บทบาทของวิถีสัญญาณ Notch ในโพลาไรเซชันของแมคโครฟาจในหนูไมซ์

นายณัฐ บุญยเตชา

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THE ROLES OF NOTCH SIGNALING PATHWAY IN POLARIZATION OF MACROPHAGES IN MICE

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วิถีสัญญาณ Notch เป็นวิถีที่เกี่ยวข้องกับการควบคุมชะตาของเซลล์ การเจริญและการพัฒนาของเซลล์ตลอดจนถึงการตายของเซลล์ การส่งสัญญาณของวิถี Notch เริ่มต้นด้วยการเกิดอันตรกิริยาระหว่างลิแกนด์กับรีเซบเตอร์แล้วก่อให้เกิดการส่งสัญญาณเข้าสู่เซลล์ซึ่งกระตุ้นให้เกิดการถอดรหัสของจีนเป้าหมาย จากการศึกษาบทบาทของวิถีสัญญาณ Notch ในแมคโครฟาจมีรายงานว่าเกี่ยวข้องในการควบคุมกระบวนการอักเสบโดยการควบคุมการสร้างและหลั่งของ TNFα และไนตริกออกไซด์ เนื่องจากแมคโครฟาจมีฟีโนไทป์ที่มีความยืดหยุ่นสูงโดยสามารถตอบสนองต่อสิ่งเร้าภายนอกได้ดีและพบว่าปัจจุบันมีแมคโครฟาจที่ทำหน้าที่แตกต่างกันอยู่อย่างน้อยสามชนิดได้แก่ แมคโครฟาจชนิด classically activated (CA) alternatively activated (AA) และ regulatory (Reg) ซึ่งในงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาบทบาทของวิถีสัญญาณ Notch ในโพลาไรเซชันของแมคโครฟาจชนิดต่างๆ และศึกษาความสัมพันธ์ที่มีต่อวิถีสัญญาณ MAPK โดยใช้แมคโครฟาจที่พัฒนาจากเซลล์ต้นกำเนิดในไขกระดูก เพื่อยืนยันการโพลาไรเซชันของแมคโครฟาจ ได้ใช้การแสดงออกของจีน *Arginase1* (*Arg1*) *il12p40* และ *il10* ซึ่งมีการแสดงออกและใช้เป็นตัวติดตามชนิดของแมคโครฟาจแบบ AA CA และ Reg ตามลำดับ จากการทดลองพบว่ารีเซปเตอร์ Notch1 มีการแสดงออกที่สูงในแมคโครฟาจชนิด CA และ Reg แต่พบในระดับต่ำในชนิด AA และนอกจากนี้ยังพบการแสดงออกของลิแกนด์ที่เป็นลักษณะที่เฉพาะในแต่ละชนิดของแมคโครฟาจอีกด้วย เมื่อยับยั้งการทำงานของวิถีสัญญาณ Notch โดยยากดเอนไซม์แกมมาซีครีเตส (GSI) ซึ่งมีฤทธิ์กดการตัดรีเซบเตอร์ของ Notch ส่งผลให้เกิดการเปลี่ยนแปลงระดับการแสดงออกของจีนที่ใช้ระบุชนิดแมคโครฟาจโดยเฉพาะในแมคโครฟาจชนิด CA และ Reg โดยมีการลดลงของระดับ mRNA ของ *il12p40* และ *il10* ในแมคโครฟาจชนิด CA และ Reg เมื่อศึกษาผลของ GSI ต่อการทำงานของวิถีสัญญาณ MAPK พบว่า GSI ส่งผลให้มีการลดลงของการทำงานของ Erk1/2 ในแมคโครฟาจชนิด CA ผลที่ได้บ่งชี้ว่าวิถีสัญญาณ Notch มีบทบาทสำคัญในการควบคุมการโพลาไรเซชันของแมคโครฟาจ โดยผ่านการควบคุมการส่งสัญญาณของวิถีสัญญาณ MAPK

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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NATT BOONYATECHA : THE ROLES OF NOTCH SIGNALING PATHWAY IN POLARIZATION OF MACROPHAGES IN MICE. ADVISOR : ASSOC. PROF. TANAPAT PALAGA, Ph.D., 79 pp.

Notch signaling is a well conserved signaling pathway which is involved in regulation of cell fate determination, differentiation, proliferation and cell death of various cell types. Interaction between Notch receptors and ligands initiates the signaling cascade, resulting in transcription of its target genes. Previous studies demonstrated that Notch signaling regulates effector functions of innate immune cells, macrophages, such as production of TNFα and nitric oxide, partly through interaction with other signaling pathways. The phenotypes of macrophages are highly plastic, depending on the microenvironment they are surrounded. At least three types of different effector macrophages have been described, *i.e.* classically activated macrophages (CA-MΦ), alternatively activated macrophages (AA-MΦ) and regulatory macrophages (Reg-MΦ). In these studies, we investigated the expression of Notch receptors and their ligands in different types of effector macrophages, and the relationship of Notch signaling pathway and other well characterized signaling pathways using murine bone marrow derived macrophages. Polarization of macrophages were confirmed by monitoring the expression of marker genes, *arginase1* (*Arg1*), *il12p40* and *il10* for AA, CA and Reg-MΦ, respectively. Western blot revealed that Notch1 is highly expressed in CA and regulatory macrophages. The level of Notch1 correlated with the appearance of cleaved Notch1 (Val1744) in these 2 types of macrophages. In addition, distinct patterns of ligand expression and Notch target gene, *Hes1*, in different types of macrophages were observed. Upon inhibition of Notch signaling by gamma secretase inhibitor (GSI), altering in gene expression of key markers for each type was observed. GSI treatment resulted in decreased *il10*, *il12p40 and il23p19* mRNA expression in CA and Reg-MΦ, but did not affect *arg1* mRNA level in AA-MΦ. Moreover, inhibiting Notch signaling affected the status of MAP kinase pathway. Reduced phosphorylation of ERK1/2 was seen in CA-MΦ upon GSI treatment. Taken together, we proposed that Notch signaling plays an essential role in regulating differentiation of effector macrophages partly by interacting and regulating MAP kinase pathway.

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

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LIST OF ABBREVIATIONS

MΦ	macrophage
CA MΦ	classically activated macrophage
AA MΦ	alternatively activated macrophage
Reg MΦ	regulatory macrophage
T_H1	T helper 1
T_H2	T helper 2
OD	optical density
qPCR	semi-quantitative real time polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
mRNA	messenger ribonucleic acid
Ab	antibody
Ag	antigen
LPS	lipopolysaccharide
IFNγ	interferon gamma
IL-10	interlukin 10
IL-12	interlukin 12
IgG1	immunoglobulin G1
IgG2a	immunoglobulin 2a
^oC	degree Celsius
HRP	horseradish peroxidase
PE	phycoerythrin
FITC	fluorescein isothiocyanate
min	minute

hr	hour
M	molar
mM	millimolar
g	gram
ng	nanogram
nm	nanometer
ml	milliliter
µl	microliter
nm	nanometer
cm	centimeter
rpm	round per minute

CHAPTER I

INTRODUCTION

1.1 Background

Notch signaling is a well conserved signaling pathway which is involved in regulation of cell fate determination, differentiation, proliferation and cell death of various cell types. Interaction between Notch receptors and ligands initiates signaling cascade, resulting in transcription of the target genes. Notch signaling regulates effectors functions of macrophages such as production of TNFα and nitric oxide, partly through interaction with other signaling pathways. The phenotypes of macrophages are highly plastic; depending on the microenvironment they are surrounded that linked to molecular pathways. At least three types of different effectors macrophages have been described, *i.e.* classically activated (CA) macrophages, and alternatively activated (AA) macrophages and regulatory (Reg) macrophages.

Because Notch receptors are expressed in macrophages and Notch signaling is reported to be involved in various cell differentiation scenarios, we hypothesized that this signaling might play a role in polarization of macrophages, and might be involved in other signaling pathways such as Mitogen-activated protein kinase (MAPK) pathway. Understanding the signaling pathway involved in this process not only lead to a new insight in macrophage plasticity but may also open a new way to engineer macrophages for controlling of immune responses for therapeutic purposes.

1.2 Objectives of the research

1. To investigate the role Notch signaling plays during polarization of macrophages in mice

2. To investigate the relationship of Notch signaling and other signaling pathways in polarized macrophages

1.3 Scope of the research

To achieve the research objectives, the following scope was set:

(I) To establish the protocol of murine macrophages polarization

(II) To examine the expression of Notch receptors, Notch ligands and Notch target genes in three types of polarized macrophages by Quantitative Real-time PCR and Western blot

(III) To investigate the effect of γ-secretase inhibitor (GSI) on the phenotypes of three types of polarized macrophages by Quantitative Real-time PCR Western blot and flow cytometry

(IV) To investigate the relationship of Notch signaling pathway and other signaling pathways such as MAPK pathway and NF-kB pathway in macrophages polarizations by Western blot

(V) To investigate the effect of GSI-treated macrophages on immune response in vivo by adoptive transfer and ELISA method

CHAPTER II

LITERATURE REVIEWS

2.1 Macrophages

2.1.1 Biology of macrophages

Macrophages are phagocytic white blood cells, which play essential roles in both innate and adaptive immunity. In innate immunity, macrophages induced inflammation by releasing some of mediators that stimulate other cells of the immune system. They also help destroy bacteria, protozoa, and tumor cells. Furthermore, they are involved in antigen presentation to T cells by MHC molecules. Macrophages development usually takes in the bone marrow and passes through the multiple steps. Firstly, hematopoietic stem cell (HSC) in bone marrow was differentiated to macrophage colony-forming unit (M-CFU) which later sequentially then its change to monoblast, pro-monocyte and monocyte in bone marrow, Monocytes move to blood periphery and migrate to tissues and advance into resident or tissue macrophages [1]. Normal resident macrophages are found in many connective tissues; they are Kupffer's cells in liver, alveolar macrophages in lung, histiocytes in skin and connective fissures. In the central nervous system (CNS) contains various macrophage subsets, including microglia, perivascular macrophages, meningeal macrophages and Choroid-plexus [1, 2].

2.1.2 Macrophages and polarizations

Plasticity is a distinctive characteristic of macrophages in responses to microenvironments in innate recognition or signals from lymphocyte subsets. It is well documented that macrophages change phenotypes in response to microenvironment. These phenotypic changes are accompanied by different gene expression profiles which can affect an outcome of a whole immune response [3]. Currently, there are at least 3 different phenotypes of effectors macrophages which have been reported in mice [4]. The first type is called classical activated (CA) macrophages, which arise in response to interferon gamma (IFN-γ) together with pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). These factors were implicated for driving M1 characterization which induced with IFNγ alone or in harmony with microbial components stimuli (e.g., LPS and CpG) or cytokines (e.g., TNF and GM-CSF) [5]. They characteristically increase expression of MHC class II molecules on surface for an antigen presentation. Moreover, they secrete pro-inflammatory mediators such as nitric oxide (NO) and reactive oxygen intermediates with microbicidal activity and pro-inflammatory cytokines. Interleukin 12 (IL-12) and Interleukin 23 (IL-23) are major cytokines produced by CA macrophages [6-8]. Interleukin 12 is composed of two subunits (p35 and p40) as well as Interleukin 23(p19 and p40), for biological functions and functions in promoting the development of Th1-type immune response [9]. Because of an effect on promoting Th1 cells, CA macrophages are effective in promoting cell mediated immune responses and provide efficient help for B cell activation and antibody class switching to IgG2a, IgG2b and IgG3 subclasses [10-12]. The second subsets of macrophages have characteristics in tissue repair function and it is called an alternatively activated (AA) macrophage or wound-healing macrophage, and or M2b.

