การพิสูจน์เอกลักษณ์ของ RGS19 เป็นโมเลกุลเป้าหมายของวิถีสัญญาณ Notch ในแมโครฟาจ
ที่ถูกกระตุ้นด้วยไลโปพอลิแซคคาไรด์โดยวิธีฟอสโฟโปรตีโอม

นางสาวนวลพรรณ แสงเพชร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2554

IDENTIFICATION OF RGS19 AS A TARGET MOLECULE OF NOTCH
SIGNALING IN LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGES
BY PHOSPHOPROTEOMIC

Miss Naunpun Sangphech

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2011

Thesis Title   IDENTIFICATION OF RGS19 AS A TARGET MOLECULE OF

                      NOTCH SIGNALING IN LIPOPOLYSACCHARIDE-

                      STIMULATED MACROPHAGES BY PHOSPHOPROTEOMIC

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นวลพรรณ แสงเพชร : การพิสูจน์เอกลักษณ์ของ RGS19 เป็นโมเลกุลเป้าหมายของวิถี สัญญาณ Notch ในแมโครฟาจที่ถูกกระตุ้นด้วยไลโปพอลิแซคคาไรด์โดยวิธีฟอสโฟ โปรตีโอม (IDENTIFICATION OF RGS19 AS A TARGET MOLECULE OF NOTCH SIGNALING IN LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGES BY PHOSPHOPROTEOMIC) อ. ที่ปรึกษาวิทยานิพนธ์ หลัก: รศ. ดร. ธนาภัทร ปาลกะ , 106 หน้า.

การติดต่อสื่อสารระหว่างเซลล์อาศัยความเชื่อมโยงระหว่างตัวรับ-ส่งสัญญาณหลายโมเลกุล การเปลี่ยนแปลงของการเติมหมู่ฟอสเฟตบนฟอสโฟโปรตีนสามารถพบได้ในหลายวิถีสัญญาณ ความ ผิดปกติในกระบวนการเติมหมู่ฟอสเฟตอาจจะนำไปสู่ความผิดปกติด้านพฤติการณ์ของเซลล์ เช่น การเพิ่มจำนวนของเซลล์,การแบ่งเซลล์ และการตายของเซลล์ วิถีสัญญาณ Notch เป็นวิถีสัญญาณที่ คงอนุรักษ์ไว้ในพัฒนาการ มีความสำคัญอย่างยิ่งในการควบคุมการการเจริญพัฒนาและการทำ หน้าที่ของเซลล์ภูมิคุ้มกัน จากหลักฐานที่ผ่านมาบคความเกี่ยวข้องของวิถีสัญญาณนี้ในแมโครฟาจที่ ถูกกระตุ้น ซึ่งเซลล์นี้เป็นเซลล์ภูมิคุ้มกันแบบมีมาแต่กำเนิด โดยทำงานร่วมกันกับวิถีสัญญาณระดับ ถัดลงมาจากตัวรับสัญญาณแบบรับรู้รูปแบบของสิ่งแปลกปลอมเช่น วิถีสัญญาณของ Toll-like receptor (TLR) ในงานวิจัยนี้ รูปแบบของฟอสโฟโปรตีนในเซลล์ไลน์ของหนูจะถูกเปรียบเทียบด้วย การศึกษาการเปลี่ยนแปลงของโปรตีน เซลล์จะได้รับยากดวิถีสัญญาณ Notch (GSI) หรือตัวควบคุม ก่อนถูกกระตุ้นด้วยลิโปโพลีแซคคาไรด์ ฟอสโฟโปรตีนจะถูกตรวจสอบและเปรียบเทียบด้วยวิธีการ แยกโปรตีนบนแผ่นวุ้นด้วยกระแสไฟฟ้าสองทิศทาง (2D-GE) 2D-GE gel จะถูกย้อมด้วยสีที่จำเพาะ ต่อฟอสโฟโปรตีน, Pro-Q diamond และถูกย้อมโปรตีนทั้งหมดด้วยสี Coumassie และ ซิลเวอร์ ตามลำดับ ความแตกต่างอย่างมีนัยยะสำคัญของโปรตีนที่ถูกเติมหมู่ฟอสเฟตระหว่างการได้รับ หรือไม่ได้รับยากดวิถีสัญญาณ Notch จะถูกระบุชนิดวิธีด้วย LC-MS/MS ผลการทดลองพบว่า หนึ่ง ในโปรตีนนั้นคือ regulator of G-protein 19 (RGS19) มีการเติมหมู่ฟอสเฟตลดลงเมื่อถูกกดวิถี สัญญาณ Notch ความเกี่ยวข้องของโปรตีน RGS19 ในวิถีสัญญาณของ TLR4 และ Notch ในแมโคร ฟาจถูกตรวจสอบด้วยการทำให้ RGS19 มีการแสดงออกลดลงด้วยเทคนิค siRNA การกดวิถี สัญญาณ Notch หรือการทำให้ RGS19 มีการแสดงออกลดลง ให้ผลเช่นเดียวกันในการเพิ่มการ ทำงานของ SAPK/JNK และ ลดการทำงานของ Akt ผลการทดลองสรุปได้ว่า วิถีสัญญาณ Notch ควบคุมการผลิต cytokine TNFα และ IL-6 และ การแสดงออกของ iNOS ในแมโครฟาจที่ถูกกระตุ้น ผ่าน TLR4 โดยการชักนำของลิโปโพลีแซคคาไรด์ผ่าน RGS19

สาขาวิชา ___เทคโนโลยีชีวภาพ___ ลายมือชื่อนิสิต..

ปีการศึกษา ___2554___ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก....................

5372452523   : MAJOR BIOTECHNOLOGY

KEYWORDS :   RGS19 / PHOSPHOPROTEOME / MACROPHAGES / NOTCH SIGNALING / LIPOPOLYSACCHARIDE

NAUNPUN SANGPHECH: IDENTIFICATION OF RGS19 AS A TARGET MOLECULE OF NOTCH SIGNALING IN LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGES BY PHOSPHOPROTEOMIC. THESIS ADVISOR: ASSOCIATE PROFESSOR TANAPAT PALAGA, Ph.D., 106 pp.

Signaling between cell to cell depends on crosstalk of many intracellular signaling molecules such as phosphoproteins. Alterations in protein phosphorylation can be found in different signaling pathways. Aberration in this process may lead to abnormal cell behaviors such as cell proliferation, differentiation and cell death. Notch signaling is an evolutionarily well conserved signaling pathway which plays critical roles in regulating development and functions of immune cells. Recent evidences suggest the involvement of this signaling pathway in activation of macrophages which are innate immune cells, by corroboratively functioning with signaling cascades downstream of pattern recognition receptors such as Toll-like receptor pathway. In this study, the patterns of phosphorylated proteins in mouse macrophage cell line were compared using a proteomics approach. Cells were pre-treated with inhibitor of the Notch signaling (GSI) or vehicle control before subjecting to stimulation with lipopolysaccharide. Phosphoproteins were examined and compared by the two-dimensional gel electrophoresis. Gels were stained with phosphoprotein-specific dye, Pro-Q Diamond, and subsequently with Coomassie stain and silver stain for total proteins. The spots with statistical significant difference in phosphorylation between treatment with or without GSI were identified by LC/MS/MS. The results identified one of the spots as regulator of G-protein signaling 19 (RGS19) which showed decreased phosphorylation when Notch signaling is inhibited. The involvement of RGS19 in TLR4 and Notch signaling in macrophages was investigated by siRGS19 silencing approach. GSI treatment and silencing of RGS19 yielded similar results of increased activation of SAPK/JNK and decreased activation of Akt The results suggested that Notch signaling regulates production of cytokine TNFα, and IL-6 and expression of iNOS in LPS induced activation of TLR4 in macrophage via RGS19.

Field of Study : Biotechnology   Student's Signature
Academic Year: 2011   Advisor's Signature

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LIST OF ABBREVIATIONS

1	%	Percentage
2	/	Per
3	:	Ratio
3	®	Registration
4	°C	Degree Celsius
5	μg	Microgram
6	μl	Micro liter
7	μM	Micro molar
8	2D-GE	two-dimensional gel electrophoresis
9	A	Absorbance
10	Akt	Protein kinase B
11	APC	antigen presenting cell
12	ATCC	American Type Culture Collection
13	BCA	bicinchoninic acid
14	BSA	Bovine serum albumin
15	CD14	Cluster of differentiation 14
16	cDNA	complementary DNA

17	CHAPs	3 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
18	CSL	CBF1/RBP-Jk in mammals,Suppressor of Hairless in Drosophila,and Lag-1 in Caenorhabditis elegans
19	DEPC	Diethylpyrocarbonate
20	DMEM	Dulbecco's Modified Eagle Medium
21	DMSO	Dimethyl sulfoxide
22	dNTPs	deoxyribonucleotide triphosphates
23	DTT	Dithiothreitol
24	EGF	Epidermal-growth factor
25	EGFR1	Epidermal Growth Factor Receptor 1
26	ELISA	Enzyme-linked immunosorbent assay
27	ERK	extracellular signal-regulated kinases
28	g	gram
29	g (Centrifugation speed)	Gravity
30	GPCRs	G-protein couple receptors
31	GSI	Gamma-secretase inhibitor

32	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
33	HES	Hairy-Enhancer of Split
34	HEY1	Hairy/enhancer-of-split related with YRPW motif 1
35	HPLC	High-performance liquid chromatography
36	HRESIMS	High Resolution Electron Spray Ionization Mass Spectroscopy
37	HRP	Horseradish peroxidase
38	IFNγ	interferon-gamma
40	IKK	I kappa B kinase
41	IL-6	Interleukin-6
42	IL-10	Interleukin-10
43	iNOS	inducible nitric oxide syntase
44	IPG	Immobilized pH gradient
45	IκB	I kappa B
46	JNK	c-Jun NH2-terminal kinase
47	LBP	LPS binding protein

48	LC-MS/MS	Liquid Chromatography- Mass Spectrometry and Liquid chromatography - Tandem Mass Spectrometry
49	LPS	lipopolysaccharides
50	MAPK	mitogen-activated protein kinase
51	MD2	Lymphocyte antigen 96
52	min	minute
53	MIP-1α	Macrophage inflammatory protein-1α
54	mRNA	massenger RNA
55	mTOR	Mammalian target of rapamycin
56	NCBI	National Center for Biotechnology Information
57	NF-κB	nuclear factor- κB
58	nm	nano-metre
59	PBS	Phosphate buffer saline
60	PCR	Polymerase chain reaction
61	PEST	Proline-,glutamine-,serine-,threonine rich
62	PI3K	phosphatidyllinositol 3-kinase

63	psi	Pounds per square inch
64	qPCR	Semi-quantitative polymerase chain Reaction
65	RAM	RAM32 domain
66	RGS	regulator of G-protein signaling
67	RNA	Ribonucleic acid
68	SAPK	stress-activated protein kinase
69	SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
70	STAT	signal transducer and activator of transcription
71	TACE	Tumour-necrosis-alpha-converting enzyme
72	TAD	Transcriptional activator domain
73	TAK1	Transforming growth factor β–activated kinase 1
74	TIR	Toll-IL-1 receptor
75	TIRAP	TIR-containing adaptor inducing IFNβ
76	TLRs	Toll-like receptors
77	TMB	3,3",5,5"-Tetramethylbenzidine

78	TNFα	Tumor necrosis factor alpha
79	Tpl2	tumor progression locus 2
80	TRAF6	TNF receptor-associated factor 6
81	v	Volt
82	v/v	volume by volume
83	Val	Valine
84	x	Fold

CHAPTER I

BACKGROUND

Phosphoproteins exhibited interested protein to educated cross talk signaling between cells to cell. Because of about 30% of protein in Eukaryotic cells are phosphoprotein. Recently reports showed the phosphorylation level of numerous proteins changed during inflammatory response in LPS-stimulated macrophage. TLR4 recognized LPS and sent the signals to regulated immune response such as cytokines production TNFα, IL–6, IL-10 and nitric oxide. Not only TLR4 modulated immune response, but also Notch signaling. Notch is conserve signaling pathway. It plays an important role to fine tune many signaling pathways such as cell differentiation, proliferation, tumorigenesis including immune response. Changes in expression of Notch resulted in different response. But how Notch regulated LPS-stimulated macrophages remain unclear. This research will find the phosphoprotein that involved in TLR4 and Notch signaling using proteomic approach. Two-dimensional gel electrophoresis was selected to investigate involvement of them.

Objective

To identify and characterize phosphoproteins differentially modified in stimulated macrophages treated with inhibitor of Notch signaling pathway

CHAPTER II

LITERATURE REVIEWS

2.1 Phosphoproteins in cell signaling pathway

Signaling between cell to cell depends on crosstalks among many proteins. Phosphoproteins are major types of proteins critical for intracellular signaling. Phosphorylation is a reversible post translational modification as show in Figure 2.1 (1), (2) which alter the properties of protein for appropriate biological functions (1). In eukaryotes, phosphorylation is found on many amino acid residues such as glutamic acid, histidine, aspartic acid but mainly found on serine, threonine and tyrosine with the ratio of 1800:200:1(3). Phosphorylation at tyrosine residues of protein is frequently found in signaling adaptor proteins, while phosphorylation at serine and threonine are often found in active site of proteins. Alteration in phosphorylation may result in conveying different signal and affect outcomes of cell responses. These changes may lead to abnormal cell proliferation, maturation, development and cell death.

3
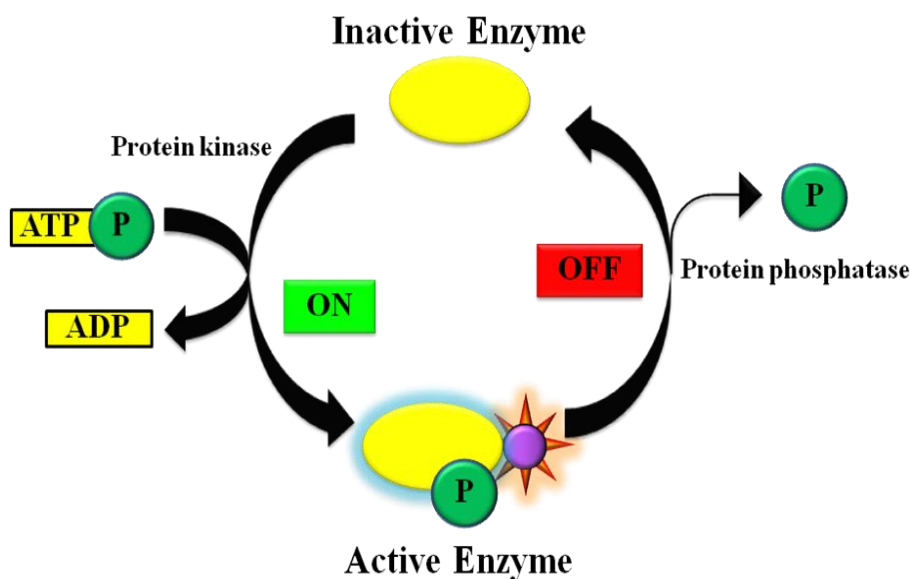
Figure 2.1 Protien phosphorylation and dephosphorylation. (Modified from David S, 2009)

The schematic reversible processes for adding or removing phosphate from protein. Phosphorylation is the process of adding phosphate to protein using an enzyme, protein kinase, while dephosphorylation is the process of displacing phosphate from phosphoprotein using an enzyme phosphatase.

2.2 Biological of Notch signaling pathway

Notch signaling plays critical roles in regulating development, differentiation and cell proliferation in various cell types (4). In mammals, Notch signaling consists of four receptors (Notch1-4) and five ligands (Jagged1, Jagged2, Delta-like1, Delta-like2 and Delta-like4) (5). Notch receptor is made up from three conserved domains, i.e. intracellular, transmembrane and extracellular membrane domains (6, 7). Extracellular domain of Notch receptor contains multiple epidermal-growth factor

Notch signaling pathway
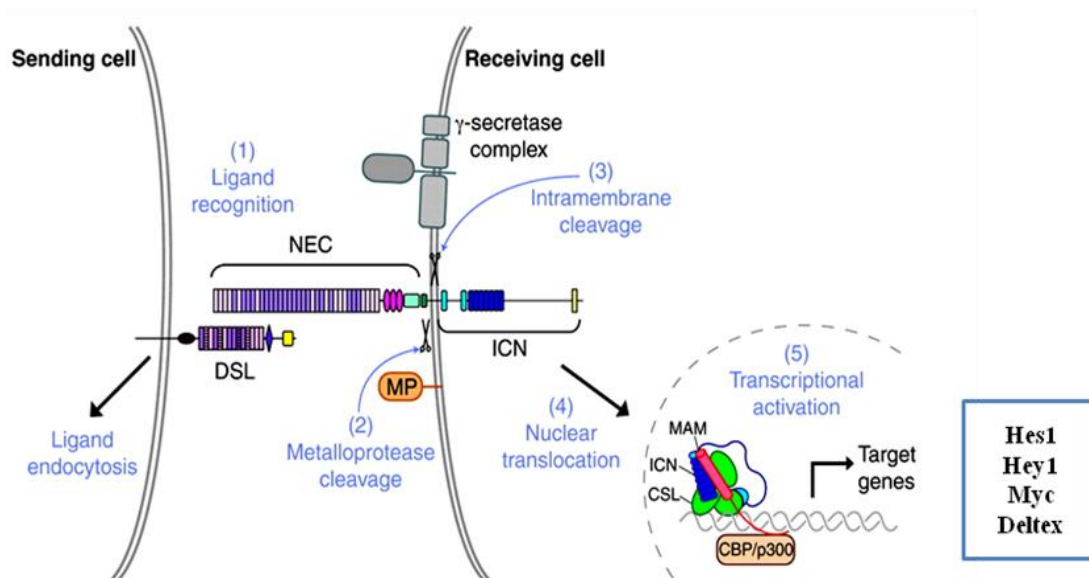
Figure 2.2 Schematic diagram of transcriptional activation of Notch signaling. (Modified from Gordon W. R. *et al*, 2008) (8)

(EGF)-like repeats that are responsible for ligand binding whereas an intracellular domain contains a RAM domain, the ankyrin repeats, a transcriptional activator domain (TAD) and the proline-, glutamine-, serine-, threonine rich (PEST) sequences (9). The activation of Notch signaling begin when extracellular domain of Notch receptor interacts with Notch ligand, a protease tumour-necrosis-alpha-converting enzyme (TACE) cleaves extracellular domain and an enzyme gamma-secretase cleaves between intracellular domain and transmembrane. These enzymatic cleavages allow an intracellular domain of Notch receptor to translocate into nucleus and bind to a transcription factor CSL (for CBF1/RBP-Jk in mammals, Suppressor of Hairless in *Drosophila*, and Lag-1 in *Caenorhabditis elegans*) (Figure2.2). After a transcriptional

complex is formed by recruitments of p300 and transcriptional co-activators, the target genes are expressed (6). Many Notch target genes are reported which includes basic helix loop helix transcription repressor in the family of Hairy-Enhancer of Split (HES), Hairy/enhancer-of-split related with YRPW motif 1 (HEY1) (6).

