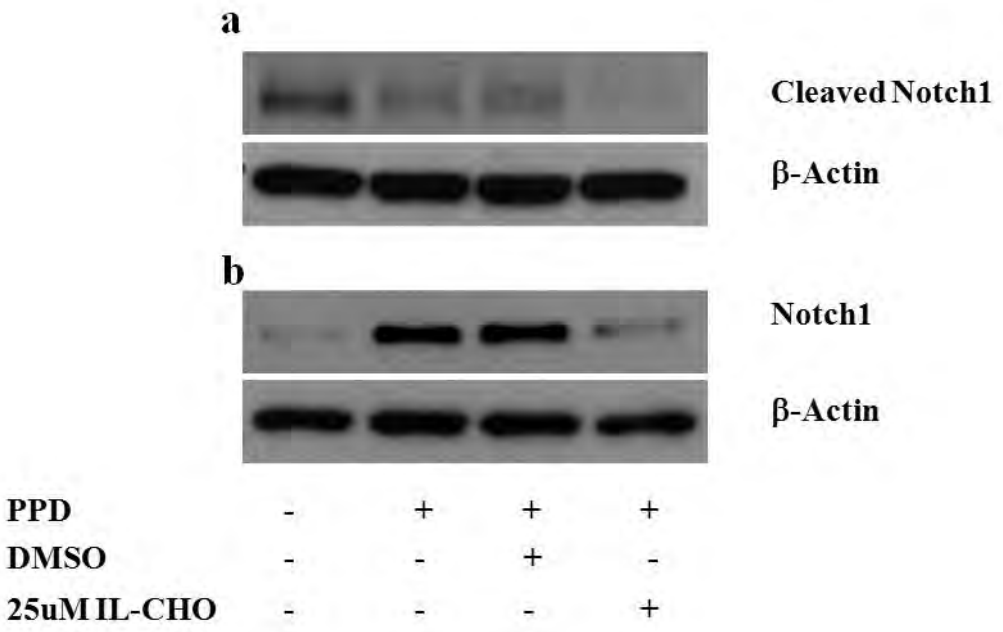

Figure 4.6 Effects of GSI treatment on cleaved Notch1 and Notch1 in BMMϕ.

BMMϕ were pretreated with IL-CHO or DMSO vehicle control for 1 hr prior to treatment with PPD for 24 hr. Cell lysates were analyzed for the presence of cleaved Notch 1 (a) and Notch1 (b) by Western blot. β-actin was used as loading control.

Upon PPD stimulation, Mcl-1 was increased at 24 hr after treatment as shown previously (Figure 4.1a). In contrast, GSI treatment resulted in complete elimination of Mcl-1 expression at 24 hr after PPD stimulation (Figure 4.7). Therefore, decreased Notch1 expression upon stimulation with PPD correlated well with decreased expression of Mcl-1. Hence, Notch signaling may affect Mcl-1 expression in macrophages.

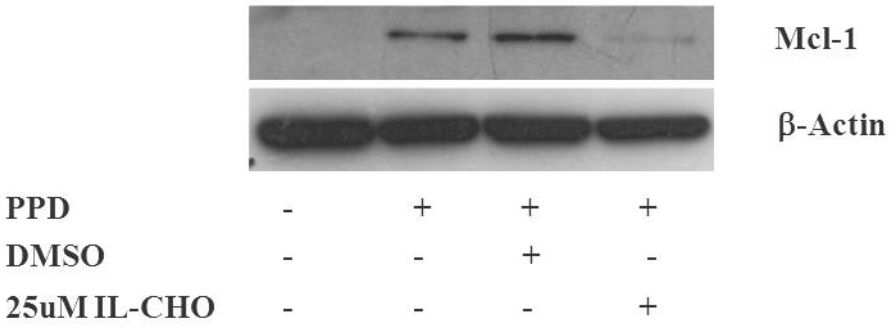

Figure 4.7 Effects of GSI treatment on Mcl-1.

BMMϕ were pretreated with IL-CHO or DMSO vehicle control for 1 hr prior to treatment with PPD for 24 hr. Cell lysates were analyzed for expression of Mcl-1 by Western blot. β-actin was used as loading control.

4.2.3 Effect of GSI on *mcl-1* mRNA expression in BMMϕ stimulated with PPD

To investigate the effect of GSI on *mcl-1* mRNA expression, BMMϕ were pretreated with 25μM IL-CHO or vehicle control DMSO for 1 hr prior to PPD stimulation for 1 hr and *mcl-1* mRNA was measured by real time RT-PCR. Upon PPD stimulation, *mcl-1* expression was upregulated as early as 1 hr after treatment as shown previously (Figure 4.2) but GSI treatment resulted in significant reduction in *mcl-1* expression (Figure 4.8). This result suggested that GSI had a negative effect on *mcl-1* expression at both transcriptional and translational levels.

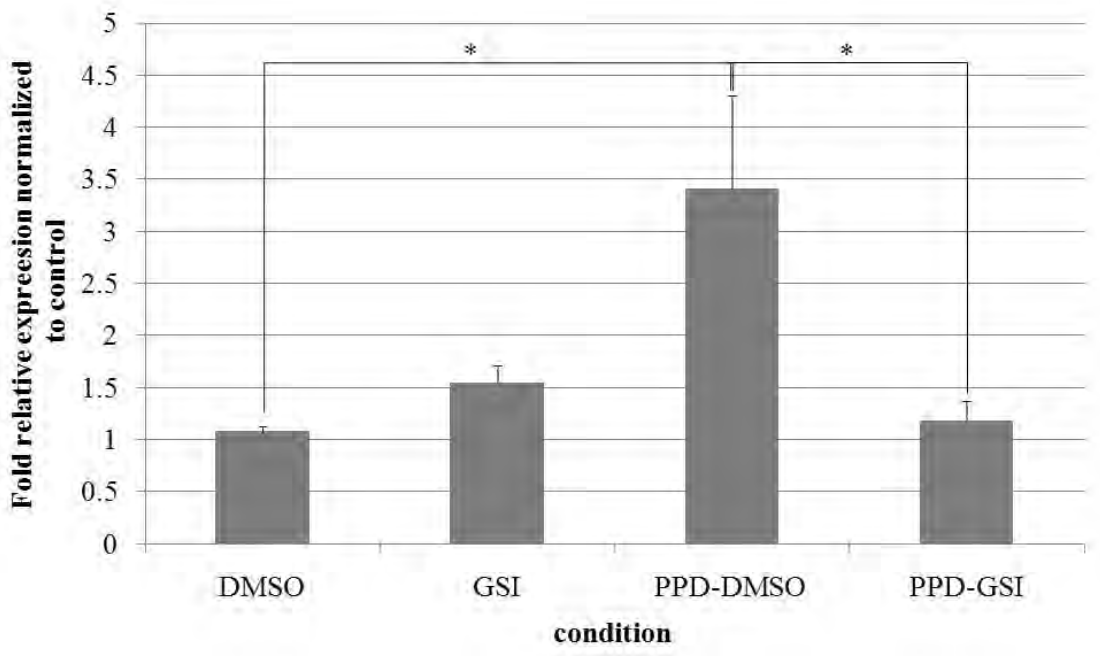

Figure 4.8 Effects of GSI treatment on *mcl-1* mRNA expression.

BMMϕ pretreated with IL-CHO or DMSO vehicle control for 1 hr, and treated with PPD for 1 hr. Expression of *mcl-1* was analyzed by semi quantitative real-time RT-PCR. β-actin was used as reference control. * indicated the statistic significance ($p < 0.05$) analyzed by student's t-test, as compared with unstimulated cells. The results represented two independent experiments, in triplicates.

