การติดต่อกันระหว่างวิถีสัญญาณนอตช์และตัวรับแบบโทลล์-ไลค์ในแมโครฟาจ และบทบาทในโรคภูมิคุ้มกันต้านเนื้อเยื่อตนเอง

นางสาววิภาวี วงศ์ชนะ

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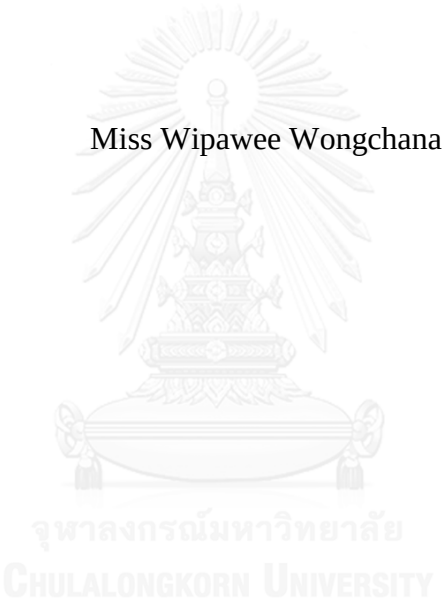
CROSSTALK BETWEEN NOTCH AND TOLL-LIKE RECEPTOR SIGNALING PATHWAYS IN MACROPHAGES AND ITS ROLES IN AUTOIMMUNE DISEASES

Miss Wipawee Wongchana



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Field of Study	Biotechnology
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วิภาวี วงศ์ชนะ : การติดต่อกันระหว่างวิถีสัญญาณนอตช์และตัวรับแบบโทลล์-ไลค์ในแมโครฟาจและบทบาทในโรคภูมิคุ้มกันต้านเนื้อเยื่อตนเอง (CROSSTALK BETWEEN NOTCH AND TOLL-LIKE RECEPTOR SIGNALING PATHWAYS IN MACROPHAGES AND ITS ROLES IN AUTOIMMUNE DISEASES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ธนาภัทร ปาลกะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. บาร์บารา เอ. ออสบอน, 109 หน้า.

แมโครฟาจเป็นเซลล์ที่จัดอยู่ในระบบภูมิคุ้มกันแต่กำเนิด ซึ่งการตอบสนองของแมโครฟาจมีความยืดหยุ่นตามแต่จุลภาพทางสิ่งแวดล้อม ส่งเร้าชนิดต่างๆ สามารถเหนี่ยวนำวิถีสัญญาณในแมโครฟาจซึ่งรวมถึงวิธีสัญญาณ Notch ทำให้เกิดการผลิตไซโตไคน์และสารตัวกลางของการอักเสบต่างๆ ด้วยเหตุนี้แมโครฟาจที่ก่อให้เกิดการอักเสบจึงมีความสัมพันธ์กับการเกิดโรคภูมิต้านทานเนื้อเยื่อตนเองซึ่งรวมถึง โรคมัลติเพิลสเกลอโรซิส การกระตุ้นแมโครฟาจด้วย IFNγ และไลโปโพลีแซคคาร์ไรด์ (LPS) นำไปสู่การตอบสนองและหลั่งไซโตไคน์ที่เกี่ยวกับการอักเสบหลายชนิด รวมไปถึง IL-12 และIL-10 ในงานวิจัยนี้ ได้ทำการศึกษาผลของความบกพร่องในการแสดงออกของ Notch1 ในแมโครฟาจที่ถูกกระตุ้นด้วย IFNγ และLPS ในแบบจำลองหนูเม้าส์ของโรคภูมิต้านทานเนื้อเยื่อตนเองชนิด Experimental autoimmune encephalomyelitis (EAE) รวมทั้งศึกษาบทบาทของวิถีสัญญาณ Notch ในการควบคุมการแสดงออกของ IL-12p40 นอกจากนี้ และศึกษาบทบาทของวิถีสัญญาณ Notch ต่อการผลิต IL-10 ของแมโครฟาจที่กระตุ้นด้วย IFNγ และ LPS ร่วมกับสารประกอบอิมมูน . การศึกษาผลของการบกพร่องในการแสดงออกของ Notch1 ในแมโครฟาจที่ถูกกระตุ้น IFNγ/LPS โดยแมโครฟาจจากหนูเมาส์สายพันธุ์ *Notch1*$^{fl/fl}$X *Mx1cre*$^{+/-}$ (N1KO) หรือสายพันธุ์ควบคุม ถูกโอนถ่ายให้หนูเม้าส์ผู้รับก่อนการเหนี่ยวนำให้เกิด EAE จากผลการทดลองพบว่า กลุ่มหนูเม้าส์ที่ได้รับแมโครฟาจจาก N1KO มีการเกิดอาการที่ช้าลง และมีความรุนแรงน้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ การทดลองในระดับ *in vitro* พบว่าหลังจากการกระตุ้นเซลล์ม้ามด้วย MOG_{35-55}เพปไทด์ เซลล์ม้ามจากหนูเม้าส์ที่ได้รับการโอนถ่ายแมโครฟาจ N1KO ผลิต IL-17 ที่น้อยกว่ากลุ่มควบคุม ขณะที่มีการผลิต IFNγ ที่ไม่แตกต่างกัน การทดลองในระดับ *in vitro* ด้วยการกระตุ้นแมคโครฟาจ N1KO พบว่ามีการผลิต IL-6 ที่ลดลง และมีการแสดงออกของ CD80 ลดลง เมื่อเปรียบเทียบกับแมโคฟาจควบคุม แต่แมโครฟาจ N1KO ไม่มีความบกพร่องในการผลิต IL-12p40/70 ขณะที่แมโครฟาจจากหนูเม้าส์สายพันธุ์ *CSL/Rbp-jκ*$^{fl/fl}$ X *Mx1cre*$^{+/-}$ (CSL/RBP-Jκ KO) มีฟีโนไทป์เหมือนกับการใช้ยากดวิถีสัญญาณ Notch (GSI) โดยมีการผลิต IL-12p40/70 ที่ลดลง ในระดับโมเลกุลพบว่าการเคลื่อนที่สู่นิวเคลียร์ของ c-Rel ซึ่งเป็นหน่วยย่อยของวิถีสัญญาณ NF-κB ซึ่งมีบทบาทในการควบคุมการถอดรหัสของ *il12p40* น้อยลงในแมโครฟาจที่ได้รับ GSI และแมโครฟาจ CSL/RBP-Jκ KO แต่ไม่มีการเปลี่ยนแปลงในแมโครฟาจ N1KO จากผลการทดลองแสดงให้เห็นว่า Notch1 ในแมโครฟาจมีผลกระทบต่อการพยาธิสภาพและความรุนแรงใน EAE ซึ่งอาจผ่านทางการลดลงของ IL-6 และการแสดงออกของ CD80 ซึ่งเกี่ยวข้องกับการตอบสนองของ Th17 แต่ไม่เกี่ยวกับการตอบสนองของ Th1 นอกจากนี้ผลนี้ยังบ่งชี้ว่า Notch1 ไม่จำเป็นต่อการผลิต IL-12p40/70 ในแมโครฟาจที่ถูกกระตุ้นด้วย IFNγ และ LPS ในขณะที่ CSL/RBP-Jκ หรือโมเลกุลเป้าหมายอื่นของ GSI มีผลต่อการผลิต IL-12p40/70 สำหรับแมโครฟาจที่ถูกกระตุ้นด้วย IFNγ LPS ร่วมกับสารประกอบอิมมูน มีฟีโนไทป์ที่ต่างไปเมื่อเปรียบเทียบกับแมโครฟาจที่ถูกกระตุ้นด้วย IFNγ และ LPS โดยที่มีการแสดงออกของ IL-10 มากกว่า IL-12 และพบว่าการผลิต IL-10 ในแมโครฟาจนั้นขึ้นตรงกับ LPS และการกระตุ้นแมคโครฟาจด้วย LPS ร่วมกับสารประกอบอิมมูน มีผลกระทบโดยตรงต่อการผลิต IL-10 IL-12 และ IL-6 แต่ไม่มีผลต่อ TNFα แมโครฟาจที่ถูกกระตุ้นด้วย IFNγ/LPS และสารประกอบอิมมูนนี้มีการเหนี่ยวนำการแสดงออกขององค์ประกอบต่างๆ ของวิถีสัญญาณ Notch แต่วิถีสัญญาณ Notch เริ่มขึ้นเมื่อมีกระตุ้นด้วย LPS เท่านั้น โดยการกระตุ้นวิถีสัญญาณของ Notch ขึ้นกับวิถีสัญญาณ NF-κB และ MAPK เช่นเดียวกับที่เกิดขึ้นในการกระตุ้นแมโครฟาจด้วย LPS เพียงลำพัง การใช้ยากดวิถีสัญญาณต่างๆ ยืนยันได้ว่าการผลิต IL-10 ในแมโครฟาจที่ถูกกระตุ้นด้วย IFNγ/LPS และสารประกอบอิมมูนมีการควบคุมผ่านวิถีสัญญาณ NF-κB, MAPK และ PI3K และยังพบว่าพบว่าการผลิต IL-10 ลดลงเมื่อใช้ GSI ในแมโครฟาจที่ถูกกระตุ้นด้วย IFNγ/LPS และสารประกอบอิมมูนโดยพบว่าวิถีสัญญาณ Notch มีผลเชิงบวกต่อการกระตุ้นวิถีสัญญาณ NF-κB และ MAPK ซึ่งนำไปสู่การผลิต IL-10 แต่ ไม่มีผลกระทบต่อการกระตุ้นวิถีสัญญาณ PI3Kในสภาวะการกระตุ้นเช่นนี้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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WIPAWEE WONGCHANA: CROSSTALK BETWEEN NOTCH AND TOLL-LIKE RECEPTOR SIGNALING PATHWAYS IN MACROPHAGES AND ITS ROLES IN AUTOIMMUNE DISEASES. ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., CO-ADVISOR: PROF. BARBARA A. OSBORNE, Ph.D., 109 pp.

Macrophages are innate immune cells which their plasticity depends on the microenvironment. Various activators act on macrophages by induction of cascade signaling pathways including Notch signaling, resulting in secretion of diverse cytokines and inflammatory mediators. Consequently, inflammatory macrophages are associated with autoimmune diseases including multiple sclerosis. Stimulation of macrophages by IFNγ and lipopolysaccharide (LPS) leads to activation and secretion of various inflammatory related cytokines, including IL-12 and IL-10. In this study, we investigated the impacts of Notch1 deletion in LPS-activated macrophages in an Experimental autoimmune encephalomyelitis (EAE) model and the role of Notch signaling in regulating the expression of IL-12p40. Moreover, the role of Notch signaling in mediating IL-10 production in LPS/immune complex-activated macrophages is investigated. To examine the impact of *Notch1* deficiency in activated macrophages, an adoptive transfer of activated macrophages derived from *Notch1*$^{fl/fl}$ X *Mx1cre*$^{+/-}$ (N1KO) mice or wild type mice was performed before the induction of EAE. Mice receiving activated N1KO macrophages showed a delay in the onset and decreased severity of EAE, compared to mice receiving wild type activated macrophages. *In vitro* re-stimulation of splenocytes by MOG_{35-55} peptide from these mice revealed that cells from mice receiving N1KO macrophages produced significantly less IL-17, compared to the control mice whereas IFNγ production was similar in both conditions. Furthermore, activated N1KO macrophages *in vitro* produced less IL-6 and exhibited lower CD80 expression. Activated N1KO macrophages did not exhibit any defect in IL-12p40/70 production whereas activated macrophages derived from *CSL/Rbp-jκ*$^{fl/fl}$ X *Mx1cre*$^{+/-}$ (CSL/RBP-Jκ KO) mice phenocopied that of the gamma secretase (GSI) treatment which inhibits activation of Notch receptors. Furthermore, the nuclear translocation of NF-κB subunit c-Rel, one of the key regulatory factors in controlling *il12p40* transcription, was compromised in GSI-treated and CSL/RBP-Jκ KO, but not in the N1KO macrophages. These results suggest that Notch1 expression in macrophages may affect the onset and severity of EAE through decreased IL-6 and CD80 which is involved in Th17 but not in Th1 response. Moreover, our findings suggest that Notch1, but not CSL/RBP-Jκ or gamma secretase activity is dispensable for IL-12p40/70 production in macrophages. Unlike the phenotype of LPS-activated macrophages, activation of macrophages with LPS in the presence of immune complex results in high level of IL-10 and low level of IL-12 production. Moreover, we investigated and shown that the production of IL-10 in macrophages was LPS-dependent and the activation of macrophages by IFNγ/LPS together with immune complex impacted the production of IL-10, IL-12, IL-6 but not TNFα. IFNγ/LPS+immune complex activated macrophages expressed Notch signaling molecules and the activation of Notch signaling was initiated after LPS-stimulation. The activation of Notch signaling depended upon NF-κB and MAPK pathways similar to that of LPS stimulation alone. Using pharmacological inhibitors to inhibit key signaling pathways, we confirmed that IL-10 production in LPS/immune complex activated macrophages were regulated by NF-κB, MAPK and PI3K. Furthermore, inhibition of Notch signaling using GSI resulted in the reduction of IL-10 production in the all LPS-stimulated condition. Finally, we found that Notch signaling affected the activation of NF-κB and MAPK signaling but not PI3K in this setting.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	Percentage
:	Ratio
×	Fold
®	Registration
°C	Degree Celsius
µg	Microgram
µL	Micro liter
µM	Micro molar
µm	Micrometer
A	Absorbance
Ab	Antibody
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC (dye)	Allophycocyanin
ADE	Antibody-dependent enhancement
BMDMs	Bone marrow derived macrophage
Bp	Base pair
C/EBP	CAAT enhancer-binding protein
CD	Clustter of differentiation
cDNA	Complementary DNA
CNS	Central nerve system
CoA	Co activator

CoR	Co repressor
CO_2	Carbon dioxide
CSL/RBP-Jκ	CBF-1/RBPJk in mammals, Su(H) in Drosophila, Lag-1 in C. elegans
DAMP	Damage associated molecules patterns
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNMAML	Dominant negative Mastermind-like
dNTP	DATP, dCTP, dGTP and dTTP
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FcγR	Fc gamma receptor
g (centrifugation speed)	Gravity
g	Gram
GSI	Gamma secretase inhibitor
HDAC	Histone deacetylase
Hes1	Hairy/Enhancer of Split1
hr	Hour
HRP	Horse radish peroxidase
IC	Immune complex
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell

IRF	Interferon regulatory factor
i.p.	Intra peritoneal
kDa	Kilo dalton
LPS	Lipopolysaccharide
M	Murine
mA	Milliampere
MAPK	Mitogen activated protein kinases
MFI	Mean fluorescence intensity
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
ng	Nanogram
NIC	Intracellular domain of Notch
NK	Natural killer
nm	Nanometer
No	Number
OD	Optical density
OVA	Oval albumin

PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PBS-Tween20	Phosphate buffer saline – Tween
PCR	Polymerase chain reaction
PE (dye)	Phycoerythrin
PI3K	Phosphoinositide 3-kinases
PRRs	Pattern-recognition receptors
PVDF	Polyvinylidine fluoride
r	Recombinant
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
rpm	Round per minute
RT	Reverse transcription
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SLE	Systemic lupus erythematosus
TAM	Tumor associated macrophage
Th	T helper cell
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
Treg	T regulatory cell
™	Trade mark
U	Unit

v/v	Volume by volume
-ve	Negative control
WT	Wild type
w/v	Weight by volume
α	Alpha
β	Beta
γ	Gamma

CHAPTER I
INTRODUCTION

CROSSTALK BETWEEN NOTCH AND TOLL-LIKE RECEPTOR SIGNALING PATHWAYS IN MACROPHAGES AND ITS ROLES IN AUTOIMMUNE DISEASES

Background

Macrophages are innate immune cells which mediate innate and adaptive immune responses. Signaling through TLRs triggers macrophage activation, leading to the cellular activities and the production of pro-and anti-inflammatory cytokines. The activation of macrophages is influenced by the downstream signaling of other receptors including Fc gamma receptors (FcγR) as well. Not only signaling pathway through TLR and FcγR has been studied but the crosstalk among TLR, FcγR and other signaling pathways also warrant investigation in the field.

The Notch signaling pathway is highly conserved in all metazoans and has important roles in development, differentiation, activation and apoptosis in various types of cells including macrophages (1). The binding of Notch receptors to their ligands initiates the Notch signaling cascade. This event results in releasing of intracellular domain of Notch receptor (NIC) which translocates to nucleus and binds to CSL/RBP-Jκ which is a DNA binding protein, and recruits other co-activators to the promoter of the target genes (2).

Murine and human macrophages from various tissues express Notch ligands and receptors. Previous reports showed that the involvement of Notch signaling in

regulation of biological activities of LPS/TLR4 activated macrophages called M1 macrophages, leads to cytokine expression such as IL-10, IL-6 and TNF-α. The activation of macrophages by TLR stimulation affects Notch signaling through NF-κB and/or MAPK activation and vice versa (3). These observations strongly imply that TLR signaling cross regulate the Notch signaling in macrophages.

Dysregulation in production of cytokines and biological functions in macrophages are linked with many inflammation-related diseases. These diseases are also associated with TLR-activated macrophages. In previous studies, the Notch signaling pathway has been documented in close relationship with TLR-activated macrophages in diseases such as SLE (4). Consequently, Notch signaling pathway collaborates with TLR signaling in macrophages in pathological conditions which may lead to severe diseases, especially autoimmune diseases.

In experimental autoimmune encephalomyelitis (EAE), which is a murine model of multiple sclerosis (MS), deletion of infiltrated macrophages in central nervous system (CNS) suppress EAE (5) and LPS/immune-complex-activated macrophages, which produce high levels of IL-10, also suppress the development of EAE (6). However, there has been no investigation about Notch signaling pathway in macrophages in EAE.

Macrophages express various types of FcγRs (7). Activation of macrophages by LPS together with an immune complex leads to the macrophage activation called Type II macrophages (also called M2b and M(IC) macrophages) (8, 9). This type of macrophage produce high level of IL-10, but low level of IL-12 which allow them to function as regulatory cells during activation . However, this phenotype of macrophage can cause autoimmune diseases such as in SLE. It has been shown that

Notch signaling is also involved in regulating the expression of IL-10 in T cells and macrophages (3, 10, 11). However, how Notch signaling in macrophages is activated by LPS and immune complex and whether Notch signaling regulates IL-10 production are still undocumented.

To understand molecular mechanisms and the involvement of Notch signaling in macrophage activation and cytokine production, we performed experiments to study the involvement of Notch signaling pathway in TLR activated macrophages using EAE as a model. Moreover, we observed the crosstalk among Notch signaling, downstream of FcγR signaling and TLR signaling which cooperates in the activation of IL-10 producing macrophages.

Hypothesis

(1) The Notch signaling pathway in LPS/TLR4-activated macrophages is involved in determining the outcome of EAE through the regulation of cytokine production

(2) The Notch signaling pathway regulates IL-10 production in IFNγ/ LPS + immune complex-activated macrophages

Objectives

(1) To determine the roles of LPS/TLR4-induced Notch activation in macrophages in EAE

(2) To investigate the signaling downstream of TLR and FcγR which mediates Notch activation and the involvement of Notch signaling in the production of IL-10 in IFNγ/LPS+immune complex-activated macrophages

CHAPTER II
LITERATURE REVIEW

Macrophages

Macrophages are white blood cells which originate either from the yolk sac during embryonic development or from adult blood monocytes. These cells play important roles in inflammation, infection and cancer (12-14). Several surface markers such as F4/80, CD11b and Ly6C are commonly used to identify and distinguish the macrophage population (15). Macrophages are highly plastic cells which play multiple functions; from development, homeostasis to immune response depending on their microenvironments. Occasionally, dysregulation in the function of macrophages are involved in several diseases such as infectious diseases, metabolic disorders, autoimmune diseases and tumor developments. Therefore, it is critical to understand the molecular mechanisms of activation and phenotypic diversities of macrophages (Fig.1).

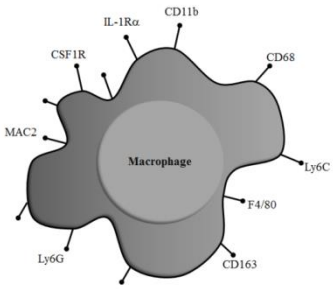


Figure 1 Macrophage surface markers

Macrophages are distinguished from DCs and other myeloid cell by differential expression of surface makers such as F4/80 and CD11b Nevertheless, macrophages share some markers with myeloid DCs because their development are from common progenitors (15)

Macrophages Mediate Immune Response

Macrophages mediate both innate and adaptive immune responses. In the infectious condition, macrophages eliminate foreign subjects by phagocytosis. The signaling through various receptors on macrophages such as Toll like receptors (TLRs) leads to their activation which results in enhanced phagocytic activities. They produce chemical mediators such as nitric oxide to kill invasive pathogens. The activation also leads to increased expression of molecules on cell surface of macrophages which enable them to present peptide antigens to activated T cells. Furthermore, the macrophages which are activated by different stimulators produce diverse pro-and anti-inflammatory cytokines which initiated the activation and the differentiation of many cell types such as T helper cells. (16). These processes facilitate a cascade of immune responses, including immunoglobulin secretion from B cells to enhance the clearance of foreign antigens.