2.3 Toll-like receptor 4 signaling pathway
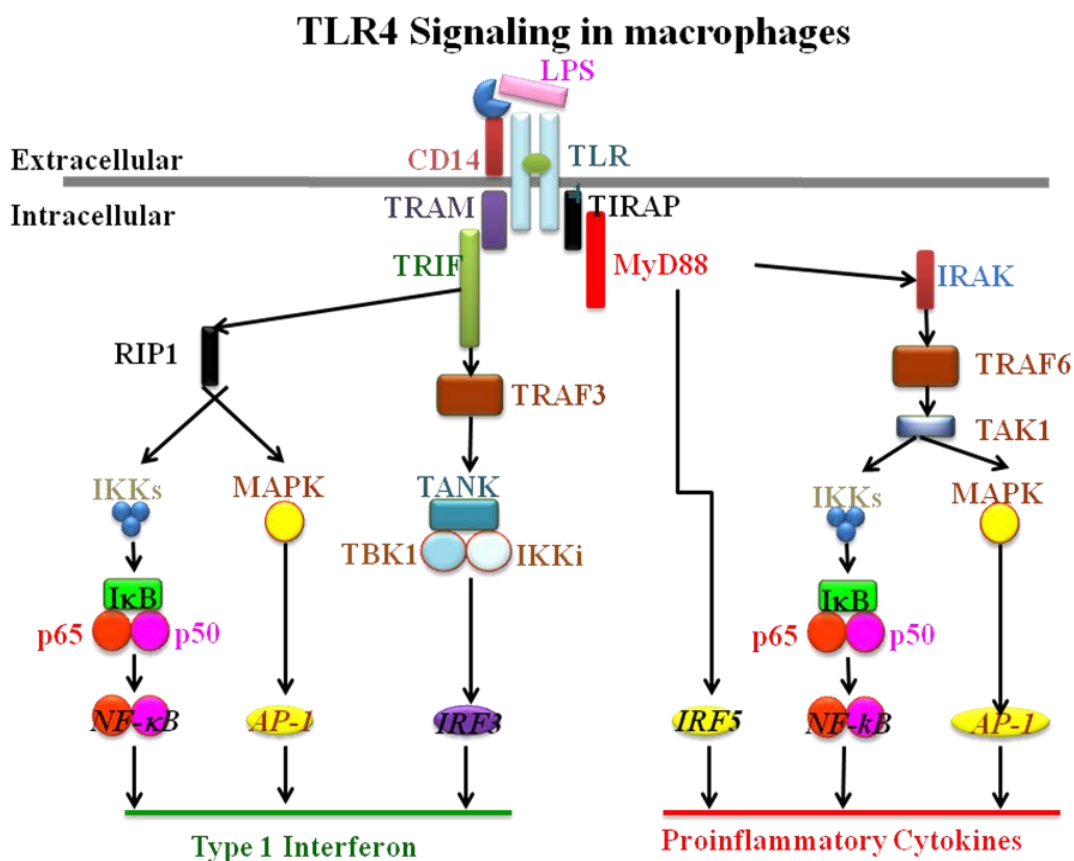
Figure 2.3 TLR4 signaling in macrophage. (Modified from Lu Y.C., *et al*, 2008)(10)

Toll-like receptors (TLRs) are pattern recognition receptors, which have more than 12 members in mammals (11). They function for recognition of molecular patterns such as lipopolysaccharides (LPS) and peptidoglycan. TLRs are expressed mostly on antigen presenting cell (APC) such as macrophages and dendritic cells (12). Stimulated macrophages using agonists of TLRs induces cytokine secretion and regulates inflammatory responses. In this study, the focus is placed on LPS (TLR4 antagonist) in macrophages. The mechanism of TLR4 signaling in mcrophage is shown in Figure 2.3. Beginning of TLR4 activation, LPS bind to LPS binding protein (LBP) which transfers LPS to CD14-accessory molecule. CD14 assist LPS bind to hydrophobic pocket of MD2, the complexes were essential for interaction with two molecules of TLR4(13). These LPS-TLR4 complexes recruit TIR-domain-containing adaptor protein (TIRAP), myeloid differentiation factor88 (MyD88), TIR-containing adaptor inducing IFNβ (TRIF) and other molecules such as TNF receptor-associated factor 6 (TRAF6), transforming growth factor β–activated kinase 1 (TAK1) to mainly activate the nuclear factor-κB (NF-κB), phosphatidyllinositol 3-kinase (PI3K)/Akt pathways and mitogen-activated protein kinase (MAPK). MAPK are classified into 3 types; extracellular signal-regulated kinases (ERK), stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) and p38 (14). However these pathways cooperated to response in TLR siganlaing pathway. For instance, the activation of NF-κB via IKK could activate ERK signaling (Figure 2.4) (15). Tpl2 is an intermediate protein between NF-κB and ERK signaling pathways (15). Tpl2 binds with NF−κBp105 in unstimulated macrophages to prevent Tpl2 get at MEK1-Tpl2 substrate (16). When macrophages are stimulated with LPS, NF−κBp105 is

phosphorylated by phospho-IKKβ which leads to partial degradation of NF−κBp105 via proteosome. Tpl2 was phosphorylated on serine290 for disassociation from NF−κBp105 by phospho-IKK (16). In addition, the phosphorylation on serine400 of Tpl2 leads to activate MEK1 before its degradation by proteosome. Activated MEK1 can activate ERK signaling pathway, resulting in increased cytokine production such as IL-10.
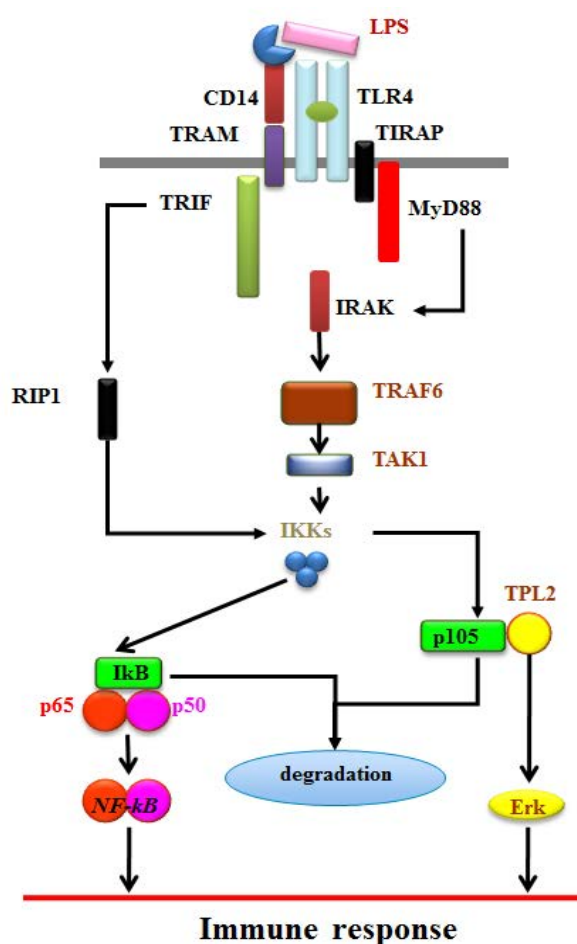
Figure 2.4 Cooperation of NF-κB and MAPK (ERK) during LPS-stimulated macrophage

Recently evidences reported that TLR4 could modify G-protein couple receptors (GPCRs) by activated regulator of G-protein signaling (RGS) in dendritic cells to control chemokine production (17). Furthermore G-protein-coupled receptor kinase 2 (GRK2)-function in phosphorylation or dephosphorylation of GPCRs is a negative regulator of NF-kB and ERK. Macrophages from GRK2 deleted mice exhibits enhanced p105 and ERK activity and macrophage inflammatory protein-1α (MIP-1α), interleukin-10 (IL-10), tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) production. These pathways regulate the balance between pro-and anti-inflammation (14).

2.4 Crosstalk between Notch and TLR4 signaling

Previous reports indicated the crosstalk between TLR4 and Notch signaling (18, 19). LPS induced Notch1 up-regulation through TLR4 both in murine bone marrow-derived macrophages and RAW264.7-macrophage cell line (18). When LPS-stimulated macrophage was treated with Notch inhibitor, IL-10 was increased but IL-6 and inducible nitric oxide synthase (iNOS), TNFα were decreased (18). Notch signaling fine tunes the responses to LPS by inducing HES1 and HEY1 to decrease expression of IL-6 and interleukin-12 (IL-12) (19). While interferon-gamma inhibited Notch2 and downstream transcription process, resulting in higher inflammatory responses (19). Both results indicated that Notch signaling regulates inflammatory responses in macrophages by cross regulating with the TLR signaling.

TLR signaling is involved more than 340 proteins and 400 reactions (20). Study from stable isotope labeling with amino acids in cell culture followed by Liquid

Chromatography–Mass Spectrometry and Liquid chromatography-Tandem Mass Spectrometry (LC-MS/MS) demonstrated that LPS-stimulated macrophages increased phosphoproteins by 24% and decreased 9% (20). Main pathways which are well known to regulate responses of LPS-stimulated macrophages are mTOR, EGFR1, cytoskeleton proteins, TNFα and MAPK (20). NF- κB pathway is the core pathway in response to recognition of microbial antigens by TLRs. It requires phosphorylation of IκB, a suppressor molecule, and targets it for ubiquitin-mediated degradation (21). Taken together, these results strongly support that phosphorylation is important post-translation modification which controls responses to LPS through TLR. However the crosstalk between TLR and Notch signaling via phosphoproteins has not been reported.

2.5 Two-dimensional gel electrophoresis (2D-GE)

There are several techniques available to dates to analyze phosphorylation of proteins. The key technique is the method of enrichment of phosphoproteins in samples (22). Phosphoprotein-specific antibody is wildly used to enrich phosphoproteins (23). This method depends on specificity and affinity of antibody to phosphoprotein, precipitate phosphoprotein-antibody complex. Therefore, it requires highly specific antibody to phosphoprotein and it is difficult to detect low level of proteins. Moreover the complexity and resemblance of proteins lead to hard distinguish them. 2D-GE is well performance technique to resolve this problem. It able to separate proteins according to iso-electric focusing point follows to molecular weight of proteins. 2D-GE composes of five steps; first, sample preparation; second,

iso-electric focusing (IEF); third, equilibration; forth, sodium dodecyl sulfate polyacrylamide gel electrophoresis; and fifth, protein detection (24).

First, sample preparation is critical step for this technique. It has not standard method for all protein types. By the way, the important point is to solubilize the most of protein in solution (24). The component of reagents in this step contains five substances. The substance which disrupts hydrogen-bond of protein is widely used such as urea and thiourea. Zwitterionic detergents (non-ionic) such as 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPs), sulfobetaine (SB3-10), TritonX-100 and amidosulfobetaine (ASB-140) are used to break hydrophobic interaction. Reducing agent such as dithiothreitol (DTT) and tributyl phosphine (TBP) not only apply to reduced disulfide bond to sulhydryl group but also assist to soluble proteins. The trace amount of bromophenol blue is used as monitoring dye. Finally, ampholyte is used to increased protein solubility. Furthermore, the contaminants-DNA, salts, lipid- need to remove from protein for good separating on 2D-GE (24).

Second, iso-electric focusing step generally apply as describe in user manual. The concerns in this step are avoided high temperature during running IEF because it breakdown urea. The breakdown of urea will be produced cyanate that reacts with the protein result in more acidic of protein. Moreover, salts are important one. High concentration of salts in samples leads to high electric current that increase temperature generation cause urea breakdown (24).

Third, equilibration step is performed to treat the focused protein in IEF gel with the solution that contains SDS, DTT and iodoacetamide. SDS is necessary added because of an iso-electric focusing point, proteins do not move along the electric current. Saturation proteins with SDS result in proteins contain positive charge so proteins can be displaced from anode to cathode when apply current. DTT is used as the same object in IEF step and iodoacetamide assists to prevent recombination of sulfhydryl groups (24).

Forth, SDS-PAGE can be done as same as the laboratory protocol. In this step, protein will be separated base on their molecular weight. Long distance of separation gel and low electric current give the better proteins separation (24).

The last step, the detection of proteins in gel has several method such as colloidal Coomassie Brilliant Blue (CBB), SYPRO Ruby, silver stain etc. These dyes have the different sensitivity to detection. CBB is the lowest sensitivity about 10 ng protein/spot but it useful for endpoint detection. SYPRO Ruby, fluorescence dye, can detect 1 ng protein/spot. Nevertheless SYPRO Ruby staining requires special equipment to signal detection. Silver stain is equal sensitivity to SYPRO Ruby and can be visualized by eye but this staining is difficult to reproducible (24). The selection of staining method depend on experiment not is the best for all.

In this study phosphoproteome in murine macrophage cell line, RAW264.7, stimulated with LPS was studied which compared the phosphoproteomic patterns in the presence and absence of inhibitor of Notch signaling pathway.

CHAPTER III

MATERIALS AND METHODS

3.1 Cell culture

3.1.1 Cell line

RAW264.7, a mouse macrophage cell line, (ATCC TIB-71), was used in this study. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Logan, UT, USA) which was supplemented with 10% (v/v) fetal bovine serum (Hyclone), 1% (w/v) sodium pyruvate (Hyclone), 1% (w/v) HEPES (Hyclone), 100 U/ml penicillin (M&H manufacturing Co. Ltd, Bangkok, Thailand) and 0.25 mg/ml streptomycin (M&H manufacturing Co. Ltd) and incubated at 37°C in a humidified 5% (v/v) CO_2 incubator.

3.1.2 Cell preparation

RAW264.7 cell line was cultured in non-treated tissue culture plate (Hycon, Germany) in 7 ml complete DMEM. To prepare cells for experiment, cells were collected by cold PBS (Appendix D). Cells were centrifuged at 1000 rpm for 5 min (Hettich ROTOFIX 32 Benchtop Centrifuge, USA). The culture supernatant was discarded and cell pellets were resuspended in complete DMEM. Viable cell numbers were counted using trypan blue dye (Hyclone, England) exclusion method and plated into the tissue culture treated plates.

3.1.3 Cell preservation for storage

After harvesting, RAW264.7 cell line was resuspended in DMEM freezing medium containing 10% DMSO (v/v) (Sigma Aldrich, USA) in complete DMEM and kept in cryogenic vials (Corning Incorporation, USA). The vials were stored at -80°C for short term storage. For long term storage, the vials were kept at -80°C overnight and transferred to the liquid nitrogen tank (Taylor Wharton, USA).

3.1.4 Thawing cells

Stored RAW264.7 cell line was rapidly thawed in the water bath (Memmert, Germany) at 37°C, and 9 ml of serum-free DMEM media were added. Cells suspension was centrifuged at 1000 rpm for 5 min. The supernatant was discarded and replaced with 7 ml of complete DMEM. Cells were maintained and cultured as described above.

3.2 Western Blot

3.2.1 Protein extraction and measurement

Cell lysates from cell treated as indicated in each experiment were extracted using method that described previously by Palaga et al. (2003). In short, culture supernatant was removed from plate and cells were washed by 1 ml of cold PBS and subsequently by 200 μl buffer A (Appendix D). The proteins were extracted using 30-150 μl buffer B (depending on the condition of an experiment). Cell lysates were transferred to 1.5 ml microcentrifuge tubes (Axygen Scientific, USA) and mixed by

vortex mixer for 1 min before centrifugation at 6000Xg for 5 min at 4°C. The clear supernatants were kept on ice until analysis or -80°C for further analysis.

Measurement of protein concentration in samples was performed using BCA (bicinchoninic acid)^{TM} protein assay kit (PIERCE, USA), according to manufacturer's guideline. Bovine serum albumin (BSA) was used as protein standard at 1, 0.5, 0.25, 0.125, 0.063 and 0.031 mg/ml and the samples were diluted in sterile double distilled water at 1:10 in 96-well microtiter plate (Corning Incorporation, USA). Two hundred microliters of working reagent which contained reagent A and reagent B mixed at the ratio of 50:1 was added into each well. The plate was incubated at 37˚C for 30 min before measuring the absorbance at 540 nm using microplate reader (Anthos 2010, UK).

3.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared following the recipe shown in Appendix D. One hundred to thirty micrograms of protein samples and Laemmli buffer (Appendix D) were mixed and heated at 100˚C for 5 min in the heat box (Thermomixer Compact, Eppendorf, Germany) before loading to the gels. Protein ladder (Fermentas, Canada) was used as a reference of molecular weight. The samples were separated using a constant volt for at least 90 min in running buffer for SDS-PAGE using mini Protein III system (Bio-Rad, USA).

3.2.3 Western Blot

After separating proteins by SDS-PAGE, gels were equilibrated in transfer buffer (Appendix D) for 5 min. Polyvinylidene fluoride (PVDF) membranes (GE Healthcare, USA) were prepared by soaking in absolute methanol (Merck, Germany), and rinsed by double distilled water twice before submerging in transfer buffer.   Six pieces of Whatman filter paper were prepared and immersed in the transfer buffer. For protein transfer, three pieces of filter paper, PVDF membrane, gel and 3 pieces of filter paper were set up in the semi-dry transfer Transfer-Blot$^{®}$ SD (Bio-Rad, USA) instrument and air bubbles were carefully removed. The transfer was performed using a constant current at 90 mA (for one gel) or 150 mA (for two gels) for 90 min.

3.2.4 Antibody probing

After protein transfer, PVDF membranes were blocked twice in blocking solution (Appendix D) for 5 min each on rocking platforms (Labnet Rocker 25, Labnet International Inc, USA). The PVDF membranes were later probed with primary antibody at 4°c in refrigerator overnight. The working dilution of the primary antibodies that were used in this study was summarized in Table 1. After this step, the membranes were gently shaked for 1 h on a rocker at room temperature, and the primary antibody was removed. The PVDF membrane was washed with PBST (Appendix D) for 5 min and 15 min twice for each step before it was probed with secondary antibody that conjugated with horse-radish peroxidase (Amersham Biosciences, UK). The working dilution of the secondary antibodies that were used in

this study was summarized in Table 1. The blots were shaked for 1 h on the rocker and washed with PBST 5 min twice and 15 min for 3 times before detection.

3.2.5 Protein detection by chemiluminescence and autoradiography

The recipe of substrates for chemiluminescent detection was shown in Appendix D. In short, substrate solution composed of solution A and solution B was freshly prepared and mixed immediately before incubation with the blots. After one minute of incubation, the blots were wrapped with plastic wrap and set in Hypercassette (Amersham Bioscience, UK) for X-Ray film (for High Performance Chemiluminescence Film: Amersham Hyperfilm^{TM} ECL (Amersham Bioscience, UK) exposure in the dark room. The exposure time of each protein varied depend on experiment. The exposed film was developed in an X-ray film developer solution for 5 second, washed with water, and immersed in X-ray film fixer solution for 1 min and washed again with water in the final step.

Table 2.1 Antibodies used in Western Blot

Antigen	Working Dilution of primary antibody	Working Dilution of secondary antibody
Cleaved Notch1 (Val 1744)[1]	1:1000	1:2000 (Donkey anti rabbit)
Notch1[2]	1:2000	1:2000 (Donkey anti rabbit)
β-actin[3]	1:10000	1:5000 (Goat anti mouse)
Phosphos-p65[1]	1:2000	1:2000 (Donkey anti rabbit)
Total-p65[1]	1:2000	1:2000 (Donkey anti rabbit)
Phospho-IkBα[1]	1:2000	1:2000 (Donkey anti rabbit)
Total-IκBα[1]	1:2000	1:2000 (Donkey anti rabbit)
Phospho-ERK[1]	1:4000	1:4000 (Donkey anti rabbit)
Total-ERK[1]	1:4000	1:4000 (Donkey anti rabbit)
Phosphos-JNK[1]	1:2000	1:4000 (Donkey anti rabbit)
Total-JNK[1]	1:2000	1:2000 (Donkey anti rabbit)
Phospho-p38[1]	1:4000	1:4000 (Donkey anti rabbit)
Total-p38[1]	1:4000	1:4000 (Donkey anti rabbit)
Tpl2[1]	1:1000	1:1000 (Donkey anti rabbit)

Antigen	Working Dilution of primary antibody	Working Dilution of secondary antibody
Phospho-IKKα/β[1]	1:500	1:1000 (Donkey anti rabbit)
Total- IKKα[1]	1:4000	1:4000 (Donkey anti rabbit)
Total- IKKβ[1]	1:4000	1:4000 (Donkey anti rabbit)
Phospho-Akt[1]	1:2000	1:4000 (Donkey anti rabbit)
Total-Akt[1]	1:2000	1:4000 (Donkey anti rabbit)
Tpl2[2]	1:1000	1:1000 (Donkey anti rabbit)

[1]. antibodies obtained from Cell signaling Technology, USA

[2]. antibodies obtained from Santa Cruz Biotechnology, USA

[3]. antibody obtained from Chemicon International,USA

3.3 Two-dimensional gel electrophoresis (2D-GE)

3.3.1 Protein clean-up

One hundred and fifty micrograms of proteins from indicated condition were subjected to removal of lipid, nucleic acids, salts and other contaminants by 2D clean-up kit (Bio-Rad, USA). The process was followed according to the manufacturer's instruction. Briefly, cell lysates were transferred to a 1.5 ml microcentrifuge tube and 300 μl of precipitating agent 1 were directly added into the lysates. The mixture was mixed well by vortexing, and incubated on ice for 15 min. Next, 300 μl of precipitating agent 2 were added to the protein mixture, and then it was mixed well by vortexing and centrifuged at 12,000Xg for 5 min. The supernatants were carefully removed using a pipette and the microcentrifuge tubes were centrifuged again at 12,000Xg for 15 second. The residual liquid was removed without disturbing pellet. After this step, the wash reagent 1 was added on the top of pellet. The microcentrifuge tubes were centrifuged at 12,000Xg for 5 min and the liquid was carefully removed. Twenty-five μl of Ready-Prep proteomic grade water was added to the pellets, and the mixture was mixed well by vortexing before adding 1 ml of wash reagent 2 (prechilled at -20°c at least before used) and 5 μl of wash 2 additive to the protein pellet. The mixture was mixed well by vortexing. The protein mixture was incubated at -20°C for 30 min with occasional mixing every 10 min. After that, the microcentrifuge tubes were centrifuged at 12,000Xg for 5 min. The supernatants were carefully removed using a pipette, the microcentrifuge tubes were centrifuged again at 12,000Xg for 15 second and the residual liquid was removed without disturbing pellet. The pellets were air dried at room temperature for 2-3 min, before dissolving in

rehydration solution (Appendix D) containing 7M urea (GE Healthcare Bio-Science, Sweden), 2M thiourea (Merck, Germany), 0.2 %w/v 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPs) (Bio-Rad), 50 mM Dithiothreitol (DTT) (Sigma Aldrich), IPG buffer pH 4-7 (GE Healthcare Bio-Science) and 0.002 % w/v bromophenol blue. The proteins were incubated at room temperature for 3-5 min and mixed well by vortexing for dissolving the protein pellet. Finally, the proteins were centrifuged at 2000Xg for 5 min and the clear supernatants were used for further analysis.