4.2.4 Effect of γ-secretase inhibitor on Notch1 and Mcl-1 expression in *M. bovis* BCG-infected BMMϕ

To study the effect of GSI treatment on Mcl-1 and Notch1 expression in infected macrophages, BMMϕ were pretreated with 25μM IL-CHO or vehicle control DMSO for 1 hr prior to *M. bovis* BCG infection. Expression of Mcl-1 was detected at 24 hr (Figure 4.9a). In contrast *M. bovis* BCG infection resulted in Mcl-1 expressed at 24 hr (Figure 4.3a). Macrophages treated with GSI exhibited decreased Notch1 protein expression (Figure 4.9b).Therefore decreased Notch1 expression upon stimulation is associated with decreased

expression of Mcl-1. Thus, Notch signaling may involve in regulating Mcl-1 expression in *M. bovis* BCG-infected macrophages.

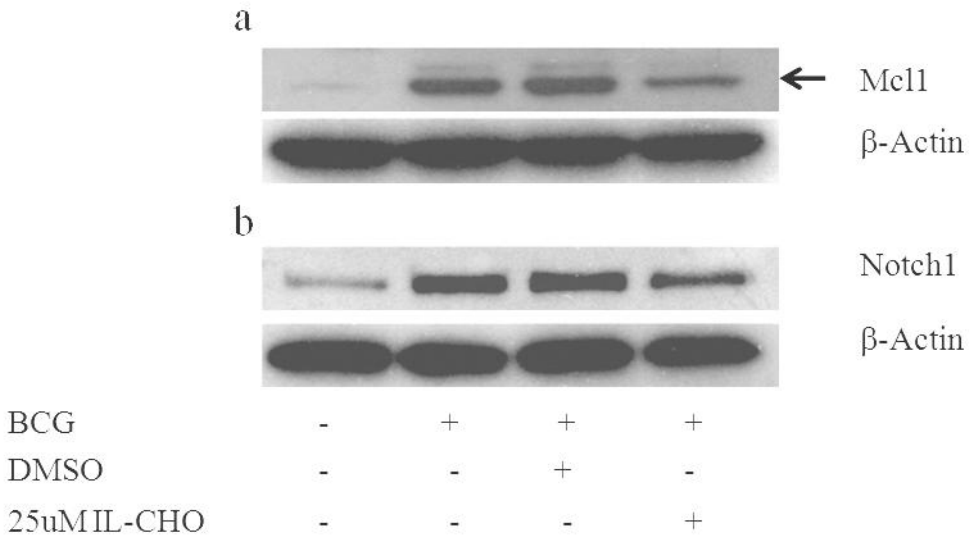

Figure 4.9 Effects of GSI treatment on Mcl-1 and Notch1 expression in *M. bovis* BCG-infected BMMϕ.

BMMϕ were pretreated with IL-CHO or DMSO vehicle control for 1 hr, and infected with *M. bovis* BCG at of MOI of 10 for 24 hr. Cell lysates were analyzed for Mcl-1 (a) and Notch1 (b) by Western blot. β-actin was used as loading control.

4.2.5 Effect of GSI treatment on *mcl-1* expression in *M. bovis* BCG-infected BMMϕ

To investigate the effect of GSI on *mcl-1* expression in infected macrophage, BMMϕ were pretreated with 25μM IL-CHO or vehicle control DMSO for 1 hr prior to *M. bovis* BCG infection and *mcl-1* expression was analyzed by real time RT-PCR. Upon *M. bovis* BCG infection, *mcl-1* was significantly upregulated at 6 hr after infection (Figure 4.4 and 4.10). In contrast, GSI treatment resulted in significant decreased *mcl-1* expression at this time point

after *M. bovis* BCG infection (Figure 4.10). This result strongly suggested that activation of Notch signaling may positively regulate Mcl-1 expression at the transcriptional level.

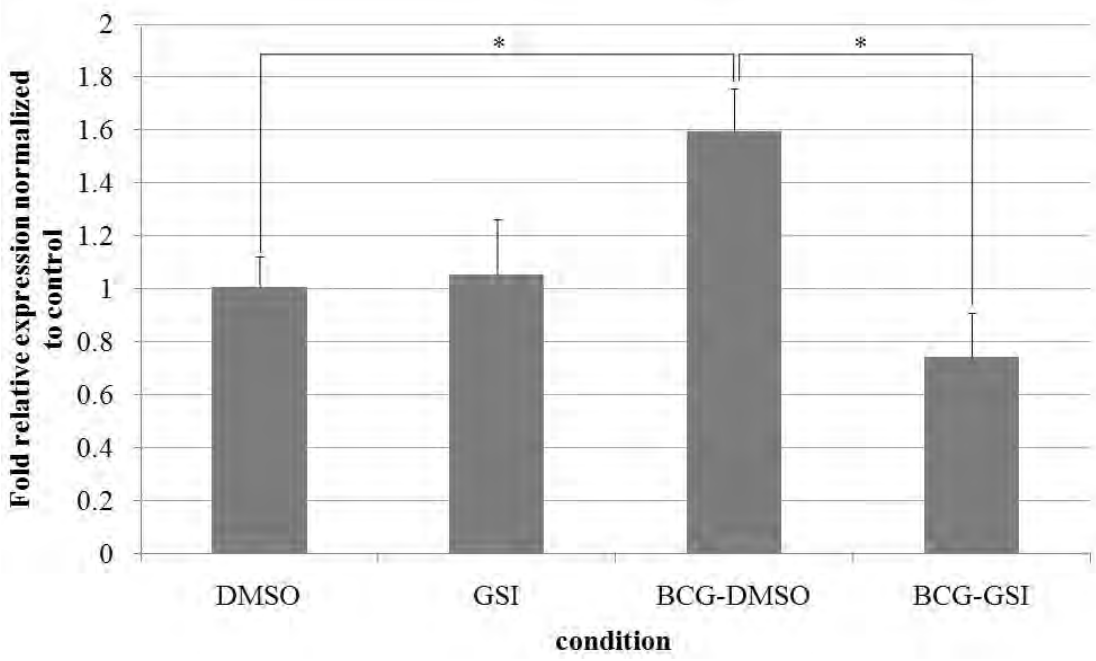

Figure 4.10 Effects of GSI treatment on *mcl-1* expression in BMMϕ infected with *M. bovis* BCG.

BMMϕ were pretreated with IL-CHO or DMSO vehicle control for 1 hr, and infected with *M. bovis* BCG at an MOI of 10 for 6 hr. Total RNA were analyzed for *mcl-1* expression by semi quantitative real-time RT-PCR. β-actin was used as reference control. * indicated the statistic significance ($p < 0.05$) analyzed by student's t-test, as compared with unstimulated cells. The results represented two independent experiments, in triplicates.

4.3 Effects of Notch1 silencing on RAW264.7

To study the role of Notch signaling pathway, in particular Notch1, in regulating Mcl-1 expression, Notch1 expression was silenced using a plasmid pShNotch1 containing an insert targeting Notch1 and the control plasmid pShLuc with luciferase targeting insert in RAW264.7 macrophage-like cell line.

4.3.1 PPD treatment or *M. bovis* BCG infection upregulated Notch1 and Mcl-1 in RAW264.7

To confirm that Notch1 and Mcl-1 are similarly upregulated in RAW264.7 as in primary macrophages, Western blot were performed. Notch1 and Mcl-1 were detected at 1, 3, 6, 12 and 24 hr after stimulation with PPD. As shown in Figure 4.11a, RAW264.7 treated with PPD expressed increasing level of Mcl-1 at 1, 3, 6 and 12 hr after stimulation. The expression fell to the basal level at 24 hr after stimulation. Expression of Notch1 was detected at 6 hr and remained high at 24 hr after treatment (Figure 4.11b). This result indicated that PPD treatment upregulated Notch1 and Mcl-1 in RAW264.7 as in BMMϕ.