After an adaptive immune response is initiated, macrophages receive a feedback signal resulting in enhancing their activations and effector functions. Interferon (IFN)γ, a major cytokine produced from T helper 1 (Th1) cells and NK cells is able to prime and promote chromatin remodeling and augments the pro-inflammatory cytokine productions in activated macrophages (17). Immune complexes, of antigen-antibody complexes which occur after antigen encounter also regulate the activation of macrophages via their Fcγ receptors (FcγRs) leading to cellular activities in macrophages subsequently either by boosting the phagocytotic processes or dampening inflammatory responses (7)

Macrophages also play important roles in the resolution of destructive inflammation and can act to control homeostasis. Moreover, macrophages drive tissue

repairing processes (18, 19). In intestinal homeostasis, microbes in gastrointestinal tract induce macrophages to produce the pro-inflammatory cytokine, IL-1β which activates type 3 innate lymphoid cells (ILC3 cells). This cytokine, in turn, regulates the production of IL-10 and the retinoic acid receptor in intestinal DCs and macrophages which is responsible for maintaining colonic T_{reg} cells. (20). Moreover, to control self-reactivity to apoptotic cells, macrophages, located in the marginal zone of spleen, play a role to suppress immunity. This vital role was confirmed in the absence of marginal zone macrophages which lead to the formation of DNA-specific antibodies and induces a systemic lupus erythematous-like autoimmune syndrome (21).

Taken together, most of the activities in the immune system require systemic interaction and macrophages play a central role in these activities (Fig.2).

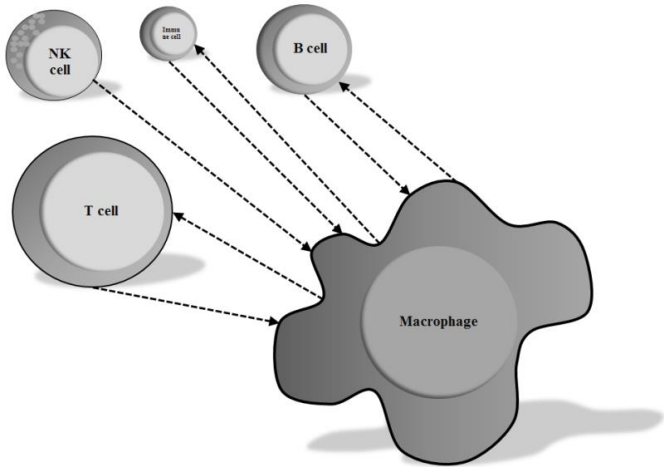


Figure 2. Macrophages function as a bridge between innate and adaptive immune response

Activated macrophages produce various cytokines and express surface molecules which trigger the activation or polarization of cells in immune system.

Activation of Macrophages and Its Molecular Mechanisms

The activation of macrophages is triggered by their environmental cues such as pathogens, cytokines and immune complexes. Activations may occur in a steady state, inflammation state or pathological state.

The response of the innate immune cells to clear foreign antigens is initiated when pattern-recognition receptors (PRRs) bind to specific pathogen-associated molecular patterns (PAMPs). One of the PRRs, which play a critical role in inflammatory response, is Toll-like receptor (TLR) protein family. More than 10 TLRs are identified in mammals (22). Like other PRRs, inflammatory response through TLRs is initiated by interaction between TLRs and their ligands, e.g. TLR4 which recognizes lipopolysaccharide (LPS) found in Gram negative bacteria, and TLR2 which recognizes peptidoglycan found mainly in Gram positive bacteria and fungus (23). This interaction results in activation of a canonical TLRs signaling pathway, following by activation of nuclear factor kappa light chain enhance of activated B cells (NF-κB), mitogen activated protein kinases (MAPKs), as well as the interferon-regulatory factors (IRFs) through the adaptor protein, Myeloid differentiation primary response gene 88 (MyD88)-dependent and -independent manner. These signaling cascades initiate the activation of macrophages and control the production of various cytokines as well as promote the expression of molecules on macrophages that play crucial roles in adaptive immune response (24)(Fig.3). In the absence of MyD88, macrophages fail to produce pro-inflammatory cytokines and exhibit resistance to LPS (25). Similar to $MyD88^{-/-}$ macrophages, the phagocytosis activity of $TRIF^{-/-}$ macrophages is impaired and they are susceptible to gram negative bacterial infection (26).

Signaling through LPS/TLR4 augments phagocytosis and killing activities in macrophages (27). The cascade of downstream signaling molecules which are induced by TLR4 ligand facilitates the transcriptional activities of the inflammatory-associated cytokine productions such as TNFα, IL-1β, IL-6, IL-12, type I Interferon, and low amounts of IL-10. Additionally, priming of macrophages by IFNγ results in increasing TLR-induced cytokine gene expression. IFNγ priming facilitates the remodeling of chromatin to increase chromatin accessibility, enhances the recruitment of TLR-induced transcription factors and is involved in epigenetic changes (28). These macrophages are well characterized and called M1 or M (IFNγ+LPS) macrophages (9).

It has been shown that self-antigens such as DNA and RNA which are damage associated molecules patterns (DAMPs) can be recognized by TLRs resulting in pathology of autoimmune diseases such as in RA patients (29). Like other TLR ligands, DAMPs initiate signaling through TLRs on macrophages and induce pro-inflammatory cytokines, resulting in improper activation of the immune cells. It is not only signaling pathway through TLR activation alone that is interesting, but the crosstalk between TLRs and other signaling pathways is of strong interest in the field.

Macrophages can be activated by signaling through FcγRs. Immune complex and IgG-opsonized pathogens or particles bind to FcγRs which are functionally characterized as activation and inhibitory receptors (7). The binding of immune complex share FcγR but the molecular mechanisms underline the internalization of activating FcγR and inhibitory FcγR are different, resulting in the difference in outcome of cellular activities (30) (Fig.4).

Activating FcγRs have immuno-receptor tyrosine-based activation motif (ITAM) which associate with the ITAM-containing signaling subunit FcR common γ-chain. The internalization of immune complexes through activating FcγRs is dependent on the tyrosines of the ITAM present in the FcγR complex. The signaling downstream of the ITAM cascade activates via SRC family kinases (SRC) and spleen tyrosine kinase (SYK) which in turn activates other signal-transduction molecules such as phosphoinositide 3-kinase (PI3K) and the Son of Sevenless homologue (SOS). As a result of these signaling events, immune complex-bearing activating FcγR increase cellular activities such as antigen uptake and production of pro-inflammatory cytokines (31)

On the other hand, inhibitory FcγRs have an immuno-receptor tyrosine-based inhibition motif (ITIM) which recruits SH2 domain-containing inositol 5'phosphatase I (SHIP-1) (32). SHIP-1 can reduce the activity of activating FcγRs (31). To emphasize the important role of inhibiting FcγRs, a gene knockout animal model such as FcγRIII$^{-/-}$ mice are used. The evidence suggests that in the absence of FcγRIII, animals suffered from inflammatory-related diseases such as sepsis and arthritis (33, 34). Moreover, in some cases such as tuberculosis (TB), FcγRIII$^{-/-}$ macrophages which are infected by *Mycobacterium tuberculosis* are able to induce increasing amount of IL-12 production and resistance to infection, whereas the absence of the FcR common γ chain is associated with increased susceptibility to infection. (35)

In macrophages, both activating FcγRs and the inhibitory FcγRs were expressed. The antigen size, concentration, IgG subclass affinities and IgG in the immune complex are factors that impact macrophage activation through FcγR. Moreover, density of Ig affects the immune response. In low density of Ig,

macrophages increase phagocytosic activity and produce low amount of IL-10 whereas high density of Ig increases the activation of MAPK/Erk which modulates the production of IL-10 in situations where immune responses need to be terminated (36). Additionally, the ratio between activating and inhibitory FcγRs is a factor that regulates the degree of the inflammatory response mediated by immune complex (37).

Crosstalk between Downstream Signaling of TLR and FcγR in macrophages

Macrophages, which receive signaling from immune complex alone, are able to produce baseline activation of inflammatory mediator, nitric oxides and the uptake of the immune complexes or IgG opsonizing antigen of macrophages can induce degradation of antigen (38). Mosser *et al.* proposed a new phenotype of activated macrophages which were triggered by LPS together with immune complex. This stimulation leads to the macrophage activation called Type II macrophages (also called M2b and M(IC) macrophages) (8, 9) (Fig.5). These macrophages produce a high level of IL-10, but low levels of IL-12. IL-10 is considered to be a functional cytokine marker of LPS/immune complex activated macrophage which marks them to function as the regulatory cells during the activation state. However, this phenotype of macrophages can cause autoimmune diseases, especially in SLE and RA (39, 40). Since IL-10 is a regulatory cytokine which is important to control the inflammatory process, the molecular mechanism of IL-10 regulation is extensively studied in immune cells including macrophages (41). Although signaling through TLR and FcγR has been reported for the regulation of IL-10, other signaling pathways including Notch signaling also play a role. Therefore, crosstalk among TLR, FcγR and other signaling needs further investigation.

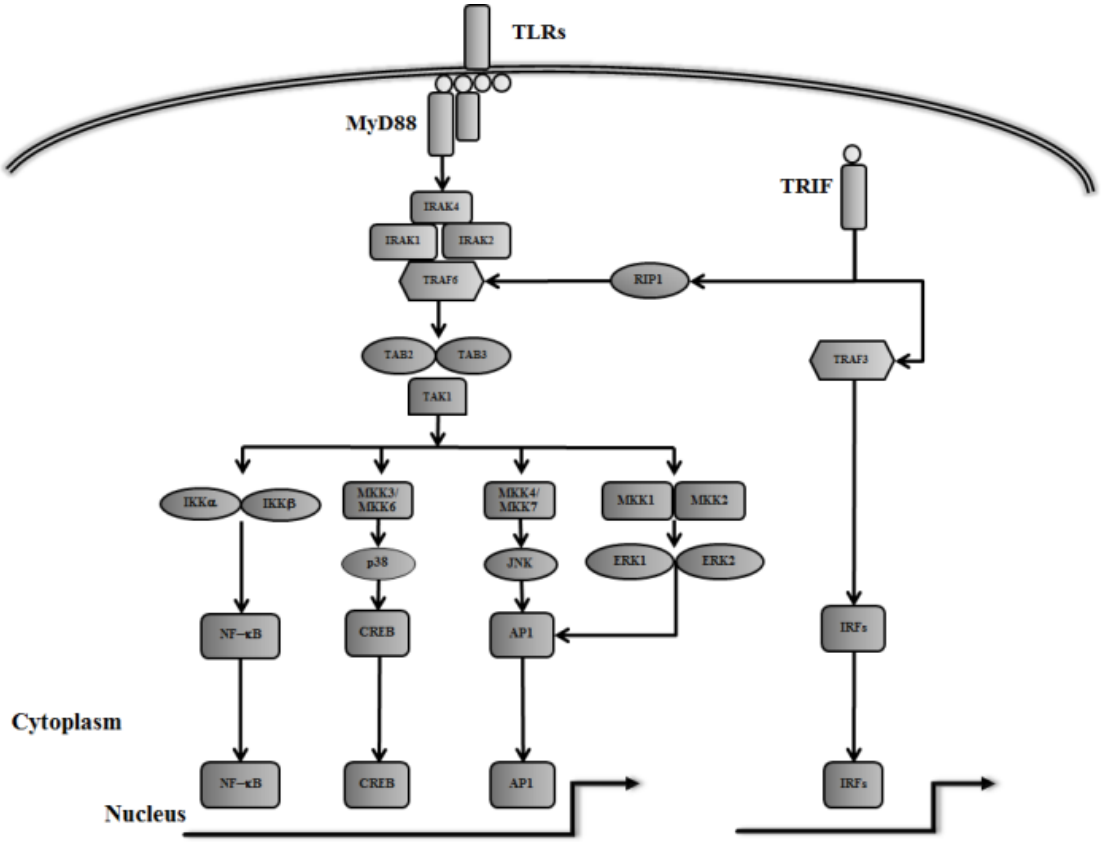


Figure 3. TLR and its downstream signaling; NF-κB, MAPK and IRFs

The binding of TLR ligand and receptor through MyD88 or TRIF leads to cascade activation NF-κB, MAPKs and IRFs. The downstream signaling of TLRs bring macrophage to the activation stage which are important to eliminate pathogens as well as trigger an adaptive immune response

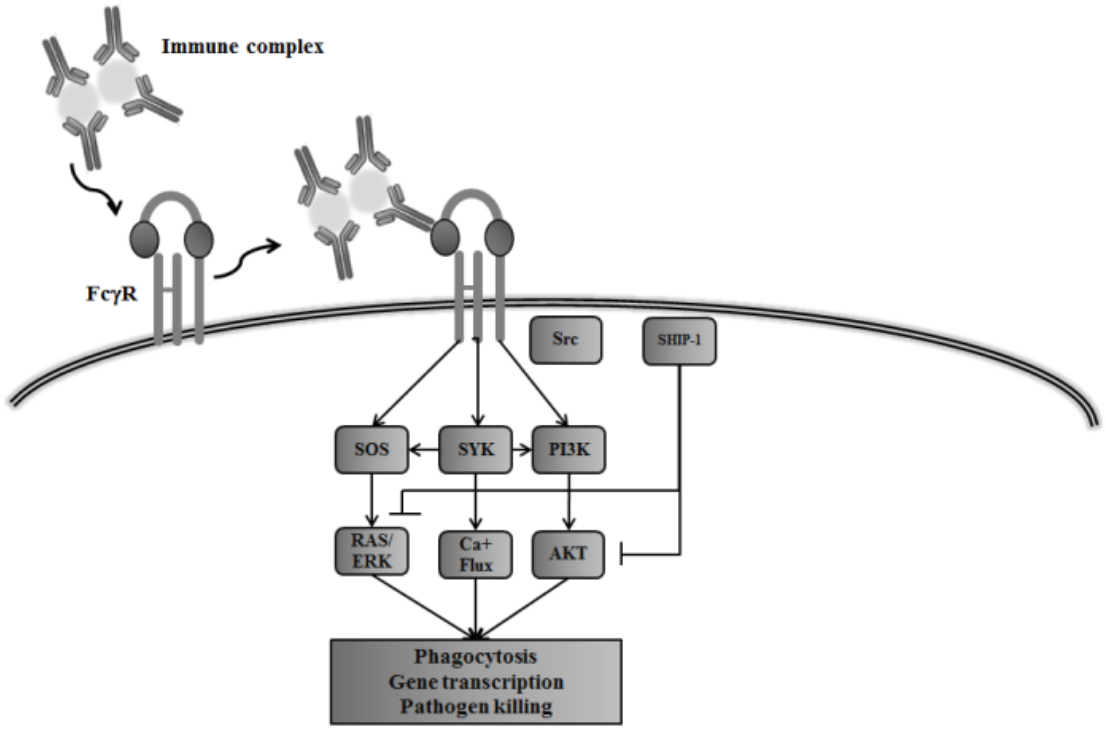


Figure 4. Downstream signaling of Fcγ receptor

An immune complex binds to activating Fcγ receptor and inhibiting Fcγ receptor on the surface of macrophages. Downstream signaling through FcγR ligation either triggers the phagocytosis activity and cytokine production to eliminate pathogen by activation of Src and SYK or inhibits pathogen killing activity through the recruitment of SHIP-1.

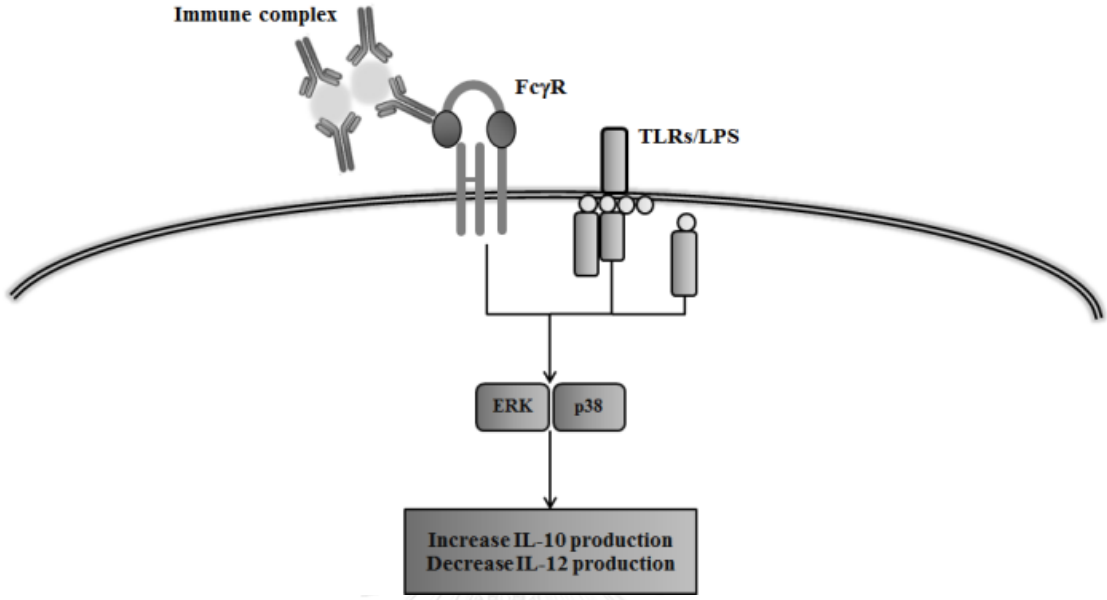


Figure 5. Crosstalk between TLR and FcγR signaling

Triggering macrophage by LPS together with an immune complex results in the increase of IL-10 production and the reduction of IL-12, Erk and p38, components of the MAPK signaling pathway are well known to regulate IL-10 production in this type of macrophages(42)

Notch Signaling Pathway and Its Signaling Crosstalk in Activated Macrophages

The Notch signaling pathway regulates the differentiation, proliferation, survival and cell fate decision in both myeloid and lymphoid lineage cells (1). There are four mammalian Notch receptors (Notch1-4) and five ligands (Delta-like 1, 3 & 4 and Jagged 1 & 2). The interaction between Notch ligands and receptors induces the enzymatic cleavage of Notch receptors, first by ADAM protease and subsequently by

gamma secretase, resulting in the release of the Notch intracellular domain. The cleaved intracellular domain of Notch translocates to nucleus and forms a complex with the DNA binding protein, CSL/RBP-Jκ which initiates the transcription of the Notch target genes (2) (Fig.6). Notch1 is the most extensively studied Notch receptor and it has been shown to play critical roles in regulating effector function of immune cells and to be involved in diseases such as cancers and autoimmune diseases (4, 43-45). In macrophages, Monsalve *et al*, demonstrated the expression of Notch family members and ligands in resting macrophages and also showed the effect of Notch1 in macrophages activation (46). Subsequently, it has been shown in several reports that Notch signaling is associated with TLR signaling in TLR activated macrophages. Palaga *et al.* demonstrated that TLR-activated macrophages expressed both intact Notch1 receptor as well as the cleaved forms, suggesting the activation of Notch signaling are triggered (3, 47) in this cell type. Recently, Foldi *et al*. showed that molecular mechanism in which the activation of Notch signaling was initiated by the Notch ligand, Jagged1, through NF-κB and MAPK signaling pathways in TLR-activated macrophages (48). The activation of Notch through TLR signalingalso is important in the activation and the production of cytokines in TLR-activated macrophages. The involvement of Notch signaling in regulation of key cytokines such as TNFα, IL-6, IL-12 and IL-10, was reported and Notch has been shown to control expression of key transcription factors, such as IRF8. Thus, Notch signaling is proposed to play the important roles in polarization of macrophages (10, 47, 49-51) .

As mentioned above, Notch signaling appears to control the production of cytokines in macrophages and mis-regulation of this function may end up by the initiating of diseases.