This type of macrophages specifically upregulate *arginase I (ArgI)* expression, which is not found or found at low level in other types of macrophages. IL-4 and IL-13, two signature cytokines of Th2 immune response, polarize macrophages to an AA type. Both cytokines promote arginase–dependent formation of L-ornithine and, eventually, fibroblast proliferation and collagen production for tissue repair [5]. When macrophages receive an immune complex in the presence of PAMPs, they change phenotype and express high levels of MHC class II and IL-10 but decrease IL-12 and lower Arg1. This type of macrophages is called type-2 activated macrophage (Reg) or regulatory macrophage, and or M2a. This subset has capacity to present Ag to T cells more efficiently than the other two types and seems to induce T cell response toward a Th2 like response, as characterized by Ig class switching to IgG1 and IgE in *vivo* [4, 13]. IL-10 secreted by regulatory macrophage have been implicated in differentiation of T regulatory (Tregs) and are linked to immune suppression, such as decreased antigen presentation and major histocompatibility complex (MHC) class II expression in dendritic cells. It also can cause downregulation of pathogenic Th1, Th2, and Th17 responses [14]. Present day, macrophages were classification with two major groups that are M1 and M2. CA macrophages are member of M1 macrophage subset that secrete pro-inflammatory cytokines, promote T_H1 responses and antitumoral function. IL-12^{high} and IL-10^{low} are characteristics of M1. Expression IL-12/IL-10 ratio is higher than M2. In particular, alternatively activated macrophages member in M2a, regulatory macrophages member in M2b and M2c are deactivated macrophages. IL-12/IL-10 ratio is lower than M1. Both M2a and M2b had immunoregulatory properties and induced T_H2 responses, but deactivated macrophages (M2c) macrophages are more related to immunosuppression [15].

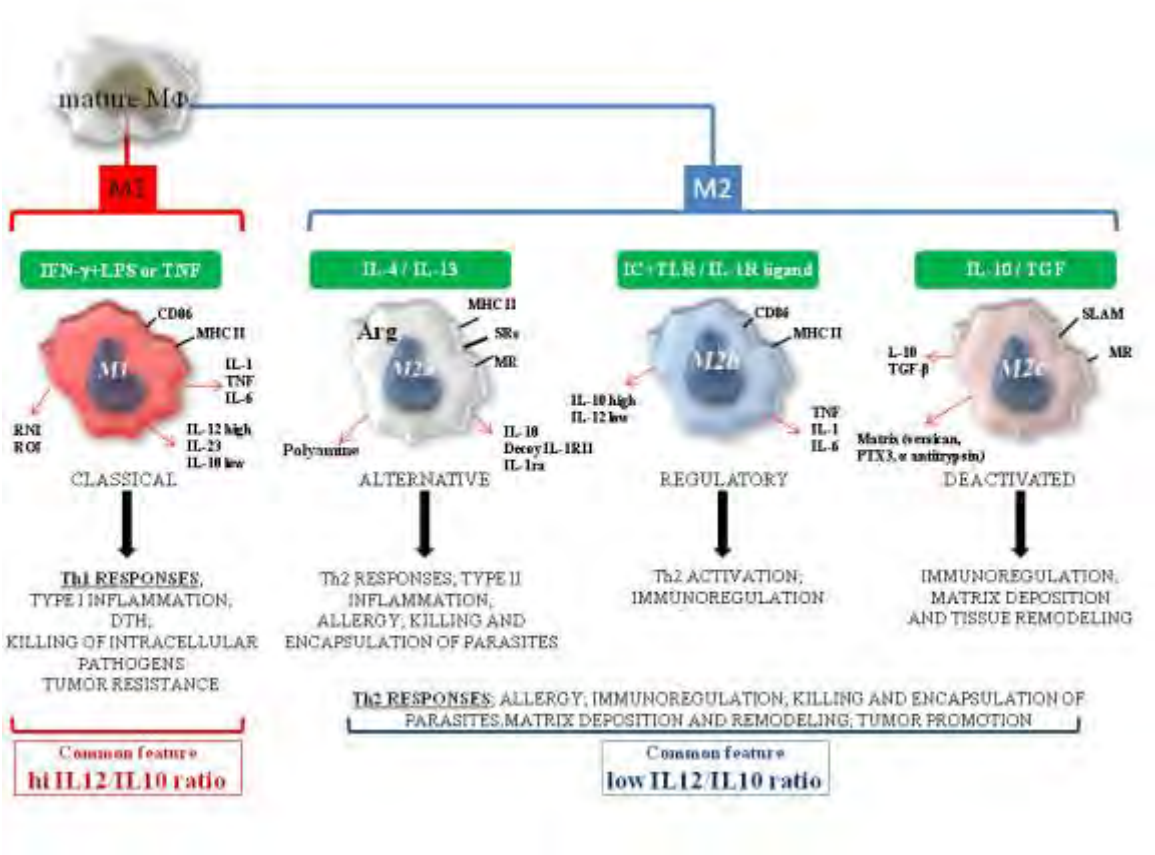

Figure 2.1 Populations of macrophage and their biological properties. Diagram shows subsets of effector macrophages and their biological properties in M1 and M2 system. High IL-12/IL-10 ratio is a common feature of M1, but low IL12/IL10 ratio is a common feature of M2.

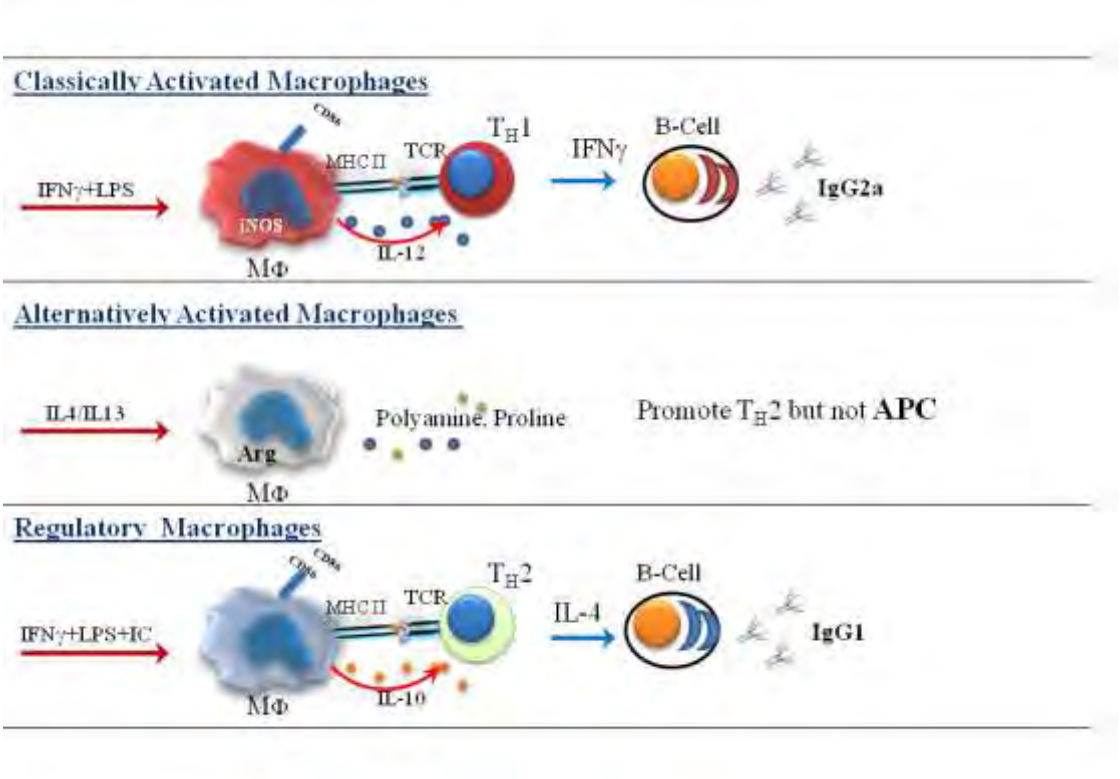

Figure 2.2 Polarization of macrophage and their effects on outcome of immune response. CA subset of M1 macrophages express cytokines such as IL-12, which is one of important cytokines promoting T_H1 and providing efficient help to B-cell activation and production of IgG2a isotype. Regulatory subset has capacity to present antigen with mediators and induce T_H2 responses which lead to antibody class switching to IgG1. AA macrophages contribute to T_H2 responses but lack MHC class II that lead to deficiency of antigen presentation and IgG class switch.

2.1.2 Molecular pathways of macrophage polarization

For CA macrophages subset of M1, LPS and Type 2 IFN stimulate resting macrophages to CA macrophages though toll-like receptor4 (TLR4) and IFN-γ receptor (IFNGR). This stimulation induces activation of the transcription factors NF-κB (p65 and p50) and downstream STAT1, leading to expression of M1 specific genes such as *il12p40* and *NOS2* [4, 16]. In contrast, IL-4, IL-13 and IL-10 are M2 inducing cytokines. AA macrophages, IL-4 and IL-13 transfer signal through common subunit of IL-4 receptor α chain (IL-4Rα) to activate STAT6 phosphorylation before interaction with IRF4 transcription factor for inducing expression of target genes such *argI* and *fizz1*. On the other hand, activation via IL-10 receptor induces STAT3. IL-4/IL-13 and IL-10 are effective in suppression of M1 signature gene expression, but instead induce the transcription of M2 type genes [17-19]. Regulatory macrophages are derived by exposure to immune complexes (IC) in the presence of PAMPs and interferonγ. Immune complexes trigger signaling via FcγR activating ITIM (immunoreceptor tyrosine-based inhibition motif) and ITAMs (immunoreceptor tyrosine-based activation motif), that induces MAPK pathways such as ERK, and NF-κB p-50 homodimer. FcγR-induced and ERK-mediated phosphorylation of histone 3 on Ser10 promotes increased chromatin accessibility at the *il10* locus, which facilitates recruitment of TLR-induced transcription factors. The signaling downstream leads to increase expression of various genes such as *socs3* and *il10*, resulting in negative regulation of the TLR4 or IL-1R and interferon-signaling pathway [20].

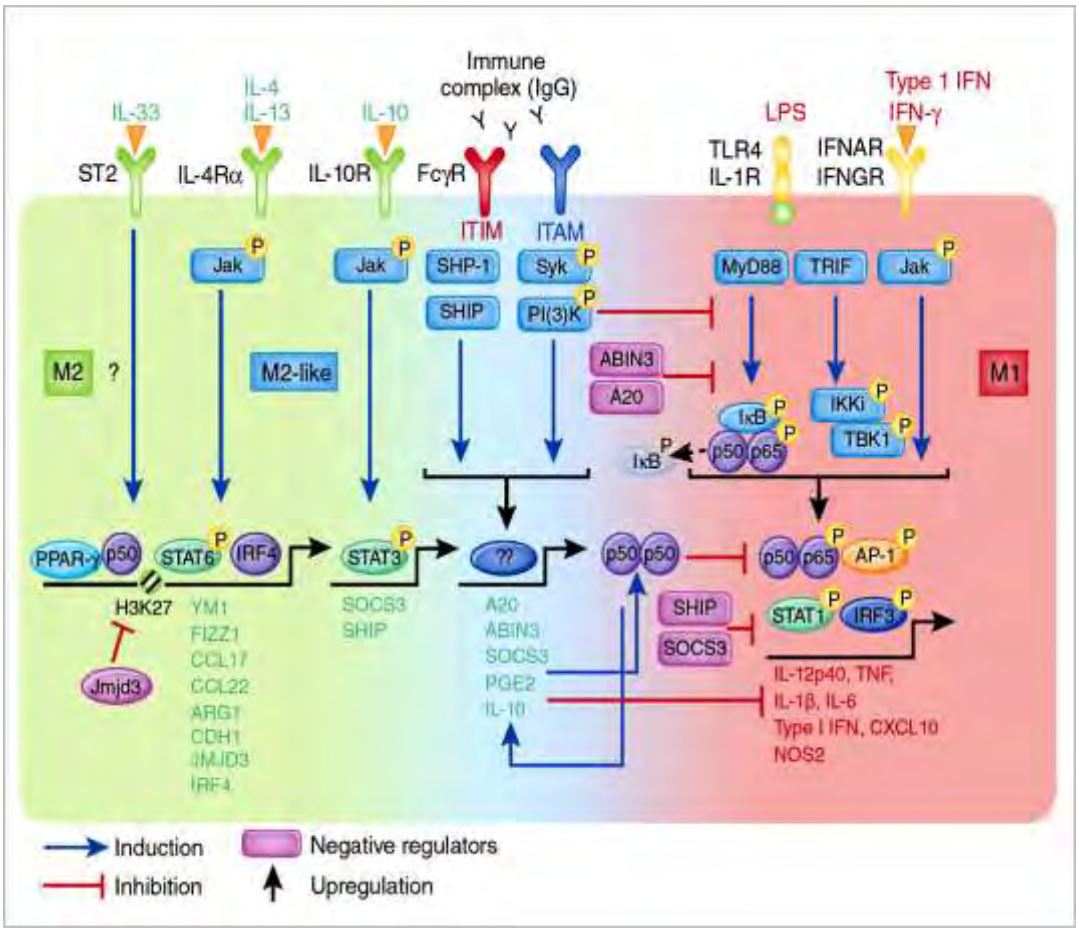

Figure 2.3 Signaling pathways involved in macrophage polarization. LPS and IFN-γ transmit signals through the TLR4, IFN-α, or IFN-β receptor (IFNAR). The IFN-γ receptor (IFNGR) pathway induces activation of the M1-specific transcription factors that composed of NF-κB (p65 and p50), AP-1, IRF3 and STAT1. M2 macrophages are induced by IL-4 and IL-13 which drive signaling through IL-4Rα to activate STAT6. IL-13 transduces signal through ST2 leading to activation of PPAR-γ and NF-κB (p50) [21]. In M2-like macrophages, immune complexes trigger activation of ITIM and ITAM. This activation induces MAPK and homodimerization of NK-κB (p50-50) which promote IL-10 and suppress p50-65 of NF-κB [3].