3.3.2 Iso-electric focusing

Clear solutions obtained as described above were loaded into IPG strips pH 4-7 (GE Healthcare Bio-Sciences) and incubated at room temperature in an equilibration tray (Bio-Rad, USA) for 1 h. The IPG strip and the remaining solution were transferred to an iso-electric focusing tray. The strips were overlaid with 1 ml of mineral oil. Five micro-Ampere of electric current was applied onto the tray to rehydrate the strips for 16 h. Before moving to the iso-slectric focusing steps, the mineral oil was carefully removed from the strips in the iso-electric focusing tray using forceps by holding the strips vertically about 7-8 seconds. The remaining oil was absorbed using wet Whatman filter paper. Next, wet paper wicks were placed on both electrodes before putting the strips on them. One milliter of fresh mineral oil was overlaid again on the strip. The current was applied to the strips according to the following steps; step 1 applying linear current from 0v to 250v within 20 min, step 2 applying linear current from 250v to 4000v within 2 h and finally, step 3 applying rapidly current from 4000v to 12,000v.

3.3.3 IPG Equilibrations

After completing the iso-electric focusing step, the mineral oil was carefully removed from the IPG strips using forceps by holding the strips vertically about 7-8 second, and the residual oil was absorbed onto the wet Whatman filter paper. Next, the IPG strips were equilibrated in 4 ml Equilibration buffer I (Appendix D) which composed of 6 M urea, 2% SDS (Bio-Rad, USA), 0.375 M Tris-HCl pH 8.8 (Bio-Rad, USA), 20% glycerol (Merck, Germany) and 2% (w/v) DTT which was added immediately before used and it was shaked with low speed on the rocker at room temperature for 10 min. The strips were gently moved from Equilibration buffer I to Equilibration buffer II (Appendix D) which contained 6 M urea, 2% SDS (Bio-Rad, USA), 0.375 M Tris-HCl pH 8.8 (Bio-Rad, USA), 20% glycerol (Merck, Germany) and 1% (w/v) iodoacetamide which was added immediately before used and the strips were shaked with low speed on the rocker at room temperature for 10 min

3.3.4 SDS-PAGE for 2D-GE

Twenty-seven milliliter of 10% SDS-PAGE gels were prepared following the recipe in Appendix D. The side-up strips were laid on the back plate of SDS-PAGE gel using forceps and gently in contact with the gel without air bubbles between them. The protein ladder was added to the filter paper (0.5x1 cm.) and it was placed near the strip. After this step, an overlay agarose (Bio-Rad, USA) which contained 0.5% low melting point agarose in 25 mM Tris, 192 mM glycine, 0.1% SDS and a trace of Bromophenol blue was added into the gel to covere the strips. The step was let stand

at room temperature for 5 min before setting the equipment PROTEIN® II xi Cell (Bio-Rad, USA). The proteins were separated using the constant current at 20 mA for 60 min and 25 mA for 210 min.

3.3.5 Phosphoprotein staining and detection

After the 2D-GE step, phosphoproteins in the gels were stained using ProQdiamond phosphoprotein gel stain (Invitrogen, UK) according to the manufacturer's instruction. In brief, gels were fixed in a fix solution that contained 50% methanol and 10% acetic acid 30 min twice with gently shake, and the gels were immersed in fix solution overnight. Next, gels were washed with sterile deionized ultrapure water for 10 min for 3 times and stained with ProQdiamond phosphoprotein gel stain for 60 min in dark. After that, gels were destained using destaining solution that composed of 20% acetonitrile and 50 mM sodium acetate pH 4, 30 min for 3 times. Finally, the gels were washed twice with sterile deionized ultrapure water for 5 min for each wash. Fluorescent signals were visualized using Phosphoimage Typhoon Trio^{TM} (GE Healthcare) (Central Instrument Facility, faculty of Science, Mahidol University).

3.3.6 Total protein staining

3.3.6.1 Coumassie blue R-250

After ProQdiamond staining, the gels were stained by Coumassie blue R-250 (Bio-Rad, USA) for total protein detection and visualized by ImageScanner™ II (GE Healthcare) (Faculty of Medicine, Chulalongkorn University).

3.3.6.2 Silver staining

Gels were stained with Silver Stain Plus (Bio-Rad, USA) by following the manufacturer's instruction. In brief, all containers were rinsed with 50% nitric acid after cleaning with detergent and wash thoroughly with sterile deionized ultrapure water at least 3 times. Gels were placed in Fixative Enhancer Solution, which contained 50% v/v methanol, 10% v/v acetic acid, 10% v/v Fixative Enhancer Concentrate and 30% sterile deionized ultrapure water, and gently shake for 20 min. Secondly, Fixative Enhancer Solution was decanted and replaced with sterile deionized ultrapure water gently shake 10 min for 2 times. During this step, the Staining Solution was prepared by adding 35 ml sterile deionized ultrapure water, 5 ml Silver Complex Solution, 5 ml of Reduction Moderator Solution and 5 ml of Image Development Reagent and gently agitated. The Development Accelerator Solution was prepared by dissolving 2.5 g of Development Accelerator reagent in 45 ml water and the volume was adjusted to make 50 ml. After this step, the staining solution was immediately added into the Development Accelerator solution, and swirled well before directly pouring on to the gels. Gels were incubated and gently shaken for 17 min. Next, the staining step was stopped by incubating gels in 5% v/v acetic acid in deionized ultrapure water for 15 min and the gels were briefly rinsed with deionized ultrapure water. The gels were photographed by ImageScanner™ II at Faculty of Medicine, Chulalongkorn University.

3.3.7 2D-GE analysis using Image 2D platinum

The intensity of phosphoproteins and total proteins in 2D-GE gel were analyzed by ImageMaster™ 2D Platinum v7.0.

3.4 In-gel digestion

The in-gel digestion procedure was done by following Shevchenko et al, 2006 (25). In brief, the gels were rinsed with sterile deionized ultrapure water for a few hours, and the interested spots were excised using sterile pipette tip. The spots were transferred to a microcentrifuge tubes and washed with sterile deionized ultrapure water several times. This step was followed by addition of 500 μl of acetonitrile into the gels and the spots were incubated for 10 min until the gels became white and sticky. All liquid were removed and 30 μl of 10 mM DTT in 100 mM ammonium bicarbonate (freshly prepared before used) (Appendix D) were added. The mixture was incubated at $56^{o}C$ for 30 min. The microcentrifuge tubes was chilled to room temperature before adding 500 μl of acetonitrile,. The mixture was incubated at room temperature for 10 min and all the liquid were removed. Thirty μl of 55 mM iodoacetamide in 100 mM ammonium bicarbonate (freshly prepared before used) (Appendix D) was added to the gels and the gels were incubated at room temperature in dark for 20 min, condensed with 500 μl of acetonitrile and all liquid were removed. Next, the gels mixed with 100 μl of 100 mM ammonium bicarbonate/acetonitrile ratio 1:1 (v/v) (Appendix D), incubated for 30 min and mixed by vortexing every 10 min. Afterward 500 μl of acetonitrile were added to the gels, and the mixture was incubated at room temperature with vortexing every 10 min for 30 min. All liquid was

removed and replaced with 5 µl trypsin buffer (Appendix D), which contained 10 mM ammonium bicarbonate containing 10% v/v acetonitrile, kept them on ice box for 30 min. Lastly, all solution were removed by decantation and the gels were saturated with 5 µl 13 ng/µl trypsin (Appendix D), incubated on ice for 60 min, and 3 µl trypsin buffer was added at lateral surface of microcentrifuge tubes. The tubes were incubated at 37°c in incubator overnight. The next day, the tubes was cool down to room temperature before adding 100 µl of extraction buffer (Appendix D) that composed of 5% formic acid/acetonitrile ratio 1:2 (v/v) incubated at 37°c in the shaker for 15 min. The supernatants were withdrawn using fine pipette tip and dried in a vacuum centrifuge Eppendorf Concentrator 5301 (Eppendorf, Germany). The tryptic peptides were analyzed by HRESIMS nano-LC (EASY-nLCII, Bruker Daltonics, German)-MS/MS (microTOF-QII, Bruker Daltonics, German) at Department of Chemistry, Faculty of Science and Faculty of Medicine, Chulalongkorn University.

3.5 LC-MS/MS data analysis

The results from LC-MS/MS were subjected to database search by online on Mascot database. The outputs of these results were compared between conditions, cross checked with iso-electric focusing point and molecular weight of the excised spots.

3.6 RNA extraction

Cells were treated as indicated experiment, culture supernatant had collected and RNA was extracted using TRIzol® (Invitrogen, UK). In brief, cells were lysed directly using 1 ml TRIzol®, and cell lysate were aspirated up and down for 7-8 times and the

total cell lysates were transferred to the microcentrifuge tubes. The samples mixture was left at room temperature for 5 min before adding 200 µl chloroform per 1 ml TRIzol®. The mixture was vigorously shaken for 15 seconds and incubated at room temperature for 3 min. Samples were centrifuged at 12,000Xg for 15 min at 4°C, and the aqueous phase was carefully transferred to the new microcentrifuge tube. After this step, 0.5 ml iso-propanol per 1 ml TRIzol® was added to the samples and the tubes were inverted to mix. The samples were incubated at room temperature for 10 min prior to centrifugation at 12,000Xg for 10 min at 4°C. Supernatants were discarded and washed with 75% ethanol in DEPC water (Appendix D). The microcentrifuge tubes were mixed by vortexing and centrifuged at 7,500Xg for 10 min at 4°C. Finally, RNA were air dried at room temperature before adding 20 µl DEPC water and incubated for 10 min at 55-60°C to dissolve an RNA pellet. Total RNA samples were kept at -80°c until use.

3.6.1 RNA quantitation using Quant-iT™ RNA Assay Kit (Invitrogen, UK)

According to manufacturer's protocol, Quant-iT™ working solution per reaction was prepared by mixing Quanti-iT reagent to Quanti-iT buffer at the ratio of 1: 200 (1 µl of Quanti-iT reagent and 199 µl Quanti-iT buffer). Ten microliter of RNA standard (1 and 2) was added to 190 µl of Quant-iT™ working solution to make an RNA concentration of 0 and 10ng/µl respectively. One microliter of RNA samples were diluted in 9 µl DEPC water, mixed well and added 2 ul of diluted RNA samples into 198 µl Quant-iT™ working solution. The tubes were mixed well by vortexing and subjected to measurement of RNA concentration using Qubit™ fluorometer

which was calibrated by RNA standard. Samples' RNA concentration was calculated using the following equation.

$$\text{Concentration of RNA (ng/}\mu\text{l)} = \text{measured RNA x dilution factor}$$

3.7 Reverse transcription for complementary DNA (cDNA) synthesis

One hundred ng to 1 μg of sample RNA were used as template to synthesize cDNA by mixing with 0.2 μg Random hexamers (Qiagen, Germany). DEPC water was used to adjust the volume to 12.5 μl. The solutions were heated at 65°C for 5 min, and cooled down to 4°C on ice. After this period, 1xRT buffer (Fermentas, Canada), 1 mM dNTP mix (Fermentas, Canada), 20U of RNase inhibitor (Fermentas, Canada) and 200U of Reverse transcriptase (Fermentas, Canada) were added into each reaction. All tubes were mixed well before put into the PCR machine Bioer Life Express (Bioer technology, China) using following steps ; 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 Semi-Quantitative polymerase chain reaction (qPCR)

The qPCR was carried out according to manufacturer's protocol. Two μl of cDNA solution was added to qPCR solution that composed of 1xMaxima$^{\text{TM}}$ SYBR Green/ROX qPCR (Fermentas, Canada) or iQ™ SYBR® Green Supermix, BioRad, USA), 0.3 μM forward and reverse primer and 9 μl of nuclease free water (Each sample was performed in triplicate). The qPCR was performed in the MJ Mini personal Thermal Cycler (BioRad, USA). The nucleotide sequences of primers used in this study and the annealing temperatures of qPCR conditions were summarized in

Table 2.2 The nucleotide sequence of primers and annealing temperature of qPCR condition.

Gene	Primer sequence		Annealing Temperature (°C)	PCR product (bp)	Reference
mβ-actin	Forward	5'-ACCAACTGGGACGACATGGAG-3'	55	385	Palaga et al. (2008)
	Reverse	5'-GTGGTGGTGAAGCTGTAGCC-3'			
rgs19	Forward	5'- AGGTTTATAGCAGGAGAA-3'	52	180	-
	Reverse	5'- TTGGAATGTAGAGGAGTA-3'			
mtnfα	Forward	5'-CCTGTAGCCCACGTCGTAGC-3'	55	374	Lee et al, (2007)
	Reverse	5'- TTGACCTCAGCGCTGAGTTG-3'			
mil-6	Forward	5'- CATGTTCTCTGGGAAATCGTGG-3'	52	78	Palaga et al. (2008)
	Reverse	5'- AACGCACTAGGTTTGCCGAGTA-3'			
mil-10	Forward	5'- TCAAACAAAGGACCAGCTGGACAACATACTGC-3'	58	430	Palaga et al. (2008)
	Reverse	5'- CTGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3'			
miNOS	Forward	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'	60	499	Lee et al, 2007
	Reverse	5'-GGCTGTCAGAGCCTCGTGGCTTTG-3'			

Table2. The relative expressions of mRNA levels were calculated according to Livak K.J., et al 2001(27).

3.9 siRNA silencing

siRNAs were custom synthesized by Qiagen (QIAGEN, Germany). NCBI reference sequence for siRNA synthesis is NM_026446.3. The sequence of each oligo was shown in Table 3. The transfection of siRNA was carried out using HiPerFect Transfection according to the manufacturer's guideline. In brief, cells (1×10^5 cells) were plated in 24-well tissue culture plates (Corning, USA) and transfection experiment was immediately performed by diluting 375 ng of synthesized siRNA in 100 µl serum free medium (Opti-MEM) and 6 µl of HiPerFect Transfection reagent (Qiagen, Germany) was added to the siRNA. The mixture was briefly mixed by vortexing and spined down. The mixture was incubated at room temperature for 5 min before pipetting onto cells dropwise. The plates were gently swirled and incubated at 37 $^\circ$C in a humidified 5% (v/v) CO_2 incubator for 6 h before add 400 µl complete DMEM.

Table 2.3 Target sequences of siRNA for RGS19 silencing

siRGS19 no.	Target sequence
siRGS19 no.1	AAGCCTCTGTTTATCCCACAA
siRGS19 no.4	AAGGCTTCAGATGCAAGTAAA
siRGS19 no.5	CACAGTGTCATCTTAATCTTA
siRGS19 no.6	TTCCAAAGACATGAAATTGTA

3.10 Measurment of TNFα by Enzyme-linked immunosorbent assay (ELISA)

Culture supernatant from indicated experiments was collected and subjected to detection of TNFα by ELISA (PeproTech, UK). Briefly, ELISA MaxiSorp™ plates (Corning Incorporation, USA) were coated with 1 μg/ml capture antibody against TNFα in refrigerator overnight. Capture antibody were removed and the plates were washed 4 times with 200 μl PBST. Two-hundred microliters of blocking solution were added into each well incubated for 1 h at room temperature. Then plates were washed 4 times by PBST. One hundred microliters of murine TNFα standard was dilute to 2, 0.62, 0.2, 0.06 and 0 ng/ml and the samples were diluted in blocking solution to appropriated dilution. Plated were incubated 2 h at room temperature before washed 4 times by PBST. Detecting antibody (0.25 μg/ml) were added 100 μl per well incubate 2 h at room temperature. Plates were washed 4 times by PBST. Avidin-HRP Conjugate dilution were diluted in blocking solution at 1:2000 and added

100 µl per well and incubated at room temperature for 30 min. Plates were washed 4 times by PBST before added ELISA substrated (Appendix D) 100 µl per well. Plates were incubated in dark for color development. After that the reactions were stopped by 100 µl of 1M H_2SO_4. The absorbance was measured at 450 nm by microplate reader (Anthos 2010, UK).

3.11 Propidium iodide staining for cell cycle analysis

Cells were treated as indicated and harvested at indicated times. Cells were washed by cold PBS once before resuspend in 200 µl cold 70%Ethanol (Appendix D). The fixed cells were incubated on ice at least 4 h or kept at -20°C until use. After that 70% ethanol was removed from cells by centrifugation at 2000g for 5 min and washed with 500 µl PBS for 1 time. Cells pellet were resuspend in 250 µl PBS which contained 0.1 mg/ml RNaseA (Appendix D) incubated at 37°C for 30 min. Finally twelve point five microliters of 1 mg/ml propidium iodide was added into each sample incubate at room temperature in dark at least 30 min before cell cycle were analzed by Flow cytometer FC500 (Beckman Coulter, USA). The data were analyzed by CXP analysis software.

3.12 Statistical analysis

The data in this research was calculated the level of significant using one-way ANOVA and pair sample t-test. A p-value less than 0.05 was determined significant.

CHAPTER IV

RESULTS AND DISSCUSSIONS

4.1 Detection of cleaved Notch1 in RAW264.7 activated by LPS

Activation of Notch signaling could be examined by various ways such as the expression of Notch target genes, *Deltex* (28), *Hes1*(28) and the detection of cleaved form of Notch receptors (29). Previous studies showed that the expression of these markers increased during LPS stimulation of macrophages. However, TLR signaling in response to LPS rapidly induced many signaling pathways such as NF-κB, MAPK, Akt and mTOR (30). In this study, we investigated the events during immediate early stages after LPS stimulation of macrophages which led to activation of Notch signaling. RAW264.7, a mouse macrophage cell line, were stimulated with LPS for 0, 30, 60, 90, 120 and 180 min and protein lysates were extracted and the appearance of cleaved Notch1 was detected by Western blot. The results in Figure 1 showed that the presence of cleaved Notch1 (Val1744) was readily detected at 1 h after LPS stimulation while the level of total Notch1 remained unchanged.
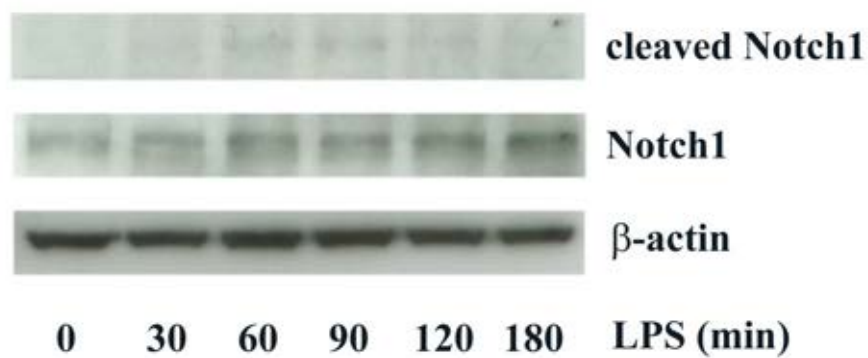
Figure 4.1 Cleaved Notch1 (Val 1744) and total Notch1 in LPS stimulated RAW264.7

Cell lysates from LPS-stimulated RAW264.7 cells for 0, 40, 60, 90, 120 and 180 min were separated by Western Blot. Antibody against cleaved Notch1 (Val1744) and total Notch1 were probed subsequently and β-actin was use as a loading control.