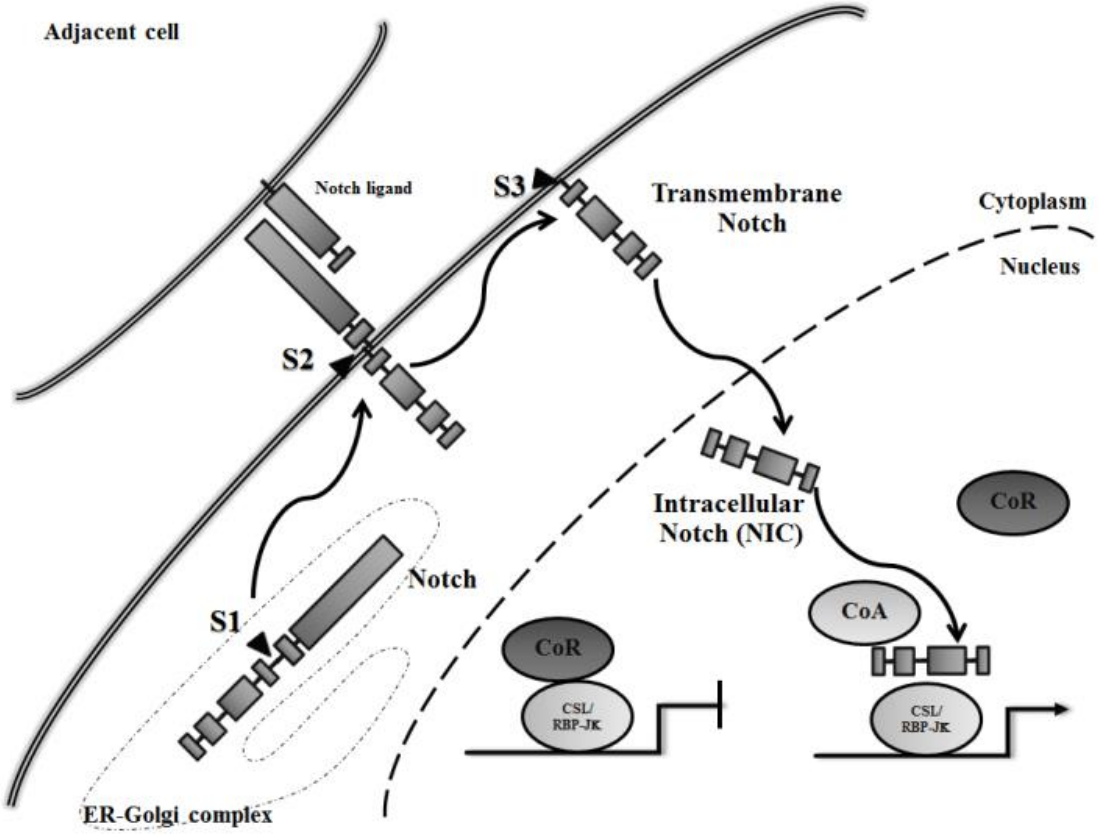


Figure 6. Notch signaling pathway

Interaction between Notch receptors and ligands trigger proteolitic cleavages of Notch, which releases truncated intracellular domain of Notch (NIC). NIC translocates into nucleus and removes CoR and forms a transactivation complex with CoA on DNA-binding protein CSL/RBP-Jκ . Adapted from Osborne and Minter.(52)

Macrophages and Diseases

Macrophages can be activated by various stimulators and this activation assists to eliminate the targets. This activation triggers other immune responses and promotes tissue repairing. The processes of activation must be tightly regulated because of the potential harmful side effect. Recently, several reports showed that uncontrolled macrophage activation leads to broad tissue damage and appearance of pathological states of many inflammation-related diseases.(Fig. 7) .For example, macrophages have been proposed to play a major role in sepsis (53, 54).

In certain cases, macrophages facilitate viruses, parasites and bacteria such as dengue virus and *Leishmania major* to escape the immune system. For example, the intrinsic antibody-dependent enhancement (ADE) of infected macrophages alters the severity of diseases such as dengue haemorrhagic fever. The immune complexes of infectious agents with non-neutralizing antibodies lead to increasing antigen uptake and induce the production of anti-inflammatory mediators such as IL-10 resulting in pathogen persistence (55).

In metabolic disorders, a number of studies show that, in obesity,macrophages, which accumulate in adipose tissue and surround adipocytes, induce chronic inflammation and insulin resistance resulting in type 2 diabetes (56). In atherosclerosis, arteriosclerotic vascular disease, macrophages and foam cells (low density lipoprotein-containing macrophages) are the cause of the pathophysiology of disease by their accumulation in an artery wall resulting in chronic inflammation and thicken of blood vessels (57). Likewise, macrophages are recruited and infiltrate livers and induce inflammation in non-alcoholic fatty liver disease (58).

Several investigations in animal models reveal the important role of macrophages in the pathology of autoimmune disease including experimental autoimmune encephalitis (EAE), animal model representative of multiple sclerosis in human. In addition, rheumatoid arthritis (RA) and systemic lupus Erythematous (SLE) are also associated with improper activation of macrophages through TLRs and FcγR.

The study of RA patients have observed the presence of activated macrophages (59). In SLE patients, the phagocytosic activities of monocytes and macrophages to remove apoptosis cells are compromised (60, 61) as well as atypical in other immunological functions including cytokine productions in macrophages were investigated (62). Moreover, macrophages are recruited to renal tissues where failure of kidney by accumulation of immune complexes, was initiated. Currently, in tumor pathology, macrophages called tumor associated macrophages (TAMs) are highlighted and extensively investigated (13).

Taken together, many examples indicate that failure in properly regulating the activations and functions of macrophages can result in disease. Therefore, the study of the molecular mechanisms of macrophage activation and function may shed a new light of further understanding in pathology of diseases and may lead to a novel therapeutic intervention.

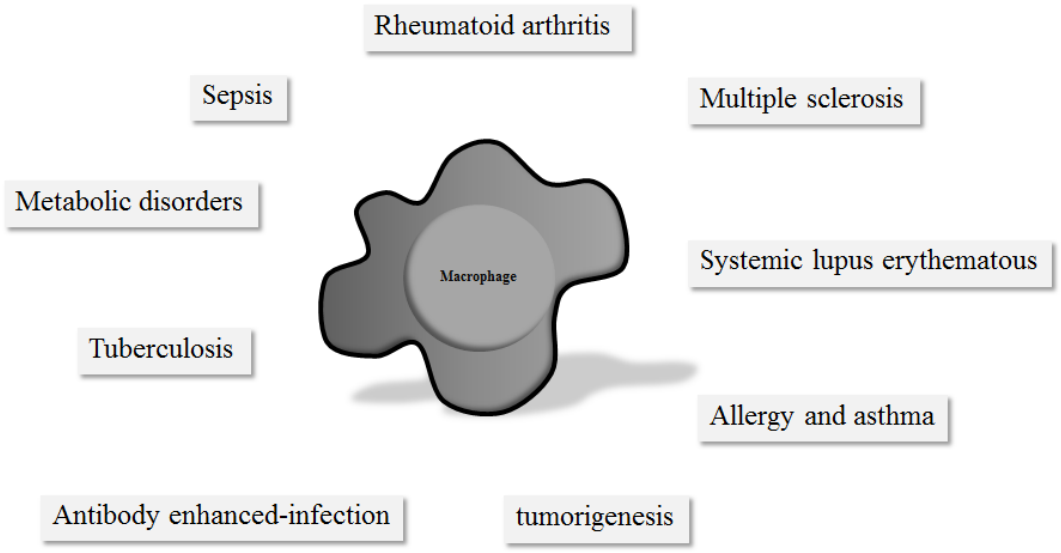


Figure 7. Macrophages and diseases

Dysfunction of macrophages results in inflammatory-related diseases such as infectious diseases, metabolic diseases, autoimmune diseases and cancer.

Notch Signaling and Experiment Autoimmune Encephalomyelitis (EAE)

It has known that the Notch signaling pathway collaborates with TLR signaling in macrophages under pathological conditions that can lead to autoimmune disease in particular in SLE (4). EAE, an animal model for multiple sclerosis, is well known to be mediated by both Th1 and Th17 CD4+ T cells for the progression and the severity of the disease. IL-12 (p40 and p35) and IL-23 (p35 and p19) plays important, but different, roles in polarization of T cells in EAE induction.(63) Recently, several studies have shown that not only auto-reactive T cells but also other cells types, play crucial roles in sustaining the disease by expressing pro- and anti-inflammatory cytokines, chemokines, and co-stimulatory molecules (64-68).

Several reports revealed the roles of Notch signaling in EAE, especially in T cells. Using a gamma-secretase inhibitor (GSI) incorporated in rodent chow (LY-chow) to inhibit Notch signaling *in vivo*, it was demonstrated clearly that Notch signaling regulates the Th1 and Th17 responses in the EAE model. A reduction in the severity of EAE-induced inflammation in GSI-treated animals was observed as well as decreases in the signature cytokines of the Th1 (IFNγ) and Th17 (IL-17) *in vitro* re-stimulation cultures of activated T cells (69, 70). In addition, the Notch ligand, Delta-like 4, on APCs, including macrophages, interacts with Notch receptors on Ag-specific T cells and regulates the traffic and accumulation of T cells to the site of central nervous system (CNS) (71). These studies show the similar result as the recent report which revealed that induction of EAE in mice overexpressing dominant-negative form of Mastermind-like 1 (DN-MAML) in CD4+ T cells or mice with the targeted deletion of CSL/RBP-Jκ in myelin-reactive T cells were prevented in more than 95% of animals, compared to the controls (72).

Macrophages also play important roles in EAE. Reduced severity of EAE was observed upon removal of the infiltrated macrophages in the CNS (5), deleting *IL-4R* in macrophages (73) and transferring of immune-complex/LPS-activated macrophages to EAE-inducing mice (6). Furthermore, it has been shown that expression of Delta-like 4 on macrophages is important in the development of EAE (71). However, the impact of signaling initiated by Notch1 in activated macrophages on EAE has not been documented.

Notch Signaling and Cytokines Production in Activated Macrophages

IL-12 Production in Macrophages

Recently, several studies have reported on the involvement of Notch signaling in regulation of cytokine production in TLR-activated macrophages such as IL-10, IL-6 and IL-12 (3, 47). We previously reported that Notch1 directly regulates the *il-6* promoter and its expression in IFNγ/LPS activated macrophages (49). Furthermore, using GSI to inhibit the activation of Notch signaling in macrophages, decreased IL12p40/70 protein was observed correlating with defects in activation of the MAPK signaling pathway and NF-κB subunit, c-Rel, nuclear translocation (50). IL-12, one of the pro-inflammatory cytokines, is produced mainly by dendritic cells and activated macrophages. IL-12p70, a biological active form of IL-12, is composed of two subunits (p40 and p35) and is essential for driving type I immune response (74, 75). IL-12 plays an essential role in T helper type 1 cell differentiation (76). The regulation of *il12p40* expression requires various transcription factors, including the NF-κB, c-Rel and p50 containing complex (77). NF-κB/Rel family promotes transcriptional induction of *il12p40* during activation of macrophages, but is not involved in nucleosome remodeling of *il12p40* promoter (78). The regulation of the *il12p40* promoter by histone deacetylation (HDAC3) during transcription in macrophages has been observed. Furthermore, IL-10, the anti-inflammatory cytokine, plays an antagonistic role to control the hyper inflammatory condition (79). However, the role of Notch1 and CSL/RBP-Jκ in regulating IL12p40/70 production in macrophages has not been clearly investigated.

IL-10 Production in Macrophages

Signaling through TLR triggers the activation of Notch signaling in macrophages. The activation of Notch affects cytokine productions which associated in polarization of TLR activated macrophages. However, the study of Notch activation in LPS/immune complex activated macrophages has not been elucidated. The expression of *IL-10* is selectively regulated by many transcription factors, however, Erk is commonly found to regulate IL-10 production in various cell types, including macrophages (41). IL-10 can be induced by TLR-dependent and –independent manner in macrophages. In LPS activated macrophages, IL-10 is produced in low amount and is controlled by NF-κB, especially p50 and p65, MAPK and STAT pathways (80-82). Unlike LPS activated macrophages, macrophages which are primed and activated by IFNγ together with LPS and immune complex produce large amounts of IL-10 (8). Signaling through FcγR amplifies the activation of Erk/MAPK and p38/MAPK signaling, resulting in the augment of chromatin remodeling and binding of Sp1 on *IL-10* promoter (42). Furthermore, PI3K signaling which is a downstream signaling of FcγR is responsible for IL-12 and IL-10 expression (83). IL-10 enhances its own expression by feedback mechanisms via downstream signaling through STAT3 from IL-10R.

It has been shown that Notch signaling also is involved in the expression of IL-10. Notch promotes *IL-10* expression via STAT4 in Th1 cell (11) and induces the transcriptional activation of *IL-10* mRNA in IFNγ/LPS-activated macrophages (3) Previously, our group demonstrated that Notch signaling affects the activation of NF-κB p50 and p65 in LPS activated macrophages, implying that Notch signaling may involve in the production of cytokines which are targets of NF-κB signaling. However,

how Notch signaling in macrophages activated by LPS and immune complex regulates IL-10 production is still undocumented.

CHAPTER III
METHODOLOGY

Animals

Wild type C57BL/6 mice were purchased from Harlan Laboratories (Wilmington, MA) or National Laboratory Animal Center (Salaya, Thailand). All transgenic mice used in this study were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). N1KO and CSL/RBP-Jκ KO mice were generated by breeding *Notch1*$^{fl/fl}$ (*Notch1*$^{tm2Rko/GridJ}$) or *Rbp-jκ*$^{fl/fl}$ (*Rbpj*^{tm1Hon}) mice to *mx1Cre*$^{+/-}$ [B6.Cg-Tg9mx1cre]1Cgn/J] mice. To conditionally delete *Notch1* or *CSL*/*RBP-Jκ*, female mice with the genotype of *Notch1*$^{fl/fl}$ X *Mx1 cre*$^{+/-}$ (N1KO), *Notch1*$^{fl/fl}$ X *Mx1 cre*$^{-/-}$ mice (control), *Rbp-jκ*$^{fl/fl}$ X *Mx1 cre*$^{+/-}$ (CSL/RBP-Jk KO), *Rbp-jκ*$^{fl/fl}$ X *Mx1 cre*$^{-/-}$ (control) were injected with 12-15 μg/gram body weight of Poly I: Poly C (GE Healthcare, Imgenex) every other day for 5 days. Animals were rested for 3 weeks prior to sacrifice and use in experiments. Female mice aged 7-12 weeks were used for all experiments. Ten weeks old animals were used for EAE induction. All animals were housed in animal facilities according to the guideline approved by the Institutional Animal Care and Use Committee at the University of Massachusetts at Amherst and Chulalongkorn University (Protocol Review No. 1323007).

Generation of bone marrow derived macrophages (BMDMs)

Bone marrow cells were flushed from femur cavities of mice. Five x 10^6 cells of bone marrow were plated in non-tissue culture treated plates (Hycon, Thailand) with 8 mL of media containing DMEM (LONZA, USA or Hyclone, England)

supplemented with 10% Fetal Bovine Serum (GIBCO, USA or Hyclone, England), HEPES (LONZA, USA or Hyclone, England), sodium pyruvate (LONZA, USA or Hyclone, England), streptomycin/penicillin G (LONZA, USA or Hyclone, England), 5% (v/v) horse serum (Thermo Scientific, USA) and 20% (v/v) L929-conditioned media in Forma Direct Heat CO_2 Incubator HEPA class 100 (Thermo Electron corporation, USA) at 37°C, 5% CO_2. Three mL of fresh DMEM media supplemented with 20% L929-conditioned media and 5% horse serum was added to the culture at day 4. Cells were harvested at the day 7 using ice-cold PBS (Appendix A). Cell surface staining with anti-F4/80 and CD11b antibodies (BioLegend, USA) was used to confirm macrophage phenotype. The obtained BMDMs were cultured in DMEM complete media without horse serum and L929-conditioned media before activation at least 6 hr.

BMDMs preservation

After 7 days, BMDMs were harvested by ice cold PBS and centrifuged at 1000 rpm, 7 min. Cells were resuspended in one part of 80% DMEM media supplemented with 20% FBS and one part of 60% DMEM media supplemented with 20% FBS and 20% DMSO. Cells were stored in cryogenic vial (NUNC, USA) and kept at -80°C until use.

BMDMs thawing

After quick thawing at 37°C in water bath and centrifugation in cold serum free media, culture supernatants were removed and 8 mL of DMEM media supplemented with 20% L929-conditioned media and 5% horse serum was added.

Cells were plated on non-tissue culture treated plates for 3 days before performing experiments.

Activation of BMDMs

BMDMs were primed overnight with recombinant murine IFNγ (10 ng/mL) (BioLegend, USA) and washed twice by medium and warm PBS respectively. Salmonella LPS (100 ng/mL) (Sigma-Aldrich, USA), Pam_3Cys-SK (10 ng/mL) (EMC microcollections, Germany), Rabbit IgG against OVA (GeneTex, USA) and IgG-opsonized OVA (immune complex) were added to activate macrophages for indicated experiments. DAPT (25 μM) (Calbiochem, USA) and DMSO (0.01%) (Signma-Aldrich, USA) were added overnight before activation. BAY-11(10μM) (Calbiochem, USA), SB203580 (10 μM) (Calbiochem, USA) U0126 (10 μM) (Calbiochem, USA), LY294002 (50 μM) (Calbiochem, USA) or DMSO (0.01%) (Sigma-Aldrich, USA) were used to treat macrophages for 30 min before activation. To determine the polarization of macrophages, *il-12* and *il-10* mRNA expression were examined by quantitative RT-PCR

Preparation of immune complex

An immune complex was made by mixing a 10-fold molar excess of Rabbit IgG against OVA (GeneTex, USA) to OVA (Sigma-Aldrich, USA) for 30 min at room temperature. Ratio volume of immune complex to media is 1:100 (Appendix A).

Intracellular staining and cell surface staining

BMDMs were activated as described above. For intracellular staining, brefeldin A (for IL12p40/70 and IL-6 detection) or monensin (for IL10 detection) was added at the starting of the activation. Briefly, cells were harvested by ice cold PBS, washed by 200 uL of FACs staining buffer (1% FBS in PBS) and pretreated by FACs staining buffer containing Fc blocker (0.5 μg) (BD Bioscience, USA), followed by the surface staining and fixation/permeabilization using the BD Cytofix/Cytoperm kit (BD Biosciences, USA) according to the manufacturer's instructions.Anti-mouse CD11b Alexa fluor® 647 (0.5 μg) (BioLegend, USA), Anti-mouse F4/80 Alexa fluor® 488 (0.2 μg) (BioLegend, USA), anti-mouse IL12p40/70-PE (0.2 μg) (BD Bioscience, USA and BioLegend, USA) and anti-mouse IL10-APC (0.4 μg) or anti-mouse IL-10-PE (0.4 μg) (BioLegend, USA) were used. Anti-mouse Notch 1-PE (clone N1A) (0.4 μg) (ebioscience, USA) was used to stain for Notch1 using the FoxP3 staining buffer set (ebioscience, USA) according to the manufacturer's instructions. In some experiments, anti-mouse CD80, anti-mouse CD86, anti-mouse MHCII (BD Pharmingen, USA) and anti-mouse PDL-1 (BioLegend, USA) antibodies were used for cell surface staining. Cells were acquired on FACS LSR II (Becton Dickinson, USA) or Cytomics FC 500 MPL (Beckman Coulter, USA) and analyzed on the FlowJo software (Trestar, CA USA)

Western Blot

At time indicated, activated BMDMs ($2.5x10^5$ cells) were washed twice by cold PBS and proteins were extracted by 40 uL of ice cold Buffer B (1mM EGTA, 1mM DTT, 50mM This-HCl pH 7.2, 0.14M KCl, 2.5mM Mg_2Cl, 0.1% NP-40

containing Phosphatase inhibitor from Sigma-Aldrich, USA, USA and mini pack Protease inhibitor from Roch Diagnostic, USA). Samples were transferred to new 1.5 mL microcentrifuge tubes (Axygen Scientific, USA) and mixed by vortex mixer model G560E (Scientific Industries, USA) for 15 sec. Samples were cleared by centrifugation for 10 min at 10,000 rpm 4C using Centrifuge 5424 R (Eppendorf ,Germany). The supernatants as cell lysate samples were transferred into new 1.5 microcentrifuge tubes and stored at -80C until use.

A forty micro liter of 2x loading dye (100mM Tris pH 6.8, 4% (w/v) SDS, 20% (v/v) Glycerol, Bromphenol blue containing 10% (v/v) β-mercaptoethanol purchased from Sigma-Aldrich, USA) was added into 40 uL of protein lysates as mention above. Samples were boiled at 100C for 5 min on Thermomixer Compact (Eppendorf, Germany). Equal amount of loading volume containing from 1.25-1.56 x 10^5 cells of total cell protein extract and prestained molecular weight markers (Fermentas, Canada) were loaded on 8% SDS-Polyacrylamide gel (Appendix A). Proteins were separated at 60-100 volt at least 120 min by using Protein III system (BioRad, USA) and running buffer (Appendix A). Separated proteins were transferred into PVDF membrane (Millipore, USA) by using a semi-dry transfer Trans-Blot® SD (BioRad, USA) and transfer buffer (Appendix A) under the following condition; current at 80 or 160 mA for 90 min for one gel or two gels, respectively. The PVDF membranes were blocked with 3% skim milk (BD, USA) in PBS-Tween20 (0.1%) (Appendix A) and probed with primary antibody as following, rabbit anti Notch1 (1:2000) which was purchased from Santa Cruz Biotechnology, USA, rabbit anti Cleaved Notch1 (1:1000), rabbit anti phospho-p38 (1:2000), rabbit anti p38 (1:2000), rabbit anti phospho-p44-42(1:4000), rabbit anti p44-42(1:4000), rabbit anti phospho-

SAPK-JNK(1:2000), rabbit anti SAPK-JNK(1:2000), rabbit anti phospho-AKT (1:2000), rabbit anti AKT (1:2000) and rabbit anti RBPJSHU (1:1000) which were purchased from Cell Signal Technology, USA and mouse anti β-actin (1:1000), was purchased from Chemicon-Millipore, USA at 4°C refrigerator overnight. The probed membranes were further incubated on a rocker for 1 hr at room temperature. The primary antibody solution was discarded, and the membrane was washed with PBS-Tween20 for 5 min 2 times and 15 min 2 times. After washing, PBS-Tween20 was discarded, and donkey anti rabbit (1:2000-1:4000) and sheep anti mouse (1:5000) which were secondary antibody against rabbit or mouse immunoglobulins conjugated with horse-radish peroxidase (HRP) which were purchased from Amersham Biosciences, UK were added. The PVDF membrane was incubated for 1 hr with rocking before washing with PBS-Tween20 as follow described above.