2.1.1 Macrophages and diseases

Previous report indicated that uncontrolled macrophages were linked to various diseases such as arthrosclerosis and tumor progression [22]. Atherosclerosis is a chronic inflammatory disease, where macrophages are proposed to have malfunction in uptaking and removing cholesterol. In the atherosclerotic lesion, macrophages appear as giant foam cells, can cause plaques blocking blood flow and accelerate inflammation [23, 24]. In breast cancer setting, tumor associated macrophages (TAMs) should have an antitumor properties to benefit the host. Instead, TAMs often promote tumor growth by secreting breast tumor mitogens that direct tumorigenesis and metastasis [25]. In Whipple's disease (WD) is a uncommon systhemic disease, the hallmark of the disease that found encapsulated bacilliform bodies in macrophages cause the accumulation of periodic-acid Schiff (PAS)-stained foamy macrophages in lamina propria [26, 27]. Previous studied in rheumatic diseases in childhood, the hallmark of this syndrome is inordinate activation from T lymphocytes along with macrophages with massive amount of cytokines, including high level of of IL-1β, IL-6, IFNγ and TNFα leading to overwhelmingly activated immune response with potential fatal inflammation [28]. Moreover, in human chronic kidney disease, macrophage infiltration is involved in capillary density in the kidney which exhibit proteinuria. MCP-1/CCL2 are important for macrophages recruitment in initiate disease [29].

2.2 Notch signaling pathway

2.2.1 Biology of Notch signaling

Notch is a family of evolutionarily conserved transmembrane receptors that regulate various processes including controlling of cell fate decisions, development and cell death. In addition to its well characterized roles in development, these receptors also play roles in regulating effector functions of many immune cells, such as in effector phases of T lymphocytes [30]. The signaling is initiated by interaction between Notch receptors and its ligands on the plasma membrane. Activated Notch signaling generates cleaved intracellular Notch which can directly enter the nucleus and bind specifically to DNA binding protein, CSL or RBPJκ. Together with CSL, they regulate the transcription of target genes such as *hes1* and *deltexI* [31]. In mammals, there are four Notch receptors (Notch1, Notch2, Notch3 and Notch4) and five ligands (Jagged1, Jagged2, Delta-like 1(Dll1), Dll3 and Dll4) [32]. All Notch receptors are proteolytically cleaved and activated by the multi-subunit enzyme complex called γ-secretase [33]. γ-secretase cleaves Notch1 between Gly1743 and Val1744 upon ligand interaction [34, 35]. The resulting activated cytosolic fragment translocates to the nucleus where it activates transcription (Figure 2.4, 2.5, 2.6). This proteolytical process can be suppressed by the use of γ-secretase inhibitors (GSI) [36].

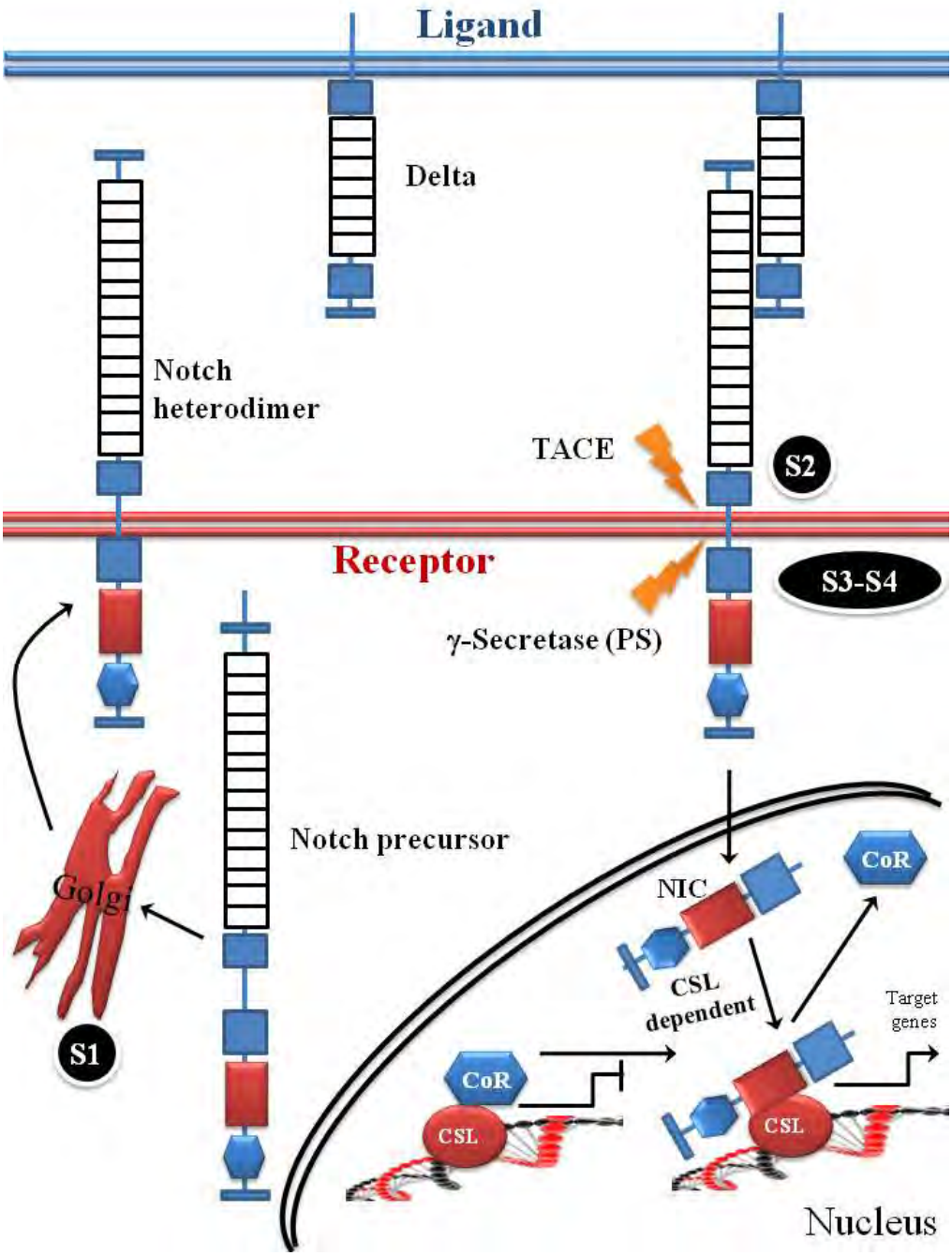

Figure 2.4 Notch signaling pathway. Notch receptor precursors are S1 cleaved by furin, and glycosylated in the Golgi apparatus before presenting on the cell surface. Interaction of Notch receptors with their ligands lead to proteolytic cleavage at S2 site by TACE (tumour-necrosis factor-α-converting enzyme), and S3-S4 sites by γ-secretase complex in the transmembrane, which leads to release of Notch intracellular

domain (NIC). NIC enters the nucleus and binds to CSL or RBPJκ transcription factor. This interaction removes co-repressor (Co-R) and recruits co-activator (Co-A) for activation of the target genes [32, 33, 37].

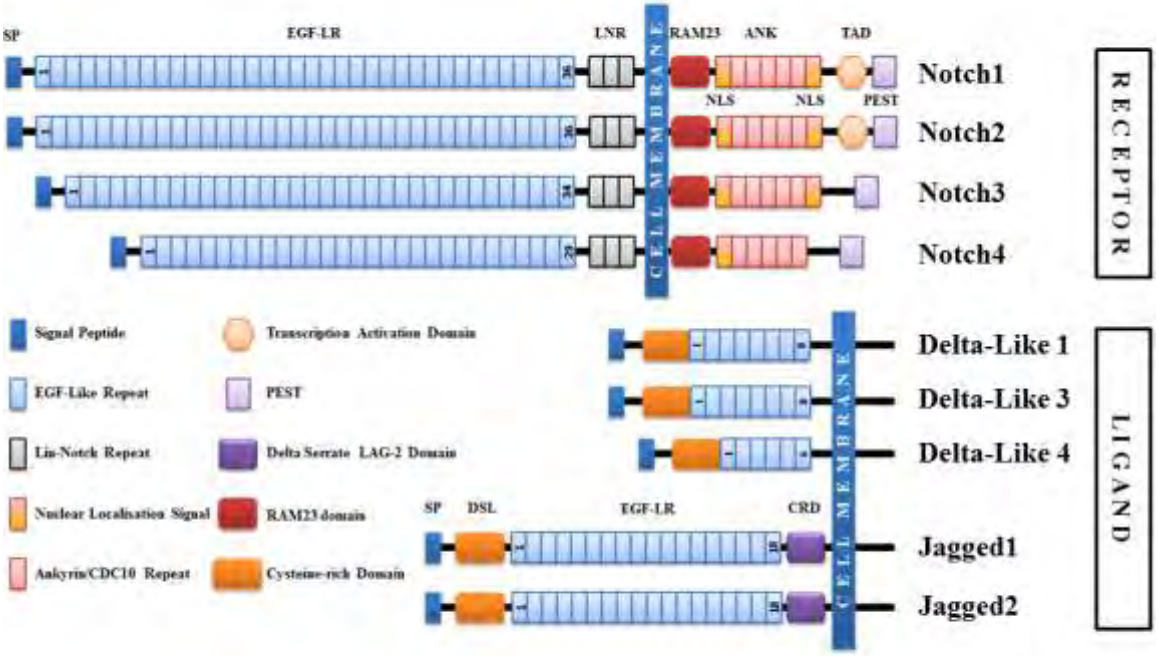

Figure 2.5 Protein structures of Notch receptors and their ligands. Diagrammatic representation of Notch receptors and their Ligands in mammals. All of them are type I transmembrane proteins. All of Notch receptor exists as heterodimer of covalently linked extracellular domain and the intracellular domain. The Notch extracellular region contains epidermal growth-factor-like repeats (EGFR) and three LIN Notch (LNR) repeats. The intracellular domain contains the RAM23 domain and seven Ankyrin/CDC10 repeats (ANK), necessary for protein- protein interactions. In addition, Notch receptors 1-3 contain two nuclear localization signals (NLS) compared to one NLS in Notch4. Both of Notch1 and Notch2 that found the

transcriptional activation domain (TAD) Whereas Notch3 and Notch4 not founded TAD domain. All four Notch receptors contain a C-terminal Pro-Glu-Ser-Thr (PEST) sequence for degradation. In Mammals have five ligands members, Delta-like 1, 3, 4 and Jagged1 and Jagged2.The number of EGF-like repeats and a cysteine rich N-terminal DSL domain are specially a property. The DSL domain is a conserved motif found in all DSL ligands and required for their interaction with Notch receptors. The cysteine rich domain (CRD) is found only in Jagged1, and Jagged2 [38, 39].

2.2.2 Target genes of Notch signaling

Hes-1 (hairy and enhancer of split homolog 1) is a transcription co-repressor (Co-R), both murine and human homolog to *Drosophila*. Hes-1 is one of proteins in hairy-related protein family of the basic helix–loop–helix (bHLH). Nuclear protein Hes1 interacts with TLE/Groucho family of corespressor to repress of the transcription of target genes. [8]. Moreover, Hes1 subunit can interact with another member of subfamily forming a heterodimer such as Hes1-Hey1. These interaction synergized to increase DNA-binding and repression activity [40]. Hes1 is a well known canonical Notch target gene and can be used to monitor Notch activation [41]. Recent studies in immune cells reported that when macrophages were activated though TLR4, expression of Hes1 was induced. Furthermore, Hes1 is critical for development of T-lymphocyte at an early stage of T cell development and Hes1 deficiency resulted in thymic hypoplasia [42, 43].