4.2 LPS-stimulated Notch1 cleavage in RAW264.7 were inhibited by GSI (DAPT) treatment

Generally, Notch signaling could be inhibited at different steps that were previously described. In this experiment, the inhibition of Notch signaling was performed by targeting the gamma-secretase to prevent cleavage between Notch intracellular domain and Notch transmembrane using a specific GSI, DAPT. Pretreatment of RAW264.7 with 25 µM DAPT or vehicle control DMSO for 1 h was performed before stimulation of cells with LPS for another 1 h. The level of cleaved Notch1 (Val1744), total Notch1 and β-actin (used as loading control) were detected by Western blot. The results are shown in Figure 4.2. From these results, the level of cleaved Notch1 (Val1744) dramatically decreased when pretreated with DAPT and the level of total Notch1 also slightly decreased. Therefore, treatment with DAPT is effective in inhibiting the activation of Notch signaling.
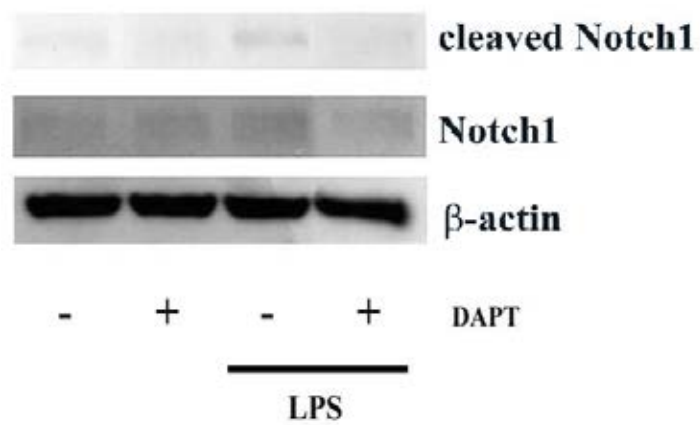
Figure 4.2 Inhibition of Notch signaling in LPS-stimulated macrophages by DAPT

RAW264.7 cells were pretreated with 25 μM for 1 h before stimulation with 100 ng/ml LPS for 1 h. Protein lysates were collected to investigate the appearance of cleaved Notch1 (Val1744) and total Notch1 expression by Western blot. β-actin was used as a loading control.

4.3 Two-dimensional gel electrophoresis of phosphoproteins and total proteins from LPS stimulated RAW264.7 in the presence or absence of DAPT

Previous studies showed that Notch signaling plays a role in regulating the responses of LPS-activated macrophages via stimulation of TLR signaling by fine tuning these signaling for appropriate responses. The lack of Notch signaling brings about decreasing of pro-inflammatory cytokine production such as IL-6 (29), TNFα (28) and nitrosative mediators such as nitric oxide (28) but anti-inflammatory cytokine, the level of *il-10* mRNA was increased (28). Nevertheless, how Notch signaling regulates TLR signaling remained unclear. This research was designed to investigate the phosphoproteins involved in LPS-stimulated macrophage treated with GSI, DAPT using two-dimensional gel electrophoresis (2D-GE). The 2D-GE gels which were stained for phosphoproteins were showed in Figure 4.3. The results of the total proteins staining by silver staining were exhibited in Figure 4.4. The representative of Coumassie stained gels were shown in Appendix A.
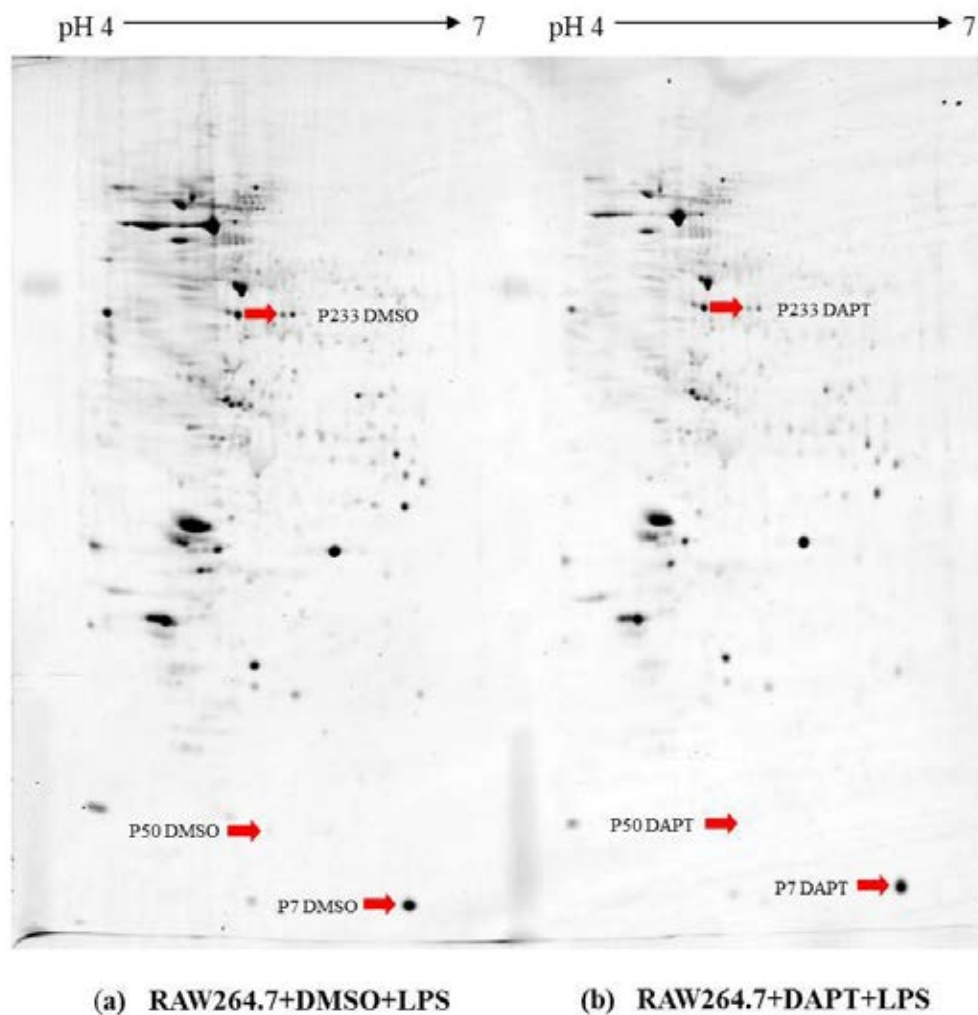
Figure 4.3 Patterns of phosphoproteins on 2D-GE gel.

Separated pattern of phosphoproteins on 2D-GE: (a) RAW264.7 were pretreated with DMSO (used as vehicle control) (b) RAW264.7 cells were treated with 25 μM DAPT for 1 hr. Cells were stimulated with 100 ng/ml LPS. Phosphoproteins of both conditions were separated by 2D-GE and stained by ProQdiamond dye and detected with Typhoon image scanning machine. The result is representative of three independent experiments. Red arrows indicated spots which were excised for protein identification.
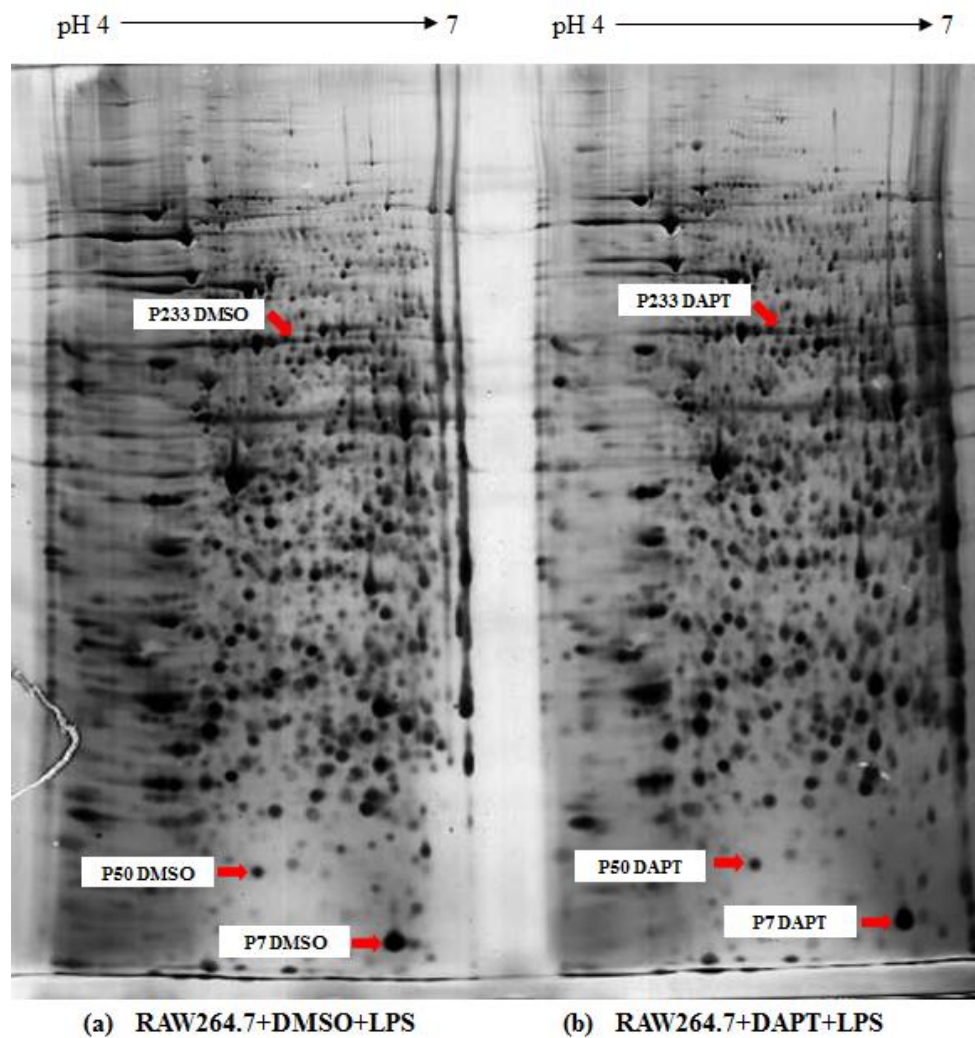
Fig 4.4 Patterns of total proteins pattern from silver staining on 2D-GE gel.

Gels from 2D-GE described in Figure 4.3 were stained by silver staining and the protein patterns were detected by ImageScanner™ II. The result is representative of three independent experiments. Red arrows indicated spots which were excised for protein identification.

4.4 Statistical analysis of different phosphoproteins from 2D-GE

The intensity of spots protein on gels was analyzed by ImageMaster™ 2D Platinum v7.0. The intensity of phosphoprotein spots on ProQ diamond stained gel were subtracted by the intensity of individual total protein spot on silver stained gel (or Coumassie blue stained gel for some spot that could be visualized by Coumassie staining). After that, the significant different in phosphorylation level of each spot were calculated using pair sample t-test. A p-value less than 0.05 was determined significant. The results of analysis were shown in Table 4. The Figure 4.5 represented the differences in phosphorylation level of one spot from triplicate experiment. The results exhibited that phosphorylation level of spot ID 50 and 233 in DAPT treated LPS-stimulated RAW264.7 were less than untreated (DMSO, vehicle control). But the phosphorylation level of spot ID 7 in DAPT treated LPS-stimulated RAW264.7 was decreased when compared with vehicle control.
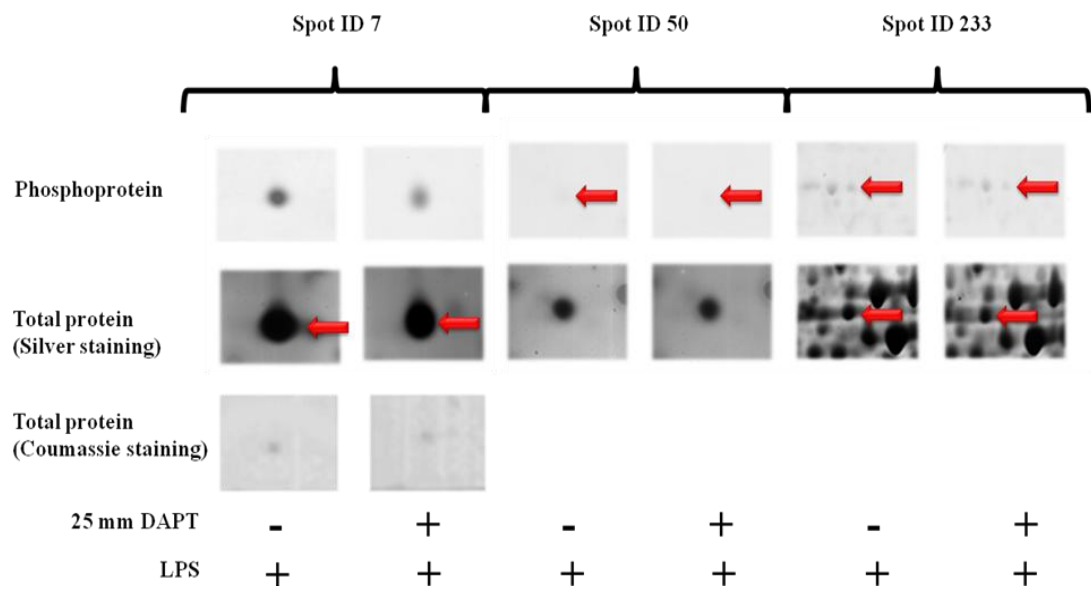
Figure 4.5 Comparison of representative spots with difference in phosphorylation levels with or without DAPT treatment.

Upper panels are phosphoproteins, middle and lower panels are total proteins of each spot. Red arrows indicated spots which were excised for protein identification. (Spot ID 50 and 233 could not be detected by Coumassie staining.)

Table 3.1 Intensity analysis of representative 2D-GE spots by ImageMaster™ 2D Platinum v7.0.

Spot ID	Phosphorylation level (relative fold)						P-value
	DMSO (1st gel)	DMSO (2nd gel)	DMSO (3rd)	DAPT (1st gel)	DAPT (2nd gel)	DAPT (3rd)	
7	0.419	0.288	0.096	0.405	0.280	0.088	0.038
50	0.121	0.135	0.134	0.106	0.118	0.118	0.001
233	2213.9	1141.4	1361.4	1280.0	571.4	549.0	0.019

4.5 Results of protein identification by LC-MS/MS

The spots with statistically significant differences in phosphorylation levels were identified by LC-MS/MS. The results revealed that spot ID 7 was a Regulator of G-protein Signaling 17 (RGS17), 19 (RGS19) or 20 (RGS20)**.** The MS results, MS/MS results and database search of spot ID 7 from DMSO pretreated LPS-stimulated RAW264.7 was shown in Appendix B and DAPT pretreated LPS-stimulated RAW264.7 was exhibited in Appendix C. The expressions of these genes were further investigated in the next experiments. Unfortunately, other spots did not yield any results from analysis by LC-MS/MS.

4.6 *rgs19* mRNA expression in LPS-stimulated RAW264.7 with or without DAPT were investigated by qPCR

Previous studies reported that RGS17, 19 and 20 were identified as the A/RZ class of RGS which has the similarity of reversibly palmitoylated, amino-terminal cysteine string (31). Due to the fact that RGS is expressed in a cell type-specific manner (32), the confirmation of *rgs17* and *rgs19* mRNA expression in macrophages was performed. RGS20 did not translate to protein in bone marrow derived macrophage from C57BL/6 (33). While the slightly expression of *rgs17* mRNA was detected by PCR (data not shown) which correlated with previous study that *rgs17* mRNA had low level expression in monocytes (34). Furthermore, the result of the expression of *rgs19* mRNA was investigated by qPCR (Figure 4.6). Interestingly, *rgs19* mRNA was found to be abundant in RAW264.7 even without any stimuli and the level declined slightly after LPS stimulation. However, the expression of *rgs19* mRNA remained unchanged in DAPT pretreated RAW264.7 when compared with DMSO (Figure 4.6). These results also correlated well with the public database on gene expression in immune cells, Immunological Genome Project (www.immgen.org). Therefore, DAPT treatment does not influence the transcription of *rgs19* in macrophages.
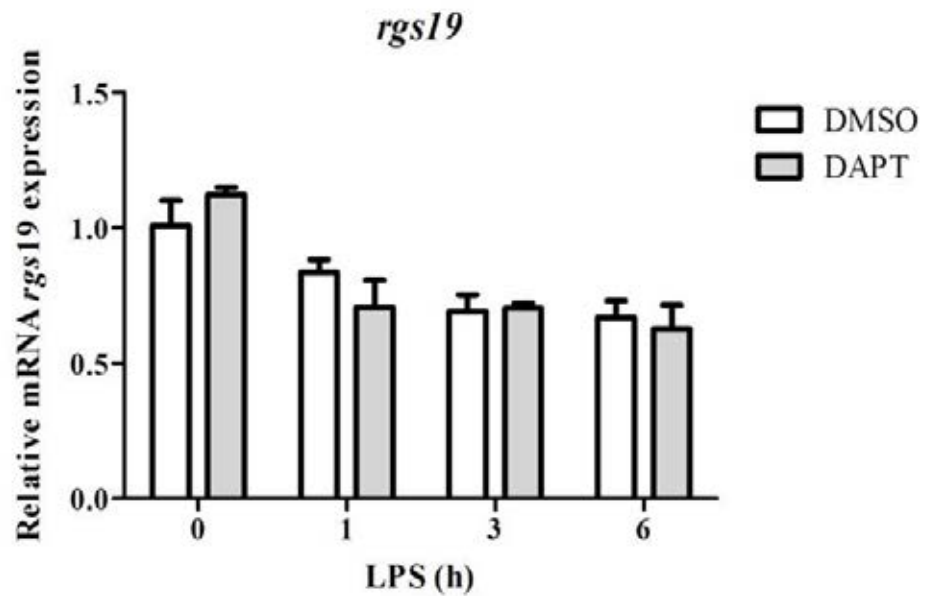
Figure 4.6 The expression level of *rgs19* mRNA in LPS-stimulated RAW264.7 treated with or without DAPT

RAW264.7 cells were pretreated with 25 μM DAPT for 1 h before stimulation with 100 ng/ml LPS for 0, 1, 3 and 6 h. The expression of *rgs19* mRNA is detected by qPCR. The result represented two independent experiments, each experiment performed in triplicate.