The solution A (100 mM Tris-HCl, pH 8.5, 196.5 μM coumaric acid and 1.24 mM luminal) was mixed with solution B (100 mM Tris-HCl pH 8.5 and 0.009% H_2O_2). PBS-Tween20 was removed from the container carrying the PVDF membranes. The membrane was poured by solution A and B and incubated for 1 min. The membranes were wrapped in the plastic wrap and placed in Hypercassette (Amersham Biosciences, UK) to expose to High Performance Chemilumunescence Film: Amersham Hyperfilm™ ECL (Amersham Biosciences, UK) in the dark. Exposed film was developed in X-ray film developer, washed with tap water, fixed for 3 min in the fixer and finally washed with tap water.

RNA extraction and quantitative RT-PCR

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

Measuring amount of RNA were performed by using Nanodrop™ (Thermoscientific, USA) and some experiment Quanti iT were carried out according to the manufacturer's instruction. Briefly, Quanti-iT reagent and Quanti-iT buffer (Invitrogen, UK) were calculated and prepared to be Quanti-iT working solution. Ten µL of RNA standards composing of 0 ng/µL of RNA and 10 ng/µL of RNA were mixed with 190 µL of working solution. RNA samples were diluted to 10-fold

dilution in Hypure® water PCR grade (Hyclone, England) and 2 µL of diluted RNA was mixed with 198 µL of working dilution. Calibrations of RNA standard were performed by Quanti-iT and concentrations of RNA samples were measured. The concentrations of RNA were calculated in µg/mL.

Five hundred ng to 1 µg of obtained total RNA was used to generate cDNA. Total RNA was mixed with 0.2 µg of random hexamer (Qiagen, Germany), and the volume was adjusted to 12.5 µL by 0.01% DEPC treated water. The RNA mixture was heated at 65°C for 5 min and placed on ice for 5 min. Then, 1×Reverse transcriptase buffer (Fermentus, Canada), 1 mM dNTP mix (Fermentus, Canada) and 20 U of RNase Inhibitor (Fermentus, Canada) were added in the mixture and followed by incubation at room temperature for 5 min. Reverse transcriptase (Fermentus, Canada) was added to final amount of 200 U per reaction, and the reaction was manipulated on Bioer Life Express (Bioer technology, China) at 25°C for 10 min, 42°C for 60 min, 70°C for 10 min and 25°C for infinity. The cDNA was stored at -20°C until use. Some experiments total RNA was isolated by using the RNAqueous kit (Ambion, Austin, TX). cDNA was synthesized, and 100 ng of cDNA transcripts were amplified by Q-PCR Stratagene MX3000P. Primer sequences for *IL23p19* were: (forward): 5'-AGC GGG ACA TAT GAA TCT ACT AAG AGA-3' (reverse) 5'-GTC CTA GTA GGG AGG TGT GAA GTT G-3'. *IL12p40* were: (forward) 5'-AAC CTC ACC TGT GAC ACG CC-3' (reverse) 5'-CAA GTC CAT GTT TCT TTG CAC C-3' *IL-10* were: (forward) 5'- TCA AAC AAA GGA CCA GCT GGA CAA CAT ACT GC-3' and (reverse) CTG TCT AGG TCC TGG AGT CCA GCA GAC TCA A-3' and β-actin were: (forward) 5'- ACC AAC TGG GAC GAC ATG GAG AA -3'

and (reverse) 5'-GTG GTG GTG AAG CTG TAG CC-3'. The expression of each gene was normalized to the expression of β-actin by the $2^{-\Delta\Delta CT}$ method.

ELISA

Culture supernatants from activated BMDMs as described were harvested at 6 or 24 hr after stimulation. Culture supernatants were collected after treatment as indicated and kept at -70°C until use. ELISA was carried out according to the manufacturer's instructions. Briefly, ELISA plate was coated with 100 µL/well of capture antibody in coating buffer and incubated for overnight at 4°C. Unbound antibodies were removed and the wells were washed with 200 µL/well of wash buffer for 6 times. During each washing step, 1 min soaking was performed to increase the effectiveness of the washing. Wells were blocked with 200 µL/well of 1x Assay Diluent or 10% FBS in PBS and incubated for 1 hr at room temperature. Wells were aspirated and washed as described above. The samples which were diluted to 1:40 (IL12-p70), 1:20 to 1:40 (IL-17), 1:20 to 1:40 (IFNγ), 1:20 (TNFα), 1:20 (IL-6), 1:10 (IL-10) and standards were prepared in 2-fold serial dilutions in new 1.5 mL microcentrifuge tube. One hundred µL of diluted samples and standards were added in the wells. The plates were sealed and incubated overnight at 4°C. Following this step, wells were emptied and washed as detailed above. Biotinylated anti-antibody was used as a detection antibody and was added in each well at 100 µL and incubated for 1 hr at room temperature. After that, the wells were washed as described above and avidin-HRP (100 µL/well) was added and the plates were incubated at room temperature for 30-60 min. Wells were washed for 7 times after this step. TMB Substrate solution (100 µL/well) (Appendix A) was added and the plates were

incubated for 5-15 min at room temperature. Finally, the reactions were stopped by addition of 50 μL of 2N H_2SO_4 (Appendix A) and the absorption was read at 450 nm.

Secreted IL-12p70, IL-10, TNFα and IL-6 levels were detected by using IL-12p40/70 ELISA (BD Pharmingen, USA), IL-10, TNFα and IL-6 ELISA (Biolegend, USA). For an EAE experiment, supernatant from re-stimulated splenocytes were collected and subjected to detection for IL-17 and IFNγ by ELISA according to the manufacturer's instruction (BD Bioscience, USA).

Immunofluorescent staining

Cells were cultured in 8 wells slide chambers and activated as indicated. After washing with PBS, cells were fixed with 4% paraformaldehyde for 10 min at room temperature before washed 5 min twice following by incubation with 0.2% Triton-x100 for 2 min at room temperature. Cells were washed 5 times in 5 min and blocked by Fc Blocker (0.5 μg) for 10 min. Then the samples were washed and incubated for 1 hr with rabbit anti-c-Rel polyclonal antibody (1:100) or mouse anti NF-κB p50 monoclonal antibody (1:100) (Santa Cruz Biotech, CA, USA), Followed by washed 5 min 3 times, then incubation for 1 hr with anti-rabbit IgG (H + L $(Fab)_2$ fragment) conjugated with PE (1:500) or anti-mouse IgG (H+L) (Fab)2 fragment) conjugated with Alexa Fluor® 488 (Cell Signaling Technology, MA, USA) (1:500). The samples were observed under an inverted fluorescent microscope or a confocal microscope.

Adoptive transfer of activated macrophages and EAE Disease Score Evaluation

BMDMs obtained from control and N1KO mice were activated by IFNγ/LPS as described above for 30 min. Cells were washed 3 times using warm PBS and the cell number was adjusted to $2x10^6$ cells in 200 μL PBS. Two hundreds μL of

activated BMDMs or PBS were intraperitoneally (i.p.) injected to the naïve C57BL/6 mice. After 4 hr, an emulsion obtained as EAE induction Hooke Kits™ (Hooke Laboratories, Lawrence, MA) was administered in the flanks of the animals according to the manufacturer's instructions. Pertussis toxin was i.p. injected twice at 2 hr and 24 hr after immunization. The progression and the severity of EAE were monitored and scored from 0-5 as follows: Score 0-no disease; 1-limp tail, 2-hind limb weakness; 3-hind limb paralysis; 4-hind and fore limb paralysis; 5-morbidity and death. Data is reported as the mean daily clinical score (70). Mice were euthanized during the peak of the disease (day 15-16 post immunization) and spleens were collected. Splenocytes were cultured at 37°C with medium alone or with different concentrations of MOG_{35-55} antigen (Hooke Laboratories) for 3 days. The culture supernatants from re-stimulated splenocytes were subjected for an ELISA for detection of IL-17 and IFNγ as described above.

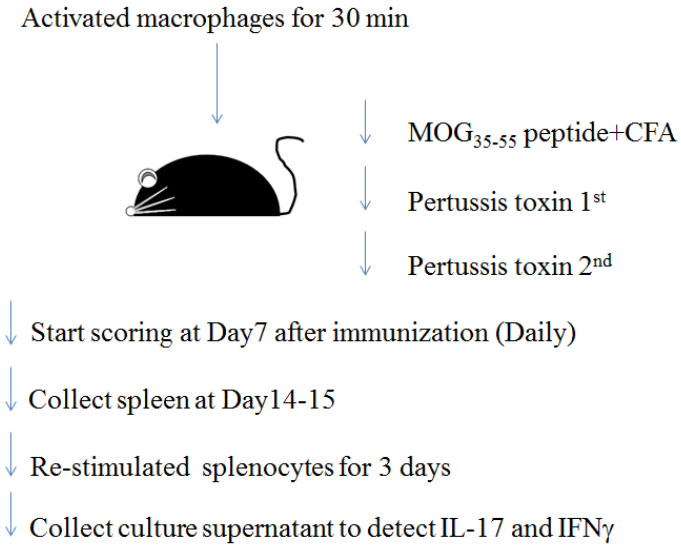


Figure 8. Experiment design on adoptive transfer of activated macrophages

Experimental design for BMDMs transfer in EAE mice

Statistical analysis

Statistical analyses were performed using SPSS version 15.0 and GraphPad Prism version 5.0. One-way ANOVA (α =0.05) were used when comparing two conditions.

CHAPTER IV

Impacts of Notch1 Deletion in Macrophages on Pro-inflammatory Cytokine Production and the Outcome of Experiment Autoimmune Encephalomyelitis

Results

Adoptive transfer of activated macrophages lacking Notch1 decreases severity and delays onset of diseases in EAE model

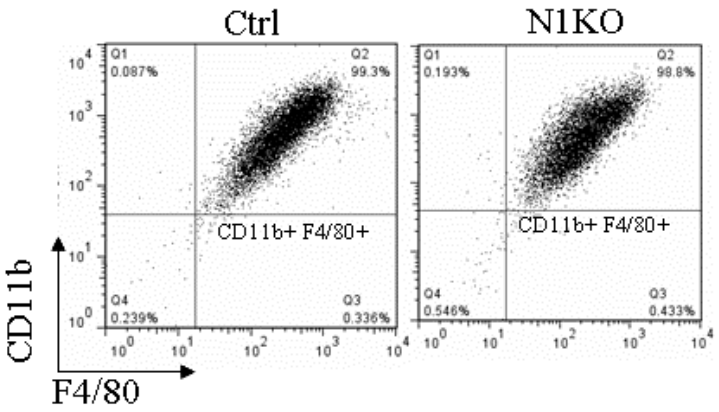
Macrophages have been identified as one of the effector cells that play critical role in EAE (5). Adoptive transfer of macrophages activated with LPS/immune complex in the EAE model resulted in delaying an onset of disease and the severity was decreased (6). Notch signaling is involved in activation of macrophages and regulates the production of cytokines such as IL-6, IL-12p40, IL-10 (3, 47, 49-51), all of which are indicated in the inflammation of EAE. Furthermore, the involvement of Notch signaling in regulating the disease severity in the EAE model by using GSI was demonstrated (69, 70). For these reasons, we hypothesized that Notch1 in macrophages may play a crucial role in regulating the disease outcome in an EAE model. BMDMs which were generated from conditional N1KO and the control wild type (WT) mice were used in this study. The expression of two macrophage markers, CD11b and F4/80, were similar in N1KO and the WT control BMDMs, suggesting that deletion of *Notch1* in this study did not interfere with macrophage differentiation (Fig. 9 A). The loss of expression of Notch1 in N1KO macrophages was confirmed by Western Blot (Fig. 10 A), and flow cytometry (Fig. 10 B, C and D). BMDMs from WT or N1KO were activated by IFNγ/LPS for 24 hr. Live cells were detected equally between WT and N1KO macrophages. Almost 50% of macrophages died under an

unstimulated condition, implying that macrophages need stimulation to survive *ex vivo* (Fig. 9 B).

BMDMs from WT or N1KO mice were activated by IFNγ/LPS for 30 min *in vitro* before adoptive transfer by i.p. injection into the wild type recipients 4 hr prior to EAE induction. We observed a significant delay in the onset of the disease and decrease in disease severity in animals which received activated N1KO macrophages, compared to those receiving activated WT macrophages (Fig. 11 A, B and Table 1). A similar disease onset and severity were observed regardless of the transferring of activated macrophages (data not shown). These data indicated that Notch1 expression in transferred macrophages influenced the outcome of EAE.

Notch signaling in macrophages is required for the optimal production of IL-12p40/70 and IL-6 and *IL-23p19* mRNA expression, which are involved in helper T cell polarization. Furthermore, EAE is a Th1/Th17-driven autoimmune disease. Therefore, we hypothesized that Notch1 in macrophages may influence the response of Th1 and/or Th17 in EAE by controlling IFNγ and IL-17 production. Splenocytes from mice with an adoptive transfer of macrophages were re-stimulated with the MOG_{35-55} peptides *in vitro* and the levels of IL-17 and IFNγ were measured in the culture supernatant by ELISA. We found that the level of IL-17 decreased significantly in peptide-stimulated splenocytes from animals which received activated N1KO macrophages, compared to those from the control mice (Fig. 12 A). Surprisingly, the level of IFNγ production did not show any statistical significant difference between the two groups (Fig. 12 B). These data imply that transfer of activated Notch1 deficient macrophages affects the onset and the progression of EAE, possibly through its influence on the activation of Th17, but not Th1 cell response.

A



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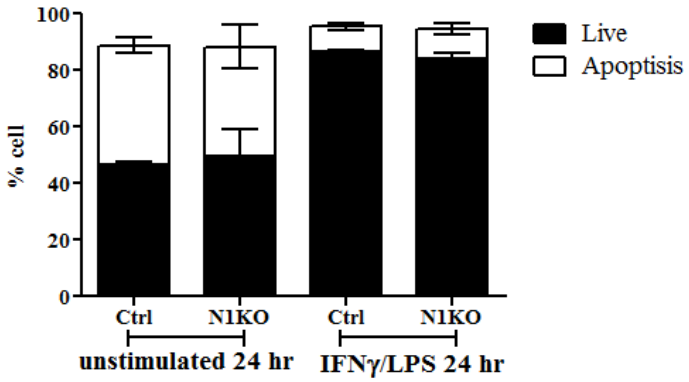


Figure 9. BMDMs from N1KO and their viability upon stimulation

(A) BMDMs were generated from bone marrow of WT or N1KO mice. Macrophage phenotype was confirmed by Cell surface staining with anti-F4/80 and CD11b antibodies (B) BMDMs from WT and N1KO mice were activated by IFNγ/LPS for 24 hr. Live and dead cells were detected by PI and Annexin V kit.

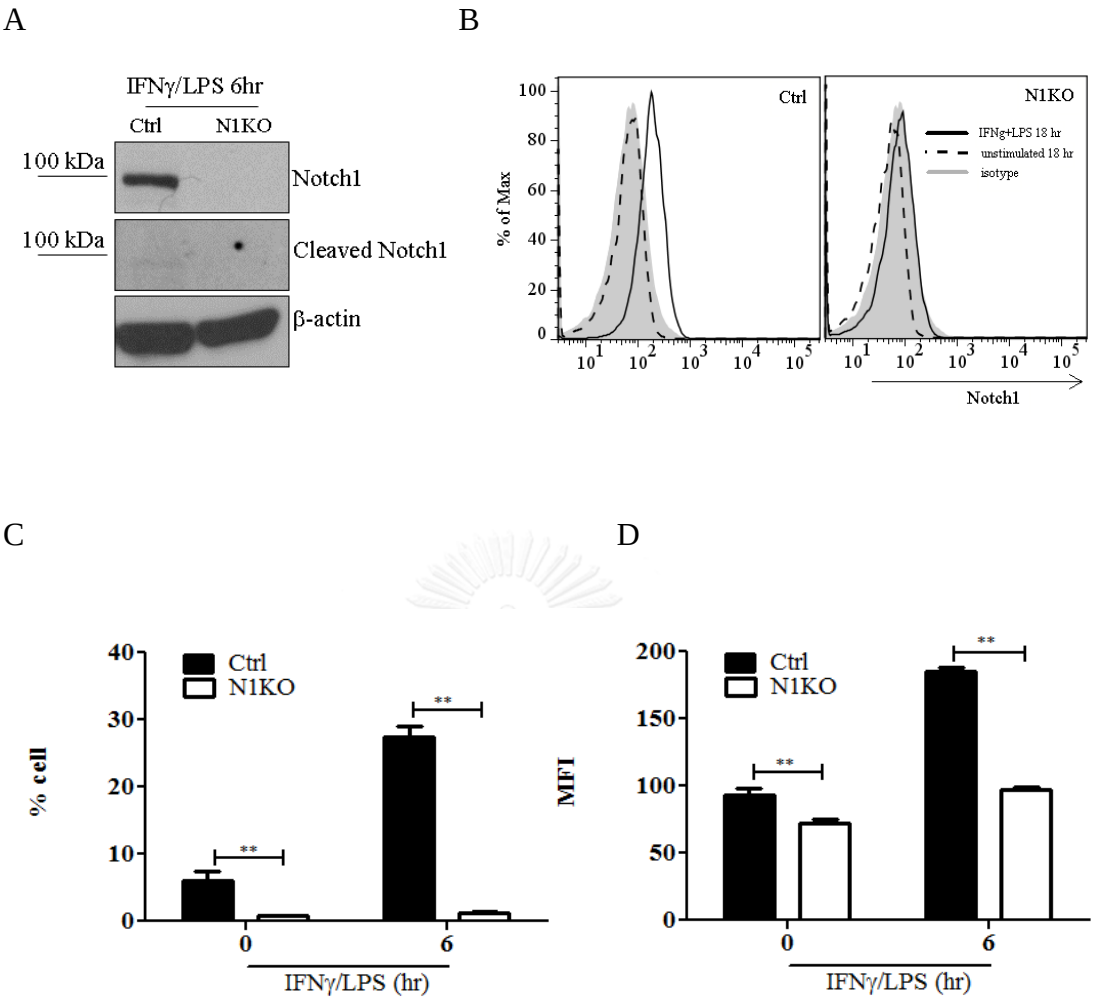
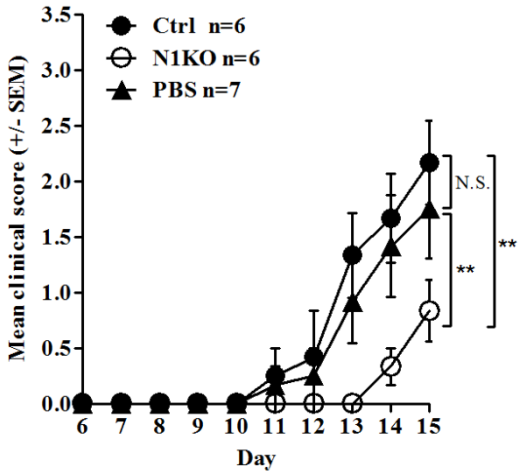


Figure 10. The expression of Notch1 in N1KO BMDMs

(A) BMDMs from WT or N1KO mice were activated by IFNγ/LPS and the level of Notch1 was measured by Western blot. The level of Notch1 was measured by Flow cytometry

(B)The percentages of $F4/80^{+}/Notch1^{+}$ cells and (C) MFI for Notch1 in $F4/80^{+}/Notch1^{+}$ population in each condition described in (A) at indicated times. ** indicated where statistical significance was observed ($p<0.05$).

A



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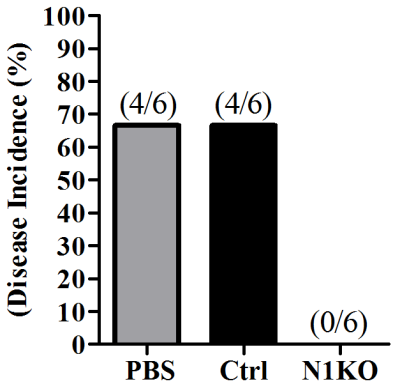


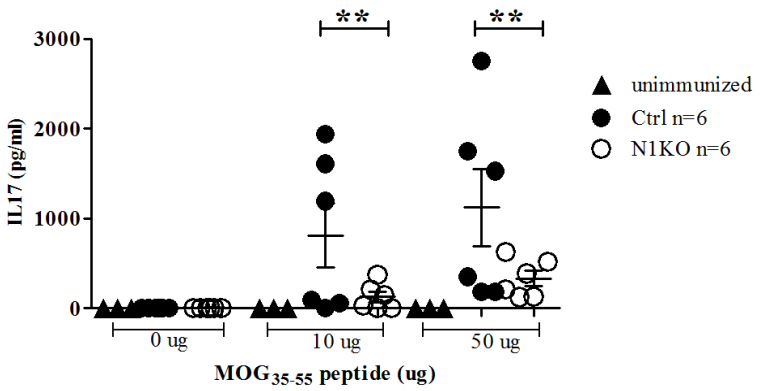
C

exp.	Incidence of Symptom (mice)		Mean Score of Severity	
	Ctrl	N1KO	Ctrl	N1KO
1st	2/3	0/3	2.0±0.9	0.3±0.6
2nd	4/6	0/6	2.2±0.9	0.8±0.7

Figure 11. Adoptive transfer of activated N1KO BMDMs delays the onset and severity of diseases in an EAE Model

(A) Mean clinical scores in EAE mice receiving WT BMDMs or N1KO BMDMs. ** indicated where statistical significance was observed (p<0.05). (B) Percentage of incidence of EAE at day 15 in animals which were scoring at ≥ 2 and the (C) Described the results on the incident of symptom and Mean clinical scores of two independent experiments.