Moreover, other target genes of Notch signaling have been reported such as *Hey1, Hey2, Hes5, Nepro and DeltexI*. Previous study showed *Hes1, Hey1, Hey2, Hes5* and *Nepro* are imvolved in the development of neocortex by inhibiting neuronal

differentiation [44]. In addition, Hey2 functions in parallel with *Hes1* and *Hes5* for mammalian auditory sensory organ development, and interacts genetically with *Hes1* for early embryonic development and survival [45]. Zhang et al. reported that Deltex1 is the target of Notch signaling and functions to inhibit invasion of osteosarcoma. The inhibitory effect of endogenous Deltex1 on Notch signaling is mediated through binding with the intracellular domain of Notch receptor and ubiquitination result in degradation of Notch receptors. However, Hes1 causes transcriptional inhibition of Deltex1 by directly binding to the promoter of *Deltex1* [46].

2.2.3 Gamma secretase complex and GSI

γ-secretase is a multi-subunit proteolytic enzyme complex integrated on the cell membrane. γ-secretase cleaves transmembrane proteins at residues within the transmembrane domain such as in the Notch receptor resulting in generating Notch intracellular domain (NIC) fragment. Substrate recognition occurs via nicastrin ectodomain binding upon the N-terminus of the target and migrate to the active site for proteolytic cleaved process. Similar to the proteolysis cleave process of NIC by γ-secretase, amyloid precursor protein (APP) can be cleaved using the same process. Once the product Aβ-peptide is released, with contributes to accumulation of insoluble toxic β-amyloid which is a pause of Alzheimer's disease [47].

The structures of γ-secretase complex is consist of four different proteins that integrate in cell membrane. The four proteins in this complex are presenilin1, nicastin (NCT), Aph-1, and Pen-2. There components are arranged in the active protease complex. N-terminal fragment (NTF) and C-terminal fracment (CTF) that remain

associated to endoproteolytic function of Presinilin1 [48]. Other studies have confirmed a model of γ-secretase are arranged within the active protease complex by NTF of presenilin1 was found to interact with Pen-2, and CTF was found to interact with NCT-Aph-1 [49]. Pesenilin1 (PS1) is a target for various GSI [50]. Many γ-secretase inhibitors have been reported such as GSI-I (Z-LLNle-CHO), GSI-IX (DAPT), and GSI-XII (Z-IL-CHO) [36]. In drug discovery research, gamma secretase inhibitor treatment can block a development of Alzheimer's disease (AD) and breast cancer [51, 52].

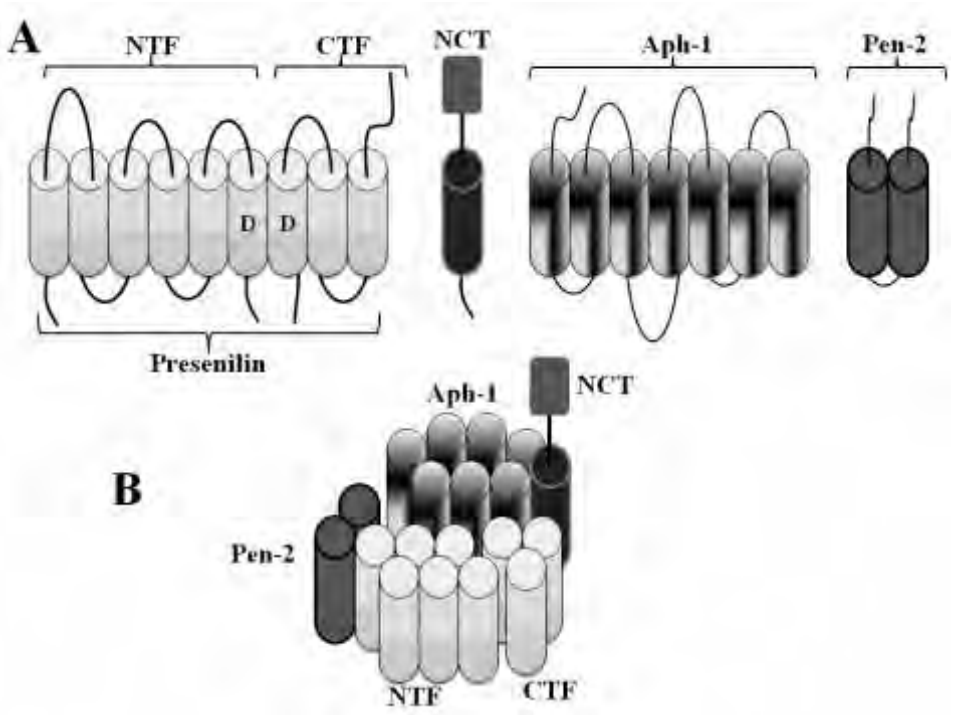

Figure 2.6 Structures of γ-secretase complex. (A) γ-Secretase consisted of four different proteins: presenilin or Pen-1; NTF and CTF domains, nicastrin (NTC), Aph-1 and Pen-2. (B) Pen-2 interact to NTF and CTF interact to NCT-Aph1 are arranged within the active structure complex [33, 49].

2.2.4 Notch signaling and Macrophages

In murine and human macrophages, Notch signaling plays a role in inflammation and responses to intracellular pathogen infection. Previously, GSI treatment suppressed Notch1 upregulation and negatively affected activation of NF-κB. While GSI treatment decreased TNFα and nitric oxide production, it increased expressions of MHC class II on cell surface of macrophages upon activation with LPS/IFNγ or CA-like macrophages condition [53]. Another study reported that GSI treatment increased mRNA expression of *il10* in *M. bovis* BCG-infected macrophages [54]. Moreover, it was found that Notch activation promoted M1 macrophages but suppression of the Notch signaling by targeted deletion of RBPJκ in mice resulted in M2-like polarization macrophage phenotype. The resulting macrophages helped tumor cells differentiation and survival [55]. In 2001, Ohishi *et al.* indicated that Notch ligand Delta like-1 inhibited differentiation of a precursor monocyte to mature macrophage but promoted differentiation to dendritic cells in GM-CSF condition, which correlated with next report by Hoshino and his team showed that Notch ligand Delta like 1 (Dll1), GM-CSF and TGF- β1as inducers of blood monocyte differated into epidermal langerhans cell [56, 57]. Moreover, both murine bone marrow-derived macrophages (BMMϕ) and human monocyte derived macrophages after treatment with a soluble egg antigen (SEA) upregulated mRNA and protein expression of the Notch ligand Jagged-1 and promoted T_H2 responses by regulating antigen presentation [58]. Study in later phase of the TLR response in primary macrophages found that autoamplification of Notch signaling was directly controlled by Jagged1 which resulted in Jagged1-RBP-J-mediated autoamplification [59]. Masuya and colleague reported that the soluble Notch ligands, Jagged1 induced expression of hes1

led to inhibit proliferation of CD34+ macrophage progenitors and macrophage colony formation [60]. Previous study in RAW264.7 after treatment with LPS and/or IFNγ found that high expression of Notch1, Notch2, and Notch4 as well as Notch ligands Jagged1 and Jagged2, but only Notch1 and Jagged2 involved in increasing the p38 MAP kinase, moreover Notch-IC stable transfection in response to IFNγ, leading to higher expression of MHC class II molecules [61].

2.3 MAPK pathway in macrophages

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that are active after cells receiving extracellular stimuli such as mitogens, osmotic stress, heat shock and pro-inflammatory cytokines. They regulate various cellular activities inclouding genes expression, mitosis cell division, differentiation, proliferation, cell survival and program cell death by apoptosis [62]. Various extracellular stimuli triggered cell surface receptors, resulting in kinase cascades, where each enzyme is phosphorylated and, thereby, activate the next enzyme of the member group. There are three major groups of MAPKs, i.e. ERK1/2, JNK and p38 pathway. Each group or family of MAPKs is composed of a set of three conserved kinases which are MAPKK kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues located in activation loop domain (Figure 2.7). Once activated, MAPKs phosphorylate target substates that are MAPK-activated protein kinas [63].

Activation of ERK1/2 pathway, potent activation of ERK1 and ERK2 can be initiated though activation of transmembrane receptors within associated protein tyrosine-kinas (PKT) such as TIM and TAM one of component Fcγ receptor [64, 65]. MEK1 andMEK2 are dual-specific protein kinases that dually phosphorylate ERK. Whereas in JNK pathway, MEK4 and MEK7 specifically phophorylate SAPK/JNK, and MEK3 plus MEK6 dual phosphorylate p38 in the p38 pathway. All of ERK1/2, SAP/JNK and p38 activation influent the efficiency of signal transduction to the nucleus [66].

Previous studies presented evidence supporting that MAPK pathway is linked to inflammation and diseases, especially JNK pathways. SAPK/JNK regulates inflammation though interaction with other target transcription factors such as AP1 and ATF2 this action is involved in the development of obesity and diabetes [67]. In macrophages, the SAPK/JNK signaling is a key regulator of TNFα production in responses to gram negative bacterial lipoplolysacharide (LPS) activated macrophages and inhibition of JNK parthway decreased TNFα production [68, 69] . In addition, in synoviocytes, JNK pathway is important for metalloproteinase expression and regulates bone destruction in rheumatoid arthritis [70]. Obesity and type 2 diabetes are closely related metabolic diseases. Interestingly, JNK is involved in TNFα production in adipocytes, which induced phosphorylation of insulin receptor substrate (IRF) that promoted insulin resistance in obese mice. However, JNK knockout mice showed reduced adiposity and improved insulin sensitivity [71-73].

Previous reported in macrophages treated with immune complex in the presence of TLR activation which induced ERK activation, resulted in phosphorylation of histone H3 at specific regions of *il10* promoter that increases

chromatin accessibility. This reaction resulted in induction of *il10* mRNA expression [74]. In 2010, O'Garra and Saraiva proposed that MAP kinases together with other transcription factors such as ERK, p38, CEBP-β and NF-κB controll or regulate a transcription of *il10* gene in macrophages. They proposed that ERK is important for *IL-10* expression in dendritic cell and T cells subsets as well [75]. In 2002, Hall and Davis reported that inhibition of the p38 pathway in myeloid M1 cell line resulted in increasing JNK and ERK activities, leading to increase IL-10 expression [76]. Previous report in macrophages indicated that both activation through TRL4 and oxidant stress such as H_2O_2 can cause phosphorylation of p38 MAP Kinase [77].

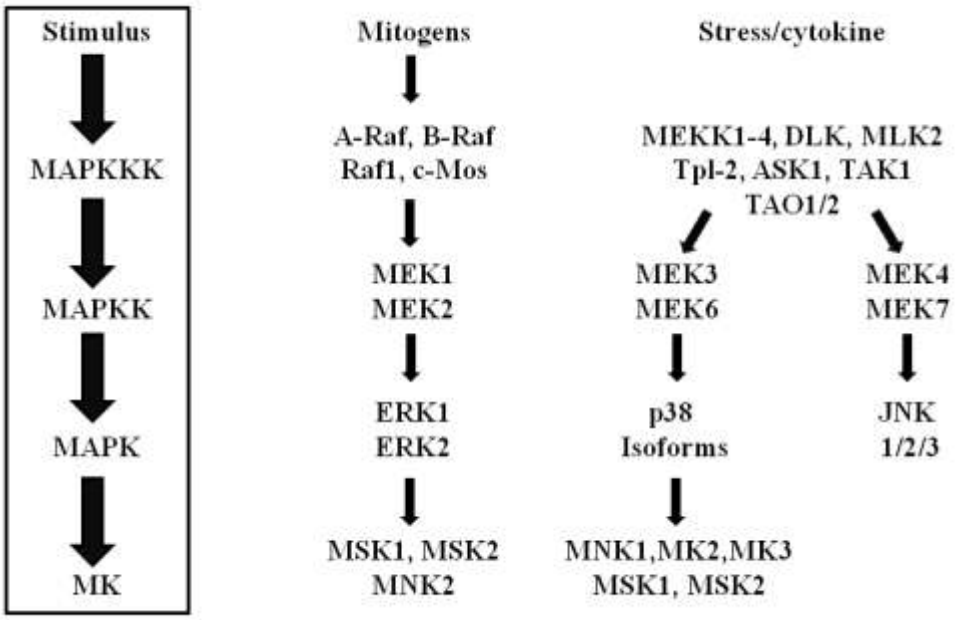

Figure 2.7 Signaling cascades leading to activation of the MAPK pathways. Mitogens stimuli lead to activation of ERK1/2 cascades, while stresses and cytokines lead to activation of p38 and JNK.