4.7 Optimizing conditions for silencing *rgs19* in RAW264.7

siRNA against RGS19 was custom synthesized as previously described (CHAPTER III). Four oligos were transfected to compare the efficiency of *rgs19* gene silencing. The results in Figure 4.7 showed the relative expression of *rgs19* mRNA in each condition by qPCR. Percents of decreasing level of *rgs19* mRNA were compared with AllStars siRNA (used as negative control) and summarized in Table 5.
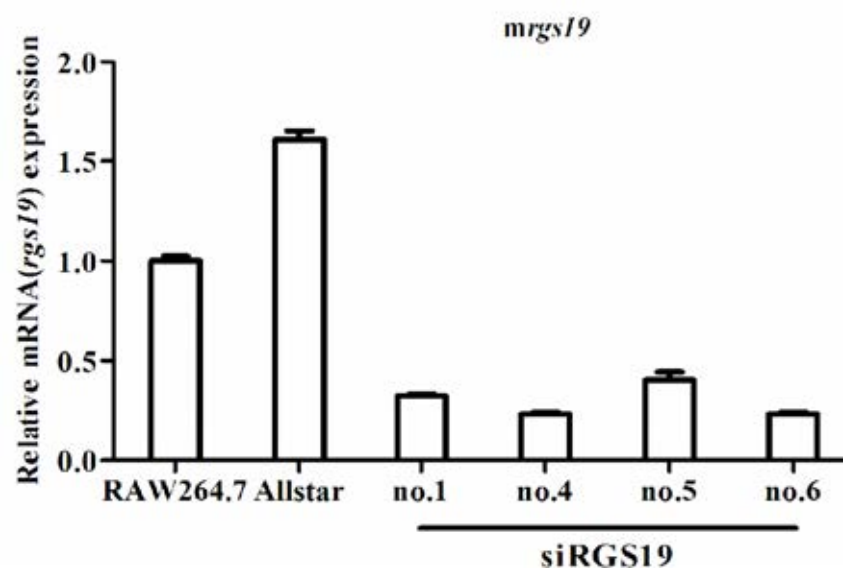
Figure 4.7 Silencing of *rgs19* mRNA expression in RAW264.7 after 42 h of transfection by four different siRNA oligos

RAW264.7 (1×10^5 cells) cells were plated immediately in 24-well tissue plate before transfection. The transfection complexes were prepared using different oligos for each reaction. Four oligos were compared for the efficiency of *rgs19* gene silencing. After 42 h of transfection, total RNA in samples were extracted and the *rgs19* mRNA expression was examined by qPCR. The experiment was performed in triplicate.

Table 3.2 Percentage of decreasing in *rgs19* mRNA expression in RAW264.7

Oligos	Percentage of decreasing *rgs19* mRNA level (%)
AllStars (negative control)	0.00
siRGS19 no.1	79.74
siRGS19 no.4	85.60
siRGS19 no.5	75.14
siRGS19 no.6	85.54

The results in Figure 4.7 and Table 5 showed that siRGS19 no.4 and 6 gave the highest silencing efficiency. They reduced 85% of *rgs19* mRNA expression compared with the AllStars. Only one oligos-siRGS19 no.6-was selected as representative oligos for optimal transfection time. The optimal transfection time of siRNA was examined by transfecting cells for 24 h and 48 h and the results are shown in Figure 4.8. This experiment revealed that after 24 h of transfection, the mRNA of *rgs*19 decreased to 85% of the negative control. However, cells appeared to partially die after 48 h of transfection, thus, this condition was not used for further experiment. Oligos siRGS19 no.4 did not test at other time point that why it was not chosen for next experiment.
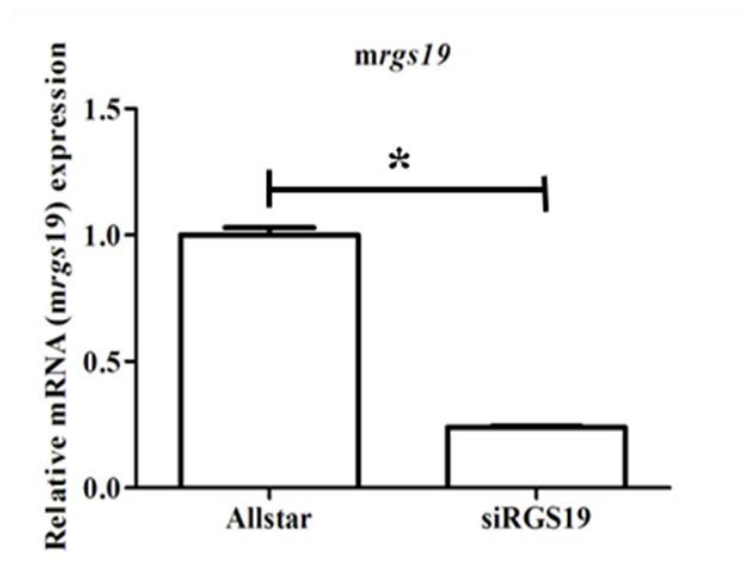
Figure 4.8 *rgs19* mRNA expression in RAW264.7 after 24 h of transfection

RAW264.7 (1×10^5 cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos. After 24 h of transfection, total RNA in samples were extracted and *rgs19* mRNA level was examined by qPCR. The experiment was performed in triplicate. * indicated the statistically significant differences between conditions at $p < 0.05$

4.8 Effects of *rgs19* gene silencing in LPS-stimulated RAW264.7

The level of *rgs19* mRNA in LPS-stimulated RAW264.7 which rgs19 was silenced was investigated by qPCR. According to the results shown in Figure 4.9, *rgs19* mRNA was significantly decreased at all time points after LPS stimulation when compared with that of the control AllStars oligos transfected cells. The expression of *rgs*19 mRNA was significant decreased after stimulated with LPS at all time points in siRGS19 oligos transfected cells.
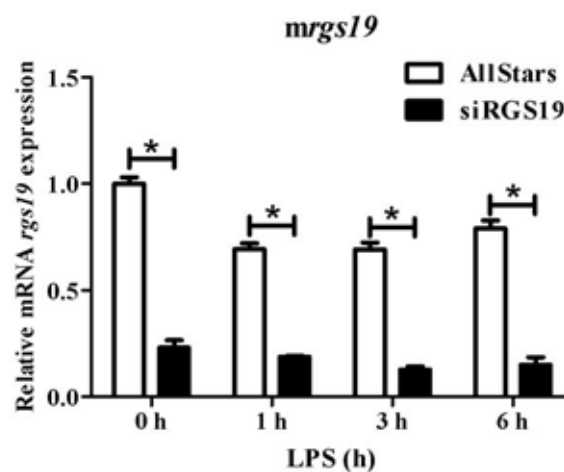
Figure 4.9 The level of *rgs19* mRNA expression in LPS-stimulated RAW264.7 which is silenced by siRGS19 no.6

RAW264.7 (1×10^6 cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos. After 28 h transfection, the cells were stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. Total RNA in samples was extracted and the level of *rgs19* mRNAexpression as examined by qPCR. The experiment was performed in triplicate. * indicated the statistically significant differences between conditions at $p < 0.05$

4.9 Effects of DAPT treatment and silencing of *rgs19* on *tnfα* mRNA levels in LPS-stimulated macrophages

The recent evidences suggested the involving of Notch signaling in TLR4 responses. TNFα is a cytokine that are secreted for inflammatory responses. The expression of *tnfα* mRNA during LPS stimulation of macrophages in the presence or absence of DAPT was investigated by qPCR. The results in Figure 4.10A demonstrated that *tnfα* mRNA expression increased at all time points tested. However, in rgs19 silencing RAW264.7, the levels of *tnfα* mRNA increased at earlier time (at 1 h after LPS stimulation) and decreased at later (3 and 6 h after LPS stimulation) (Figure 4.10B). These results correlated with TNFα protein detected in the culture supernatant by ELISA that showed in Figure 4.11A and 4.11B. Therefore, decreasing Notch signaling and *rgs19* mRNA levels yielded the similar effect on TNFα expression only at earlier time points.

These results correlated with study done by Zhang, Q. C. , et al. 2012 that overexpression of Notch1 in mouse primary peritoneal macrophage resulted in decreased TNFα secretion upon stimulation with LPS for 8 h (35). This result is in contrast with that reported by Palaga, et al, 2008 which showed that bone marrow derived macrophage from C57BL/6 mice when treated with another GSI, IL-CHO and stimulated with LPS plus IFNγ decreased the level of secretion of TNFα at 6 h. This discrepancy may be because of IFNγ functions as negative control of TNFα (36).

Figure 4.10 Level of *tnfα* mRNA in LPS-stimulated macrophage treated with DAPT or *rgs19* silencing

(A) RAW264.7 cells were pretreated with 25 μM DAPT for 1 h before being stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. (B) RAW264.7 (1.0x10^5 cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos. After 28 h transfection, the cells were stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. The levels of *tnfα* mRNA of these samples were examined by qPCR. The experiment was performed in triplicate. * indicated the statistically significant differences between conditions at *p* < 0.05

Figure 4.11 TNFα in culture supernatant of LPS -stimulated RAW264.7 treated with DAPT or *rgs19* silencing

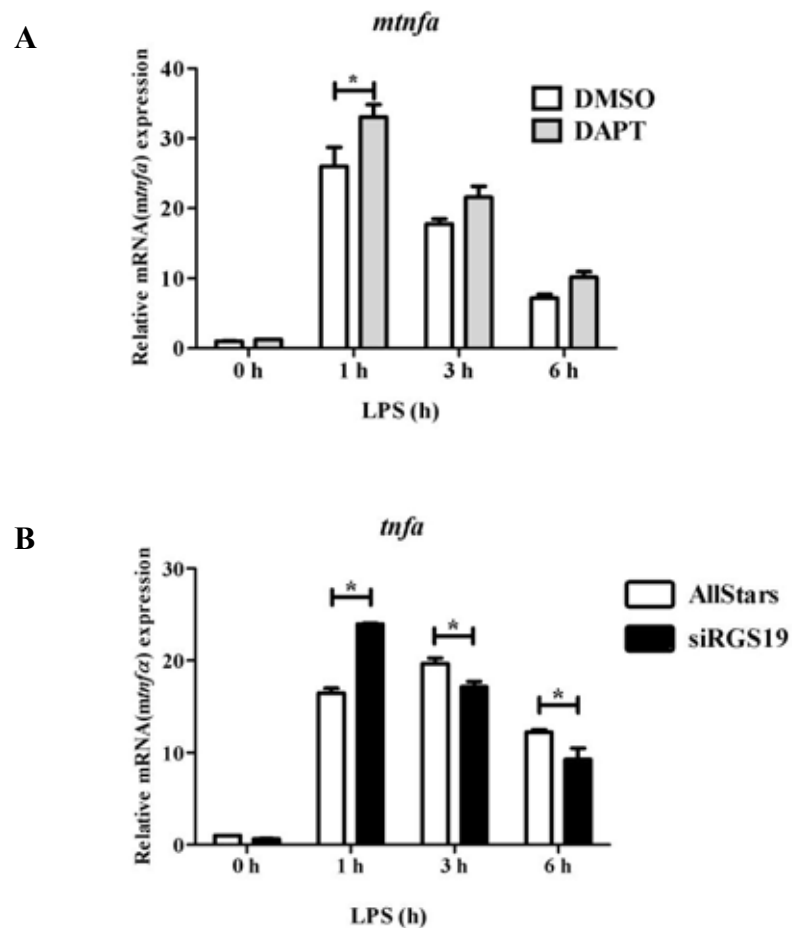(A) RAW264.7 cells were pretreated with 25 μM DAPT for 1 h after that cells were stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. (B) RAW264.7 (1.0×10^5 cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos. After 28 h transfection, the cells were stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. Culture supernatant were collected and TNFα level was determined by ELISA. The experiment was performed in triplicate. * indicated the statistically significant differences between conditions at $p < 0.05$

4.10 Effects of DAPT treatment and silencing *rgs19* on *il-10* mRNA level in LPS-stimulated macrophages

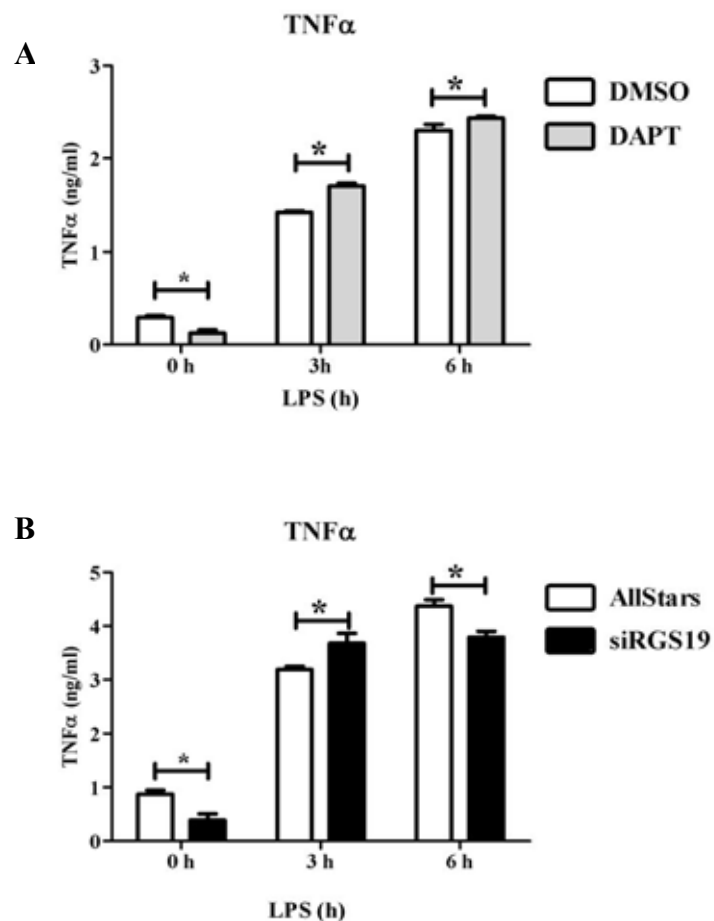Previously, IL-10 expression is reported to increase in LPS-stimulated macrophage (28). When Notch signaling was suppressed by IL-CHO, the *il-10* mRNA level increased (28). The mRNA *il-10* level in activated macrophage treated with DAPT was measured and the results are shown in Figure 4.12A. The level of mRNA *il-10* increased upon DAPT treatment, consistent with previous findings. Nevertheless, silenced *rgs19* showed diminished expression of mRNA *il-10* (Figure 4.12B). Therefore, Notch signaling suppressed *il-10* mRNA upon the activation via TLR4 and this effect was not similar to when RGS19 is silenced.
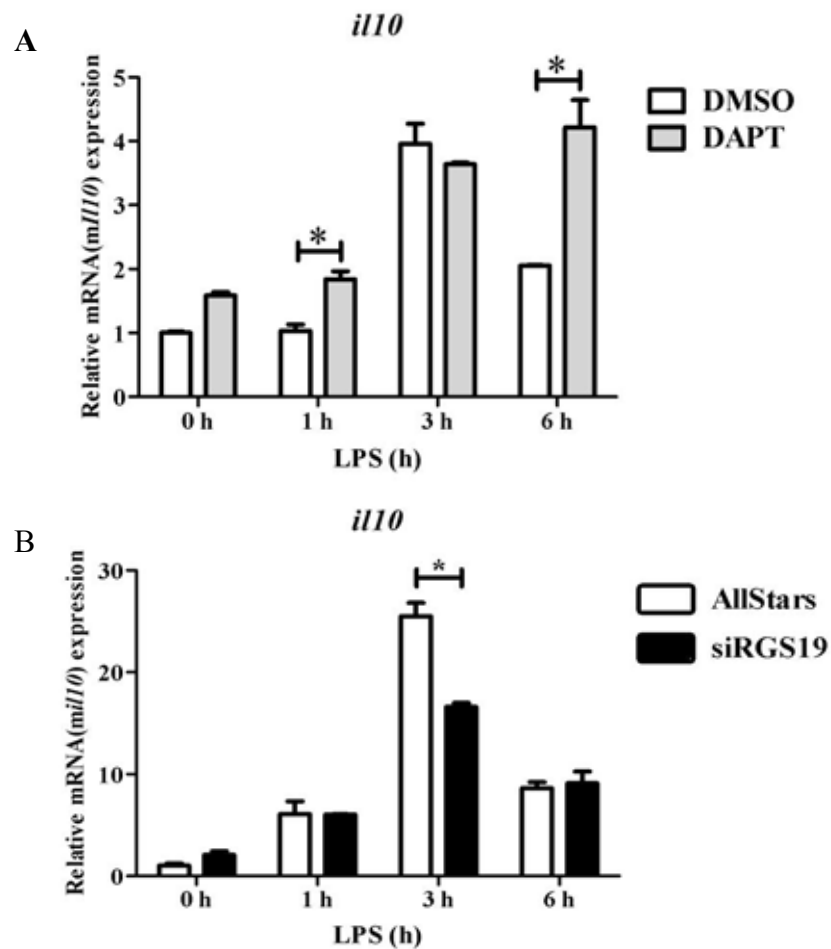
Figure 4.12 Level of *il-10* mRNA in LPS-stimulated macrophage treated with DAPT or *rgs19* silencing

(A) RAW264.7 cells were pretreated with 25 μM DAPT for 1 h before being stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. (B) RAW264.7 ($1.0x10^5$ cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos. After 28 h transfection, the cells were stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. The levels of *il-10* mRNA of these samples were examined by qPCR. The experiment was performed in triplicate. * indicated the statistically significant differences between conditions at $p < 0.05$

4.11 Effects of DAPT treatment and silencing *rgs19* on *il-6* mRNA level in LPS-stimulated macrophages

IL-6 expression was activated through NF-κB pathway upon TLR4 and Notch activation. Decreasing Notch signaling via IL-CHO treatment in LPS plus IFNγ stimulated bone marrow derived macrophages resulted in lower level of IL-6 (29). In this experiment, *il-6* mRNA decreased at 3 h after stimulation with LPS in DAPT-treated macrophage and in cells with silencing of *rgs19*. The level of *il-6* mRNA was higher than DMSO or AllStars at 6 h after stimulation (Figure 4.13A and 4.13B. These results imply that Notch signaling and RGS19 have the similar impact on *il-6* transcription.

il6

A

il6

B
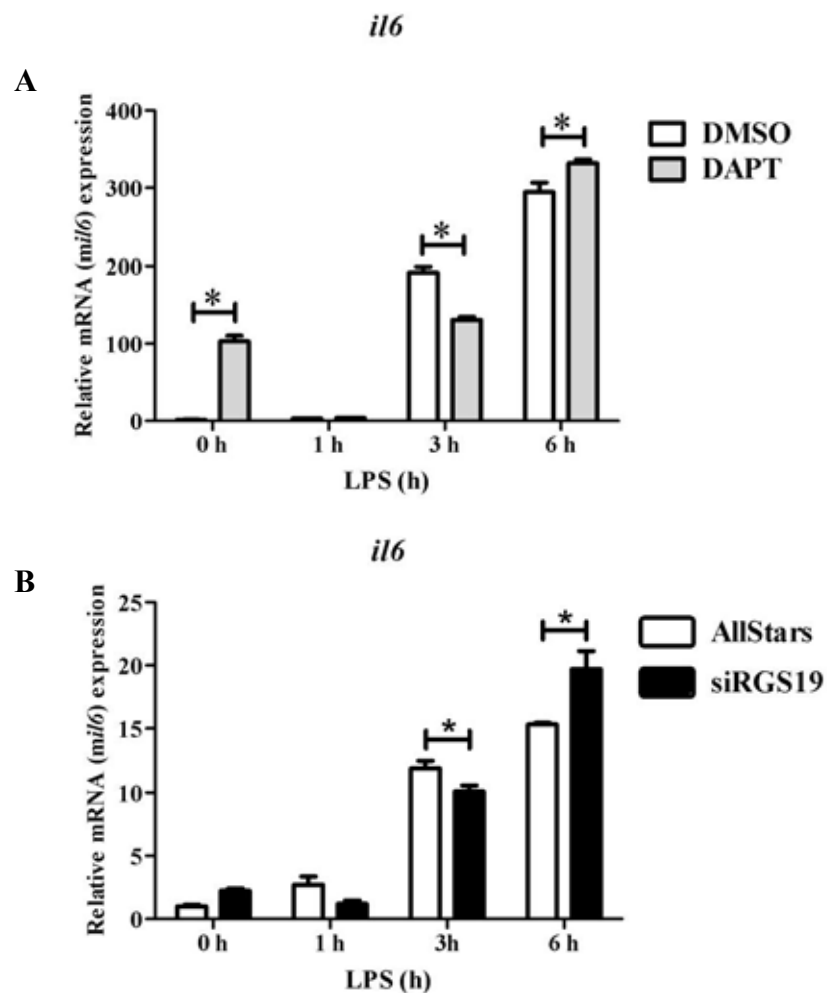
Figure 4.13 Levels of *il-6* mRNA in LPS-stimulated macrophage treated with DAPT or *rgs19* silencing

(A) RAW264.7 cells were pretreated with 25 μM DAPT for 1 h before being stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. (B) RAW264.7 (1.0×10^5 cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos. After 28 h transfection, the cells were stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. The levels of *il-6* mRNA of these samples were examined by qPCR. The experiment was performed in triplicate. * indicated the statistically significant differences between conditions at $p < 0.05$.