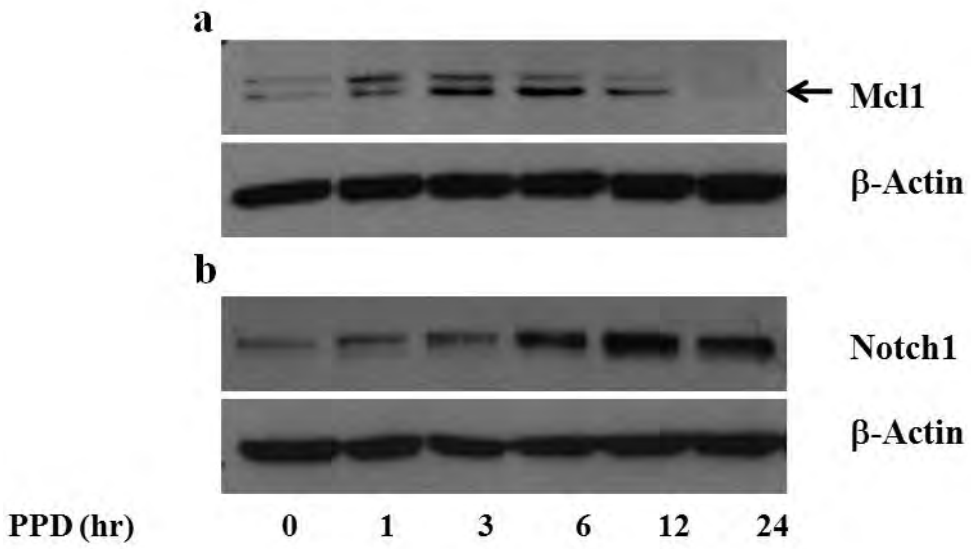

Figure 4.11 PPD treatment upregulated Notch1 and Mcl-1 in RAW264.7.
RAW264.7 were treated with 5μg/ml PPD and total cell lysates were analyzed for Mcl-1 (a) or Notch1 (b) by Western blot. β-actin was used as loading control.

To determine whether Notch1 and Mcl-1 upregulation could be observed in *M. bovis* BCG-infected RAW264.7, Western blot using antibodies specific for Notch1 and Mcl-1 were performed. Notch1 and Mcl-1 protein levels were detected at 0, 1, 3, 6, 12, 24 and 48 hr after

infection. RAW264.7 infected with *M. bovis* BCG increased Mcl-1 as early as 0 hr and the level remained high until 24 hr. However, the level decreased at 48 hr after infection (Figure4.12a). Furthermore, *M. bovis* BCG infection induced an increase in Notch1 at 12 hr and peaked at 24 hr and the level decreased at 48 hr after infection. This result indicated that BCG infection led to increased Notch1 and Mcl-1 expression.

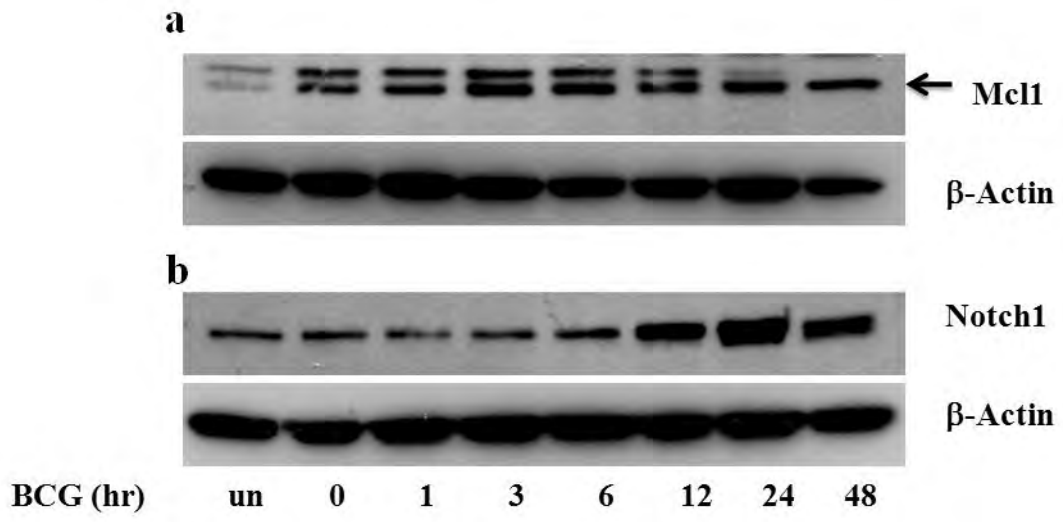

Figure 4.12 *M. bovis* BCG upregulates Notch1 and Mcl-1 expression in RAW264.7.
RAW 264.7 were infected with *M. bovis* BCG at an MOI of 10 at indicated times. Total cell lysates were analyzed for Mcl-1 (a) or Notch1 (b) by Western blot. β-actin was used as loading control

4.3.2 Morphology of RAW264.7 with Notch1 knockdown

In RAW264.7 with Notch1 knockdown, the alteration in the cellular morphology was observed (Figure 4.13). RAW264.7 were transiently transfected with pShNotch1 or pShLuc (vector control) for 36 hr prior to stimulation with PPD for 12 hr. For Notch1 knockdown, cells appeared as flat and showed multiple projections from the cell body. In addition, vacuoles in cytoplasm were also observed in RAW264.7 cells with pShNotch1 (arrows in Figure 4.13 e and f).