A



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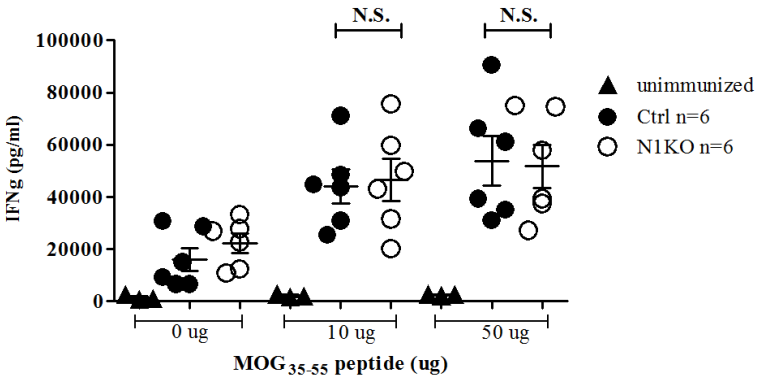


Figure 12. Adoptive transfer of activated N1KO BMDMs affects helper T cell response in re-stimulation assay

Splenocytes from animals (n=6 per group) were collected at day 15 after EAE induction and re-stimulated by MOG_{35-55} peptide (10 μg and 50 μg). Cells were cultured for 3 days and the supernatants were measured for IL-17 (A) and IFNγ (B) by ELISA. ** indicated where statistical significance were observed (p<0.05).

Notch1 is dispensable for IL12p40/70 production in IFNγ/LPS-activated BMDMs

Notch signaling has been shown to regulate IL12p40/70 production and especially Notch1 is important for the regulation *il12p35 (il12a)* and *p40 (il12b)* mRNA expression in LPS-activated macrophages (51). Based on the results obtained in this study so far in the EAE model, we asked specifically whether Notch1 is important for IL12p40/70 production in IFNγ/LPS-activated macrophages. BMDMs from conditional N1KO and control mice were activated by IFNγ and LPS and IL12p40/70 was detected. Surprisingly, there was no significant difference in both the percentage of IL12p40/70^{+} cells and the mean fluorescent intensity (MFI) between N1KO BMDMs and the control BMDMs upon activation with IFNγ/LPS (Fig. 13A, B and C). Moreover, there was no statistical difference in the level of IL12p40/70 production detected in the culture supernatants from IFNγ/LPS activated N1KO BMDMs, compared to that from the control BMDMs at any time points tested (Fig. 14A). Next, we examined whether activity of a gamma secretase in N1KO macrophages may be involved in IL12p40/70 production in IFNγ/LPS activated N1KO BMDMs using GSI treatment. We found that IL12p40/70 was reduced upon GSI treatment in both N1KO and the control BMDMs, similar to our previous results (Fig. 14B). These results implied that Notch1 is dispensable for optimal IL12p40/70 production in IFNγ/LPS activated macrophage and other gamma secretase substrates such as other Notch receptors may be essential for this regulation.

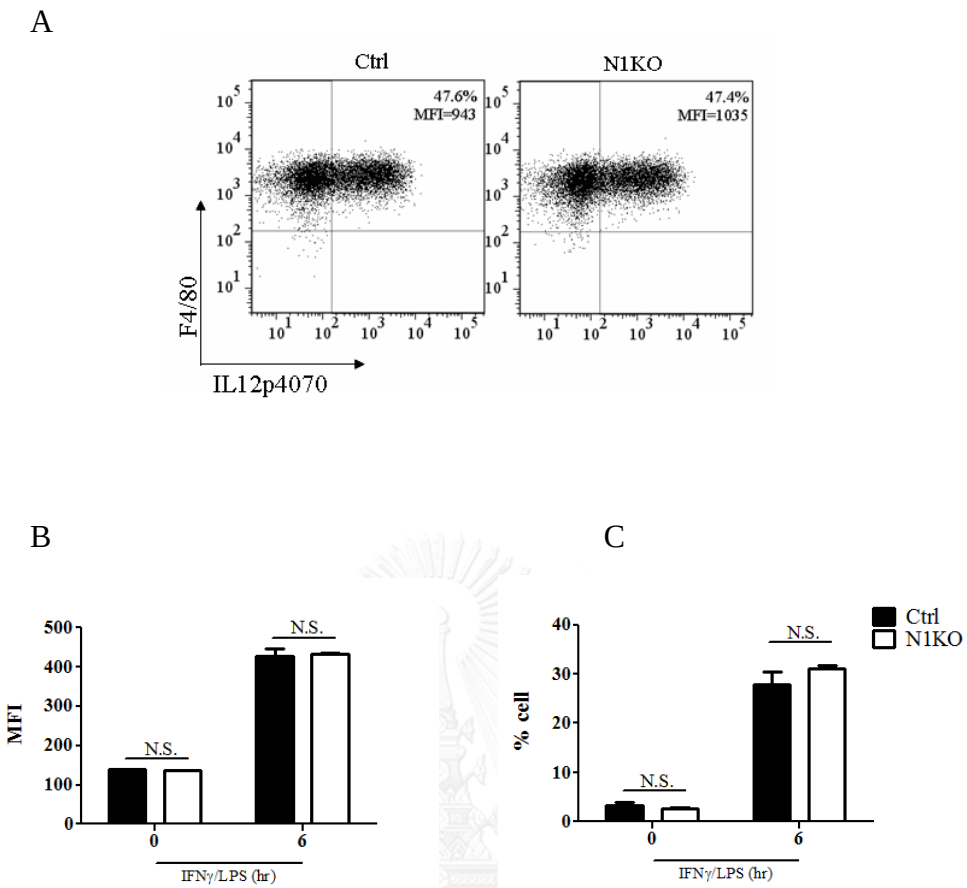
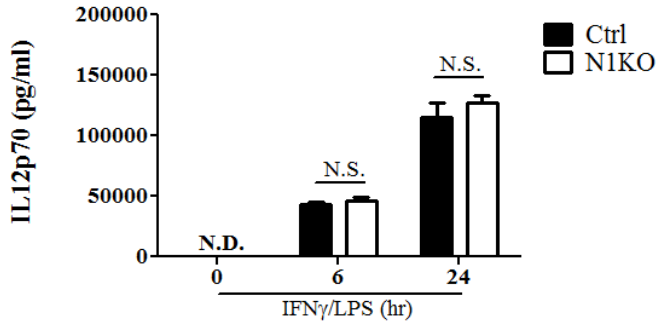


Figure 13. Notch1 is dispensable for IL12p40/70 production in IFNγ/LPS activated BMDMs

(A) BMDMs from WT or N1KO mice were activated by IFNγ/LPS for 6 hr and the level of IL-12p40/70 was measured by intracellular cytokine staining. (B) The percentages of $F4/80^{+}/IL12p4070^{+}$ cells and (C) MFI for IL12p40/p70 in $F4/80^{+}/IL12p4070^{+}$ population in each condition described in (A) were shown. All data are representative of three independent experiments where ** indicates the statistical significance with p <0.05. N.S.: no statistical significance.

A



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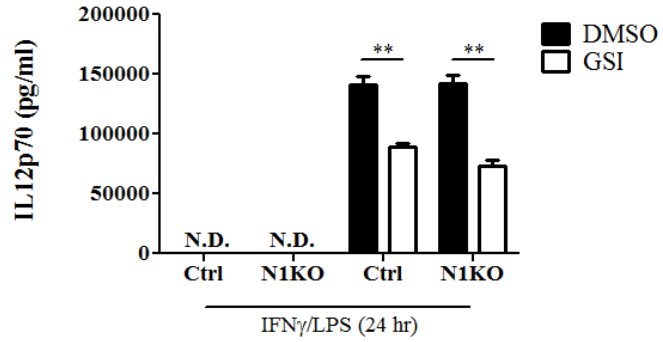


Figure 14. Notch1 is dispensable for IL12p40/70 production in IFNγ/LPS activated BMDMs

(A) The amount of IL12p40/70 production was detected from the culture supernatants at the indicated time points by ELISA. (B) WT and N1KO BMDMs were stimulated by IFNγ/LPS for 24 hr in the presence of vehicle control DMSO or GSI (25 μM). The amount of IL-12p40/70 in the culture supernatants were measured by ELISA. All data are representative of three independent experiments where ** indicates the statistical significance with p <0.05. N.S.: no statistical significance. N.D.: no detection

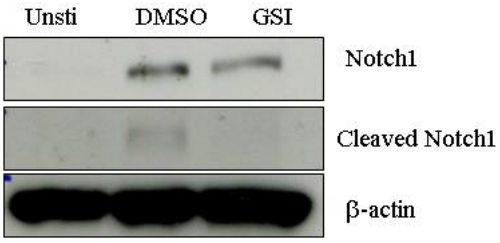
Production of IL12p40/70 in macrophages partially depends on the canonical Notch signaling pathway

Because gamma secretase has other target substrates besides all Notch receptors, the molecular mechanism for the involvement of Notch in regulating IL12p40 production is not fully understood. To address whether canonical Notch signaling is important for IL-12p40/70 production, *CSL/RBP-j*κ, a gene encoding a DNA binding protein which plays a central role in the canonical Notch signaling pathway, was conditionally deleted from BMDMs in a similar manner as the N1KO mice in order to investigate the role of canonical Notch signaling in the IL12p40/70 production elicited by IFNγ and LPS stimulation. BMDMs which were generated from conditional *CSL/RBP-Jκ* KO mice and WT control mice were compared with the GSI treatment in wild type BMDMs. The reduction of the cleaved Notch1 in GSI-treated stimulated wild type BMDMs was confirmed by Western Blot (Fig.15A). The absence of CSL/RBP-Jκ did not affect macrophage differentiation because there was no difference in the expression of CD11b and F4/80 (Fig. 16A).The level of *CSL*/*RBP-jκ* mRNA and protein in activated *CSL*/*RBP-jκ* KO BMDMs were observed by Western Blot and qPCR, respectively (Fig. 16B and C).

The production of IL12p40/70 in the wild type BMDMs which were treated with GSI showed significant reduction in both the MFI and the percentage of IL12p4070^{+}cells (Fig. 15B), compared to the vehicle control-treated BMDMs, consistent with the previous report (50). Similar reduction of IL-12p40/70 was observed in IFNγ/LPS-activated CSL/RBP-Jκ KO BMDMs (Fig. 17A, B and C). These results strongly demonstrate that the activity of gamma secretase and the

canonical Notch signaling via CSL/RBP-Jκ are required for the optimal production of IL-12p40/70 in activated macrophages.

A



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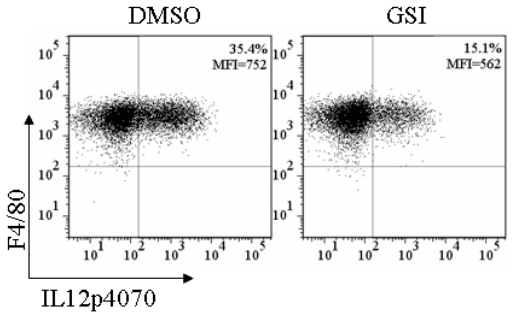


Figure 15. Notch activation in GSI treated macrophage and effects of GSI treatment on IL12p40/p70 production in IFNγ/LPS activated BMDMs

(A) BMDMs were stimulated by IFNγ/LPS for 6 hr in the presence of vehicle control DMSO or GSI (25 μM). Notch1 and cleaved Notch1 (Val1744) were detected using Western blotting. (B) The percentages of $F4/80^{+}/IL12p4070^{+}$ cells and MFI for IL12p40/p70 in $F4/80^{+}/IL12p4070^{+}$ population in each condition described in (A) were measured by intracellular cytokine staining. All data are representative of two independent experiments

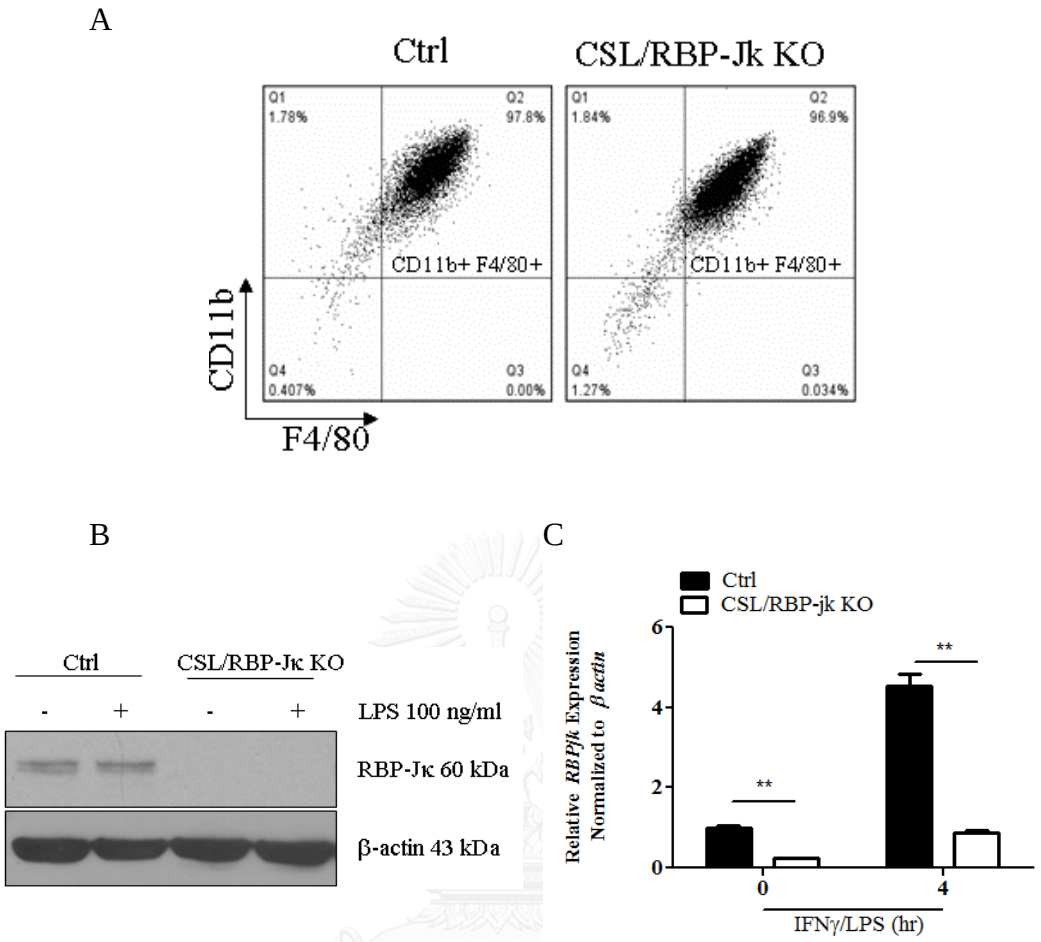


Figure 16. Generation of CSL/RBP-Jκ macrophages

(A) BMDMs were generated from bone marrow of WT or CSL/RBP-Jκ KO mice. Macrophage phenotype was confirmed by Cell surface staining with anti-F4/80 and CD11b antibodies (B) BMDMs which were generated from bone marrow of WT or CSL/RBP-Jκ KO mice were activated by LPS or IFNγ/LPS at indicated times. The level of CSL/RBP-Jκ was measured by Western blot and (C) qPCR .All data are representative of at least two independent experiments where ** indicates the statistical significance with p <0.05.

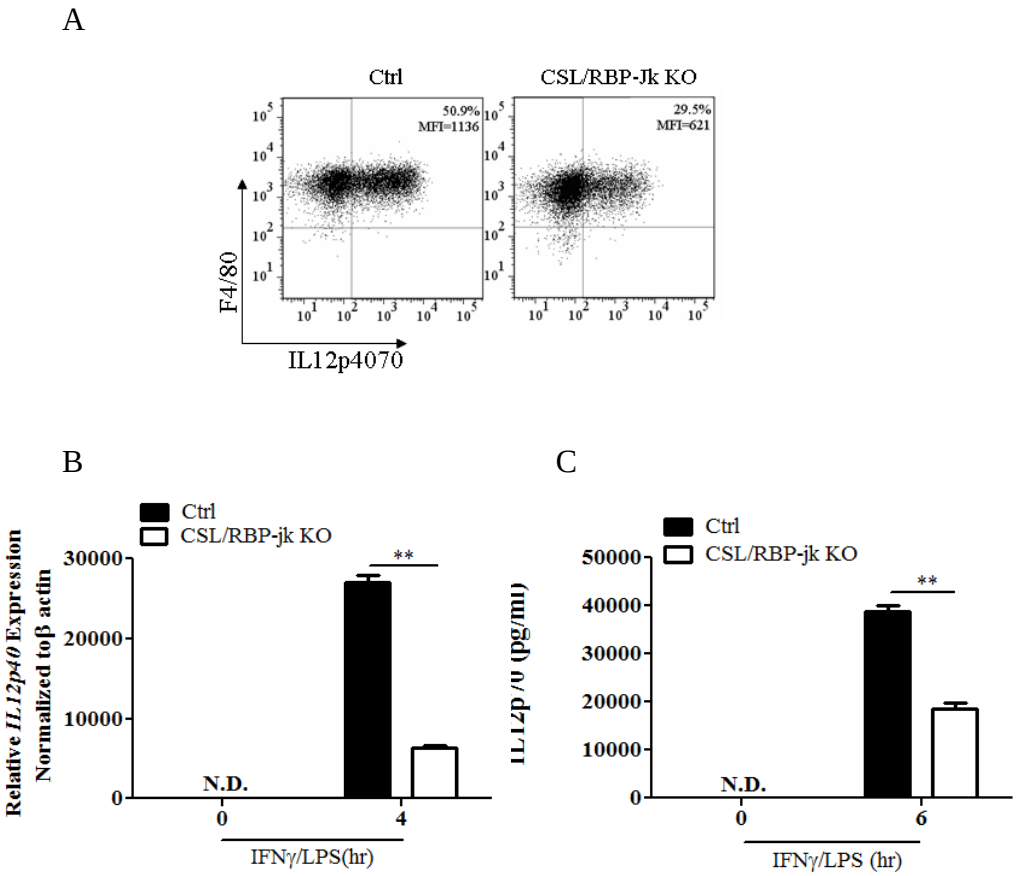


Figure 17. Effects of GSI Treatment and conditional deletion of *CSL/RBP-Jκ* on IL12p40/p70 production in IFNγ/LPS activated BMDMs

(A) BMDMs from wild type control or CSL/RBP-Jκ KO mice were activated by IFNγ/LPS for 6 hr The percentages of $F4/80^{+}/IL12p4070^{+}$ cells and MFI for IL12p40/p70 in $F4/80^{+}/IL12p4070^{+}$ population were measured by intracellular cytokine staining. (B) The level of IL12p40/70 production was detected from the culture supernatants of BMDMs treated as described in for 4 hr by qPCR and (C) 6 hr by ELISA. All data are representative of at least two independent experiments where ** indicates the statistical significance with p <0.05. N.S.: no statistical significance. N.D.: no detection

c-Rel nuclear translocation in IFNγ/LPS activated macrophages requires CSL/RBP-Jκ but not Notch1

The production of IL12p40/70 in activated BMDMs is partially c-Rel dependent (84). In our previous study, we found that GSI treatment in wild type BMDMs affects the nuclear translocation of c-Rel upon stimulation with IFNγ/LPS (50). Activation of BMDMs generated from *CSL/RBP-Jκ* KO mice showed reduction in c-Rel nuclear localization as detected by immunofluorescent staining whereas BMDMs from N1KO mice showed intense nuclear localization of c-Rel upon activation similar to the control wild type macrophages (Fig. 18). The pattern of c-Rel nuclear localization correlated well with the level of IL12p40/70, suggesting that the optimal production of IL12p40/70 in IFNγ/LPS activated BMDMs requires canonical Notch signaling, but not Notch1, in a c-Rel-dependent manner.

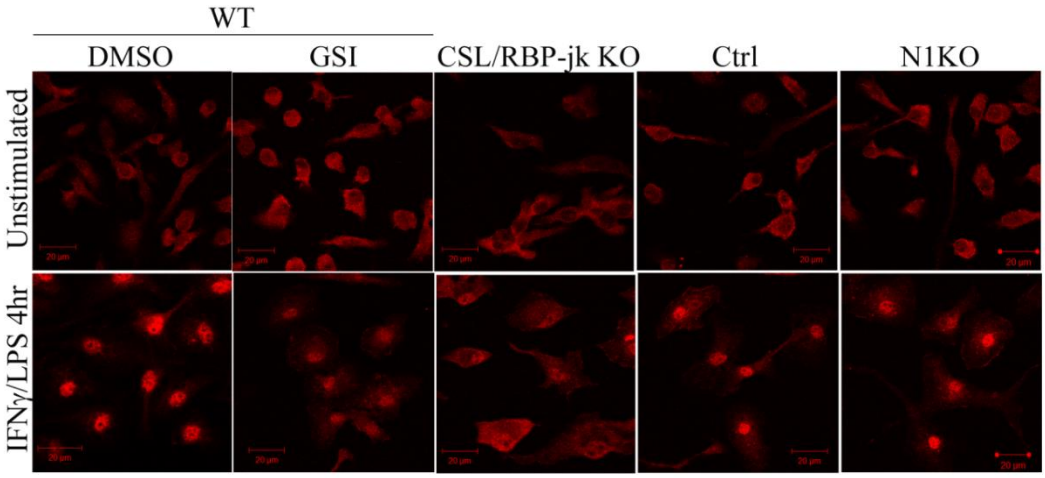


Figure 18. Reduction in c-Rel nuclear translocation was detected in GSI treatment and CSL/RBP-JκKO, but not in N1KO, macrophages

BMDMs obtained from wild type mice were pretreated with vehicle control DMSO (A and F) or GSI (25 μM) (B and G) before stimulation with IFNγ/LPS for 4 hr. BMDMs from CSL/RBP-Jκ KO mice (C and H), wild type control (D and I) and N1 KO mice (E and J) were left untreated or stimulated with IFNγ/LPS for 4 hr. Localization of c-Rel was detected using immunofluorescent staining. Data are representative of at least two independent experiments.