4.12 Effects of DAPT treatment and silencing *rgs19* on *iNOS* mRNA expression in LPS-stimulated macrophages

iNOS was an inducible enzyme responsible for nitric oxide production. Its expression is regulated by NF-κB and STAT pathways (37). The results revealed that *iNOS* mRNA level decreased in LPS-stimulated macrophages with silencing *rgs19* (Figure 4.14). The level of nitric oxide production when Notch signaling was suppressed was also decreased (data not shown). This results correlated with that reported by Palaga, et al (2008) (28). These results imply that Notch signaling and RGS19 have the similar impact on *iNOS* transcription.
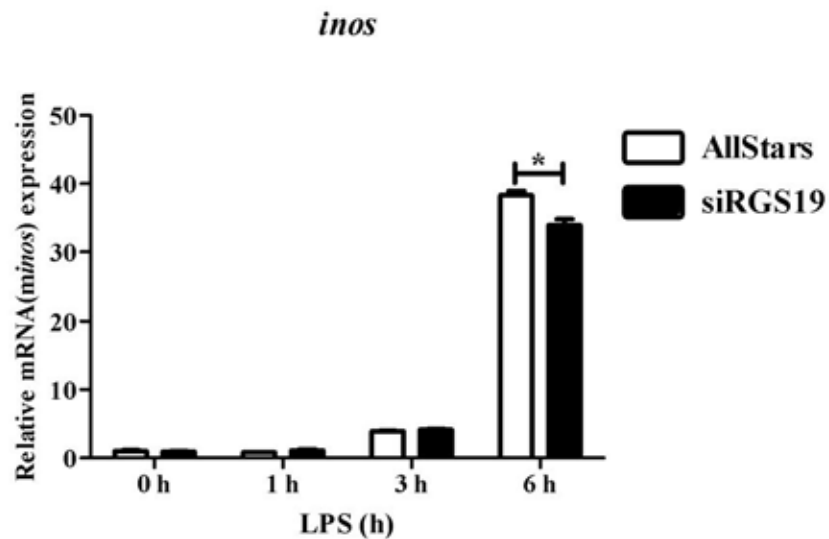
Figure 4.14 Level of *iNOS* mRNA in LPS-stimulated macrophage silenced RGS19

RAW264.7 (1.0×10^5 cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos. After 28 h of transfection, the cells were stimulated with 100 ng/ml LPS for 0, 1, 3 and 6 h. Total RNA in samples were extracted and m*inos* expression was examined by qPCR. The experiment was performed in triplicate. * indicated the statistically significant differences between conditions at $p < 0.05$

4.13 Phosphoproteins modulates cross talk between Notch and TLR4 signaling

4.13.1 Effects of DAPT treatment and silencing *rgs19* on activation of MAPK pathways in LPS-stimulated macrophages

Regulation of MAPK signaling pathway via TLR4 and GPCRs are well characterized, but the links between these pathways and Notch signaling pathway are not well documented. The findings in this study showed that inhibiting Notch signaling suppressed phosphorylation of ERK1/2 at 30 min after LPS stimulation (Figure 4.15A). On the other hand, phosphorylation of p38 did not change with DAPT treatment (Figure 4.15C). Recent reports demonstrated that expression of IL-10 in macrophages was regulated by ERK, p38, Tpl2 pathways via NF-κB (38). Therefore, the increasing mRNA *il-10* levels in LPS stimulated macrophages treated with DAPT cannot be explained by the negative effects of DAPT on these pathways. Interestingly, LPS stimulated RAW264.7 which overexpressed Notch1 resulted in increased activity of STAT1 (39), a transcription factor which plays a role in suppression of IL-10 production in human monocyte (13). Therefore, Notch signaling may regulate IL-10 expression via STAT1, instead of ERK and p38 pathway.

Furthermore, DAPT treatment increased SAPK/JNK signaling in LPS stimulated macrophages as shown in Figure 4.15B, suggesting that Notch signaling is inhibitory against this pathway. The similar results were obtained from macrophages with *rgs19* silencing. The phosphorylated level of SAPK/JNK increased in *rgs19* silencing macrophages when compared with control Allstar transfected cells (Figure 4.16). Previous evidences reported that TNFα was modulated by JNK pathway in LPS

stimulated RAW264.7 (40). Therefore, the high level of phosphorylation of SAPK/JNK may lead to the augmentation of TNFα both at mRNA and protein levels.

Taken together, Notch signaling negatively regulates SAPK/JNK signaling pathway and TNFα production possible via RGS19, but positively regulated ERK1/2 signaling and IL-10 expression via other uncharacterized pathways. In addition, LPS stimulated RAW264.7 slightly activated phosphorylation of p38MAPK but Notch signaling did not affect p38MAPK signaling.
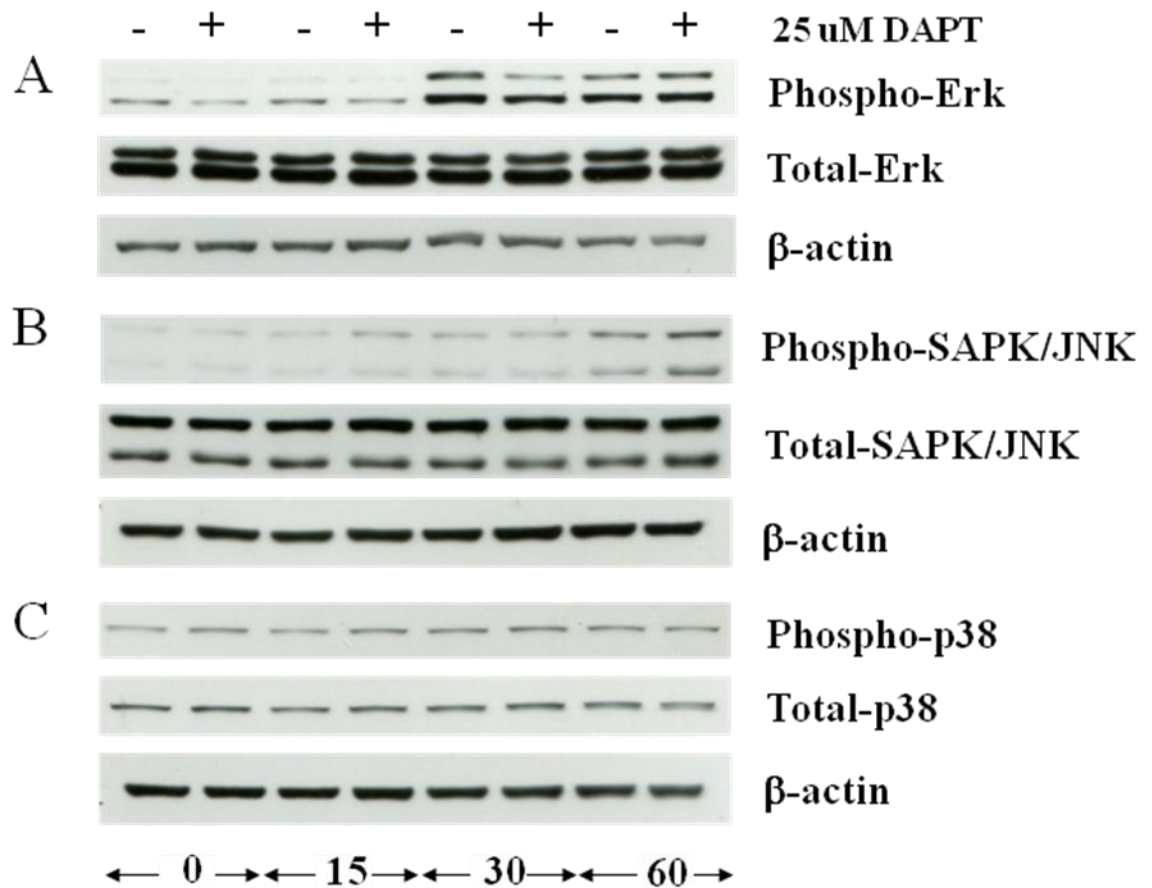
Figure 4.15 Inhibition of Notch signaling results in changing phosphorylation of MAPK signaling in LPS-stimulated macrophage.

RAW264.7 cells were pretreated with 25 μM DAPT or vehicle control DMSO for 1 h before stimulating with 100 ng/ml LPS for 0, 15, 30 and 60 min. Phosphorylation of ERK1/2 (A), SAPK/JNK(B) and p38 (C) in cell lysates were detected by Western blot.

| + | - | + | - | + | - | + | - | AllStars |
| - | + | - | + | - | + | - | + | SiRGS19 |

Phospho-SAPK/JNK

Total-SAPK/JNK

← 0 → ← 15 → ← 30 → ← 60 →   LPS (min)
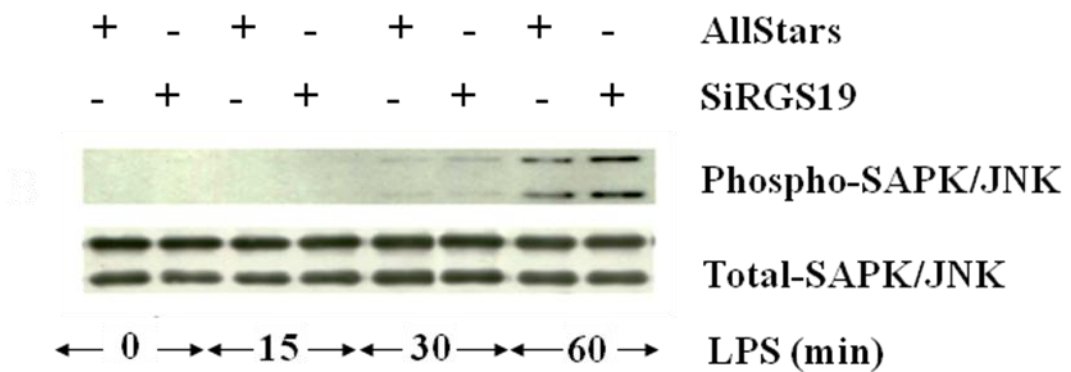
Figure 4.16 Effect of silencing *rgs19* on activation of SAPK/JNK in LPS stimulated macrophages.

RAW264.7 (1.0×10^5 cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos as described above. After 28 h of transfection, cells were stimulated with 100 ng/ml LPS for 0, 15, 30 and 60 min. Phosphorylation of SAPK/JNK and total SAPK/JNK were detected by Western blot.

4.13.2 Effects of DAPT treatment on NF-κB signaling during LPS-stimulated macrophages

NF-κB signaling plays important roles in cell differentiation, stress and inflammatory response (41, 42). Upon TLR4 activation in macrophages, NF-κB is activated via induction of TRAF6 to phosphorylate IKK. The activated IKK phosphorylates IκBα, IκBα is released from p65 and p50 complex and is degraded via proteosome pathway. p65/p50 is phosphorylated before translocation to nucleus to act as a transcription factors to regulate various cytokines such as IL-6 and iNOS (29). Inhibition of Notch signaling by DAPT resulted in decreased phosphorylation of IKKα/β (Figure 4.17), IκBα (Figure 4.18A) and p65 (Figure 4.18B).

Because the similar results of *il*-6 and *iNOS* mRNA level and the production of nitric oxide obtained by DAPT treatment and *rgs19* silencing led us to hypothesize that Notch signaling pathway may act together with TLR4 and RGS19 to activate NF-κB pathway

Although, the phosphorylation level of IKKα/β was decreased during LPS-stimulated macrophage treated with DAPT, Tpl2 was degraded faster than vehicle control (Figure 4.19) and the Western blot results did not reveal the different level of NF-κBp105 between both conditions (data not shown) but phosphorylated-ERK was decreased. From these results, it is possible that ERK activation requires phosphorylation on serine400 of Tpl2 that independent on IKK activation (16) but MEK1 could be activated by Tpl2 since Tpl2 did not bind with NF-κBp105. Therefore the activation of ERK was diminished owing to appearance of NF-κBp105.
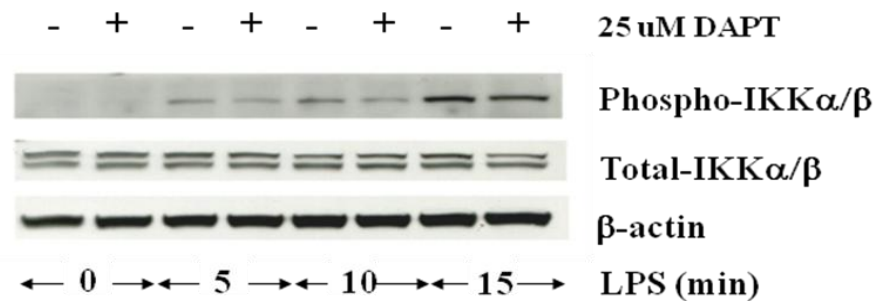
Figure 4.17 Effects of DAPT treatment on phosphorylation of IKKα/β in LPS-stimulated macrophages.

RAW264.7 cells were pretreated with 25 μM DAPT for 1 h before stimulation with 100 ng/ml LPS for 0, 5, 10 and 15 min. Phosphorylation of IKK and total IKK were detected by Western blot.
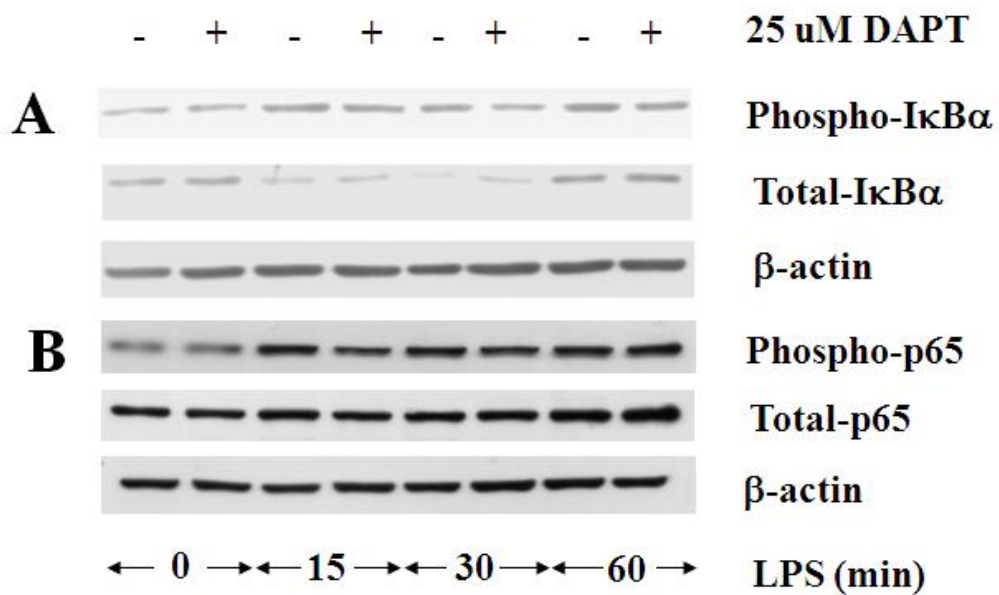
Figure 4.18 Effects of DAPT treatment on phosphorylation of NF-kB signaling molecules in LPS-stimulated macrophage.

RAW264.7 cells were pretreated with 25 μM DAPT for 1 h after that cells were stimulated with 100 ng/ml LPS for 0, 15, 30 and 60 min. Phosphorylation of IκBα (A) and phosphorylation of p65 (B) in cell lysates were detected by Western blot.
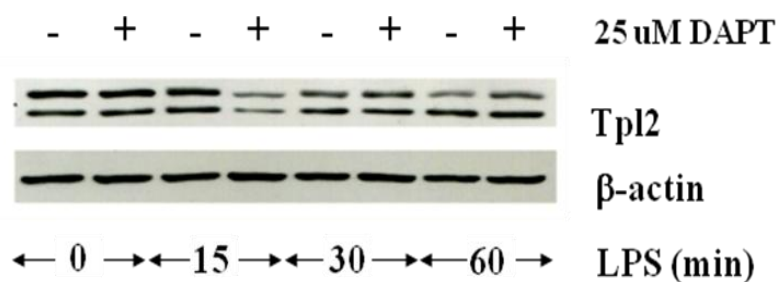
Figure 4.19 Effect of DAPT on Tpl2 level in LPS-stimulated macrophage

RAW264.7 cells were pretreated with 25 μM DAPT for 1 h after that cells were stimulated with 100 ng/ml LPS for 0, 15, 30 and 60 min. Level of Tpl2 were detected by Western blot.

4.14 Involvement of Notch and TLR4 signaling in regulating cell cycle during LPS stimulated macrophages via RGS19.

Cell cycle in macrophages is regulated through many proteins, one of which is Akt. Akt is important not only in cell cycle regulation (43) but also in inflammatory responses (30, 43). In this study, inhibition of Notch signaling resulted in decreased phosphorylation of Akt in LPS-stimulated macrophage (Figure 4.20). The similar results were obtained from *rgs19* silencing RAW264.7 stimulated with LPS (Figure 4.21). This finding was similar as previous report which demonstrated that overexpression of RGS19 in Caco-2 (human epithelial colorectal adenocarcinoma cells) and 293T (human embryonic kidney 293 cells) enhanced Akt phosphorylation (44) and silencing of *rgs19* could lead to deregulation of cell proliferation (40).

Consistent with the effects on Akt phosphorylation, cell cycle profiles of LPS-stimulated macrophages treated with DAPT showed significantly increased percentage of apoptotic cells (sub G1) compared to vehicle control DMSO treated cells (Figure 4.22).
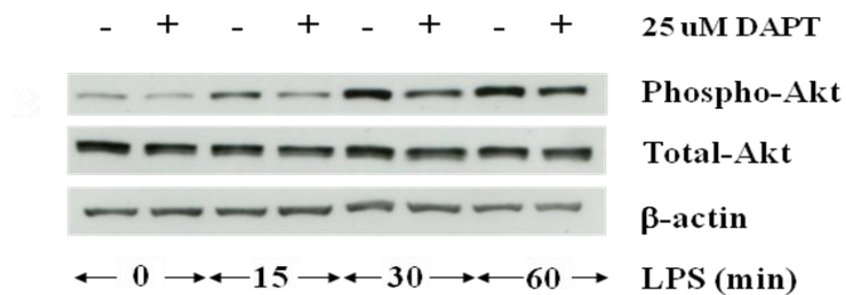
Figure 4.20 Inhibition of Notch signaling results in changing phosphorylation of Akt signaling in LPS-stimulated macrophage.

RAW264.7 cells were pretreated with 25 μM DAPT for 1 h after that cells were stimulated with 100 ng/ml LPS for 0, 15, 30 and 60 min. phosphorylation of Akt in cell lysates were detected by Western blot.

| + | - | + | - | - | + | + | - | AllStars |
| - | + | - | + | + | - | - | + | SiRGS19 |

Phospho-Akt

Total-Akt

← 0 →←15 →←30 →←60 → LPS (min)
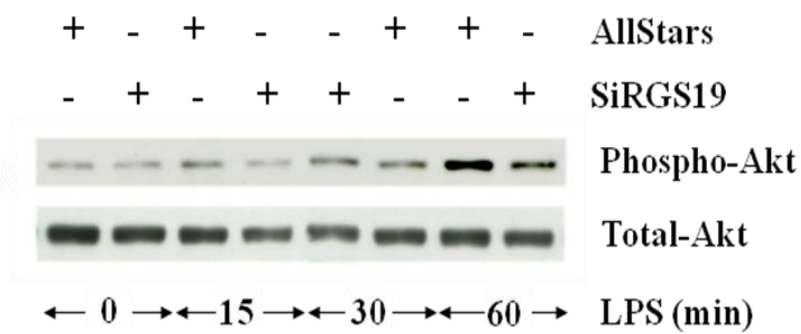
Figure 4.21 Alteration of phosphorylated Akt in LPS stimulated macrophage silenced *rgs19*

RAW264.7 (1×10^5 cells) cells were plated immediately in 24-well tissue plate before transfection. Transfection complexes were prepared using the AllStars or siRGS19 no.6 oligos as described above. After 28 h transfection, the cells were stimulated with 100 ng/ml LPS for 0, 15, 30 and 60 min. Phosphorylation of Akt and total Akt were detected by Western blot.