CHAPTER III

MATERIALS AND METHODS

3.1 Animals

BALB/c and C57BL/6 mice were purchased from national laboratory animal center, Mahidol University. The procedure for preparing primary macrophages has been described in research by Mosser and team [4]. All procedures involving laboratory animals were conducted according to the guidelines issued by IACUC of Chulalongkorn University. The animal protocols used in this study have been approved by IACUC of Faculty of Science, Chulalongkorn University (Animal Protocol Rev. No. 0923013).

3.2 Bone marrow derived macrophages (BMMΦ)

BMMϕ were prepared from femoral bone marrows of C57BL/6J or BALB/c mice. Bone marrow was flushed from the femurs of 6- to 8-week-old mice. Five $x10^6$ cell of Bone marrow were plated in petri-dishes with DMEM, 10% fetal calf serum (FCS), 5% equine serum (Hyclone, UK), glutamine, Pen/Strep, and 20% conditioned medium from macrophage colony-stimulating factor (M-CSF) secreting L929 cells line (ATCC CCL1). Fresh media condition were added at day 3 and replaced again at days 6. After 8 days, macrophages were stock frozen or used for experiments. Cells were harvested using cold PBS. In culture cell suspension was centrifuged at 1,000 rpm for 5 min and culture supernatant was discarded. Cells were re-suspended in

DMEM complete media and viable cells were counted using trypan blue dye (Hyclone, England). Cells were diluted to appropriate concentrations as indicated and plated in the tissue culture plates for the experiments.

3.3 Macrophages Polarizations

BMMϕ ($3x10^5$ cells) were seeded in 12 well plates and maintained in 1 ml DMEM supplemented with 10% fetal calf serum (FCS), glutamine, Penicillin-G/Streptomycin To generate 3 different types of macrophage, the protocol described by Edwards et al. was followed with some modification [4]. Briefly, CA macrophages were generated by priming BMMΦ with 10ng/ml of rIFNγ (R&D System, USA) overnight and re-stimulating with 100 ng/ml for LPS from Salmonella (Sigma Aldrich, USA) for 4 hr or 6 hr. RNA extraction was keep at 4hr and protein lysate were keep at 6 hr after polarization. For AA macrophages, BMMΦ were treated with rIL-4 (100 ng/ml) for duration similar to CA. For regulatory macrophages, cells were primed with rIFNγ (100 ng/ml) overnight before being subjected to stimulation with LPS (100 ng/ml) in the presence of an immune complex (Appendix A). To confirm the polarization of macrophages, the expression of *il-12* (for CA), *il-10* (for regulatory) and *argI* (for AA) were examined by quantitative RT-PCR.

3.4 Adoptive transfer of Macrophages

Refer to Edward protocol with minor modification BMMϕ from BALB/c mice were primed with IFNγ plus IL-CHO (25μM) as described above, but treated for 6 hr before activated in vitro with LPS for CA Mϕ or activated with LPS plus anti-

OVA IgG-OVA immune complex for regulatory Mϕ. One hour later, macrophages were washed twice with cold PBS, and resuspended with cold PBS. A total of $2x10^6$ macrophages was transferred *i.p.* into mice along with 100μg of hen egg lysozyme (HEL) in the absence of adjuvant. This procedure was repeated day 10 and day 20. Mice were venipuncture 5 days are the last transfer and sera were used for measurement of Ig titer by ELISA.

3.5 Western blot

3.5.1 Protein extraction

BMMΦ ($3x10^5$ cells) were treated as indicated in 3.2 and washed once with ice-cold PBS before rinsing with 200 μl of buffer A (1 mM EGTA, 1 mM DTT, 50 mM Tris-HCL (pH 7.2), 0.14 M KCl, 2.5mM $MgCl_2$). After addition of 50 μl lysis buffer (0.1% Nonidet P-40 in buffer A), supernatants were transferred to microcentrifuge tubes. Sample were gently vortexed for 15 sec and centrifuged for 5 min at 5,000 rpm, and the supernatants as cell lysate samples were stored at -80°C until use.

3.5.2 SDS- polyacryamide gel electrophoresis (SDS-PAGE)

Depending on the experiment, a loading volume containing from $1.125x10^5$ cells of total cell protein extract was boiled in denaturing buffer and loading dye, and separated on 10% polyacrylamide SDS-PAGE. Polyacrylamide gels were prepared following the formulas shown in Appendix A. Heated samples and prestained

molecular weight markers (Fermentas, Canada) were loaded to the gels. The samples were separated at 100 volt for at least 90 min in Western blot 1x running buffer (Appendix A) using Protein III system (BioRad, USA).

3.5.3 Protein transfer to PVDF membrane

After separation process, proteins were transferred to PVDF membranes (Millipore). The separating gel were equilibrated in transfer buffer (Appendix A) for 5 min. Six pieces of Whatman filter paper and PVDF membranes (Millipore) were prepared. PVDF membranes were next soaked in absolute methanol (Merck, Germany) and rinsed with deionized water and immersed in transfer buffer. Gels, PVDF membrane and filter papers were placed in a semi-dry transfer Trans-Blot® SD (BioRad, USA) apparatus and air bubbles were eliminated by gently rolling a glass test tube on the top of stacks of filter paper. The semi-dry transfer was carried out under the following condition; current at 90 or 150 mA for 90 minute for one gel or two gels, respectively.

3.5.4 Antibody probing and signal detection by X-ray film

The PVDF membranes after protein transfer were blocked in blocking solution (Appendix A) twice for 5 min each on Labnet Rocker 25 (Labnet International Inc, USA). After blocking, the PVDF membranes were probed with 1:2,000 dilution of rabbit anti Notch1 (Santa Cruz Biotechnology, USA), 1:1,000 dilution of rabbit anti Cleaved Notch1 at val1744 (Cell Signal Technology, USA) and 1:5,000 dilution of mouse anti β-actin (Chemicon International, USA) which probed with 1° antibody at

4°C overnight. The probed membranes were further incubated on a rocker for 30 min at room temperature. After that, membrane was washed with PBST (Appendix A) for 5 min 2 times and 15 min 2 times. After washing, PBST was discarded, and 1:4,000 dilution of donkey anti rabbit and 1:5,000 of sheep anti mouse which are secondary antibody against mouse or rabbit immunoglobulins conjugated with horse-radish peroxidase (HRP) (Amersham Biosciences, UK) were added. The PVDF membranes were incubated for 1 hr with rocking before washing with PBST as follow described above. After that, add ECL luminal substrate (Appendix A) and one min for rocking. Keep membrane to wrap and fixed in to cassette box, moved in to the dark room and exposed to hypersensitivity X-ray film until appeared of band. Exposed X-ray film was development of black colure band by developer solution (Appendix) and washed with water, keep a film to fixer solution (Appendex) for fix and background clearing. Finally, washed again with water and keep to dried.

3.6 RNA extraction

Total RNA from BMMΦ ($3x10^6$ cells) was extracted using TRIzol reagent following the manufacturer's instruction (Invitrogen, UK). After remove of culture supernatant, 1 ml of TRIzol reagent was added directly to cells and incubated for 5 min at room temperature. The mixed well solution was transfered to 1.5 ml microcentrifuge tube. This process use following addition, 200µl of chloroform (Lab-Scan, Ireland). Tubes were vigorously mixed by hands for 15 second and incubated at room temperature for 3 min. The samples were centrifuged using refrigerated centrifuge (model 1920 Kubota, Japan) at 12,000xg for 15 min at 4°C. Only top phase of colorless aqueous was transferred to new tubes. RNA was precipitated by invert

mixing with 500 µl of isopropanol (Merck, Germany) and incubated at room temperature for 10 min before centrifugation at 12,000xg for 10 min at 4°C. After centrifugation, the RNA pellets were visible at the bottom. The supernatants were rinsed and added 1ml of ice cold 75% Ethanol in 0.01% diethylpyrocarbonate (DEPC) treated water (Appendix A) for washed the RNA pellets. The samples were semi-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 by 0.01% DEPC treated water, and incubated for 10 min at 55°C. RNA samples were stored at –80°C until used.

3.6.1 Quantitation of RNA

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

3.7 cDNA synthesis by reverse transcriptase

Obtained total RNA (0.1-1.0 µg) 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. RNA mixture was relaxed by heated at 65°C for 5 min and placed on ice for 5 min. 1×Reverse transcriptase buffer (Fermentas, Canada), 1 mM dNTP mix (Fermentas, Canada) and 20 U of RNase Inhibitor (Fermentas, Canada) were added in the mixture and followed by incubation at room temperature for 5 min. Reverse transcriptase (Fermentas, Canada) was added to final amount of 200 U per reaction, and the reaction was manipulated on Bioer Life Express (Bioer Technology, China) at 25°C for 10 min, 42°C for 60 min, 70°C for 10 min and 25°C for infinity. The cDNA was stored at -20°C until use.

3.8 Semi-quantitative polymerase chain reaction (qPCR)

cDNA was generated from 0.1-1.0 µg of total RNA using ReverseAid reverse transcriptase (Fermentas). qPCR amplification was performed with 1x Maxima™ SYBR Green/ROX qPCR Master Mix, with 0.3 µM forward and reverse primer, RNase Free water and 2 µl obtained cDNAs according to the manufacturer's protocol (Fermentas, Canada). Realtime-PCR assays were run for 40-45 cycles. The primer sets for Notch signaling follows by Narayana and co-worker [54]. Amplification of the housekeeping gene β-actin (Forward 5'-acc aac tgg gac gac atg gag aa-3', Reverse 5'-gtg gtg gtg aag ctg tag cc-3') was used as a quality and loading control. To confirm the polarization of macrophages, the following were used primers used follows: *il10* (Forward 5'-tca aac aaa gga cca gct gga caa cat act gc-3', Reverse 5'-ctg tct agg tcc tgg agt cca gca gac tca a-3'); *il12p40* (Forward 5'-aac ctc acc tgt gac acg cc-3', Reverse 5'-caa gtc cat gtt tct ttg cac c-3') and *arg1* (Forward 5'-cag aag aat gga aga gtc ag-3', Reverse 5'-caa ata tgc agg gag tca cc-3') [4, 53, 78]. The relative expression levels were calculated from ΔCT and analyzed by $2^{-\Delta\Delta CT}$ equation [79].

3.9 Inhibition of Notch signaling by IL-CHO

To inhibit the Notch signaling, GSI (IL-CHO, a kind gift of Professor Todd Golde, Mayo Clinic, FL, and USA) was used in this study. Cells were pretreated or primed with 25 μM of IL-CHO overnight or negative control DMSO before subjecting to polarization condition as described above. To confirm that Notch signaling was effectively suppressed by GSI treatment, the expression of Notch1 and cleaved Notch1 were monitored by Western blot. The phenotypes of each type of macrophages were monitored as described above (3.3).

3.10 Immunofluorescence staining for MHC class II

Macrophages were polarized with descript above but more long time for activation and used 24 hr. After that, cells were scraped and centrifuged cell suspension at 2000 rpm for 10 minutes. The supernatant was completely removed and the pellet was resuspended in 100 μl 2.4G2 culture supernatant for blocking of Fc receptors on the cell surface. Cells were incubated for 30 minutes at 4°C and washed with 1 ml of FACS staining buffer and centrifugation as above, removed supernatant and added 0.5 ug of primary antibody master mix contained anti-mouse I-A/IE antibody conjugated with biotin (Bio Legend, United Kingdom). The samples were incubated for 30 minutes in the dark at 4°C before washing and centrifugation. In the next step, supernatants were removed and a secondary reagent which is streptavidin PE (phycoerythrin) (Bio Legend, United Kingdom). Finally, resuspened cell pellet in 600 μl of FACS staining buffer for analysis by flow cytometry.