Reduced IL-6 production and CD80 expression in activated N1KO macrophages

Based on the results from the EAE experiments above and *in vitro* re-stimulation with MOG peptide, we hypothesized that other cytokine production or co-stimulatory molecules which were produced or expressed by activated N1KO macrophages may be compromised, resulting in decrease IL-17 production. We investigated IL-6 and IL-10 production and *IL23p19 mRNA* expression in N1KO BMDMs, all of which are shown to have a role in EAE and involved in T_{reg}/Th17 polarization (63, 85). As shown in Fig. 19B and C, a significant reduction in the percentages of IL-6 producing cells was observed upon activation (Fig. 19B and C), consistent with our previous report that Notch signaling partially regulates IL-6 (3, 49) whereas no difference in IL-10 production and *IL23p19 mRNA* expression were found between N1KO and WT BMDMs (Fig. 19A and D). We further determined the expression of co-stimulatory molecules which had been reported to be important in the EAE model for T cell activation (6). Among cell surface molecules tested (CD86, CD80, MHCII, PD-L1), the level of a co-stimulatory molecule, CD80, was reduced in N1KO BMDMs (Fig. 19E). These data implies that reduction of IL-17 in MOG_{35-55}-restimulated splenocytes from the recipient mice of activated N1KO macrophages together with the reduction in co-stimulatory molecule, CD80, and IL-6 in N1KO macrophages may culminate in delaying the onset and progression of EAE.

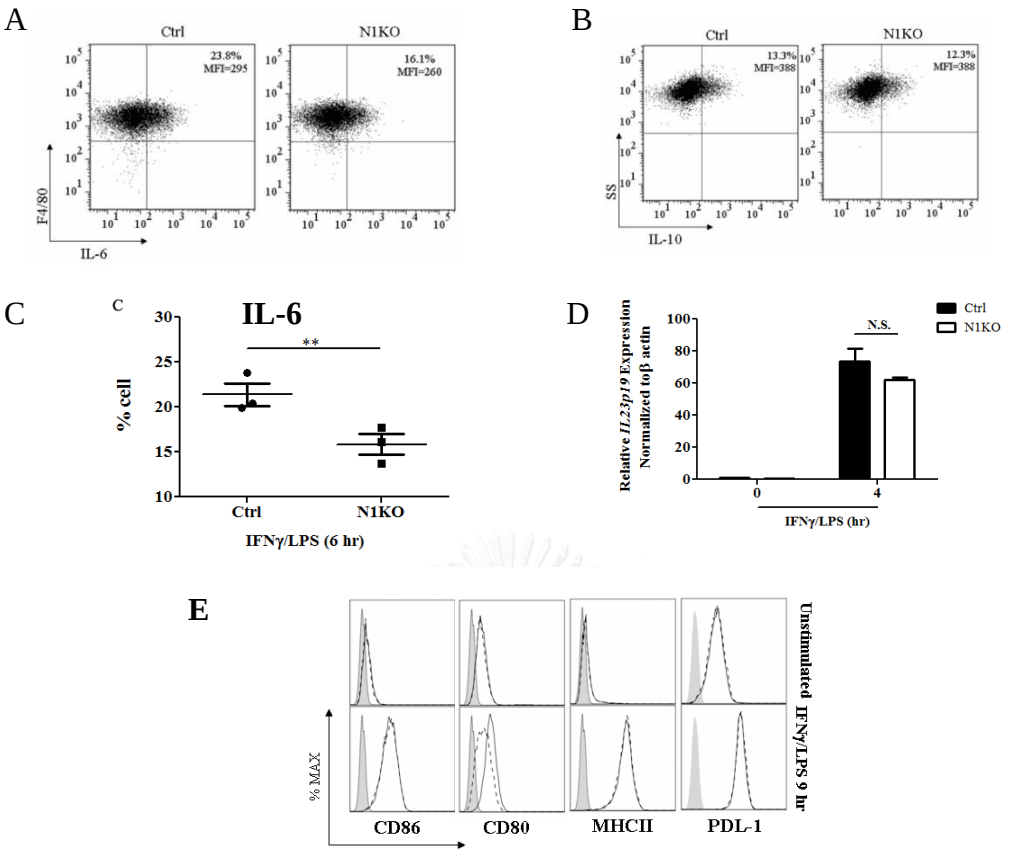


Figure 19. Reduced IL-6 production and CD80 expression in activated N1 KO macrophages

(A) BMDMs from wild type control or N1KO mice were stimulated for by IFNγ/LPS for 6 hr and the production of IL-10 and (B and C) IL-6 were detected by intracellular cytokine staining. (D) BMDMs from wild type control or N1KO mice were stimulated for by IFNγ/LPS for 4 hr and the expression *IL23p19 mRNA* was performed by qPCR (E) BMDMs treated as described in (A) for 9 hr were detected for the level of CD86, CD80, MHCII and PD-L1 by flow cytometer.Solid lines were represented wild type control macrophages and dotted lines were N1KO macrophages All data are representative of at least two independent experiments. ** indicates the statistical significance with p <0.05. N.S.: no statistical significance

Discussion

We used an animal model of EAE to evaluate the impact of *Notch1* deficiency in macrophages on the severity of this autoimmune inflammatory condition and showed that CSL/RBP-Jκ and the activity of gamma secretase are partially involved in the production of pro-inflammatory cytokine, IL12p40/70 in IFNγ/LPS stimulated macrophages. Mice which were adoptive transferred with activated N1KO macrophages showed partial reduction in the onset and progression of EAE. We found that IL-17 production, but not IFNγ, from peptide- stimulated splenocytes were compromised in mice receiving activated N1KO macrophages. Many reports revealed that Th1 and Th17 play important, but distinctive, roles in this disease model and IL-12 is important for differentiation of Th1 whereas IL-23 is essential for maintaining the Th17 phenotype (63). In our *in vitro* experiments, deficiency in *Notch1* did not show any effects on the expression of IL12p70 or *il23p19* mRNA in macrophages but the reduction of IL-6 and CD80 were detected. Previous data showed that IL-23 is not essential for Th17 differentiation whereas IL-6 and TGF-β are involved in this process (86). The blockade of IL-6 can impair differentiation of Th17 (87). In the EAE model, it has been demonstrated that IL-6 is important in this auto-inflammatory disease such as mice which were defective in IL-6 production show the resistance phenotype to the MOG peptide-induced EAE. The signaling through A2B adenosine receptor in APCs such as DCs enhances the IL-6 production and its blockade helps improve the EAE phenotype (68, 88). Taken together, these data strongly implicate that IL-6 is important for Th17 cell differentiation in EAE. We and others previously showed that Notch1 is involved in IL-6 production in activated macrophages. (47, 49) For these reasons, together with the findings in this study, it implies that Notch1 in

activated macrophages might play an important role in regulating the outcome of T cell responses through IL-6 (Th17), but not IL-12 (Th1), in the EAE model.

In the present study, endogenous macrophages were not depleted before the transfer of activated macrophages for the EAE study. Previously, similar adoptive transfer of macrophages activated by LPS and immune complex were reported in an EAE model. This type of macrophages produced high level of IL-10 and the decrease in disease severity was attributed to this anti-inflammatory cytokine production (6). Because the level of IL-10 produced by N1KO macrophages was similar to that of the wild type macrophages, it is unlikely that IL-10 is responsible for decreased severity in our study.

Activated N1KO macrophages expressed less co-stimulatory molecule CD80. It is not clear how Notch signaling regulates CD80 expression. Co-stimulatory molecule such as CD80 (B7.1) and CD86 (B7.2) interact with CD28 on T cells together with the signaling through MHC-TCR interaction are important for T cell activation. Blockage of CD80 has been reported to suppress EAE (67, 89). Furthermore, the blockage of CD28 resulting in reduction of CD80/CD86 ratio in APCs and decreasing of EAE severity was reported (66). In another auto inflammatory disease such as rheumatoid arthritis, CD80 is more important for Th1 cell differentiation than Th17 cells (90) whereas our data indicate that Notch1 in macrophages seem to be not necessary to Th1-type response in EAE. It is possible that macrophages and T cell crosstalk was interrupted during the disease induction; however, cytokines which were produced from transferred macrophages may play a role in T cell polarization and influence the EAE development. Because N1KO macrophages express less IL-6 and CD80, these defects together may lead to

decreased T cell differentiation and, resulting in decreased disease severity. In addition, N1KO macrophages may interfere with the activity of the endogenous macrophages which influence the outcome of the disease.

Previous *in vitro* studies demonstrated that activation of macrophages by IFNγ and LPS triggers cleavage of Notch receptors and activation of the Notch signaling, and the Notch signaling, in turn, directly or indirectly regulates pro-inflammatory cytokines production such as IL-6 and IL12p40/70. Inhibition of Notch signaling by using a pharmacological inhibitor, GSI, which blocks gamma secretase activity, resulted in the reduction of IL12p40/70. Our *in vitro* study revealed that deletion of *Notch1* in macrophages did not show any detectable effect on IL12p40/70 production after the activation by IFNγ and LPS. These results imply that other Notch receptors such as Notch2 may play a redundant role in regulating IL12p40 expression when *Notch1* is deleted. Notch2 is also highly expressed in macrophage (3). Xu *et al.*, however, reported that macrophages from *Notch1*$^{+/-}$ mice expressed less *il12p40* than the control wild type macrophages upon LPS stimulation, and silencing *Notch2* in these macrophages did not reduce the level of *il12p40* mRNA further, suggesting a disposable role of Notch2 for this activity (51). GSI is a pan-inhibitor of all Notch receptor processing. We found the reduction of IL12p40 in GSI-treated N1KO macrophages which implies that other Notch receptors might compensate for the loss of Notch1. The discrepancy on the effect of Notch1 deletion on IL12p40 expression between our results from N1KO macrophages and Xu *et al*. which used macrophages from Notch1 haploinsufficiency may be due to different approaches in generating *Notch1* deletion macrophages and in the use of LPS in their study *vs*. LPS with IFNγ in our system.

We observed the reduction of IL12p40/70 upon the deletion of *CSL/RBP-Jκ* in macrophages, similar to the GSI treatment. However, the level of IL12p40/70 production upon GSI treatment and deletion of CSL/RBP-Jκ showed only partial reduction, compared to the control, suggesting that Notch signaling pathway works with other signaling pathways such as NF−κB, C/EBP−β and AP1 in controlling the optimal production of IL12p40/70 in IFNγ/LPS activated BMDMs (74).

NF-κB signaling, in particular c-Rel, is required for the *IL12p40* transcription in macrophages, possibly as a dimer with p50 (77). Indeed, we observed that *CSL/RBP-Jκ* KO macrophages stimulated by IFNγ/LPS exhibited less c-Rel nuclear accumulation compared to the control wild type macrophages. This result is consistent with that observed in the GSI treated macrophages (50). In contrast, the activated N1KO macrophages showed similar c-Rel pattern as the control macrophages. How CSL/RBP-Jκ and the activity of gamma secretase regulate nuclear translocation of c-Rel upon LPS/IFNγ treatment needs further investigation. In contrast, Xu *et al.* reported that in their system that activation of *CSL/RBP-Jκ* KO macrophages by LPS alone did not have any effect on the activation of NF-κB signaling pathway (51). In their study, Notch/RBP-Jk induces the expression of a transcription factor, IRF8, which acts as a regulator of genes involved in polarization of pro-inflammatory macrophages. The detailed mechanism is proposed that CSL/RBP-Jκ selectively enhances kinase IRAK2-dependent signaling via TLR4 to the kinase MNK. This event leads to the downstream translation-initiation control through eIF4E. However, IRF8 can be regulated by IFNγ (91) and the discrepancy on the effect of CSL/RBP-JK

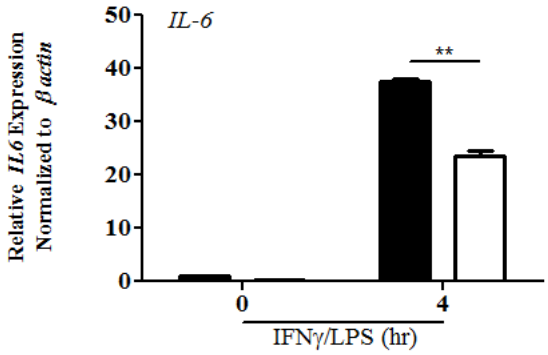
deletion on NF-κB between our study and that by Xu *et al*. is possibly due to the difference in stimuli used, i.e. LPS *vs*. LPS/IFNγ.

In T cells, Notch signaling functions to augment the NF-κB signaling by facilitating the nuclear retention of the NF-κB subunits, p50 and c-Rel. This study revealed that Notch1 interacts directly with NF-κB and competes with IκBα, resulting in the retention of NF-κB in the nucleus (92). In our study, similar mechanism may operate in driving c-Rel nuclear retention in macrophages. This possibility needs further investigation.

Our study reported a novel role of Notch1 in macrophages in EAE upon transferring into wild type mice and Notch signaling pathway in regulating c-Rel activation and IL12p40/p70 expression in macrophages upon IFNγ/LPS treatment. These findings indicate that Notch signaling in macrophages is important for the development of EAE and may have therapeutic implication for autoimmune diseases.

Supplementary

A



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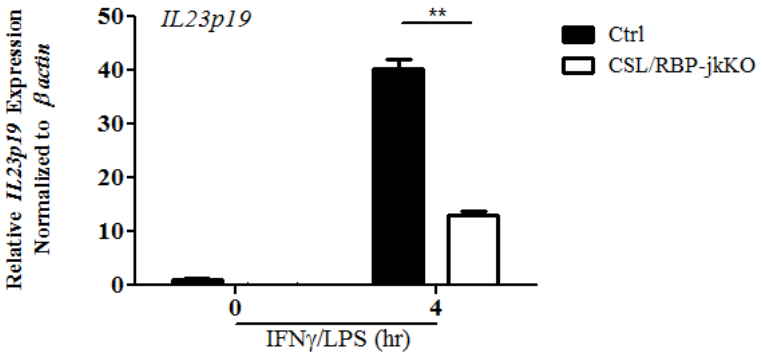
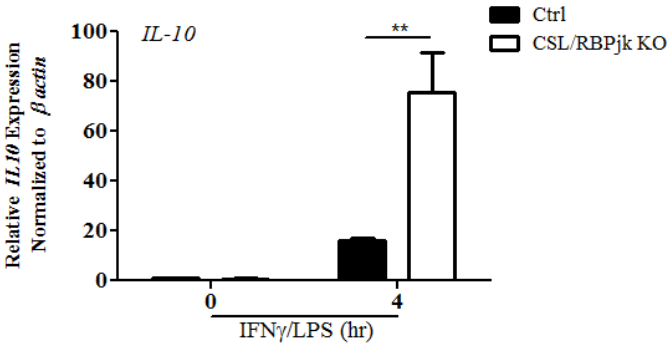


Figure 20. Effects of targeted deletion of *RBP-J*κ on *IL-6* and *IL-23p19* mRNA in IFNγ/LPS activated BMDMs

BMDMs from wild type control or RBP-Jκ KO mice were activated by IFNγ/LPS for 4 hr. The level of (A) *IL-6* and (B) *IL-23p19* mRNA was measured by qPCR. All data are representative of at least two independent experiments.** indicates the statistical significance with $p < 0.05$.

A



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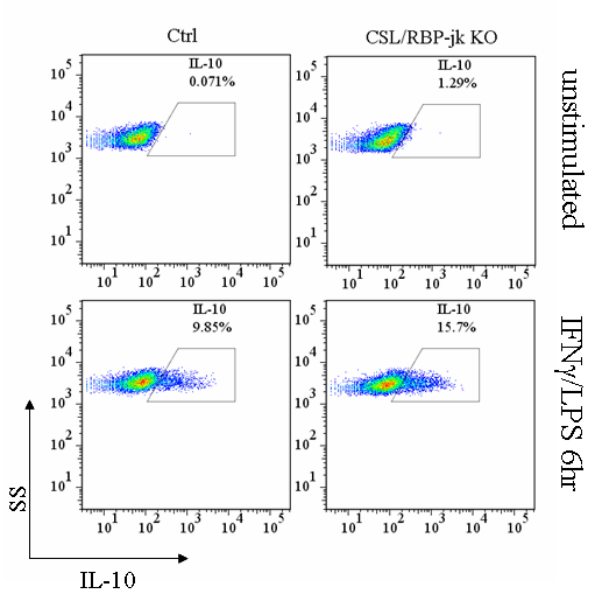


Figure 21. Targeted deletion of *RBP-Jκ* on IL-10 production in IFNγ/LPS activated BMDMs

(A) BMDMs from wild type control or RBP-Jκ KO mice were activated by IFNγ/LPS for 4 hr. The level of IL-10 was measured by qPCR. (B) The level of IL-10 production was detected by flow cytometer All data are representative of at least two independent experiments. ** indicates the statistical significance with p <0.05. N.S.: no statistical significance

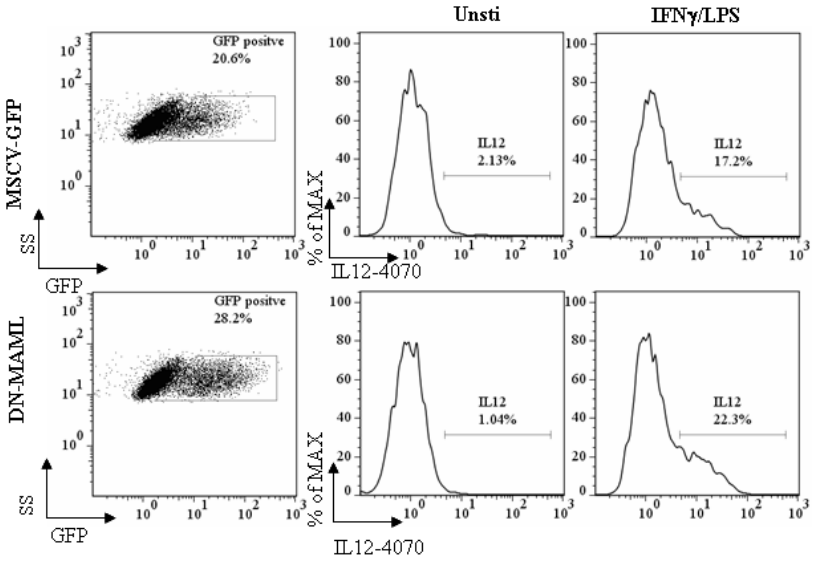


Figure 22. Effect of DNMAML overexpression on IL-12p70 production in IFNγ/LPS activated BMDMs

BMDMs were transduced by retroviral vectors as indicated and activated for 6 hr and the level of IL12p70 in GFP positive cells were analyzed by intracellular cytokine staining. All data are representative of at least two independent experiments

CHAPTER V

Regulation of IL-10 Production by Notch Signaling in Macrophages Activated with IFNγ/LPS and Immune Complex

Result

IFNγ/LPS+immune complex activated macrophages produce IL-10 and express Notch ligands and receptors

Cytokines produced from activated macrophages are determined mainly by the microenvironment (93). Signaling through TLRs by an agonist such as LPS in macrophages leads to production of large amount of pro-inflammatory cytokines, meanwhile they also produce anti-inflammatory cytokine, IL-10 at low levels to maintain homeostasis (81). In contrast, macrophages activated by IFNγ/LPS in the presence of immune complexes in which the signal is transduced via the crosslinking of the Fcγ receptors, produce a larger amount of IL-10 than cells being activated by LPS alone (41). Previously, we and others showed that Notch signaling is important in the activation and the production of cytokines such as IL-12, TNFα and IL-6 in TLR activated macrophages (47, 49, 50).

Here, we focused on the contribution of Notch signaling on macrophages which are activated by IFNγ/LPS together with immune complexes. To confirm the cytokine profiles of macrophages after stimulated by IFNγ/LPS or IFNγ/LPS with immune complexes, *IL-10* and *IL-12p40* mRNA expression were determined at 4 hr of activation. Similar to the previous reports from Mosser group, macrophages activated by IFNγ/LPS with immune complexes showed higher level of *IL-10* mRNA, compared with those activated by IFNγ/LPS whereas the relative level of *IL12p40* mRNA were lower (Fig. 23A). To directly compare the amount of IL-10 produced by

macrophages stimulated with IFNγ/LPS in the presence or absence of immune complexes, the amount of IL-10 was measured. We found that IL-10 production was detected in all LPS-stimulated conditions. As expected, the production of IL-10 was detected at highest level upon activation by IFNγ/LPS together with an immune complex, compared with other conditions (Fig. 23B). We next measured the amount of TNF-α and IL-6 which has been reported in this type of macrophages (40, 94, 95). We found that TNF-α was produced at a comparable level regardless of the presence of an immune complex, whereas the production of IL-6 was dampened in IFNγ/LPS in the presence of immune complexes, compared with stimulation with IFNγ/LPS alone (Supplement Fig. 32). These data indicated that the presence of an immune complex tips the balance of cytokine production mainly IL-6, IL-12 and IL-10 but not TNF-α.