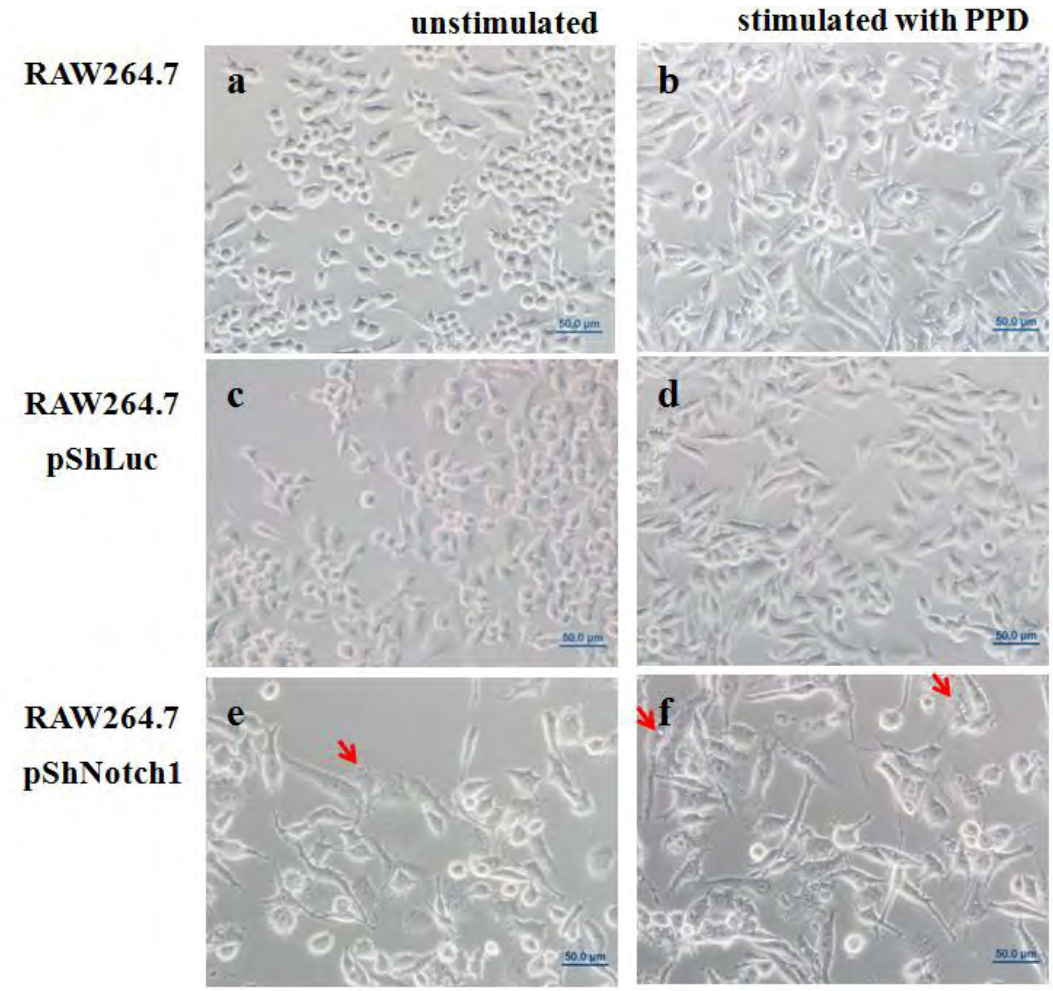

Figure 4.13 Morphology of RAW264.7 with knockdown of Notch1.

In (a), (c) and (e), cells were left unstimulated and in (b), (d) and (f), cells were stimulated with 5µg/ml of PPD for 12 hr.

4.3.3 Effects of Notch1 knockdown on Notch1 and Mcl-1 in RAW264.7 stimulated with PPD

To study the involvement of Notch1 in Mcl-1 expression in RAW264.7, RAW264.7 was transfected with a plasmid pShNotch1 or control pShLuc, prior to stimulation with 5μg/ml of PPD. To confirm that Notch1 was knockdown, the level of Notch1 was detected. As shown in Figure 4.14b, the level of Notch1 was clearly decreased as compared to control. The level of Mcl-1 was detected at 12 hr after PPD stimulation. Previously, PPD stimulation was shown to increase Mcl-1 level at 12 hr (Figure 4.11a). When Notch1 was knockdown, however, the level of Mcl-1 was also decreased. Therefore, decreased Notch1 is associated with decreased expression of Mcl-1. Thus, Notch signaling may be involved in regulating Mcl-1 expression in *M. bovis* BCG-infected macrophages.

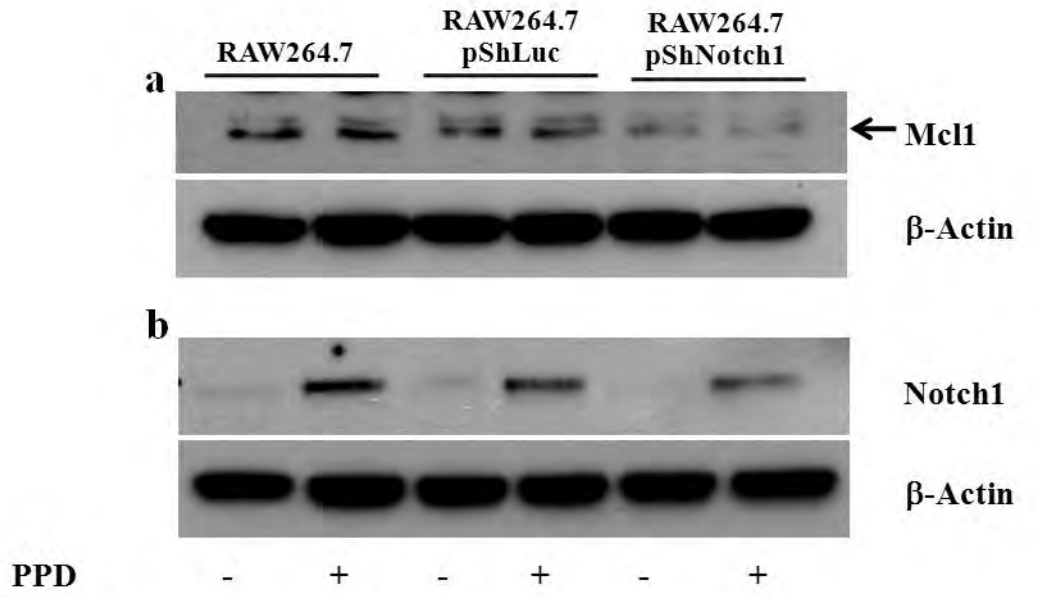

Figure 4.14 Effects of Notch1 silencing in RAW264.7 on Mcl-1 and Notch1.

RAW264.7 were transiently transfected with pShNotch and pShLuc as a control for 36 hr, and activated with 5μg/ml PPD for 12 hr. Cell lysates were analyzed for expression of Mcl-1 (a) and Notch1 (b) by Western blot. β-actin was used as loading control.

4.4 Effect of GSI on apoptosis of *M. bovis* BCG-infected macrophages

BMMϕ were pretreated with 25μM IL-CHO or vehicle control DMSO and infected with *M. bovis* BCG at an MOI of 10. The amount of apoptotic cells was detected on day 5 post-infection. Mono- and oligonucleosomes, apoptotic features, were measured in the cytoplasmic fraction of cell lysates by ELISA. As shown in Figure 4.15, in the present 25μM IL-CHO, the level of apoptotic infected macrophages were higher than in the presence of DMSO (Figure 4.15). This result strongly correlated well with decreased Mcl-1 in GSI treated macrophages.

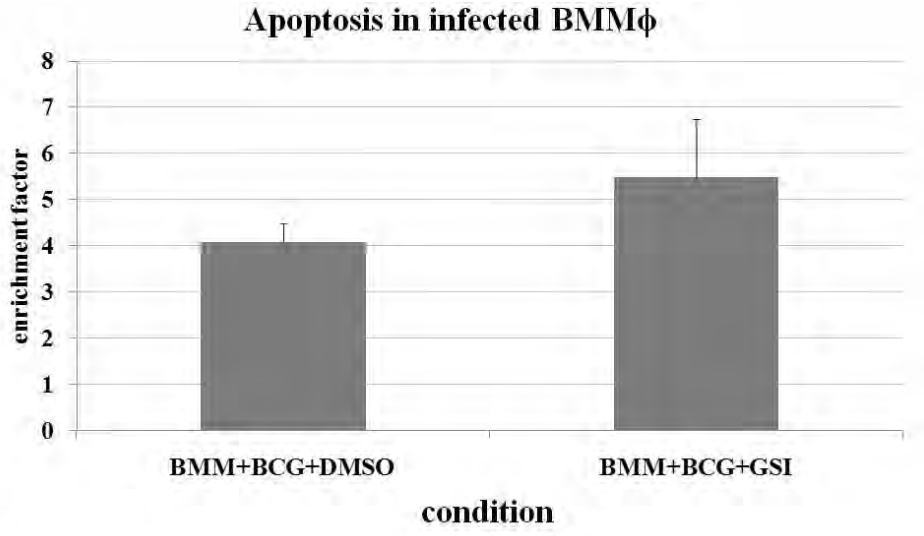

Figure 4.15 Apoptosis of *M. bovis* BCG-infected with BMMϕ.

BMMϕ infected with *M. bovis* BCG in the present or absent of 25μM IL-CHO. On day 5 apoptotic cells were determined. In the present of IL-CHO, macrophage were apoptosis more than infected macrophage that absent of IL-CHO.