3.11 ELISA for immunoglobulin subclass

After immunizations, mice were bled, and HEL-specific Ig, IgG1, and IgG2a were measured by ELISA. The titer is defined as the final dilution of serum at which the absorbance at 450 nm was equal to blank OD units. Briefly, ELISA plates were coated with 100 µl/well of 10 µg/ml HEL and kept in the refrigerator overnight at 4°C. Unbound HEL antigens were washed three times with 200 µl of washing buffer (1xPBS with Tween). All well were blocked with 200 µl of blocking solution and incubated for 1 hr in humidity chamber at room temperature. In the next step, plates were washed three times with 200 µl of washing buffer, and 100 µl of sera with $10^{1/4}$ serial dilutions (Appendix B) were added. Plates were incubated in humidified chamber at room temperature for 2 hr. After that, the plates were washed three times and 100 µl of secondary HRP conjugated antibody was added and the plates were incubated again for 1 hr. Plates were washed three times, and 100 µl of TMB substrate (Appendix A) were added and incubated in the dark until the blue color was developed. Finally, the reactions were stopped by addition of 100 µl of 1M H_2SO_4 and the absorbance was measured at 450 nm by microplate reader (BioTex Instrument, United States).

3.12 Statistical analysis

Data analysis for statistical significance was done using Student's t-test, except for comparison of expression level between phenotype of macrophages, which was done by multiple comparisons of ANOVA (SPSS). The values of $p<0.05$ were considered statistically significant.

CHAPTER IV

RESULTS

4.1 Polarization of macrophages and expression of Notch1 in each type

RNA was isolated from the unstimulated and 3 types of polarized macrophages at 4 hr after polarization for analysis of markers gene expression by quantitative RT-PCR (qPCR) (Figure 4.1). Expressions of *arg1*, *il12p40* and *il10* were first examined as they are known markers for each type of polarized effector macrophage. AA MΦ selectively upregulated *arg1* but failed to express *il10* and *il12p40* (Figure 4.1; A). In contrast, CA and regulatory MΦ expressed both *il10* and *il12p40*, but CA MΦ showed higher level of *il12p40* expression and lower level of *il10* expression, compared to regulatory MΦ (Figure 4.1; B). Conversely, regulatory MΦ expressed highest level of *il10* mRNA but almost undetectable *il12p40* (Figure 4.1; C). Unstimulated MΦ expressed all markers at undetectable level. These results confirmed the previous report by Edwards and Mosser [4].

To determine the expression protein of Notch1 and the appearance of cleaved Notch1 in polarized MΦ, protein lysates at 6 hr after stimulation were used for analysis by Western blot. As shown in Figure 4.2, Notch1 was highly expressed in CA and regulatory MΦ, but the level detected in regulatory macrophages was higher than those in CA MΦ. Activation of Notch1 was determined by the appearance of cleaved Notch1 which is cleaved at valine 1744 by gamma secretase complex. We not found the appearance of cleaved Notch1 in polarized macrophages (data not show).

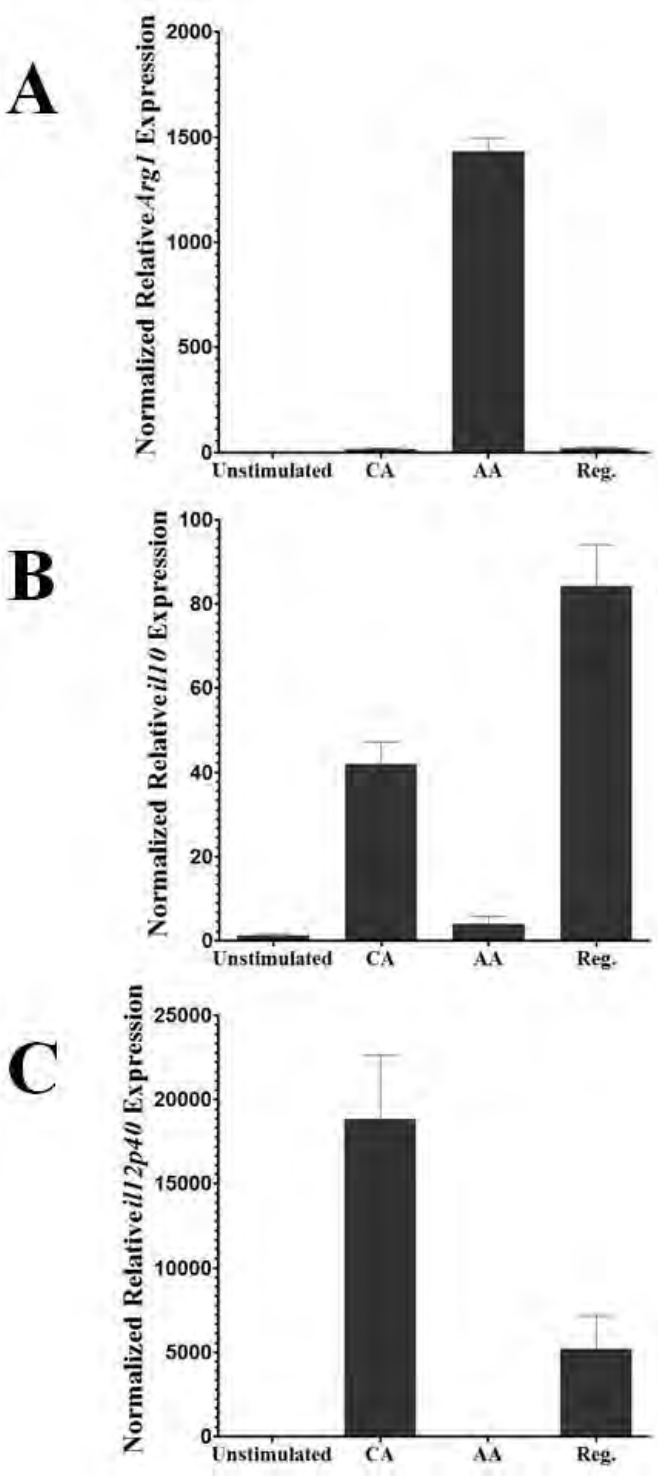

Figure 4.1 Expression of mRNA in polarized MΦ; (A) *Arg1*, (B) *il10* and (C) *il12p40*. Macrophages were polarized as described in materials and methods for 4 hr and total RNA were analyzed for expression of *argI* by qPCR. The results represented 2 independent experiments carried out in triplicate.

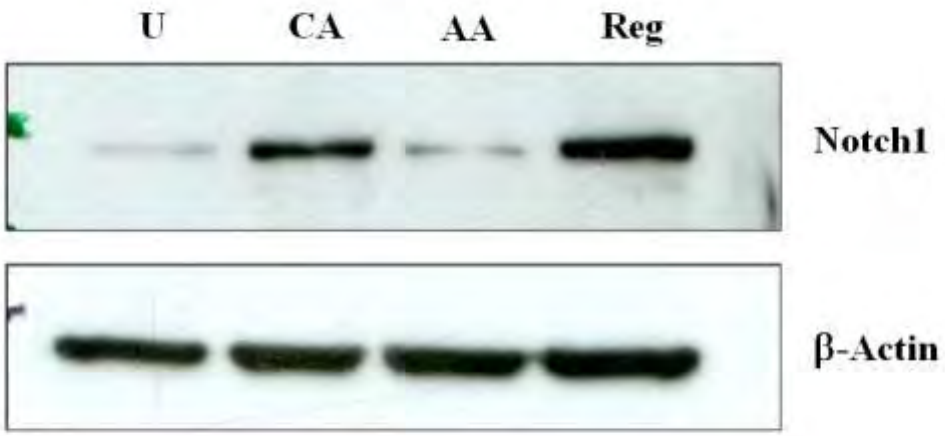

Figure 4.2 Expression of Notch1 in polarized MΦ. Macrophages were polarized as described in materials and methods for 6 hr and total cell lysates were analyzed by Western blot. The results represented 2 independent experiments. The expression of Notch1 is shown in Figure 4.4

4.2 Expression pattern of *Notch receptors* and *ligands* in polarized macrophages

In order to completely understand the involvement of Notch signaling in polarization of MΦ, the mRNA expressions of *Notch receptor* and *ligands* were detected by qPCR. For comparison of relative expression levels, unstimulated macrophages were used as a base line. The expression of *Notch2* detected at 4 hr are at high level in CA and regulatory MΦ while the level of Notch 3 decreased in CA and AA MΦ, compared to unstimulated MΦ (Figure 4.3). Expressions of *Notch4* were below detectable level in all subsets of macrophages (data not show). Similarly, expressions of *Notch ligands* were determined by qPCR. All five Notch ligands were examined, *i.e. dll1, dll3, dll4, jagged1 and jagged2* (Figure 4.4). The expression levels could be summarized as follows: *dll1*: Reg MΦ > CA MΦ > AA MΦ, *dll4*: decreased in CA MΦ, *jagged1*: CA MΦ > Reg MΦ > AA MΦ and *jagged2*: decreased in CA MΦ and Reg MΦ. The expression of *dll3* was not detectable in all subsets (data not show). The summary of the expression profiles of Notch receptors and ligands in polarized macrophages is shown in Table 1. Expression of *dll1* and *jagged1* were found to be the highest in CA and regulatory MΦ, Moreover, we found that expression of *jagged2* was significantly increased in AA.

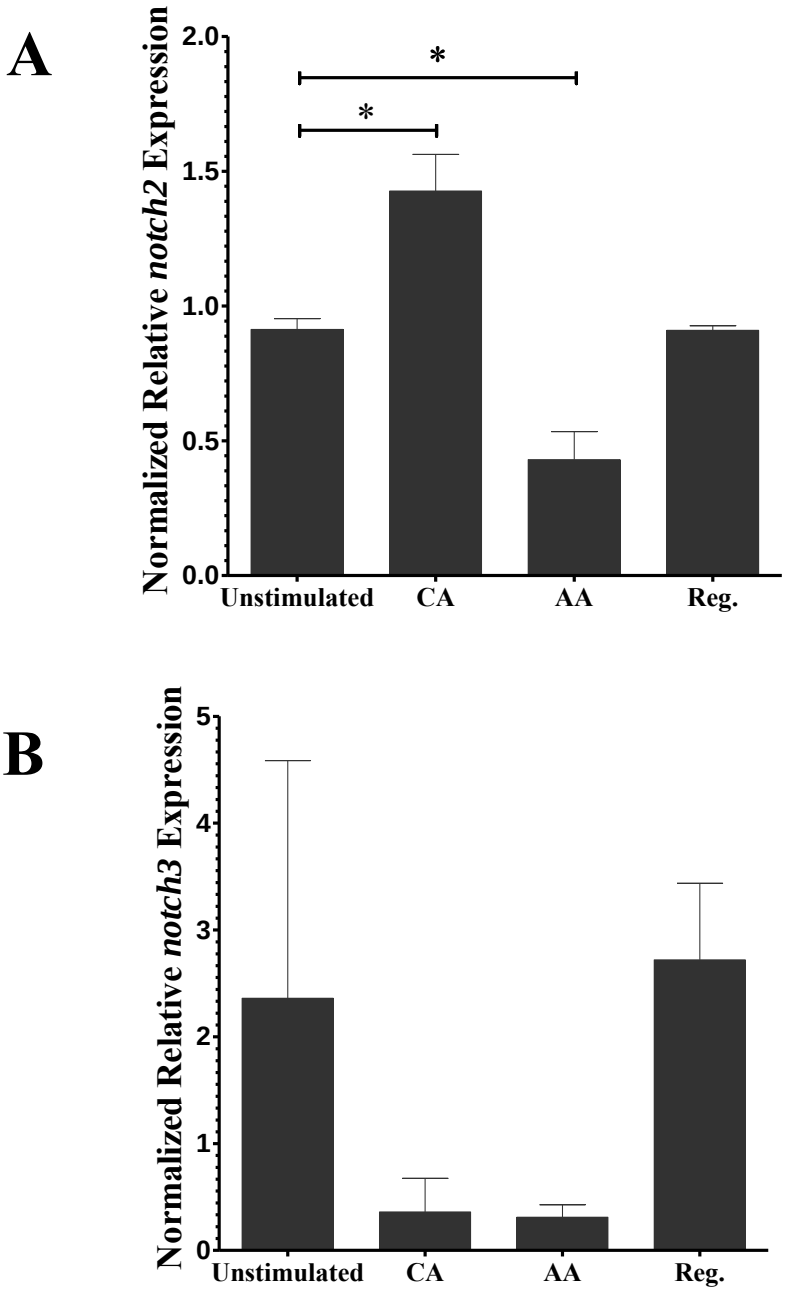

Figure 4.3 Expression of Notch receptors in polarized MΦ. Macrophages were polarized as described in materials and methods for 4 hr and total RNA were analyzed for expression of *Notch2* (A) and *Notch3* (B) by qPCR. The results represented 2 independent experiments carried out in triplicate. (*) indicated statistical significance if $p < 0.05$.