Next, we asked whether Notch receptors and its ligands were detected in IFNγ/LPS+immune complex activated macrophages. Upregulation of Notch1 and Notch2 proteins were detected at 1 hr after activation. Cleaved Notch1 (Val 1744) protein which was used to determine the activation of Notch1 also appeared after activation by IFNγ/LPS+immune complex (Fig. 23C). At 3 hour after activation, the expression of Notch ligand, Jagged1 was observed at higher level than other ligands (Fig. 23D). Therefore, macrophages activated by IFNγ/LPS+immune complexes expressed Notch receptors, Notch1 and Notch2, and Notch ligands. The activation of Notch signaling was initiated after this stimulation.

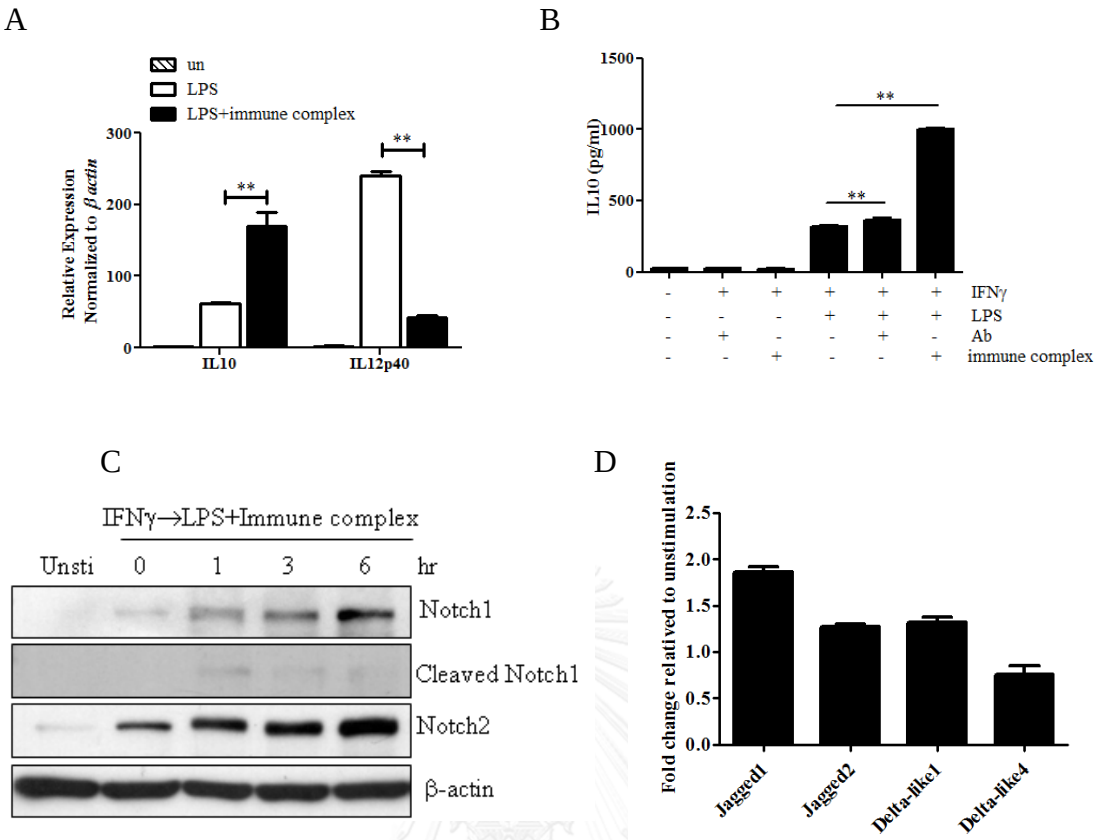


Figure 23. Expression of the key cytokines and Notch ligands and receptors in macrophages activated by IFNγ/LPS or IFNγ/LPS+immune complex

(A) BMDMs were activated by IFNγ/LPS or IFNγ/LPS+immune complex for 4 hr. The expression of *IL-12p40* and *IL-10* mRNA were measured by qPCR. (B) BMDMs were activated by IFNγ/ immune complex and anti-OVA with or without LPS for 6 hr. The production of IL-10 was measured by ELISA. (C) BMDMs were activated by IFNγ/LPS+immune complex for 0, 1, 3 and 6 hr. Notch1, Notch2 and cleaved Notch1 (Val1744) were detected using Western blot. β-actin were used for loading control. (D) BMDMs were activated by IFNγ/LPS+immune complex for 3 hr. The relative expression level of Jagged1, Jagged2, Delta-like1 and Delta-like4 were detected by flow cytometry and normalize to an unstimulated condition. ** indicated where statistical significance was observed ($p<0.05$)

LPS but not the immune complex initiated the activation of Notch signaling in IFNγ/LPS+immune complex activated macrophages

The immune complex binds specifically to Fcγ receptors on macrophages, resulting in enhancing phagocytotic activities and producing microbicidal mediators such as nitric oxide (7, 38). Macrophages which recognize immune complexes together with TLR ligand such as LPS produce more IL-10 and function as the regulatory cells in the inflammatory state (7). In LPS-stimulated conditions, Foldi *et al*. reported that the activation of Notch signaling was initiated by Jagged1 autoamplification through NF-κB and MAPK signaling pathway in TLR-activated macrophages (48). We, hence, asked whether Notch activation was initiated through the signal downstream of TLR and/or immune complex. Macrophages were activated by anti-OVA antibody alone or in an immune complex state with or without LPS. Then, the cleaved Notch1 was determined. We found that the activation of Notch signaling was detected only after stimulation with LPS whereas anti-OVA antibody or immune complex alone did not result in the appearance of cleaved Notch1 (Fig. 24). These data indicated that Notch activation in IFNγ/LPS+immune complex activated macrophages depends solely upon signaling via LPS stimulation.

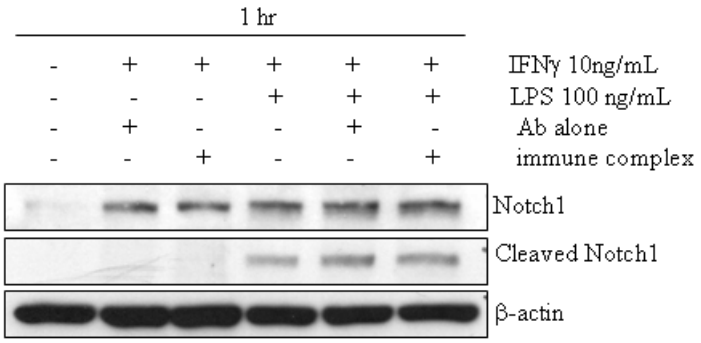


Figure 24. LPS is responsible for the activation of Notch signaling in IFNγ/LPS+immune complex activated macrophages

BMDMs were activated by IFNγ/anti-OVA antibody and IFNγ/immune complex with or without LPS. Protein lysates were collected after 1 hr activation. Notch1 and cleaved Notch1 (Val 1744) were detected by Western blot. β-actin was used as loading control

Notch activation is NF-κB and MAPK dependent in macrophages activated with IFNγ/LPS+immune complex

Stimulation of macrophages through TLR alone induces the activation of Notch signaling through NF-κB and MAPK (48). Moreover, the data from our study showed that the Notch activation in IFNγ/LPS+immune complex-activated macrophage was dependent upon LPS stimulation and the signaling downstream of TLR4. Therefore, we asked whether the activation of Notch signaling is dependent on NF-κB or MAPK pathways, similar to TLR-activated macrophages. We used pharmacological inhibitors to specifically inhibit NF-κB, MAPK and PI3K signaling and observed the activation of Notch1. Cleaved Notch1 was not detectable after pretreatment of macrophages with either IκBα or Erk inhibitor, whereas the activation of Notch1 remained intact after pretreatment with p38 or PI3K inhibitor (Fig. 25). Therefore, the activation of Notch signaling was NF-κB and/or MAPK (Erk) dependent in IFNγ/LPS+immune complex activated macrophages. In contrast, MAPKs p38 and PI3K are dispensable for this activation.

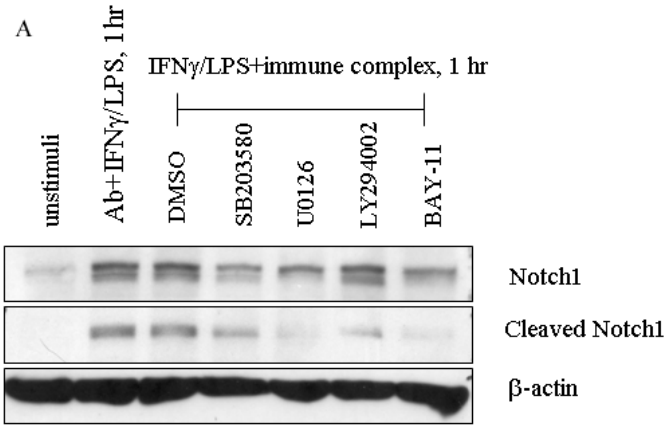


Figure 25. Activation of Notch signaling in IFNγ/LPS+immune complex activated macrophages was NF-κB and MAPK pathway dependent

BMDMs were pretreated by Bay-11 (10 μM), SB203580 (10 μM), U0126 (10 μM) and LY94002 (50 μM) for 30 min and activated by LPS+ immune complex. Protein lysates were collected after 1 hr activation. Notch1 and cleaved Notch1 (Val 1744) were detected by Western blot. β-actin was used as loading control

Inhibition of NF-κB, MAPK and PI3K signaling pathway decreased IL-10 production in activated macrophages

The regulation of IL-10 production in macrophages has been extensively studied (41). Lucas *et al.* showed that the production of IL-10 in LPS/immune complex activated macrophages was mainly controlled by p38 and Erk signaling pathway (42). Moreover, homodimers of NF-κBp50 subunits regulates the transcription of *IL-10* in LPS activated macrophages was reported (82). Then, we confirmed the role of NF-κB, MAPK and PI3K signaling on IL-10 production in LPS/immune complex activated macrophages. Specific pharmacological inhibitors were used and IL-10 production was detected at 6 hr after activation by ELISA. Using the inhibitors of MAPKs p38 (SB203580) and PI3K (LY94002) resulted in a profound reduction of IL-10. The level of the reduction was lower than the production of IL-10 in macrophages stimulated by IFNγ/LPS alone. In addition, using the inhibitors of MAPKs MEK1/2 (U0126) and NF-κB IκBα (Bay-11) resulted in a partial reduction of IL-10 level (Fig. 26). These results implied that MAPKs MEK1/2 and NF-κB are necessary for the regulation of IL-10 production when immune complexes were applied. Taken together, these data indicated that IL-10 production in LPS/immune complex activated macrophages were regulated by NF-κB, MAPK and PI3K signaling pathways with different magnitude.

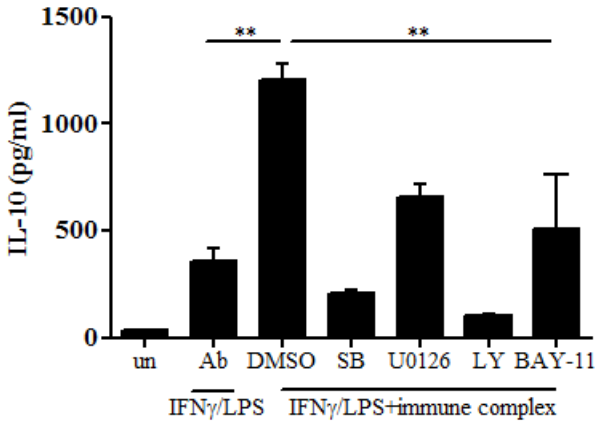


Figure 26. Inhibition of NF-κB, MAPK and PI3K signaling pathway affected the production of IL-10 in activated macrophages

BMDMs were pretreated by Bay-11 (10 μM), SB203580 (10 μM), U0126 (10 μM) and LY94002 (50 μM) for 30 min and activated by LPS+ immune complex. Culture supernatants were collected after 6 hr activation. IL-10 was determined by ELISA. ** indicated where statistical significance was observed (p<0.05)

Gamma secretase inhibitor treatment reduced IL-10 production in activated macrophages

Next, we ask whether Notch signaling in these macrophages is involved in the production of IL-10. To study the effect of Notch signaling on IL-10 production, gamma secreatase inhibitor (GSI) was used and cleaved Notch1 was examined to determine the activation of Notch signaling. Cleaved Notch1 was not detected in the GSI treatment condition compared with that of the DMSO control, suggesting that GSI treatment effectively inhibited the Notch signaling pathway (Fig 27A and B). Moreover, the production of IL-10 was decreased by almost 50% in GSI treatment, compared with the mock control in both intracellular staining assay and ELISA (Fig. 27C and D). The amount of IL-10 in GSI-treated IFNγ/LPS+immune complex activated macrophages was at the comparable level with that in IFNγ/LPS activated macrophages, implying that Notch signaling is critical for IL-10 production in macrophages activated by IFNγ/LPS+immune complex.

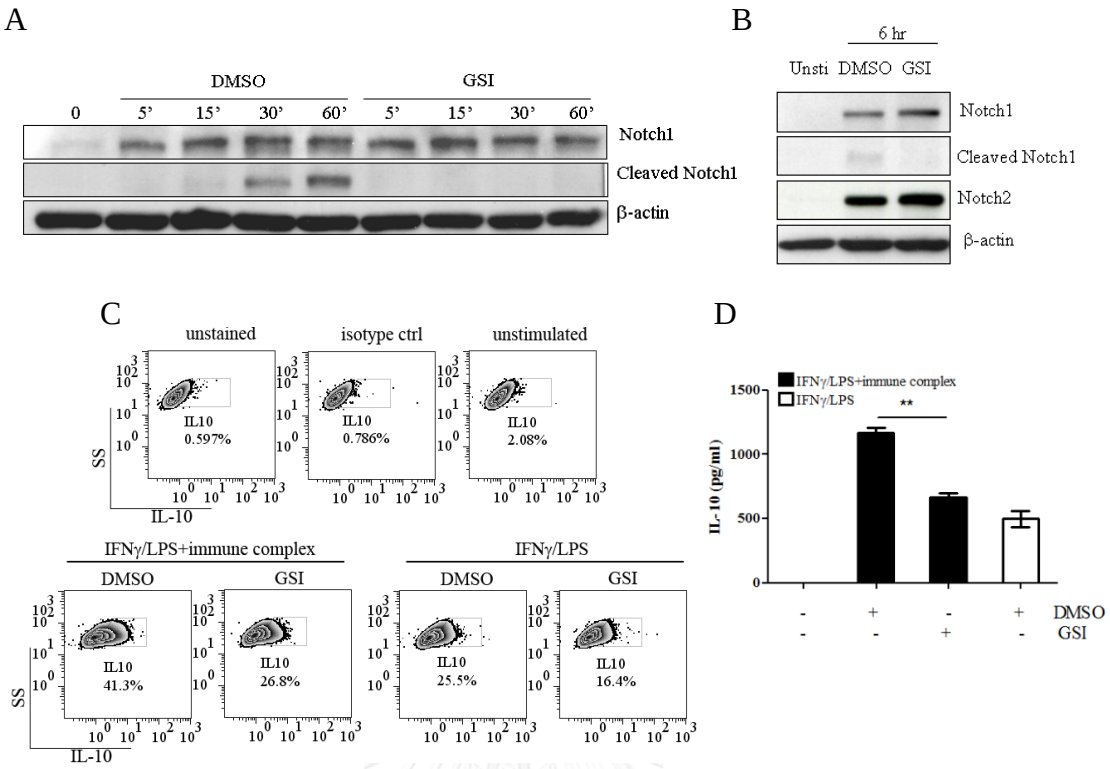


Figure 27. Inhibition of Notch signaling by GSI in IFNγ/LPS+immune complex activated macrophages reduced IL-10 production

(A) BMDMs were activated by IFNγ/LPS+immune complex for 5, 15,30 and 60 min in the presence of vehicle control DMSO or GSI (25 μM). Cleaved Notch1 (Val 1744), Notch1 and loading control (β–actin) was detected by Western blot. (B) BMDMs were activated by IFNγ/LPS+immune complex for 6 hr in the presence of vehicle control DMSO or GSI (25 μM). Cleaved Notch1 (Val 1744), Notch1, Notch2 and loading control (β-actin) was detected by Western blot. (C-D) BMDMs were activated by IFNγ/LPS+immune complex for 6 hr in the presence of vehicle control DMSO or GSI (25 μM). Expression of IL-10 was detected by intracellular cytokine staining (C) or ELISA (D). ** indicated where statistical significance was observed (p<0.05)

GSI treatment slightly affected the activation of p38, Erk and NF-κB signaling pathways

Next, we asked whether Notch signaling affected MAPK, NF-κB and PI3K signaling using GSI treatment to suppress the activation of Notch signaling pathway. BMDMS were activated by IFNγ/LPS+immune complexes at 5, 15, 30 and 60 min in the presence of GSI or DMSO. The phosphorylation of p38 and Erk downstream of MAPKs signaling was lower in the GSI treatment condition than in the DMSO control. However, the activation of SAPK/JNK remained intact. (Fig. 28A-C). Moreover, the reduction in the nuclear translocation of p50 subunit of NF-κB was observed in GSI treatment (Fig. 29). However, we did not detect the reduction in PI3K activation even when GSI treatment was carried out (Fig. 30). These data suggested that Notch signaling regulates activation of NF-κB and MAPK signaling pathways and, in turn, regulates IL-10 in IFNγ/LPS+immune complex activated macrophages.

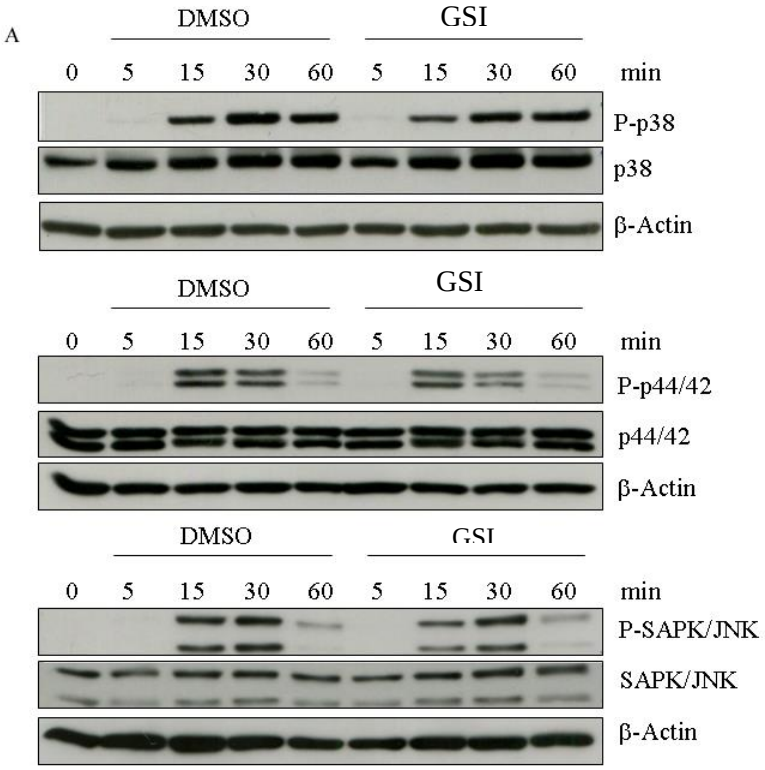


Figure 28 GSI treatment slightly reduced the activation of MAPK signaling pathways

BMDMs were activated by IFNγ/LPS+immune complex for 5, 15, 30 and 60 min in the presence of vehicle control DMSO or GSI (25 μM) (A)Phospho-p38, p38 (B) Phospho-p44-42, p44-42, (C) Phospho-SAPK/JNK, SAPK/JNK and loading control (β–actin) were detected by Western blot.