4.5 Effects of GSI treatment on survival of BCG in macrophages

To investigate the relationship between inhibition of Notch signaling and the survival of *M. bovis* BCG, BCG were measured by colony forming unit in infected macrophages. BMMϕ were pretreated with 25μM IL-CHO or DMSO vehicle control for 1 hr and infected with *M. bovis* BCG at of MOI 10. The intracellular bacteria were plated immediately after 4 hr of infection (day 0), and at day 2, day 3 and day 7 post infection. The intracellular bacterial CFUs determined at each time points were shown in Figure 4.16. The difference in numbers of intracellular bacteria were seen only at day 7 after infection, where GSI treatment resulted in decreased numbers of intracellular bacteria than in the control or untreated cells.

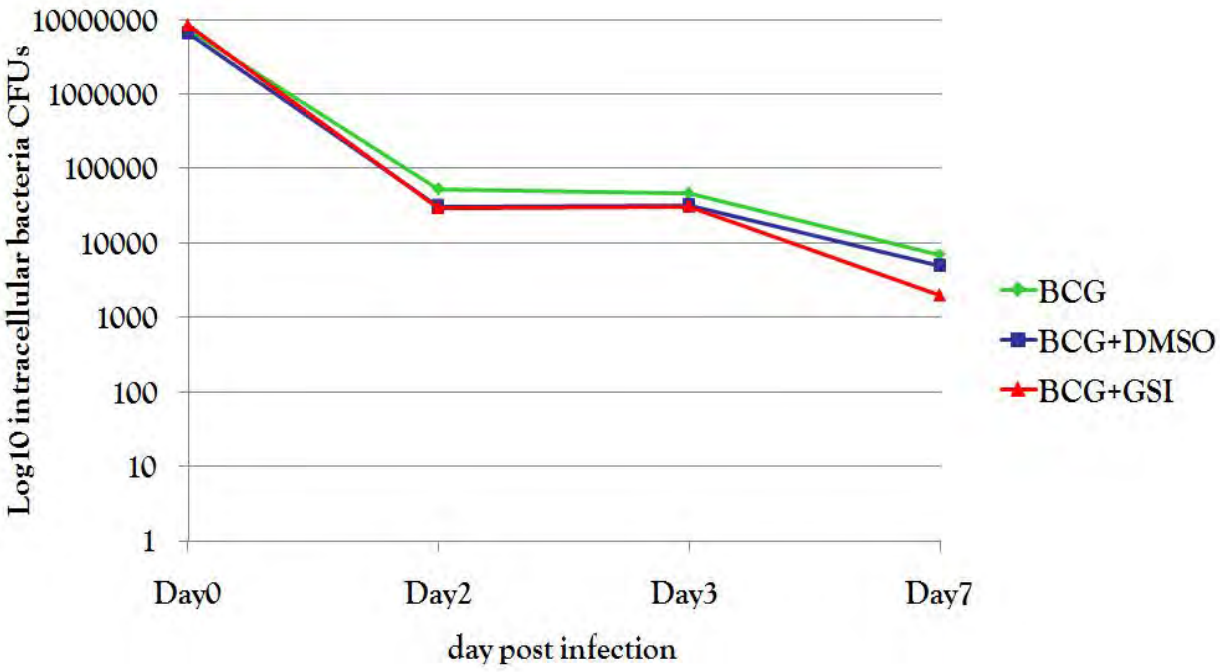

Figure 4.16 GSI treatment reduced numbers of intracellular bacteria in macrophages. BMMϕ infected with *M. bovis* BCG in the present or absent of 25μM IL-CHO. The recovery of intracellular bacterial was observed at day 0, day 2, day 3 and day 7 post infections.

4.6 Direct binding of Notch1 to promoter region of Mcl-1

4.6.1 Database search for the potential binding sites of CSL in the regulatory region of *mcl-1* promoter

To search for potential binding sites of CSL in the regulatory region of *mcl-1* promoter, program was used. The potential binding sites of various transcription factors were shown in Figure 4.17. The potential CSL binding site was analyzed using NCBI sequence viewer v.2 and EBI ClustalW2 was used to carry out the consensus sequence alignment of human and murine *mcl-1*. In the regulatory regions of *mcl-1*, consensus sites for transcription factors such as NF-κB, E2F, AP1 were found and the potential CSL binding site in both human and mouse was indicated.

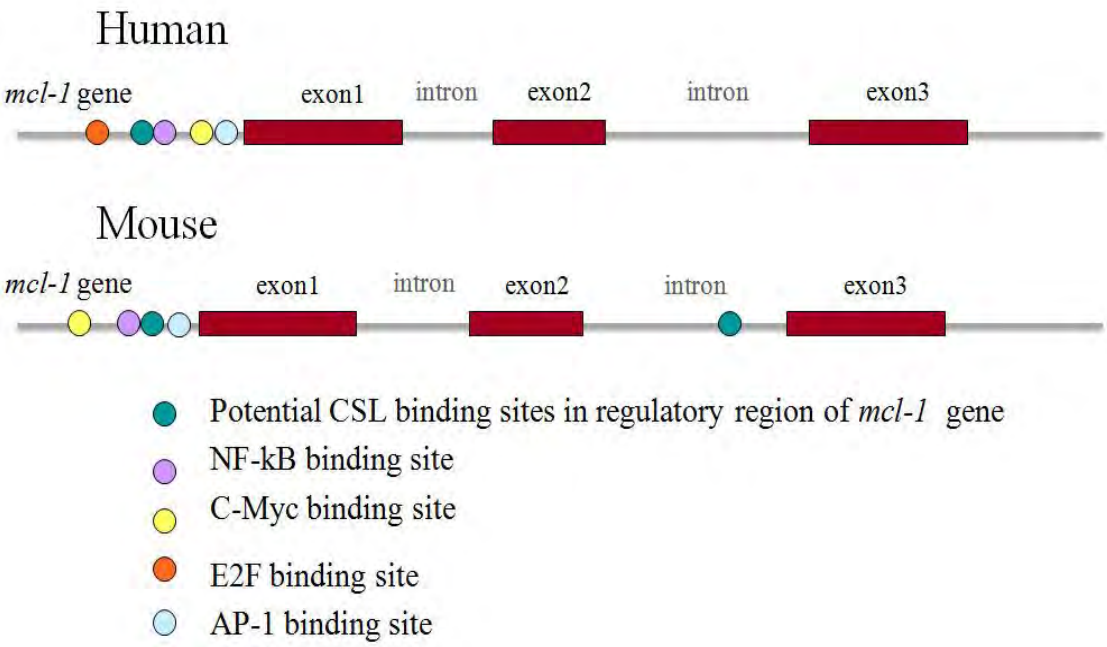

Figure 4.17 Potential binding sites for transcription factors in regulatory region of *mcl-1* gene in human and mouse genome.

In regulatory region of *mcl-1*, various consensus sites for transcription factors were found such as NF-κB, E2F, AP1 and potential CSL binding site in both human and mouse.

To compare the consensus sequence between human and murine species, Mulan program was used to identify the potential transcription factor binding sites conserved between human and murine species (Figure 4.18). The consensus sequences of potential CSL binding site was found to be conserved between species, suggesting that this site may be important in regulating Mcl-1 expression.