A

450

DMSO

F
63.9%

G
20.0%

E
1.8%

H
12.6%

0   1023

450

DAPT

J
61.0%

K
17.6%

I
3.0%

L
15.1%

0   1023

450

DMSO+LPS 36 h

AB
60.5%

AC
14.6%

AA
14.7%

AD
9.0%

0   1023

450

DAPT+LPS 36 h

AF
52.4%

AG
12.8%

AE
21.9%

AH
11.9%

0   1023

B

450

DMSO

BB
64.6%

BC
13.3%

BA
10.4%

BD
10.9%

0   1023

450

DAPT

BF
62.0%

BG
12.3%

BE
15.7%

BH
9.3%

0   1023

450

DMSO+LPS 48 h

BJ
49.0%

BK
12.0%

BI
35.4%

BL
3.6%

0   1023

450

DAPT+LPS 48 h

BN
34.0%

BO
9.3%

BM
52.7%

BP
4.2%

0   1023
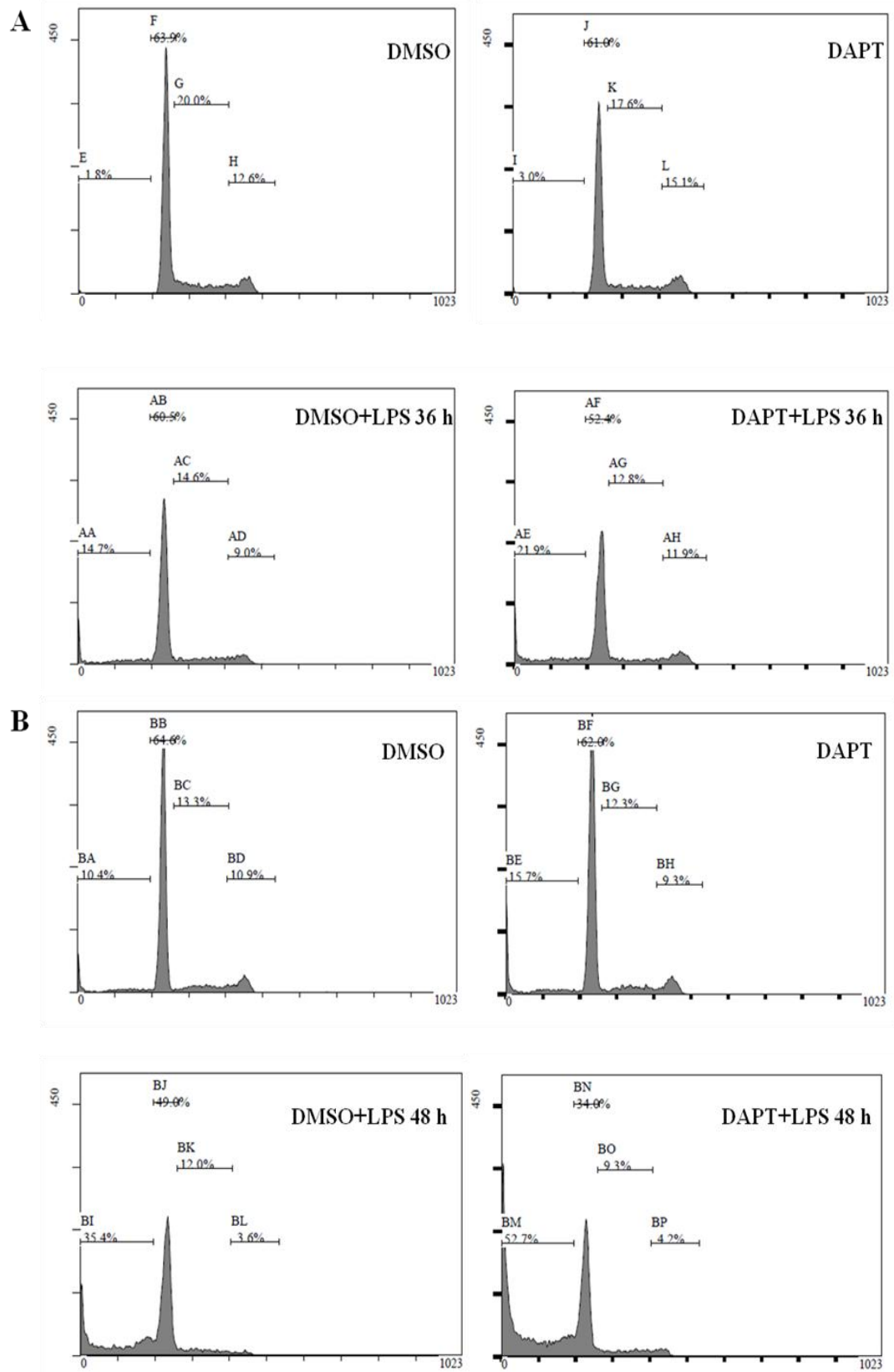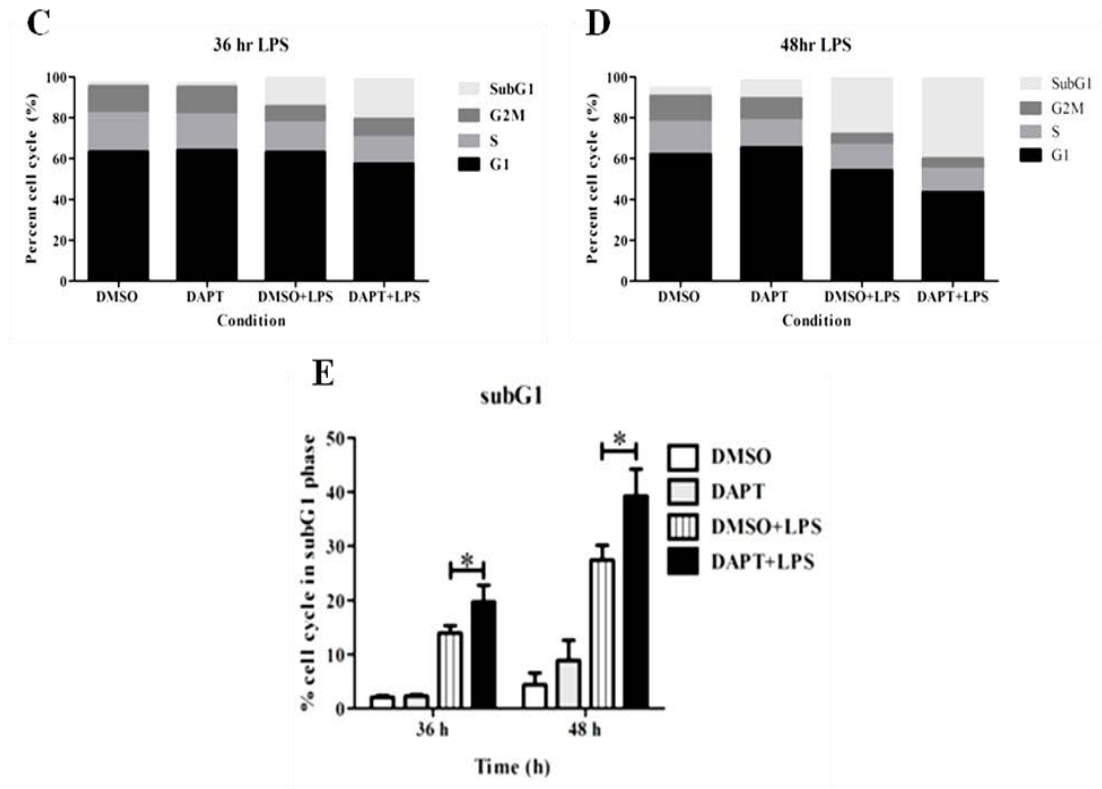
Figure 4.22 Effect of DAPT on cell cycle progression during LPS-stimulated macrophage

RAW264.7 cells were pretreated with 25 μM DAPT for 1 h .The cell were stimulated with 100 ng/ml LPS for 36 (Figure 4.22A and 4.22C) and 48 h (Figure 4.22B and 4.22D) before stained by propidium iodide staining and cell cycle were investigated by FACs. Percent cell cycle in subG1 phase in each condition was shown in Figure 4.22E. The result represented three independent experiments in triplicate. * indicated the statistically significant differences between conditions at $p < 0.05$

CHAPTER V

CONCLUSIONS

(1) Stimulation with LPS activated the Notch signaling as determined by increased expression of cleaved Notch1 as early as 1 h after stimulation in RAW264.7, a macrophage cell line.

(2) A gamma secretase inhibitor, DAPT, effectively inhibited the appearance of cleaved Notch1 in LPS stimulated RAW264.7.

(3) Decreasing phosphorylation level of regulator of G-protein 19 (RGS19) in LPS-stimulated macrophages treated with DAPT was uncovered by two-dimensional gel electrophoresis and mass spectrometry.

(4) DAPT treatment did not influence the level of *rgs19* mRNA in LPS-treated macrophages.

(5) In LPS stimulated-RAW264.7, inhibition of Notch signaling and silencing *rgs19* similarly affected the level of *tnfα, il-6* and *iNOS*, but not that of *il-10*.

(6) Notch signaling positively regulated activation of ERK, NF-κB (IKKα/β, IκBα and NF-κBp65) and Akt signaling pathway but suppressed SAPK/JNK signaling pathway during LPS-stimulation of RAW264.7.

(7) Silencing rgs19 resulted in decreasing phosphorylation of Akt and SAPK/JNK.

(8) Decreasing of Notch signaling resulted in increasing percentages of apoptotic cells.

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         Signaling following Macrophage Activation Modulates Gene
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APPENDICES

APPENDIX A
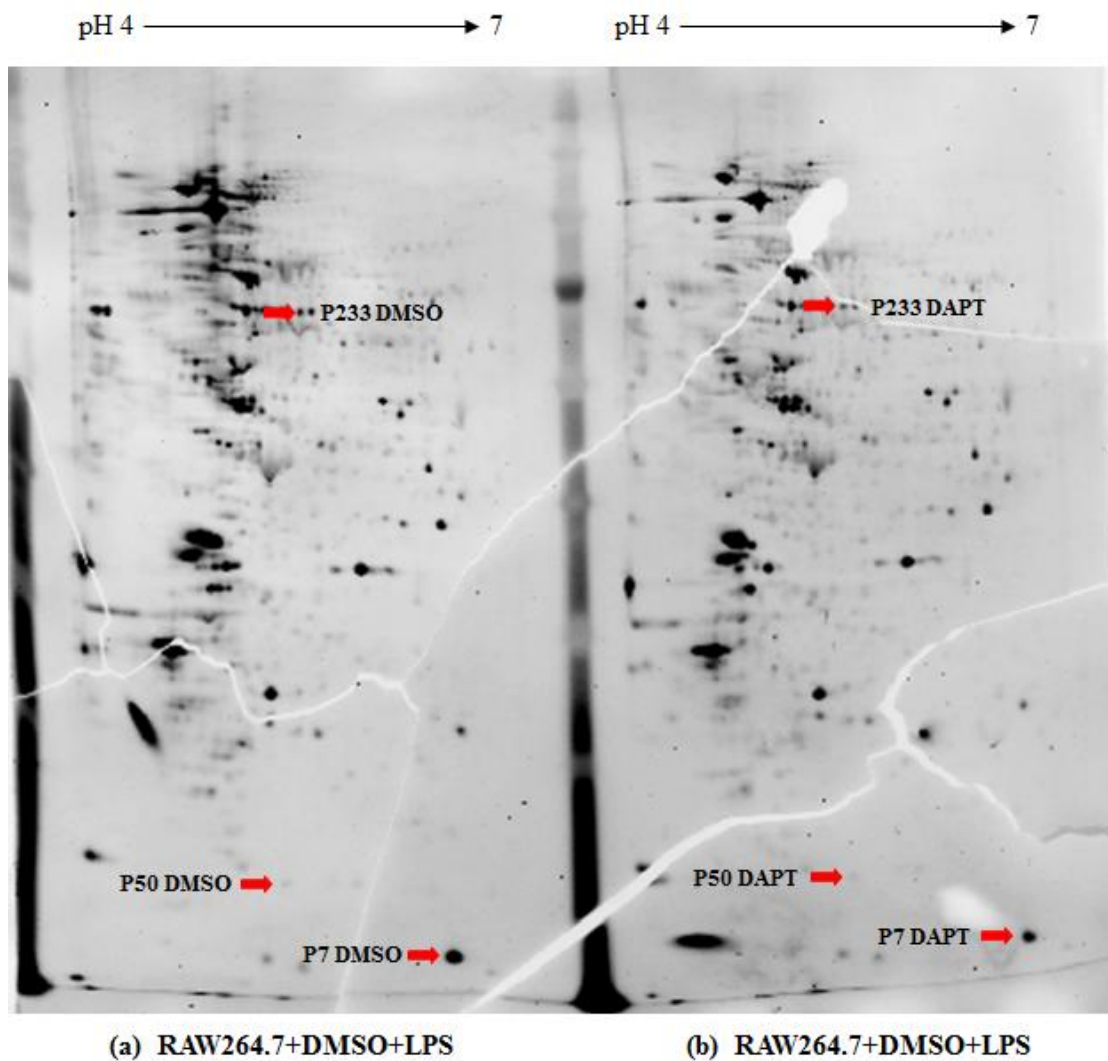
Figure 6.1 Patterns of phosphoproteins on 2D-GE gel.

Separated pattern of phosphoproteins on 2D-GE: (a) RAW264.7 were pretreated with DMSO (used as vehicle control) (b) RAW264.7 were treated with 25 μM DAPT for 1 hr. Cells were stimulated with 100 ng/ml LPS. Phosphoproteins of both conditions were separated by 2D-GE and stained by ProQdiamond dye and detected with Typhoon image scanning machine. The result is representative of three independent experiments. Red arrows indicated spots which were excised for protein identification.
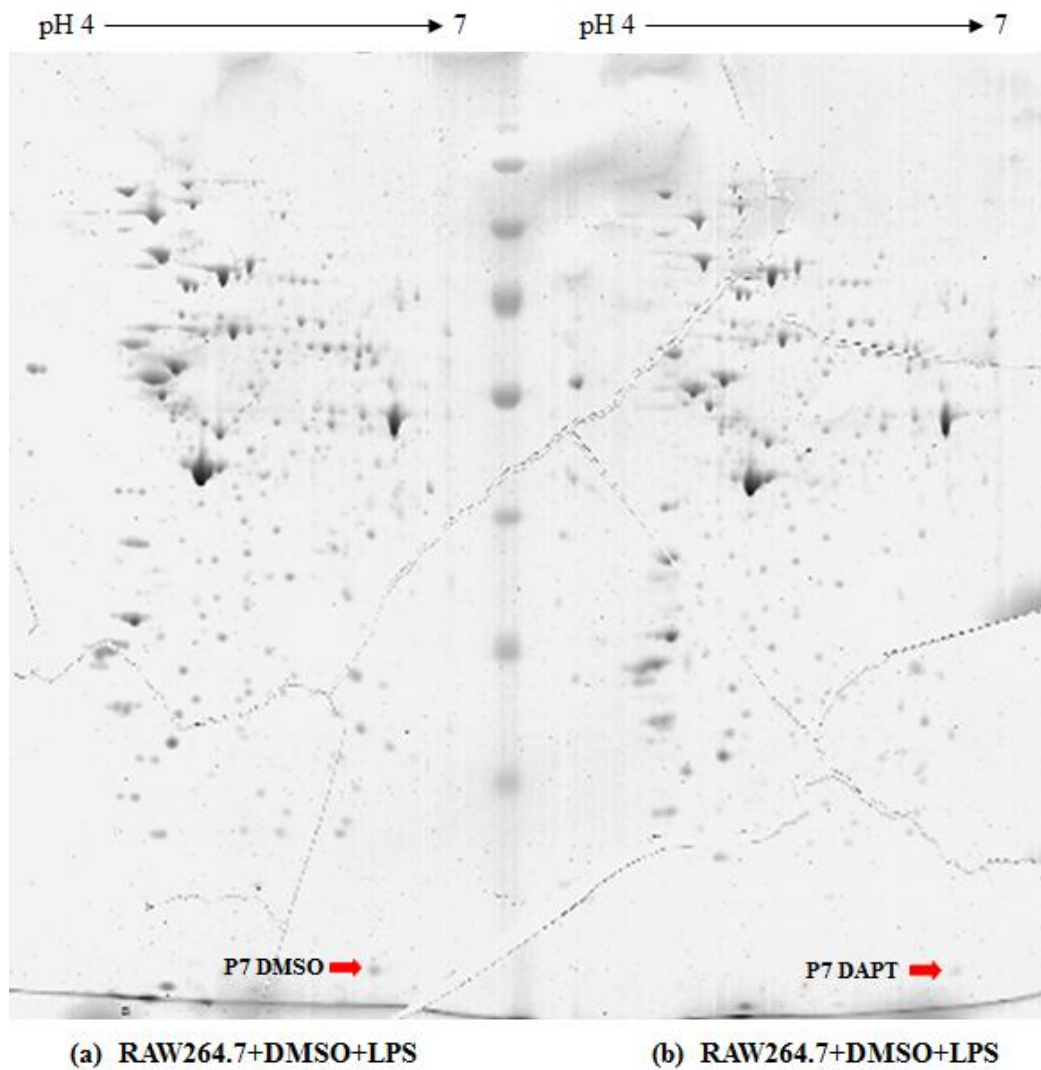
Figure 6.2 Patterns of total proteins pattern from Coumassie staining on 2D-GE gel.

Gels from 2D-GE described in Figure 6.1 were stained by Coumassie staining and the protein patterns were detected by ImageScanner™ II. The result is representative of three independent experiments. Red arrows indicated spots which were excised for protein identification.