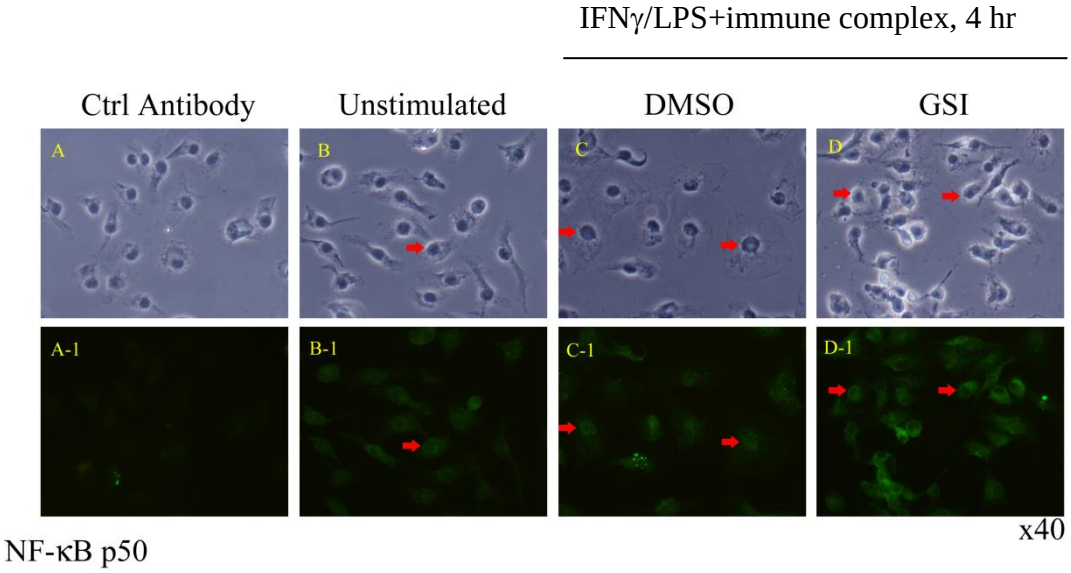


Figure 29.GSI treatment reduces the translocation of NF-κB p50 to nucleus in activated macrophages

BMDMs were activated by IFNγ/LPS+immune complex for 4 hr in the presence of vehicle control DMSO or GSI (25 μM). NF-κB p50 was detected by immunofluorescence staining. (A-D) bright Field (A-1 to D-1) fluorescence field. The arrows indicated the changes or lack of nuclear translocation of p50 (green fluorescence).

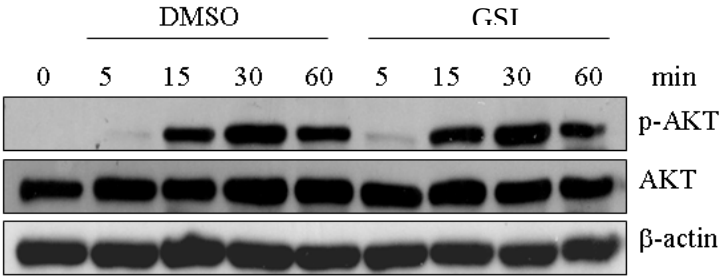


Figure 30. GSI treatments did not influence the activation of PI3K/AKT signaling pathway

BMDMs were activated by IFNγ/LPS+immune complex for 5, 15, 30 and 60 min in the presence of vehicle control DMSO or GSI (25 μM). Phospho-AKT, AKT and loading control (β-actin) was detected by Western blot.

Discussion

In this study we aimed to investigate the roles of Notch signaling in IFNγ/LPS+immune complex activated macrophages. We asked which signaling is responsible for the Notch activation in this stimulating condition. We showed that stimulation of macrophages by an antibody or an immune complex without LPS was not sufficient to initiate Notch activation. Previously, Foldi *et al* reported that the activation Notch signaling was initiated through downstream signaling of TLR4 in LPS activated macrophages, mainly the MAPK and NF-κB pathways (48). Similar results were observed in IFNγ/LPS+immune complex activated macrophages where both NF-κB and MAPK were critical for the activation of Notch signaling. Therefore, it is likely that the initiation of Notch activation in this type of macrophages is through TLR signaling in FcγR-independent manner.

The production of IL-10 in macrophages was mainly regulated by MAPKs and NF-κB signaling pathways(41). In this type of macrophages, the immune complex which triggered signaling downstream of FcγRs and LPS cooperate to enhance the production of IL-10 (8). Our study yielded similar results from previous reports that immune complex alone was inadequate for the induction of IL-10, indicating that TLR signaling was important for the initiation of IL-10 production and the immune complex helps to boost the amount of IL-10 (94). The different level of IL-10 production was observed when different specific inhibitor (MAPKs MEK1/2, MAPKs p38, NF-κB IκBα , PI3K and Notch) was used, indicating that each signaling differentially contributes to the controlling of IL-10 production in macrophages activated by IFNγ/LPS with an immune complex. Using pharmacological inhibitor of MAPK signaling, we yielded the similar results to the previous study from the Mosser

group which demonstrated the synergistic regulation of MAPKs p38 and Erk (which is downstream target of MEK1/2) signaling on IL-10 production in LPS+immune complex activated macrophages (42).

We further investigated the role of NF-κB in macrophages which were activated by IFNγ/LPS together with an immune complex due to NF-κB signaling has been suggested to control the IL-10 production under LPS simulation in macrophages (41). We found that NF-κB partially regulated the production of IL-10 in IFNγ/LPS+immune complex activated macrophages. Thereby, NF-κB appeared to regulate the production of IL-10 induced only by an immune complex in our system. A previous study demonstrated the role of NF-κB in supporting MAPK signaling in LPS-induced IL-10 production macrophages (96) whereas various DNaseI hypersensitive sites (HSS) containing the NF-κB binding motifs were identified in the *IL-10* locus, emphasizing the important role of NF-κB in the direct regulation of IL-10 production in various cell types including macrophages. (80, 82). Thus, it is possible that the regulation of IL-10 in IFNγ/LPS+immune complex-activated macrophages through NF-κB might be independent of the regulation by MAPK. Additionally, the epigenetic regulation in the *IL-10* locus controlled by various factors was reported (41). The signaling initiated by an immune complex might partially affect the recruitment of NF-κB to the nucleus or moderately impact the chromatin remodeling which alter the capability of NF-κB to assess to the binding sites on *IL-10* promoter.

According to our results, the reduction in IL-10 production was observed with GSI treatment, indicating that Notch signaling is important for the production of IL-

10 in IFNγ/LPS+immune complex activated macrophages. We further showed that the amount of IL-10 production in GSI-treated IFNγ/LPS+immune complex activated macrophages was at the comparable level with that in IFNγ/LPS activated macrophages. These data implied that Notch signaling involved in TLR and/or FcγR downstream signaling to regulate IL-10 production.

Notch signaling supported the activation of NF-κB (p50, p65 and c-Rel) signaling in LPS-activated macrophages (3). In T cell activation, Shin *et.al* showed that the intracellular domain of Notch1 in nucleus directly interacted with NF-κB (p50) and sustained NF-κB activation to maintain T cell activation. In contrast, decreasing of NF-κB activation was observed in the absence of nuclear localization of Notch1 protein. (92). Based on our observation, Notch signaling may play a similar role in activation of NF-κB in IFNγ/LPS+immune complex-activated macrophages.

Furthermore, our data showed that Notch signaling involved in the activation of MAPK signaling which similar results were observed in the absence of CSL/RBP-Jκ in LPS-activated macrophage reported by Xu (51) implied that pharmacological inhibitor (GSI) has similar results to genetic deletion of gene in the Notch signaling pathway. Taken together, Notch signaling potentially controls IL-10 production through the regulation of MAPK and NF-κB in IFNγ/LPS+immune complex activated macrophages.

We also observed that PI3K regulated the production of IL-10 in FcγR signaling. FcγR ligation by immune complex induces activation of PI3K/AKT signaling which can down regulate the production of IL-12 and increase IL-10 production (83) However, Notch signaling did not affect the activation of PI3K/AKT

or vice versa. Nevertheless, there were other molecules related to PI3K activation which may crosstalk with Notch signaling such as PTEN and affect IL-10 production. This possibility needs further investigation.

Although our observations showed similar results from other reports (8, 42, 51) and demonstrated a new finding on regulation of IL-10 production by Notch signaling in IFNγ/LPS+immune complex activated macrophages, using of pharmacological inhibitors may result in an off target effect. The genetically deletion of genes in the Notch signaling or gene silencing using specific siRNA should be used to confirm the phenomenon.

Here, we propose a model of how Notch signaling is involved in the regulation of IL-10 in IFNγ/LPS+immune complex-activated macrophages, based on the results obtained in this study. We suggest that Notch signaling is activated only via the downstream signaling of TLR4, mainly the NF-κB and MAPK pathways. Notch signaling regulates the production of IL-10 by involvement in the activation process of NF-κB and MAPK. Therefore, the production of IL-10 in IFNγ/LPS+immune complex activated macrophages was regulated by NF-κB, MAPK in a Notch-dependent manner via TLR4 signaling and by PI3K signaling via FcγR in a Notch-independent manner (Fig. 31).

Taken together, we, for the first time, demonstrated the important role of Notch signaling in the production of IL-10 and its activation in IFNγ+LPS/immune complex activated macrophages. Our investigations may help to further understand molecular mechanisms which terminate the inflammatory response and may shed a new light on inflammatory-related diseases including autoimmune disorders.

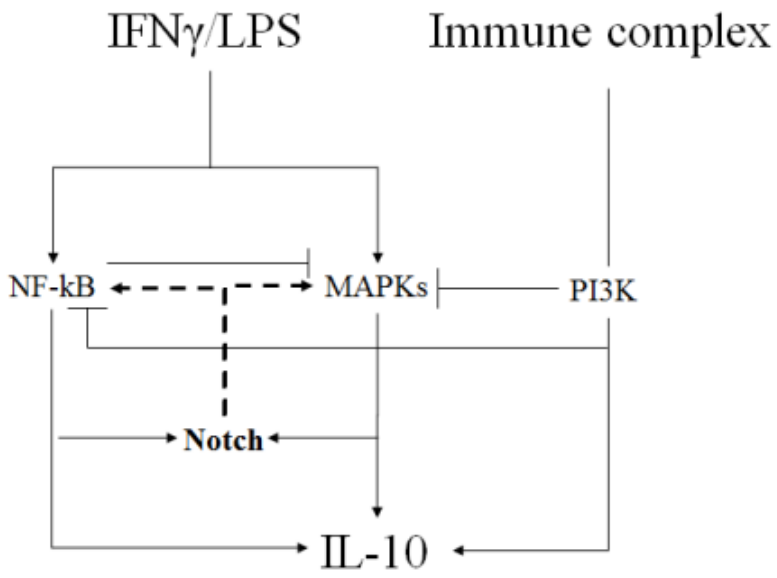
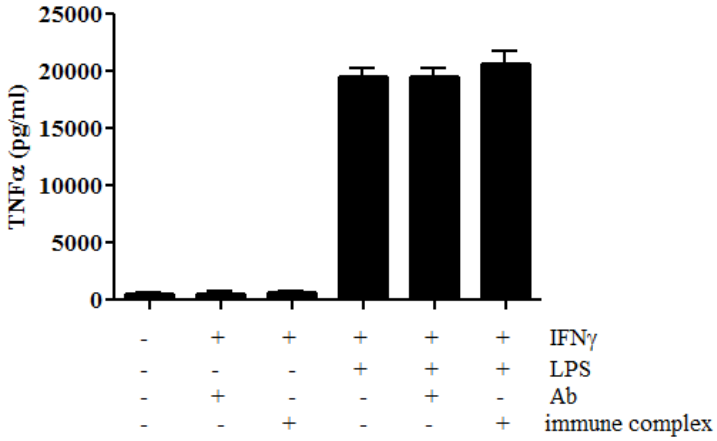


Figure 31. Proposed model of the regulation of IL-10 production by Notch signaling in macrophages activated with IFNγ/LPS and immune complex.

Solid lines depict the reported regulation or the cross talk observed in this study. The dash lines depict the unknown/unproved links.

Supplementary

A



B

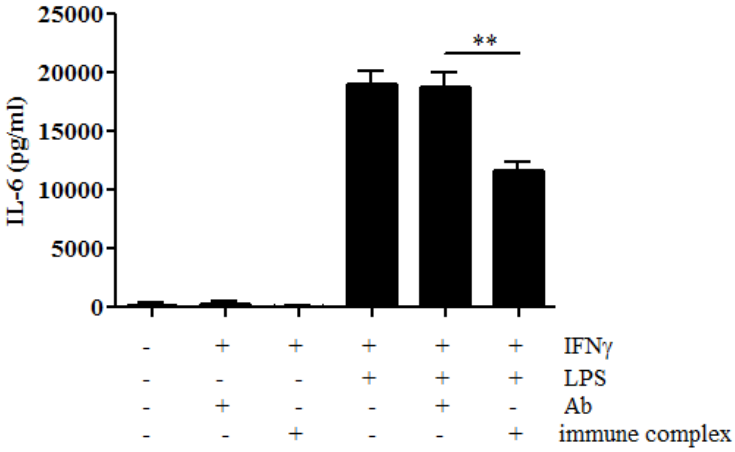


Figure 32. The production of the TNFα and IL-6 in IFNγ/LPS or IFNγ/LPS+immune complex activated macrophages

BMDMs were activated by IFNγ/LPS+immune complex and antiOVA with or without LPS for 6 hr. The production of (A) TNFα and (B) IL-6 was measured by ELISA

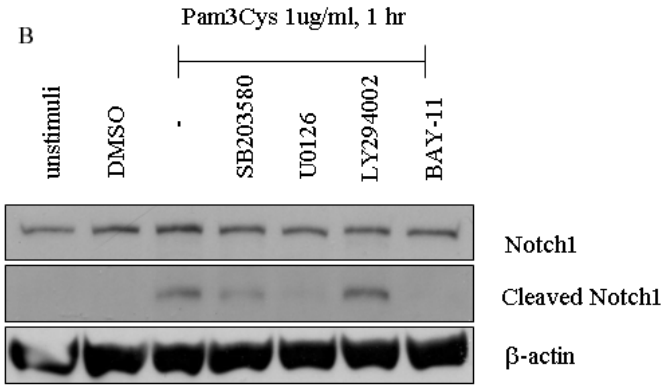


Figure 33. Activation of Notch signaling in IFNγ/LPS+immune complex activated macrophages was NF-κB and MAPK pathway dependent

BMDMs were pretreated by 10 μM of Bay-11 (IκBα inhibitor), 10 μM of SB203580 (p38 inhibitor), 10 μM of U0126 (MEK1/2 inhibitor) and 50 μM of LY (PI3K inhibitor) for 30 min and activated by IFNγ/LPS+immune complex. Protein lysates were collected after 1 hr activation. Notch1 and cleaved Notch1 (Val 1744) were detected by Western blot. β−actin was used as loading control

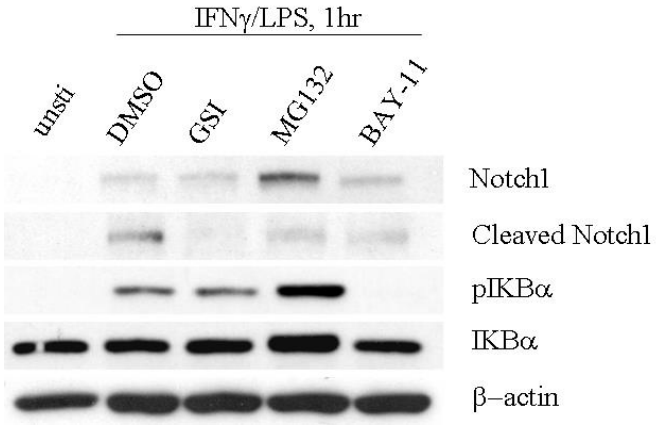


Figure 34. Effect of MG132 and BAY-11, (NF-κB inhibitor) to activation of NF-κB and Notch signaling in IFNγ/LPS activated macrophages

BMDMs were activated by IFNγ/LPS for 1 hr. Cleaved Notch1 (Val 1744), Notch1, pIκBα, IκBα and loading control (β-actin) was detected by Western blot

CHAPTER VI
CONCLUSIONS

In conclusion, Notch signaling in macrophages is mainly activated by NF-κB and MAPK signaling downstream of TLR and, in turn, regulates NF-κB and MAPK activation. The TLR-induced Notch activation in macrophages is involved in the regulation of cytokine production, including IL-12, IL-6 and IL-10 and the expression of the co-stimulatory molecule, CD80. In this respect, we demonstrated by Notch1 deficient macrophage transfer that Notch signaling pathway in IFNγ/LPS-activated macrophages plays important role in determining the outcome of autoimmune disease, EAE. Moreover, we used pharmacological inhibitors to show that Notch signaling is involved in NF-κB and MAPKs activation to regulate IL-10 production in IFNγ/LPS with immune complex-activated macrophages. However, Notch signaling appears to partially affect the production of this cytokine. Our studies suggested that under activation conditions, Notch signaling might be responsible for optimal production of cytokines and the regulation of these cytokines require the crosstalk among Notch, NF-κB and MAPKs signaling pathways. Therefore, our findings may help to further understand the molecular mechanisms which control the inflammatory response and may bring about the finding of new therapeutic drugs for inflammatory-related diseases, including autoimmune conditions.

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APPENDIX

DMEM completed media 100 mL

DMEM	90 mL
Fetal bovine serum	10 mL
Streptomycin/ Penicillin G 100x	1 mL
Sodium pyruvate 100x	1 mL
HEPES 100x	1 mL

BMDMs media 100 mL

DMEM completed media	80 mL
L929 culture supernatant	20 mL
Horse serum	5 mL

BMDMs Freezing media 10 mL

A

DMEM (w/o serum)	80%
Fetal bovine serum	20%

B

DMEM (w/o serum)	60%
Fetal bovine serum	20%
DMSO	20%

Add 500 μL of ice cold (A), followed by gently adding 500 μL of ice cold (B)

Fetal bovine serum inactivation

Commercial fetal bovine serum were kept at -20°C and thawed at 4°C for overnight followed by inactivated at 56°C for 30 min in water bath prior using

Immune complex preparation (10 uL for 1 mL of DMEM completed media)

10 μM Rabbit anti OVA (10mg/mL)	:	1xPBS	:	1μM OVA
0.75 μL	:	4.25 μL	:	5 μL

Mix gently by pipette and incubated for 30 min at room temperature

L929 culture supernatant

L929 cells were cultured in 8 mL of DMEM completed media in 5% CO_2 , 37°C. The supernatant was collected when L929 cell was 70-80% confluent in container. Supernatant was filtrated by using 0.2 μm filter and kept at 4°C least than one month

1×PBS pH 7.4, 1000 mL

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

Deionized water	1000 mL

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

PBS-Tween20

1×PBS	500 mL
Tween20	250 μL

FACs staining buffer (1% FBS in PBS)

1xPBS	99 mL
Fetal bovine serum	1 mL

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	3.4 mL

Buffer B for protein extraction

Buffer A	840 µL
Nonidet P-40	10 µL
7x protease inhibitor	150 µL

8% 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

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

2×Laemmli buffer (SDS-dye) 10 mL

1 M Tris-HCl pH 6.8	1 mL
10% SDS	4 mL
99.5% glycerol	2.01 mL
HPLC water	1.989 mL
Bromphenol blue	0.001 g

Before using, add 100 uL of β-Mercaptoethanol in 900 uL of 2×Laemmli buffer

5×running buffer for Western blot (1000 mL)

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

Transfer buffer for Western blot

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

Blocking solution for Western blot

PBS-Tween20	100 mL
Non-fat dry milk	3 g

ECL substrate of HRP for Western blot

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

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

Solution A for ECL

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

Solution B for ECL

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

Film developer and fixer

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

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.

75% Ethanol in DEPC 100 mL

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

Coating buffer for ELISA

Na_2CO_3	3.56 g
$NaHCO_3$	8.40 g

Add 1Liter of sterile water, pH. 9.5

Block solution for ELSA (10% FBS in PBS)

1x PBS	90 mL
Fetal Bovine Serum	10 mL

0.2M TMB buffer (500 mL)

33.25 g of Tripotassium Citrate Monohygrate and 19.69 g of Citric acid were dissolved in 350 mL of sterile water, followed by adding 0.2M Citric acid to adjust pH to 4. Final adjust volume to 500 mL by using sterile water

TMB substrate (for 10 mL of TMB buffer)

TMB (3,3,5,5-tetramethylbensidine)	2.5 mg
DMSO	250 µL
Freshly prepared before used	

TMB substrate solution

TMB buffer	10 mL
TMB substrate (containing TMB)	250 uL
H_2O_2	2.5 uL

Stop solution for ELISA (2N H_2SO_4)

H_2SO_4	9.808 mL
Sterile water	100 mL

VITA

My name is Wipawee Wongchana. I was born in Yala, one of the 3 Southern borders of Thailand. In 1999, I entered Chulalongkorn University to study Microbiology and it was my first time to move to Bangkok, a capital of Thailand.

I earned my undergraduate degree of Science in Microbiology from Chulalongkorn University in 2002. After graduation, I worked with Mars Cooperation as a Quality System Technician for 3 years. Later, I had opportunity to work at Itex Foods International Ltd as a Quality System Assistant. With 5 year work experiences in food quality relating to customer healthy concerns, I designed to pursue my graduate study focusing on Immunulogy for both Master's and Ph.D. degrees.

During the course of my Master's program, I had an opportunity to attend the Introductory Course in Immunology at the University of Pennsylvania, Philadelphia, USA by the American Association of Immunologist (AAI) and International Union Immunological Societies (IUIS) during 19-25 June, 2009. During 2011, my Master's degree project was published in Journal of Cell and Molecular Immunology.

In 2010, I started my Ph.D.degree and at the same year I was granted a scholarship from Royal Golden Jubilee to work on Inflammatory Macrophages and Notch Signaling projects. A year later I had an opportunity to perform in vivo experiments at University of Massachusetts at Amherst, USA for one year. During the Ph.D., I had attended both domestic and international conferences/meeting, as well as the publications shown afterwards.

In addition to academic works and projects, I have been involved in the charitable activity that is to establish some people who have a common interest to help the deserted Alzheimer patients at an old people asylum in a province near Bangkok. The activity has undergone for a few years, and hope that this would bring a little peace to our human race although it is very minor activity.