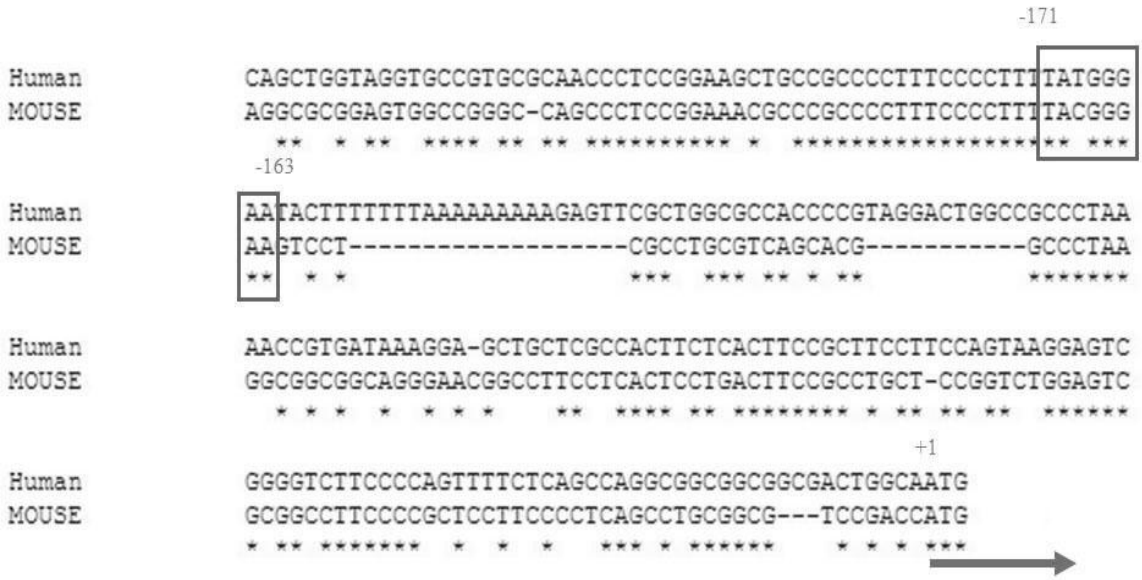

Figure 4.18 Alignment of the consensus sequences of CSL binding sites in human and murine *mcl-1*.

The consensus sequence of potential CSL binding site on regulatory region of human and murine *mcl-1* was identified at -171 to -163 from the translation start site and is well conserved between species.

4.6.2 Direct binding of Notch1 to the regulatory region of *mcl-1*

To investigate the binding of Notch1 to *mcl-1* promoter, EZ Magna ChIP™ Chromatin Immunoprecipitation Kit was used as describe in materials and methods. BMMϕ were stimulated with PPD for 24 hr. After that chromatins were sonicated as described previously, the size of shear chromatin was measured by electrophoresis (Figure 4.19a). This result showed that the sizes of shear chromatins were in the range of 200-1000 bp. To confirm that *mcl-1* was transcribed at this time point, sheared chromatin was subjected to immunoprecipitation using specific antibody for RNA polymerase II or isotype control normal mouse IgG. Precipitated and purified DNA was amplified by conventional PCR, using specific primers to murine *mcl-1* promoter. The amplified PCR products were detected by 2% agarose gel electrophoresis (Figure 4.19b). When immunoprecipitation was conducted using anti-RNA polymerase II antibody, the PCR product of murine *mcl-1* promoter was readily detected. This result implicated that RNA polymerase II was actively associated with mcl-1 promoter and suggested that *mcl-1* was actively transcribed in PPD-treated BMMϕ.

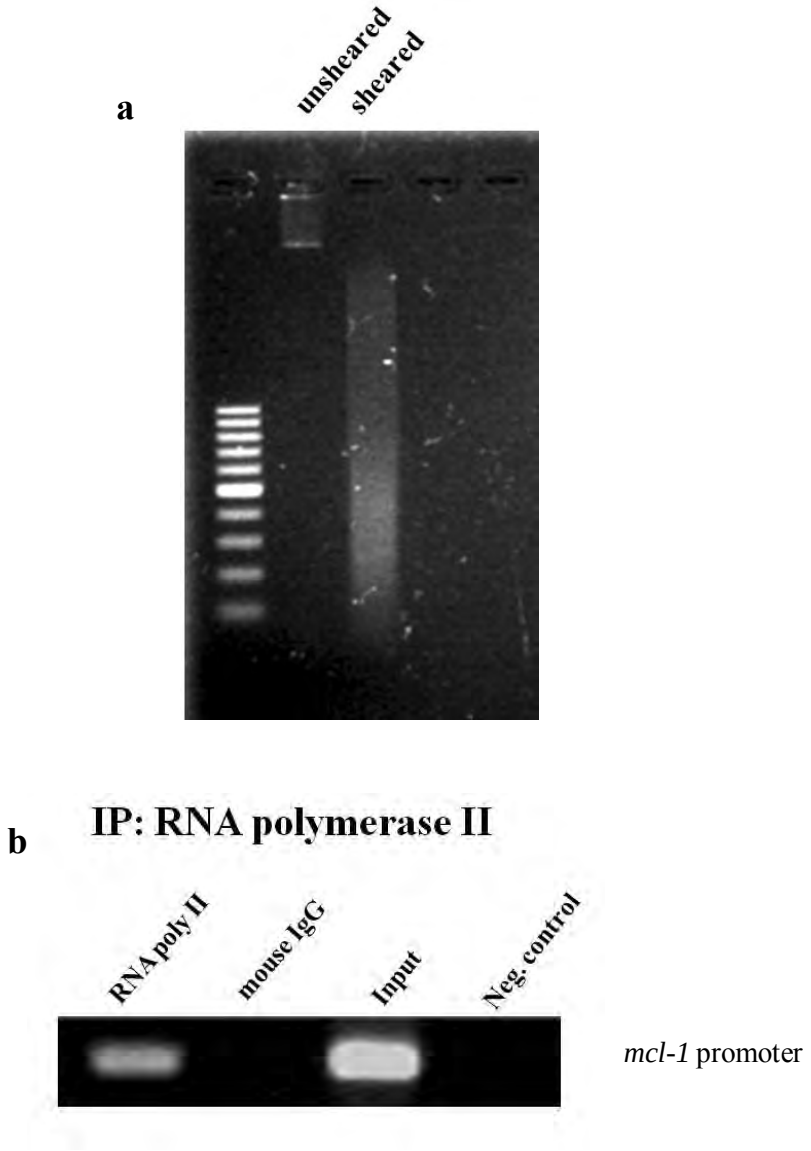

Figure 4.19 ChIP analysis of murine *mcl-1* promoter in stimulated macrophages.

BMMϕ were stimulated with 5μg/ml PPD for 24 hr. Genomes were sonicated to optimize chromatin shearing (a). ChIP assays were conducted using antibody specific for RNA polymerase II or normal mouse IgG (isotype control). Conventional PCR was performed using primer set specific for the murine *mcl-1* promoter (b).

Binding of Notch1 to murine *mcl-1* promoter was confirmed using the same sheared chromotin. Sheared DNA was immunoprecipitated using antibody specific for Notch1 or normal rabbit IgG as isotype control. Purified DNA was amplified by conventional PCR, using specific primers to murine *mcl-1* promoter. The amplified DNA products were detected by agarose gel electrophoresis (Figure 4.20). Immunoprecipitation with antibody specific for Notch1 clearly showed the PCR product, suggesting that Notch1 directly binds to *mcl-1* promoter in PPD-treated BMMϕ. This result indicated that Notch signaling pathway is involved in the direct regulation of murine *mcl-1* expression.

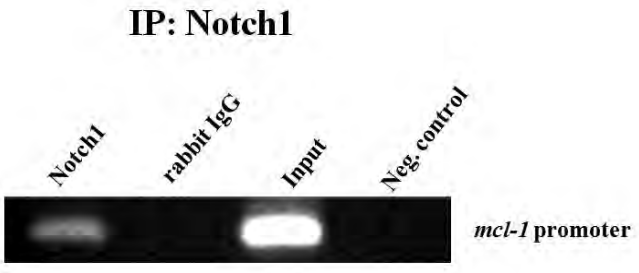

Figure 4.20 ChIP analysis of murine *mcl-1* promoter in stimulated macrophages.
BMMϕ were stimulated with 5μg/ml PPD for 24 hr. ChIP assays were conducted as described above. Immunoprecipitations were performed using antibody specific for Notch1 and conventional PCR was performed using primers specific to the murine *mcl-1* promoter.