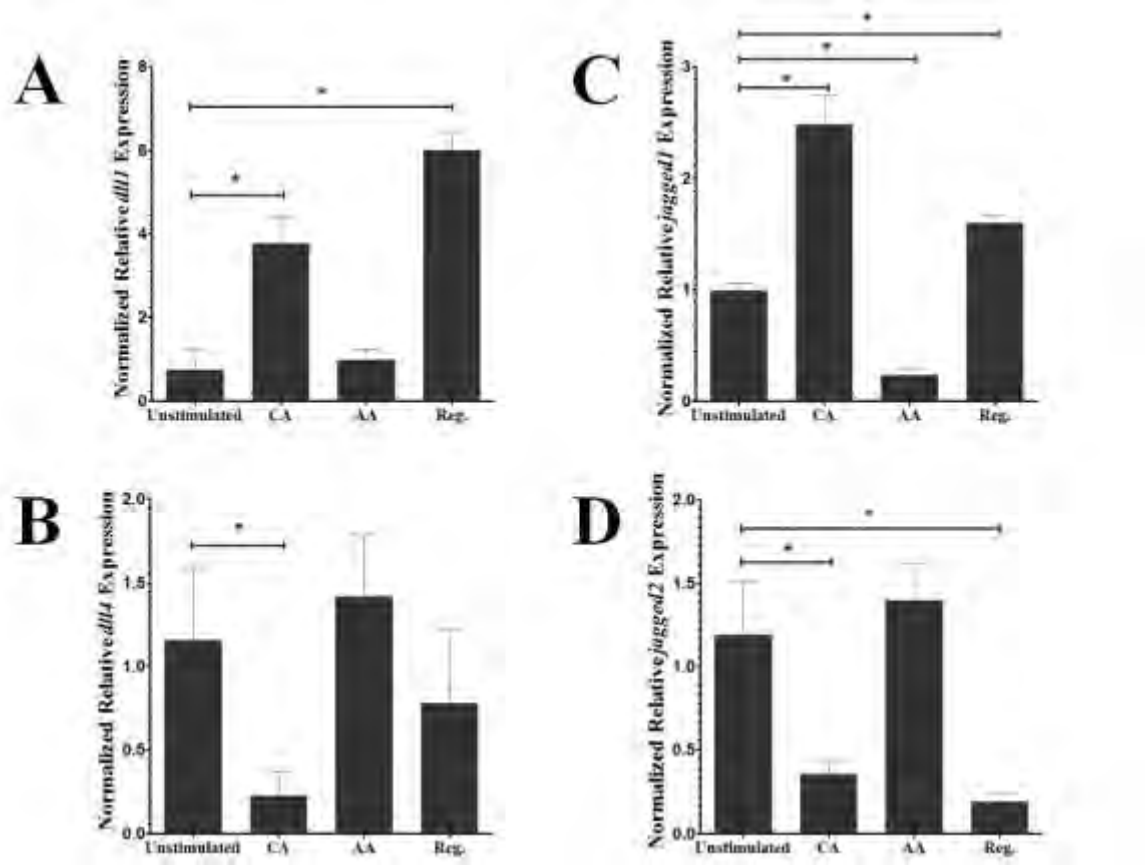

Figure 4.4 Expression of *Notch ligands* mRNA in polarized MΦ. Macrophages were polarized as described in materials and methods for 4 hr and total RNA were analyzed for expression of *dll1* (A), *dll4* (B) and *jagged1* (C) and *jagged2* (D) by qPCR. The results represented 2 independent experiments carried out in triplicate. (*) indicated statistical significance if $p < 0.05$.

notch2	CA ↑ AA ↓
notch3	CA ↓ AA ↓
jagged1	CA ↑ > Reg ↑ > AA ↓
jagged2	CA ↓ Reg ↓
dll1	CA ↑ < Reg ↑
dll4	CA ↑

Table 1. Summary of mRNA expression of *Notch receptors* and their *ligands* in polarized macrophages. (↑) indicated increase in expression and (↓) indicated decrease in expression.

4.3 Expression of MHC Class II in polarized macrophages

The level expressions of MHC class II is important and one of the defining characteristic signature of effectors macrophages in antigen presenting to T cells [80]. We wished to examine the expression of MHC Class II that is antigen presenting potential of each of the three macrophages populations. The results depicted in Figure 4.5 showed that both CA and regulatory MΦ expressed MHC class II at the highest level, while AA MΦ expressed MHC class II at basal level, similar to that found in unstimulated MΦ.

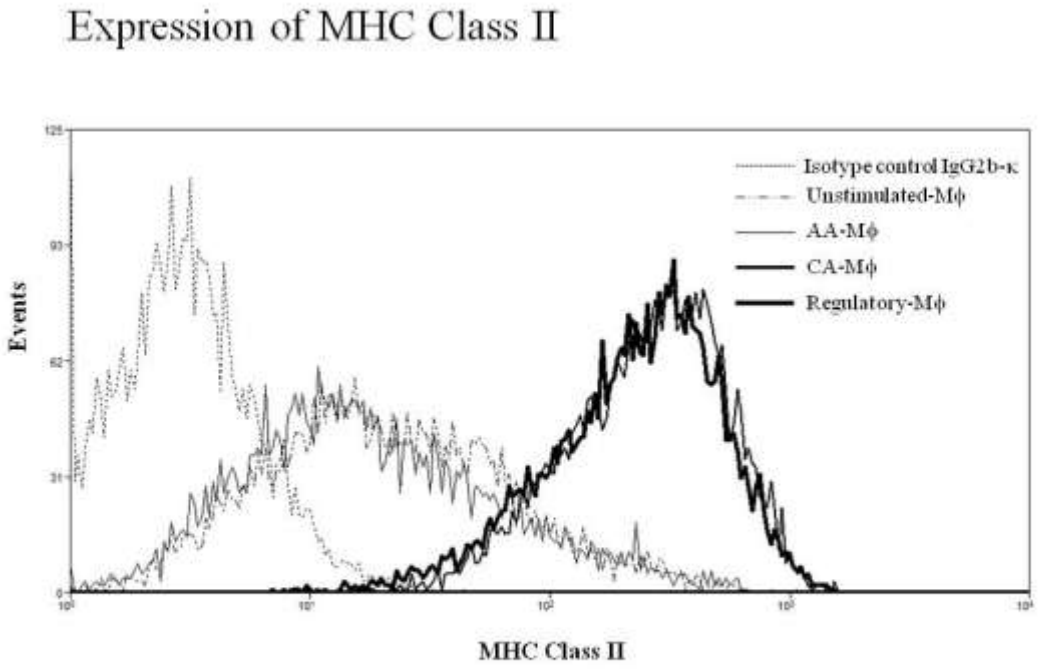

Figure 4.5 Expression of MHC class II in polarized macrophages. Macrophages were polarized as described in materials and methods for 6 hr and cell suface staining for MHC class II was carried out. The representative FACS analysis is shown. Regulatory MΦ (large thick line), CA MΦ (thick line), AA MΦ (line), unstimulated MΦ (dash rough line) and isotype control IgG2b-κ (dash smooth line).

4.4 Expression of *hes1*, the target of Notch signaling

Expression of *hes1*, a well defined target of Notch activation, was examined in 3 types of polarized macrophages. Relative expression of *hes1* expression was determined by qPCR after polarization to detect the activation of Notch signaling. The results of qPCR showed that expression of *hes1* increased in both CA and regulatory MΦ after 6 hr of stimulation and decreased at 24 hr (Figure 4.6; B, C), In contrast, the expression of *hes1* decreased dramatically in AA MΦ, at 6 hr to24 hr (Figure 4.6; A).

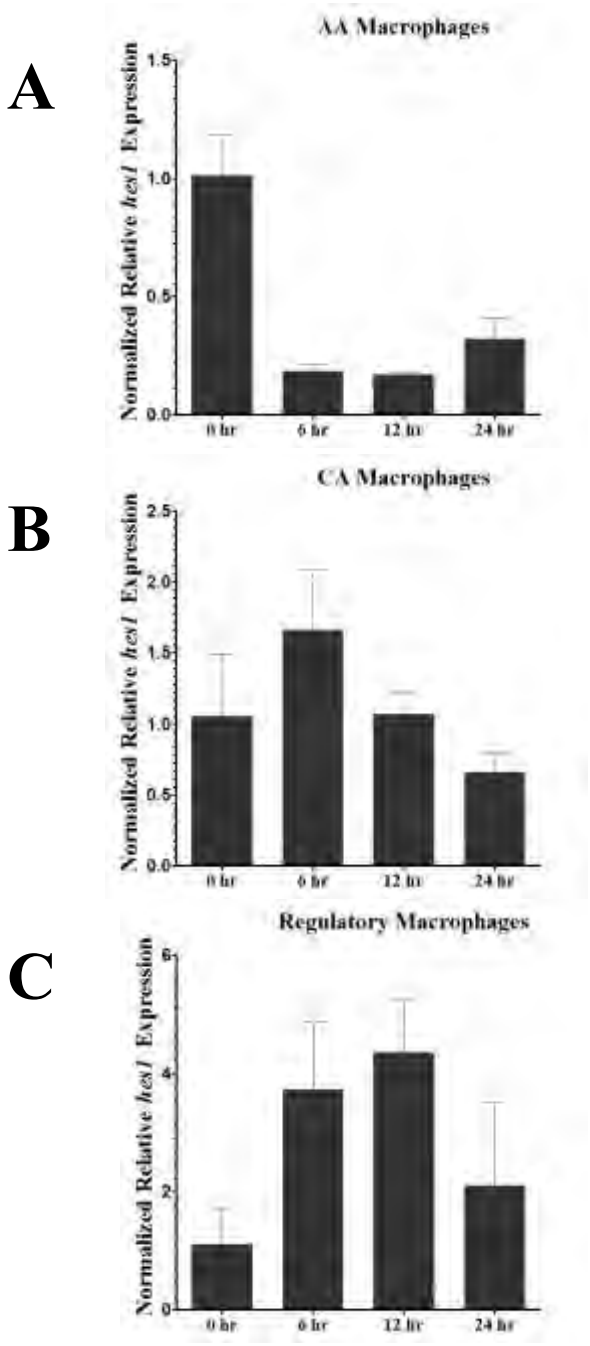

Figure 4.6 Expression of Notch target gene, *hes1*, in polarized MΦ; (A) AA-MΦ, (B) CA-MΦ and (C) Reg-MΦ. Macrophages were polarized as described in materials and methods and total RNA was isolated and analyzed at various times. Relative expression of *hes1* was determined by qPCR. The results represented 2 independent experiments carried out in triplicate.

4.5 Effects of inhibition of Notch signaling on the expression of maker genes in polarized macrophages

We generated three distinct populations of activated macrophages *in vitro* as described as above.GSI inhibits the activity of gamma-secretase and suppresses Notch receptor processing. After treatment with GSI, decreased in both Notch1 and cleaved Notch1 were observed in CA, AA and regulatory macrophages (Figure 4.7 and 4.8). Interesting, the appearance of cleaved Notch1 was detected at 1 hr after polarization both in CA and regulatory macrophages, but it was detected at 3 hr after polarization in AA macrophages. When mRNA expression profiles of marker genes were examined, it was found that GSI treatment had differential effects on marker gene expression (Figure 4.10-4.12). GSI treatment resulted in decreased *il10* mRNA expression in regulatory macrophages*, and* decreased *il12p40* mRNA expression both in CA and regulatory macrophages. However, GSI treatment did not affect *arg1* mRNA level in AA macrophages.