APPENDIX B

81

A

B

Generic Display Report

Analysis Info		Acquisition Date	12/21/2011 2:27:09 PM
Analysis Name	D:\Data\Data Service\Year 2011\21122011\P7DMSO_B1_01_604.d		
Method	proteomics_20122011.m	Operator	BDAL@DE
Sample Name	P7DMSO	Instrument	microTOF-Q II
Comment			

Bruker Compass DataAnalysis 4.0   printed: 5/5/2012 12:12:32 PM   Page 1 of 1

C
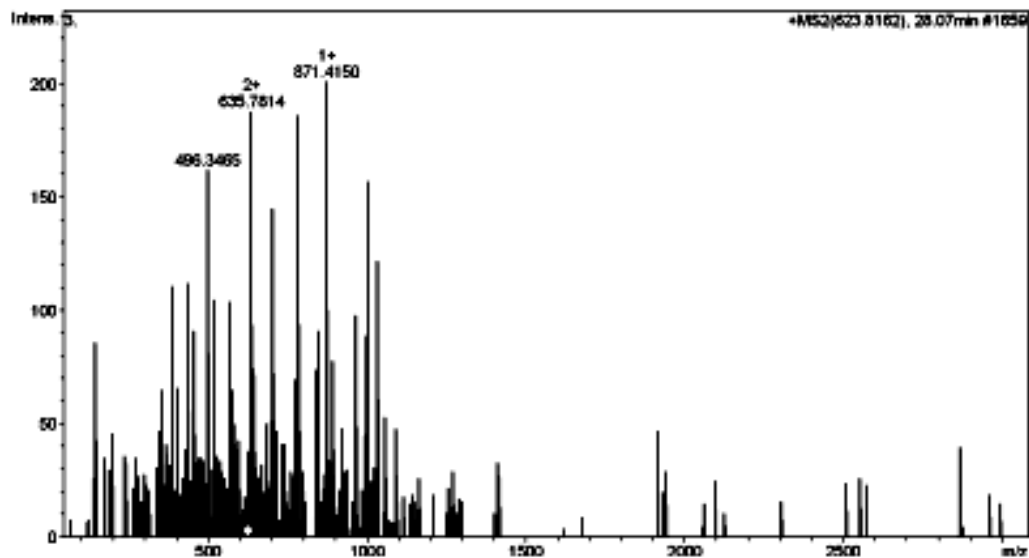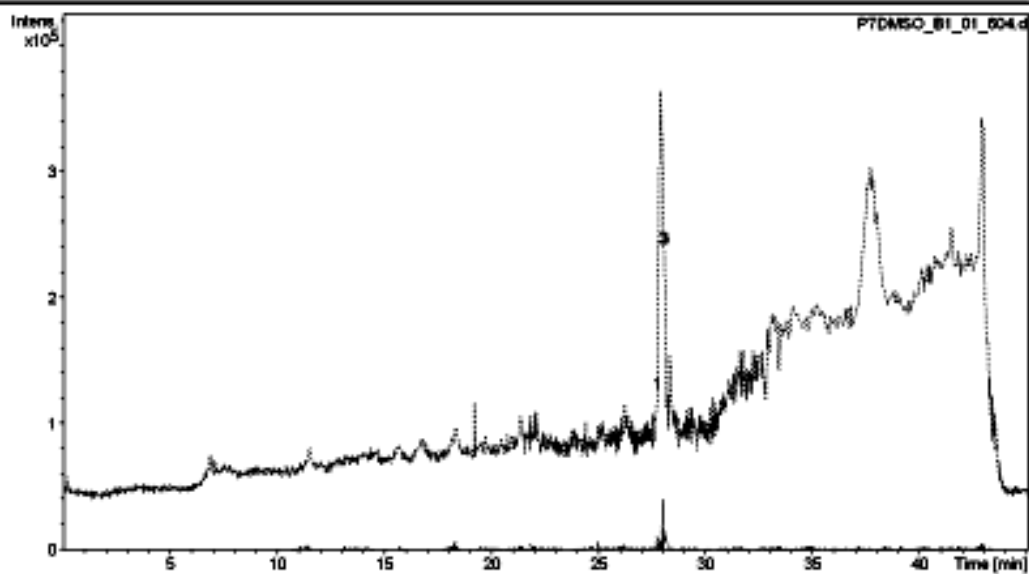
Mascot Search Results

User	: naunpun sangphech
Email	: atom_mice@yahoo.com
Search title	: P7DMSO
MS data file: P7DMSO_B1_01_614.mgf	
Database	: SwissProt 2012_04 (535698 sequences; 190107059 residues)
Taxonomy	: Mus musculus (house mouse) (16528 sequences)
Timestamp	: 4 May 2012 at 10:51:19 GMT
Protein hits :	**RGS17_MOUSE**
	MAP4_MOUSE
	TESK1_MOUSE
	VPS8_MOUSE
	S38A9_MOUSE

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.

Individual ions scores > 31 indicate identity or extensive homology (p<0.05).

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.
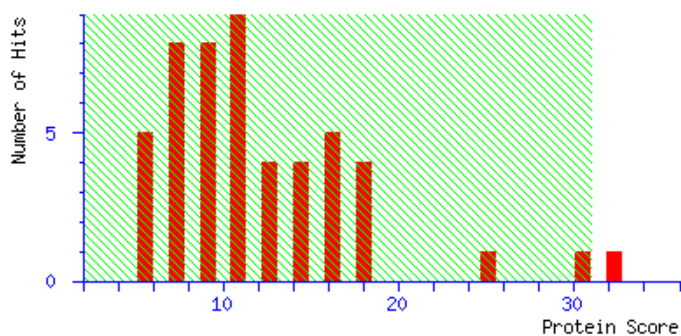
Peptide Summary Report

Significance threshold p< 0.05	Max. number of hits 5	Help
Standard scoring ⊙ MudPIT scoring ○	Ions score or expect cut-off 0	Show sub-sets 0
Show pop-ups ⊙ Suppress pop-ups ○	Sort unassigned Decreasing Score	Require bold red ☐

Search Selected ☐ **Error tolerant**

---

1. <u>RGS17_MOUSE</u> **Score:** 32 **Matches:** 1(0) **Sequences:** 1(0)

☑ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
4	403.7051	805.3957	804.3977	0.9980	0	32	0.078	1	U	K.EVSLDSR.V

Proteins matching the same set of peptides:

<u>RGS19_MOUSE</u> **Score:** 32 **Matches:** 1(0) **Sequences:** 1(0)

<u>RGS20_MOUSE</u> **Score:** 32 **Matches:** 1(0) **Sequences:** 1(0)

---

2. <u>MAP4_MOUSE</u> **Score:** 30 **Matches:** 1(0) **Sequences:** 1(0)

☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
4	403.7051	805.3957	805.3930	0.0027	0	30	0.12	2	U	K.SASADLSR.S

---

3. <u>TESK1_MOUSE</u> **Mass:** 68009 **Score:** 26 **Matches:** 1(0) **Sequences:** 1(0)

Dual specificity testis-specific protein kinase 1 OS=Mus musculus GN=Tesk1 PE=2 SV=3

☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
16	831.4301	2491.2683	2490.1749	1.0935	1	26	0.098	1	U	R.GPGPGEAPGEGPGGAGGGPGRGRPSSYR.A

---

4. <u>VPS8_MOUSE</u> **Score:** 19 **Matches:** 1(0) **Sequences:** 1(0)

☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
4	403.7051	805.3957	804.3865	1.0092	0	19	1.8	3	U	R.SDLIDDK.E

5. S38A9_MOUSE **Score:** 19 **Matches:** 1(0) **Sequences:** 1(0)

☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
4	403.7051	805.3957	805.3566	0.0391	0	19	1.8	4	U	M.ASVDGDSR.H

Peptide matches not assigned to protein hits: (no details means no match)

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	11	812.6101	1623.2057	1623.8039	-0.5982	1	17	0.95	1		QARLADTANYTCVAK
☑	13	721.6948	2162.0625	2162.2021	-0.1395	0	15	1.4	1		ELISLLNSLLLVFMTVSDR
☑	8	486.5192	1456.5357	1456.8514	-0.3157	1	14	5.9	1		FVIRNIVEAAAVR
☑	9	577.2927	1152.5708	1152.5849	-0.0141	0	13	5.1	1		DFLITQMTGK
☑	12	871.3088	1740.6029	1740.8934	-0.2904	1	9	6.3	1		DQIAYATGEKLYEIK
☑	15	1165.3291	2328.6437	2328.1559	0.4878	0	8	5.4	1		DIVYCVLSEGPGEPPPVSETLK
☑	10	678.3544	1354.6942	1354.6704	0.0238	0	7	17	1		FLACVSQDGFLR
☑	7	923.3014	922.2942	922.5600	-0.2658	0	4	34	1		KPKPAAKPS
☑	5	810.2436	809.2363	809.3780	-0.1417	0	4	33	1		GHPSQER
☑	6	899.0760	898.0687	897.5760	0.4928	1	4	29	1		KVLALQAR
☑	14	1082.0389	2162.0632	2161.2299	0.8333	0	4	22	1		APVLALSLFLAVWSLVGYSR
☑	1	483.3513	482.3440								

☑   <u>2</u> *606.0726*   *605.0653*

☑   <u>3</u> *806.0686*   *805.0613*

Search Parameters

Type of search   : MS/MS Ion Search
Enzyme   : Trypsin
Mass values   : Monoisotopic
Protein Mass   : Unrestricted
Peptide Mass Tolerance : ± 1.2 Da
Fragment Mass Tolerance: ± 0.6 Da
Max Missed Cleavages   : 1
Instrument type   : Default
Number of queries   : 16

Figure 6.3 Analysis of spot P7 obtained from DMSO pretreated LPS-stimultated macrophage (P7DMSO) by HRESIMS (nano-LC-MS/MS).

After the significant different phosphorylation spot was subjected to in-gel digestion, digested peptides were identified by HRESIMS (A) MS spectrum of spot P7DMSO. (B) HRESIMS spectrum of spot P7DMSO. The HRESIMS result of P7DMSO was quested on Mascot database was shown in (C).

APPENDIX C

A

Generic Display Report

Analysis Info
Analysis Name   D:\Data\Data Service\Year 2011\21122011\P7DAPT_B2_01_605.d
Method   proteomics_20122011.m
Sample Name   P7DAPT
Comment

Acquisition Date   12/21/2011 3:46:29 PM
Operator   BDAL@DE
Instrument   microTOF-Q II

Bruker Compass DataAnalysis 4.0   printed: 5/5/2012 12:04:15 PM   Page 1 of 1

B

C

{MATRIX SCIENCE} Mascot Search Results

User : Naunpun Sangphech

~~Email : atom_mice@yahoo.com~~

Search title : p7 dapt

~~MS data file : P7DAPT_B2_01_605.mgf~~

Database : SwissProt 2012_04 (535698 sequences; 190107059 residues)

Taxonomy : Mus musculus (house mouse) (16528 sequences)

Timestamp : 6 May 2012 at 16:24:01 GMT

Protein hits : **RGS17_MOUSE**

              MAP4_MOUSE

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.

Individual ions scores > 32 indicate identity or extensive homology (p<0.05).

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.
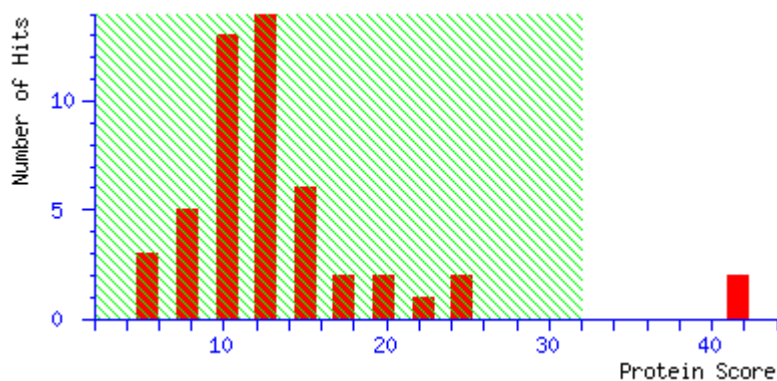
Peptide Summary Report

		Help
Significance threshold p< 0.05	Max. number of hits AUTO	
Standard scoring ⊙ MudPIT scoring ○	Ions score or expect cut-off 0	Show sub-sets 0
Show pop-ups ⊙ Suppress pop-ups ○	Sort unassigned	Require bold red ☐

☐ **Error tolerant**

1. <u>RGS17_MOUSE</u> **Score:** 42   **Matches:** 1(1) **Sequences:** 1(1)

☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	6	403.7009	805.3873	804.3977	0.9896	0	42	0.009	1	U	K.EVSLDSR.V

Proteins matching the same set of peptides:

<u>RGS19_MOUSE</u> **Score:** 42   **Matches:** 1(1) **Sequences:** 1(1)
<u>RGS20_MOUSE</u> **Score:** 42   **Matches:** 1(1) **Sequences:** 1(1)

2. <u>MAP4_MOUSE</u> **Score:** 41   **Matches:** 1(1) **Sequences:** 1(1)

☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
6	403.7009	805.3873	805.3930	-0.0056	0	41	0.0099	2	U	K.SASADLSR.S

Peptide matches not assigned to protein hits: (no details means no match)

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	10	831.7586	2492.2538	2492.3349	-0.0810	1	23	0.22	1		DRIPGIVPMQIPSPEVFEDLIK
☑	8	577.2878	1152.5610	1152.6026	-0.0416	1	22	0.65	1		SSFDDKTLLK
☑	4	655.1630	654.1558	655.3514	-1.1956	0	14	2.7	1		GGRPNR
☑	7	483.3464	964.6782	965.4679	-0.7897	1	14	6.2	1		GRGSGTFER
☑	5	768.5015	767.4942	767.4541	0.0401	0	11	6.9	1		VSLQPPK
☑	9	1783.2042	1782.1969	1782.0113	0.1856	1	4	18	1		IAILTCPFEPPKPKTK
☑	1	487.3711	486.3638								
☑	2	606.0694	605.0621								
☑	3	607.0708	606.0635								

Search Parameters

Type of search   : **MS/MS Ion Search**
Enzyme   : **Trypsin**
Mass values   : **Monoisotopic**
Protein Mass   : **Unrestricted**
Peptide Mass Tolerance : ± 1.2 Da
Fragment Mass Tolerance: ± 0.6 Da
Max Missed Cleavages : 1

Instrument type : Default
Number of queries : 10

Figure 6.4 Analysis of spot P7 obtained from DAPT pretreated LPS-stimultated macrophage (P7DAPT) by HRESIMS (nano-LC-MS/MS).

After the significant different phosphorylation spot was subjected to in-gel digestion, digested peptides were identified by HRESIMS (A) MS spectrum of spot P7DAPT. (B) HRESIMS spectrum of spot P7DAPT. The HRESIMS result of P7DMSO was questes on Mascot database was shown in (C).

APPENDIX D

1. Completed DMEM 100 ml

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

2. Freezing media 10 ml

Completed DMEM	90	%
DMSO	10	%

3. Penicillin and streptomycin

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

4. FBS inactivation

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

5. Buffer A for protein extraction

10 mM EGTA	1	ml
10 mM DTT	1	ml
500 mM Tris-HCl pH 7.2	1	ml
1.4 M KCl	1	ml
25 mM MgCl$_2$	1	ml
Sterile water	5	ml
Protease Inhibitor Cocktail Tablets	1	tablet

6. Buffer B for protein extraction

Buffer A	990	μl
Nonidet P-40	10	μl

7. 10% SDS-polyacrylamide gel 8 ml

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

8. 10% SDS-polyacrylamide gel 27 ml (for 2D-GE)

Sterile water	11.6	ml
40% Acrylamide and Bis-acrylamide solution	8.1	ml
1.5 M Tris-HCl pH 8.8	6.75	ml
10% SDS	0.27	ml
10% APS	0.27	ml
TEMED	0.014	ml

9. 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. 5×running buffer for Western blot (1000 ml)

Trisma base	15.1	g
Glycine	94	g
SDS	5	g
Deionized water was add to adjust volume into	1000	ml

11. 1.5M Tris-Cl pH 8.8 1000 ml

One point five mole of Trisma-base was dissolved in sterile deionized water 800 ml, pH was adjusted into 8.8. Finally volume was adjust into 1000 ml

12. 2×Laemmli buffer (SDS-dye) 10 ml

1 M Tris-HCl pH 6.8	1	ml
10% SDS	4	ml
99.5% glycerol	2.01	ml
Bromphenol blue	0.001	g
HPLC water was add to adjust volume into	10	ml

13. Transfer buffer for Western blot

Trisma base	5.08	g
Glycine	2.9	g
SDS	0.37	g

All reagents was dissolved in deionized water before added absolute methanol 200 ml. Volume was adjusted into 1000 ml by deionized water.

14. 1×PBS pH 7.4 1000 ml

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g

KH$_2$PO$_4$ 0.24   g

Volume was adjusted into 1000 ml by deionized water before autoclaved at 121°C and pressure 15 psi for 15 min.

15. PBST (washing buffer for Western blot)

1×PBS   500   ml

Tween20   0.05   %

16. Blocking solution for Western blot

PBST   200   ml

Non-fat dry milk   6   g

17. ECL substrate of HRP

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

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

18. Solution A

100 mM Tris-HCl pH 8.5 (stored at 4˚C)	2.5	ml
90 mM coumaric acid	11	μl
250 mM luminol	12.5	μl

19. Solution B

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

20. Film developer and fixer

Film developer and fixer were diluted in tap water at dilution 1:4.

21. 0.01% DEPC water for RNA 100 ml

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

22. 75% Ethanol in DEPC 100 ml

Twenty-five milliliter of 0.01% DEPC water was added in 75 ml of Ethonol and kept at –20°C.

23. Rehydration buffer for 2D-GE 10 ml

Thiourea	1.522	g
Urea	4.2	g
CHAP	0.2	g

Adjust volume into 10 ml, aliquoted and kept at -20°C. Immediately before used, add 0.65 µl IPG buffer, bromophenol blue solution and 50 mM DTT into 250 µl rehydration buffer.

24. Equilibration buffer for 2D-GE 200 ml

Urea	72.07	g
SDS	4	g
Glycerol	40.2	ml
Tris-Cl	0.375	M

Adjust volume into 200 ml, aliquoted and kept at -20°C.

25. Equilibration buffer I for 2D-GE

Equilibration buffer	4	ml
DTT	0.08	g

26. Equilibration buffer II for 2D-GE

Equilibration buffer	4	ml
iodoacetamide	0.1	g

27. 1M CH₃COONa , pH 4.0 1000 ml

One mole of CH_3COONa was dissolved in sterile deionized water 800 ml, pH was adjusted into 4.0. Finally volume was adjusted into 1000 ml

28. 1M Ammonium bicarbonate 1 ml

Ammonium bicarbonate	79.06	mg
Water was add to adjust volume into	1	ml

29. 1mM HCl 1ml

Conc.HCl	30.67	μl
Sterile deionized ultrapure water	969.33	μl

30. 100 mM ammonium bicarbonate 1 ml

1M Ammonium bicarbonate	100	μl
Sterile deionized ultrapure water	900	μl

31. 10 mM DTT in 100 mM ammonium bicarbonate 1 ml

DTT	1.54	mg
Sterile deionized ultrapure water	1	ml

32. 55 mM iodoacetamide in 100 mM ammonium bicarbonate

Iodoacetamide	10.17	mg
Sterile deionized ultrapure water	1	ml

33. Extraction buffer 1.5 ml

5% v/v formic acid	500	μl
Acetonitrile	1000	μl

34. 0.2M Citric acid 1000 ml

Thirty-nine point thirty-eight gram of Citric acid was dissolved in sterile deionized water and adjusted volume into 1000ml

35. 66.6 ng/µl trypsin 1.5 ml

Trypsin	100	µg
1mM HCl	1.5	ml

Trypsin were aliquoted and kept at -20°C

36. 0.2M TMB (3,3",5,5"-Tetramethylbenzidine) buffer 1000 ml

Sixty-six point five milligram of tripotassium citrate monohydrated and 39.38 g of Citric acid were dissolved in sterile deionized water. pH was adjust into 4.0. Finally volume was adjusted into 1000 ml.

37. TMB substrate 250 µl

Two point five milligram of TMB was dissolved in 250 µl DMSO. The solution was kept in dark until used.

38. ELISA substrate

TMB substrate	250	µl
30% H2O2	2.5	µl
TMB buffer	10	ml

39. 70% Ethanol for propidium iodide staining 1000 ml

Absolute ethanol	700	ml
Sterile deionized ultrapure water	300	ml

40. 10 mg/ml RNase A 1 ml

RNaseA	10	mg
Sterile deionized water	1	ml

41. 1 mg/ml propidium iodide 1 ml

Propidium iodide	1	mg
Sterile deionized water	1	ml

Biography

I graduated from the Faculty of Allied Health Science, Chulalongkorn University with a Bachelor's degree in 2008. I received a certificate in Medical Technology and worked as medical technologist in Lab Plus One for 1 year. After that I enrolled in a Graduate Program in Biotechnology, Faculty of Science, Chulalongkorn University in 2009.

In 2011, my presentation was awarded the third prize for oral presentation in the topic of "Phosphoproteome in lipopolysaccharide-stimulated macrophage treated with inhibitor Notch signaling." from the 16th Biological Sciences Graduate Congress (BSGC). This conference was held at National University of Singapore, Singapore during Dec 12th-14th, 2011

In 2012, I received the excellent oral presentation award from the 1st ASEAN Plus Three Graduate Research Congress (AGRC). This academic conference was held at Chiang Mai University, Thailand, during Mar 1st-2nd, 2012

PUBLICATIONS

1. Natt Boonyatecha(1), **Naunpun Sangphech**(1), Wipawee Wongchana, Pathipark Kueanjinda, Tanapat Palaga (2012). Involvement of Notch signaling pathway in regulating IL-12 expression via c-Rel in activated macrophages. Molecular Immunology51, 255-262((1) is equal distribution)

2. Yanin Kuncharin, **Naunpun Sangphech**, Patipark Kueanjinda, Parvapan Bhattarakosol, Tanapat Palaga (2011). MAML1 regulates cell viability via the NF-κB pathway in cervical cancer cell lines. Experimental Cell Research 317, 1830-1840