CHAPTER V

DISCUSSIONS

One of mechanisms that mycobacteria utilize to evade host defense is inhibiting apoptosis of macrophages. Recent data reported that virulent Mtb upregulated Mcl-1 expression, resulting in delay apoptosis in infected macrophages (Sly *et al.*, 2003). Regulation of *mcl-1* expression is controlled at multiple levels, including transcriptional, post-transcriptional, and post-translational levels (Le Gouill *et al.*, 2004). Extra-cellular stimuli trigger Mcl-1 expression via several signaling pathways such as JAK/STAT, MAPK, PI3K/AKT and MEK/ERK pathways (Wang *et al.*, 1999; Bingle *et al.*, 2000; Craig, 2002; Liu *et al.*, 2003; Le Gouill *et al.*, 2004) Although Mcl-1 expression is regulated by various pathways but it is unclear whether activation of Notch signaling pathway is involved in regulating Mcl-1 expression in macrophages infected with mycobacteria. In this study, we investigated whether treatment of PPD, Mtb-derived purified protein derivatives, or *M. bovis* BCG infection upregulate Notch receptor and activate Notch signaling pathway, leading to the expression of Mcl-1 and inhibition of apoptosis.

Notch signaling pathway modulates numerous cellular functions such as proliferation, adhesion, angiogenesis and apoptosis. In this study, treatment of PPD or *M. bovis* BCG infection induced Notch1 and Mcl-1 expression in activated macrophage with similar kinetics. These results were seen in both the macrophage-like cell line RAW264.7 and BM-derived primary macrophages. These findings are consistent with our unpublished data and findings from other groups, suggesting that Notch signaling is activated in macrophages during infection with *M. bovis* BCG (Jayakumar *et al.*, 2008; Narayana and Balaji, 2008) and (Palaga *et al.*, unpublished data). Previously, it was reported that during Mtb infection, macrophages undergo changes in the expression of Mcl-1, leading to host cell apoptosis and

decreased intracellular survival of bacteria (Sly *et al.*, 2003). Marriott *et al.* have recently demonstrated that dynamic changes in Mcl-1 expression during bacterial infection determine macrophage viability as well as antibacterial host defense (Marriott *et al.*, 2005). Furthermore, Swingler *et al.* reported that during infection, macrophages exhibited resistance against apoptosis by upregulation of Mcl-1 expression and host cell survival (Swingler *et al.*, 2007). In this study, Western blot for Mcl-1 proteins revealed two close bands which is consistent with Kojima *et al*. which reported that the two close bands were Mcl-1L and Mcl-1V (shorter band), a new splicing variant of Mcl-1L. Mcl-1V has anti-apoptotic activity similar to Mcl-1L. Mcl-1V more stable than Mcl-1L (Kojima *et al.*, 2010).

Notch signaling is initiated through ligand-receptor interaction that promotes proteolytic cleavages of the receptor. For inhibition of Notch signaling pathway, IL-CHO, a γ-secretase inhibitor, is routinely used to probe the biological functions of this signaling pathway. In this study, pretreatment with γ-secretase inhibitor prior to PPD treatment or *M. bovis* BCG infection resulted in decreased Notch1, cleaved Notch1 and Mcl-1 expression in both transcriptional and translational levels. Decreased Notch1 expression upon stimulation correlated well with decreased expression of Mcl-1. Hence, Notch signaling may directly or indirectly affect Mcl-1 expression in PPD-treated or *M. bovis* BCG-infected macrophages. This result is consistent with previous reports on the effect of GSI on expression of Notch receptor (Palaga *et al.*, 2008). In addition, Oishi *et al.* (2004) have reported that Bcl-2 and Mcl-1 appear to be required for the survival-promoting effect downstream of Notch1 in neural precursor cells (Oishi *et al.*, 2004).

Notch-mediated activation of a given target gene often requires the transcription factor CSL. When Notch signaling is activated through ligand-receptor interaction, this event leads to translocation of ICN to the nucleus. Nuclear ICN binds to CSL and recruit co-activators to form transcription activation complex which, in turn, induces expression of the Notch target genes. Although the relationship between Notch and Mcl-1 are not well

established, recent data suggested that *mcl-1* promoter contains potential CSL binding site and Notch was found to bind to promoter region of *mcl-1* by ChIP-on-chip assay in activated macrophages (Palaga *et al*., unpublished data). In this study, data obtained from the ChIP assay clearly demonstrated that activation by PPD in macrophages recruited ICN to the *mcl-1* promoter (Figure 4.22). Additionally, the significant reduction of Mcl-1 (Figure 4.7 and Figure 4.14) upon treatment with the γ-secretase inhibitor or Notch1 knockdown by siRNA in PPD-treated macrophages strongly suggested the involvement of Notch signaling in Mcl-1 expression. Furthermore, significant reduction of Mcl-1 upon treatment with the γ-secretase inhibitor was also found in *M. bovis* BCG-infected macrophage. Taken together, this result suggested that Notch signaling pathway may be involved in Mcl-1 expression directly in macrophages stimulated with PPD or *M. bovis* BCG infection and regulate apoptosis of macrophages.

Intracellular infection with various species of mycobacteria has been reported to cause host cell apoptosis (Kornfeld *et al.*, 1999). In infection models, macrophage apoptosis has been shown to reduce survival of intracellular mycobacteria (Molloy *et al.*, 1994). In addition, FasL-induced apoptosis of macrophage had an anti-mycobacterial effect *in vitro* against *M. tuberculosis* strains H37Ra and H37Rv as did TNF-α-induced apoptosis (Oddo *et al.*, 1998). Anti-mycobacterial activity of picolinic acid against *M. avium* was attributed to induction of host cell apoptosis (Pais and Appelberg, 2004). In this study macrophages apoptosis was determined during downregulation of *mcl-1* expression through treatment with γ-secretase inhibitor. This treatment resulted in increasing apoptosis of *M. bovis* BCG-infected macrophages. Furthermore, when intracellular *M. bovis* BCG were counted, pretreatment with γ-secretase inhibitor resulted in reduction in *M. bovis* BCG which survived within BMMϕ. Therefore, the induction of apoptosis in γ-secretase inhibitor-treated, *M. bovis* BCG-infected BMMϕ correlated well with slightly reduced recovery of CFUs from infected cells. Because Mcl-1 was regulated by many pathways including PI3K pathway, it is possible

that Notch signaling may promotes cell survival by indirectly regulating Mcl-1 expression through such pathways. Previous studies reported that modulation by Notch signaling of the activity of various signaling pathways and survival pathways in many cell types often requires the involvement of PI3K-AKT, ERK1/2, and NF-κB, as possible downstream regulators (Cheng *et al.*, 2001; Liu *et al.*, 2006; Shin *et al.*, 2006; Chan *et al.*, 2007). Together with our data, it is likely that Notch signaling pathway is involved in regulating apoptosis via Mcl-1 regulation by directly upregulating Mcl-1 and/or indirectly by activating other pro-survival pathways (Figure 5.1).

In apoptosis, Notch signaling pathway prevents apoptosis induced during murine erythroleukemia cells differentiation (Shelly *et al.*, 1999). In this study pretreatment with γ-secretase inhibitor resulted in increased apoptosis of infected macrophages. This result indicated that Notch signaling may play an important role in regulating apoptosis during innate immune response against mycobacteria infection. This result is consistent with Wang *et al.* which had reported that down-regulation of Notch-1 by siRNA resulted in the growth inhibitory activity of pancreatic cancer cells which could be attributed to increased cell apoptosis (Wang *et al.*, 2006). Mycobacterial infection induces macrophage apoptosis as a result of a beneficial gateway to promote protective immunity. This important new function of Notch signaling pathway in anti-bacterial immunity may open a new way to manipulate host response against mycobacterial infection.

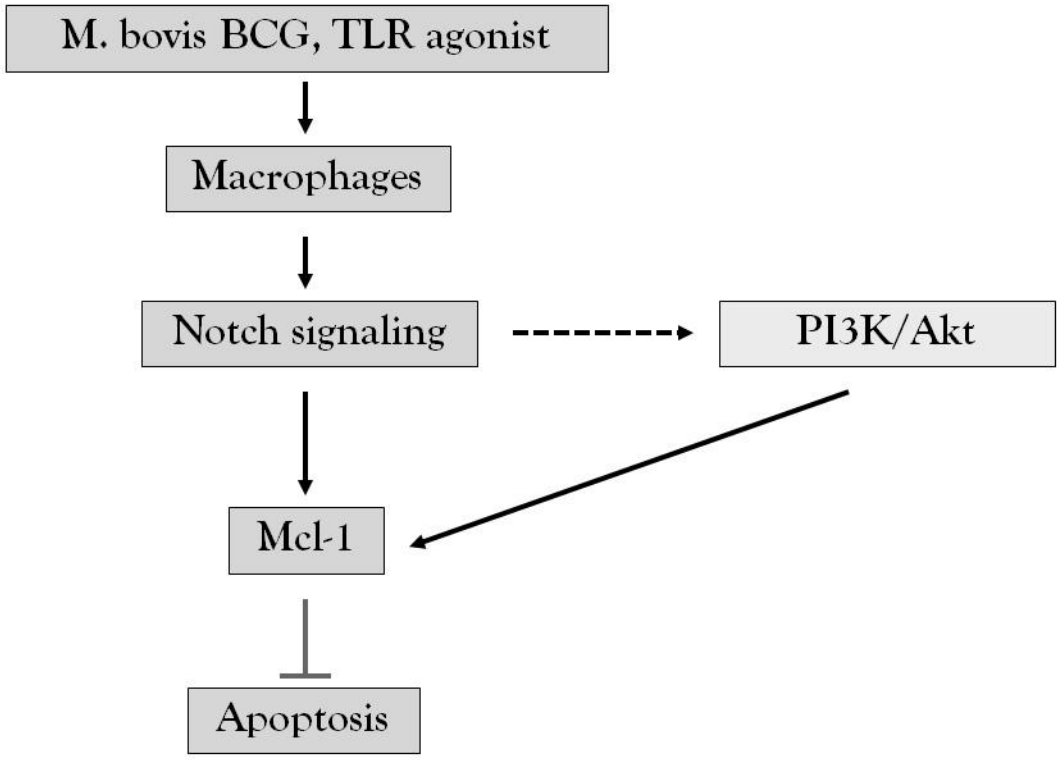

Figure 5.1 Proposed mechanism of the involvement of Notch signaling in regulating apoptosis via Mcl-1 expression in infected macrophages.

CHAPTER VI

CONCLUSIONS

Treatment with PPD or *M. bovis* BCG infection in macrophages resulted in increased Notch1 and Mcl-1 expression at both transcriptional and translational levels.

Pretreatment with IL-CHO or silencing of Notch1 led to reduction in Notch1 and Mcl-1 expression in PPD-treated or *M. bovis* BCG infected macrophages. In addition pretreatment with IL-CHO had an affect on infected macrophage apoptosis by increasing apoptotic macrophages. Furthermore, induction of apoptosis in γ-secretase inhibitor-treated correlated well with reduction in *M. bovis* BCG survived within BMMϕ.

Finally, ChIP assay were used to clearly demonstrate that activation by PPD in BMMϕ recruited ICN to the *mcl-1* promoter. Taken together, these results strongly suggested that Notch signaling pathway is directly involved in regulating Mcl-1 expression in macrophages upon PPD stimulation or *M. bovis* BCG infection.

Suggestions for the future work

1. Downstream of Notch signaling pathway such as PI3K may be involved in regulating Mcl-1 expression. The status of such pathway should be studied in infected macrophages, using specific inhibitors.

2. Study the effects of γ-secretase inhibitor on mycobacterial-infected macrophage in *in vivo* model.

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APPENDIX

APPENDIX

1. Complete DMEM 100 ml

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

2. Freezing media

2.1 DMEM freezing media

Complete DMEM	90%
DMSO	10%

2.2 BMMϕ freezing media

2.2.1 20% FBS freezing media

DMEM	80%
FBS	20%

2.2.2 20% DMSO freezing media

DMEM	60%
FBS	20%
DMSO	20%

3. DEPC water for RNA work 100 ml

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

4. FBS inactivation

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

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

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

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

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

5.1 2% agarose gel preparation

Agarose gel	2%
1×TAE	20 ml

5.2 running buffer for agarose gel electrophoresis

50x TAE was diluted to final concentration 0.5x in 500 ml of deionized water.

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

6.1 LB broth preparation

Bacto tryptone	10 g
Yeast extracts	5 g

NaCl 10 g

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

6.2 LB agar preparation

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

6.3 LB agar containing 50µg/ml ampicillin

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

7. Middlebrook 7H9 (M7H9) broth (1000ml)

M7H9 powder	4.7 g
Glycerol	2 ml
Tween80	0.05%
deionized water	900 ml

M7H9 broth was autoclaved at 121˚C and pressure 15 psi for 10 minutes. After sterilization, the M7H9 was warmed to approximately 45˚C and added with 10% Middlebrook OADC Enrichment.

8. Middlebrook 7H11 (M7H11) agar (1000ml)

M7H11 powder	21 g
Glycerol	5 ml
deionized water	900 ml

M7H11 agar was autoclaved at 121˚C and pressure 15 psi for 15 minutes. After sterilization, the M7H11 was warmed to approximately 50-55˚C and added with 10% Middlebrook OADC Enrichment.

9. SDS-polyacrylamide gel preparation

9.1 8% separating gel 8 ml

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

9.2 12% separating gel 8 ml

Sterile water	3.436 ml
40% Acrylamide and Bis-acrylamide solution	2.4 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

9.3 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

10. 2x Laemmli buffer (SDS-dye) 10 ml

1 M Tris-HCl pH 6.8	1 ml (final concentration 100 mM)

10% SDS	4 ml (4% v/v)
99.5% glycerol	2.01 ml (20% v/v)
HPLC water	2.989 ml
Bromphenol blue	0.001 g

11. Reagent for protein extraction

11.1 Buffer A (stored at 4˚C)

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

11.2 Buffer B (stored at 4˚C)

Buffer A	990 µl
Nonidet P-40	10 µl

12. PBST (washing buffer for Western blot)

1x PBS	500 ml
Tween20	0.05%

13. Blocking solution for Western blot

PBST	200 ml
Non-fat dry milk	3%

14. 1x PBS pH 7.4

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

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

15. 5x running buffer for Western blot

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

16. 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

17. ECL substrate of HRP

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

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

17.1 Solution A

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

17.2 Solution B

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

18. Bacterial glycerol stock

An inoculum preparation, a bacterial colony on agar plate was picked and cultured in 1.5 ml of LB broth overnight. The bacterial culture was added with 0.5 ml of 60% glycerol to 15% final concentration and kept at –80˚C. To measure the CFU of stock culture, the aliquot was diluted and determined the CFU by spread plate.

19. Film developer and fixer

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

20. Ampicillin, penicillin and streptomycin solution

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

BIOGRAPHY

Miss Siriluk Ratanabunyong was born on February 15, 1980 in Bangkok, Thailand. She graduated with the Bachelor Degree of Science (Biology) from the Faculty of Science, Kasetsart University in 2002. She is currently a student in the Inter-Department of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University since 2007.