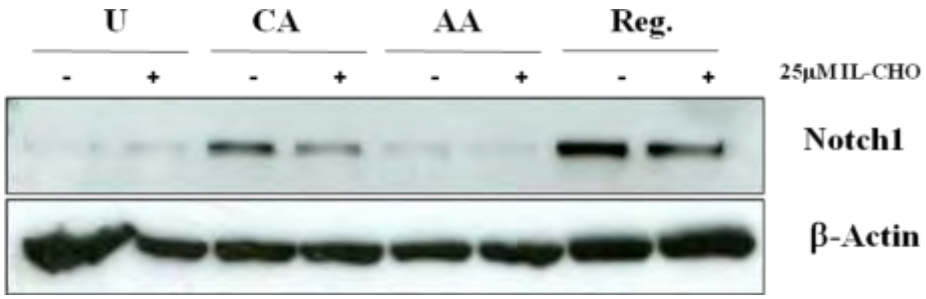

Figure 4.7 Expression of Notch1 in polarized macrophages upon treatment with GSI. Macrophages were pretreated with 25 μM IL-CHO or vehicle control DMSO for overnight as described in materials and methods before subjecting to polarization for 6 hr. Protein lysates from each time point were analyzed for Notch1 and cleaved Notch1(Val1774) by Western blot. β-Actin was used for reference of loading control.

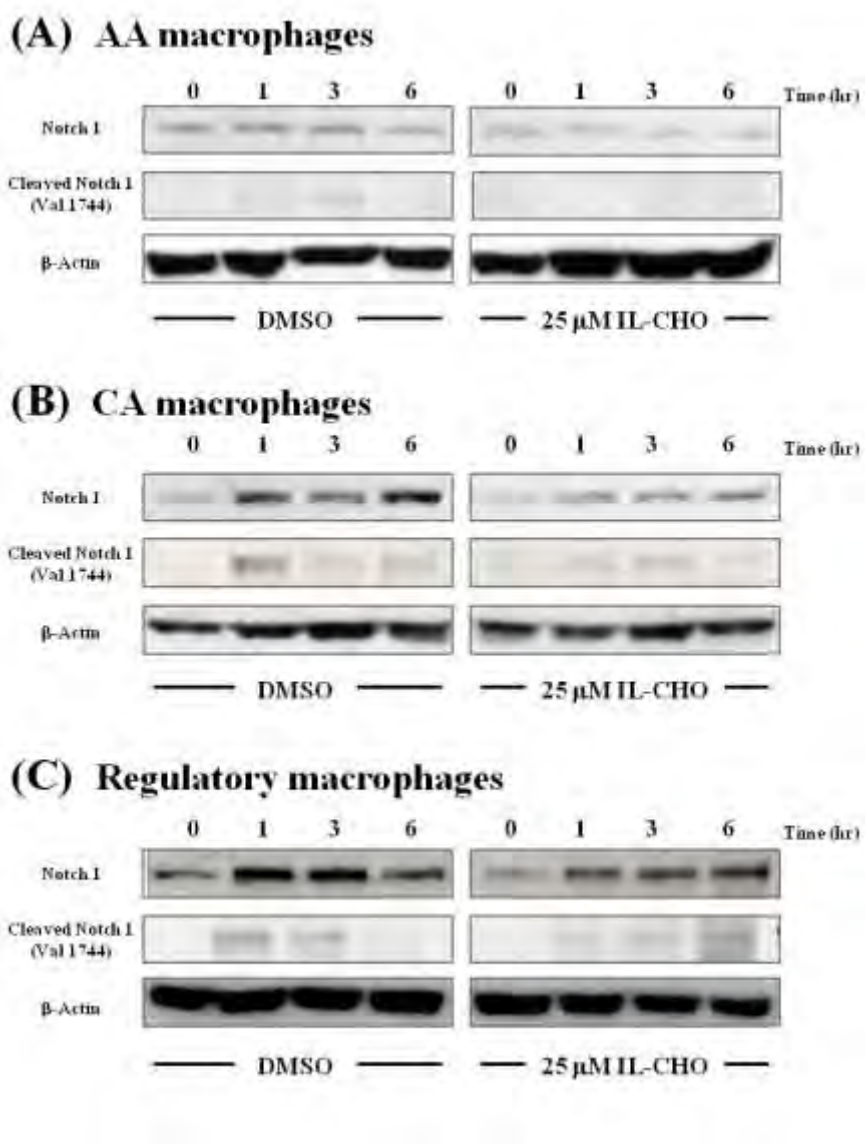

Figure 4.8 Disappearance of cleaved Notch1 (Val 1774) upon GSI treatment in polarized macrophages. Macrophages were pretreated with 25 μM IL-CHO or vehicle control DMSO overnight as described in materials and methods before subjecting to polarization for various time points. Protein lysates from each time point were analyzed for Notch1 and cleaved Notch1 (Val 1774) by Western blot. β-Actin was used for reference as loading control.

4.6 Effect of inhibition of Notch signaling on the expression of MHC class II in polarized macrophages

To explore the effect of GSI treatment on antigen presenting functions of polarized macrophage, the level of MHC class II expression was investigated. After 24 hr treatment with IL-CHO or vehicle control DMSO as described above in materials and methods (3.3 and 3.9), cells were subjected to cell surface staining for MHC class II expression and analyzed by FACS. The results indicated that the mean florescence intensity of MHC Class II in macrophages treated with IL-CHO significantly decreased (Figure 4.9), especially in regulatory MΦ. However, GSI treatment did not affect MHC class II expresses in AA or unstimulated MΦ.

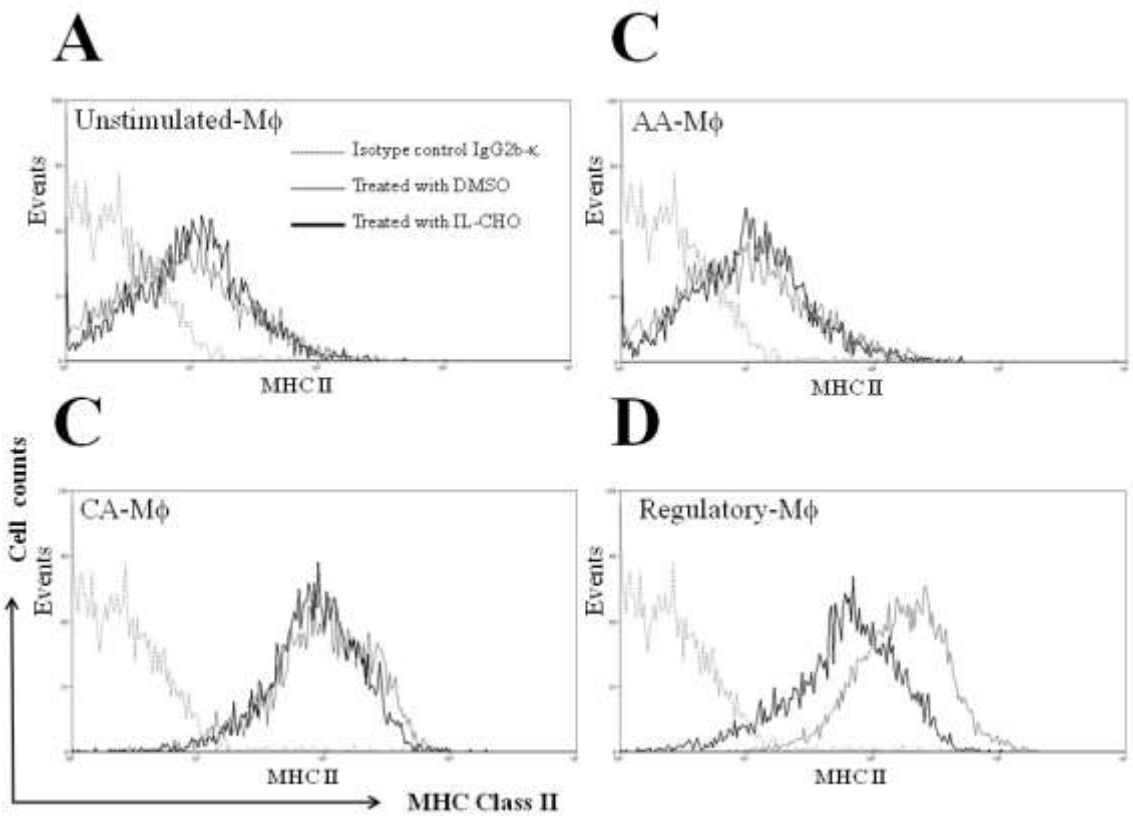

Figure 4.9 Effect of GSI treatment on expression of MHC class II. Macrophages were pretreated with IL-CHO (25 μM) or vehicle control DMSO as described in materials and methods before polarization and analysis of mean fluorescence intensity of MHC Class II by FACS.

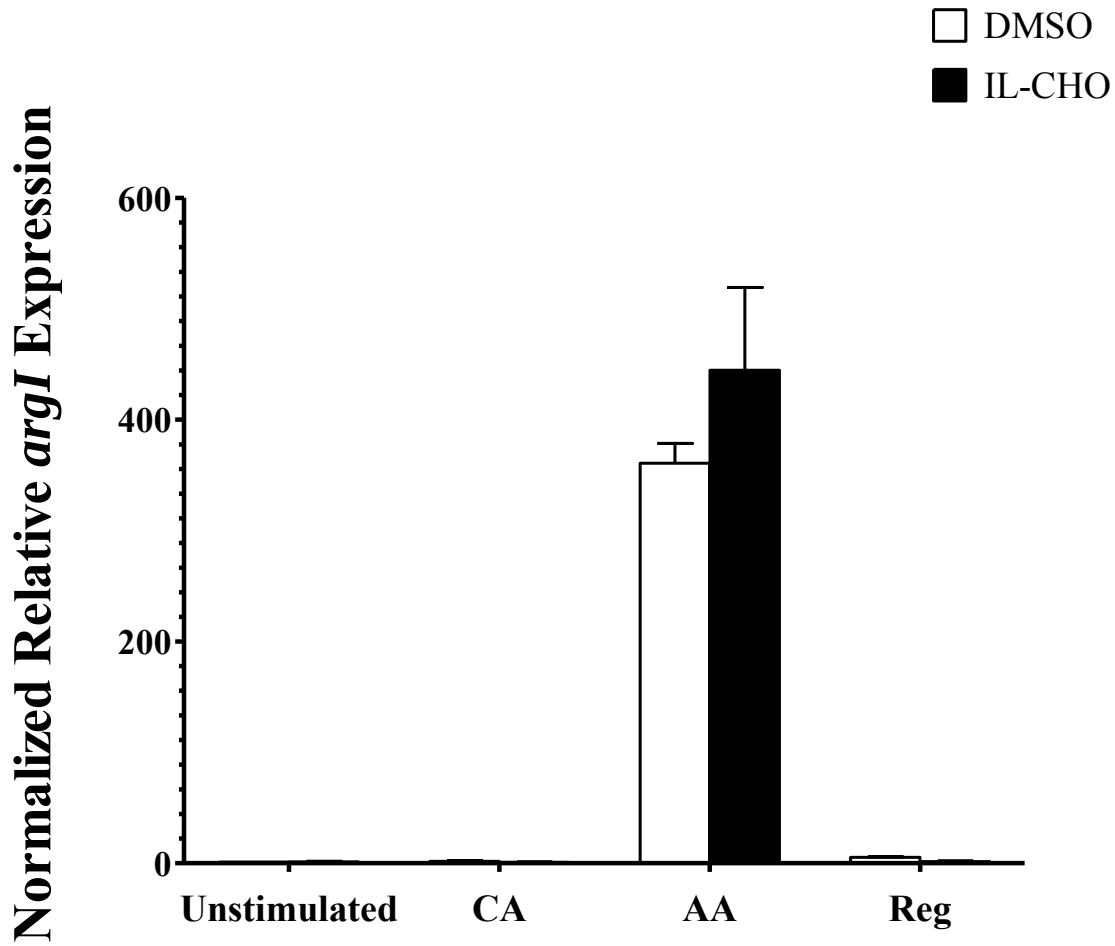

Figure 4.10 Effected of GSI treatment on *argI* expression in polarized marophages.. Macrophages were pretreated with IL-CHO (25 μM) or vehicle control DMSO as described in materials and methods before subjecting to polarization. Quantitative RT-PCR of *argI* was examined. The statistical analysis was carried out using parametric 1-tail of Student's t-Test for comparing the effect of treatments. (*) indicated where statistical significance, if $p < 0.05$.

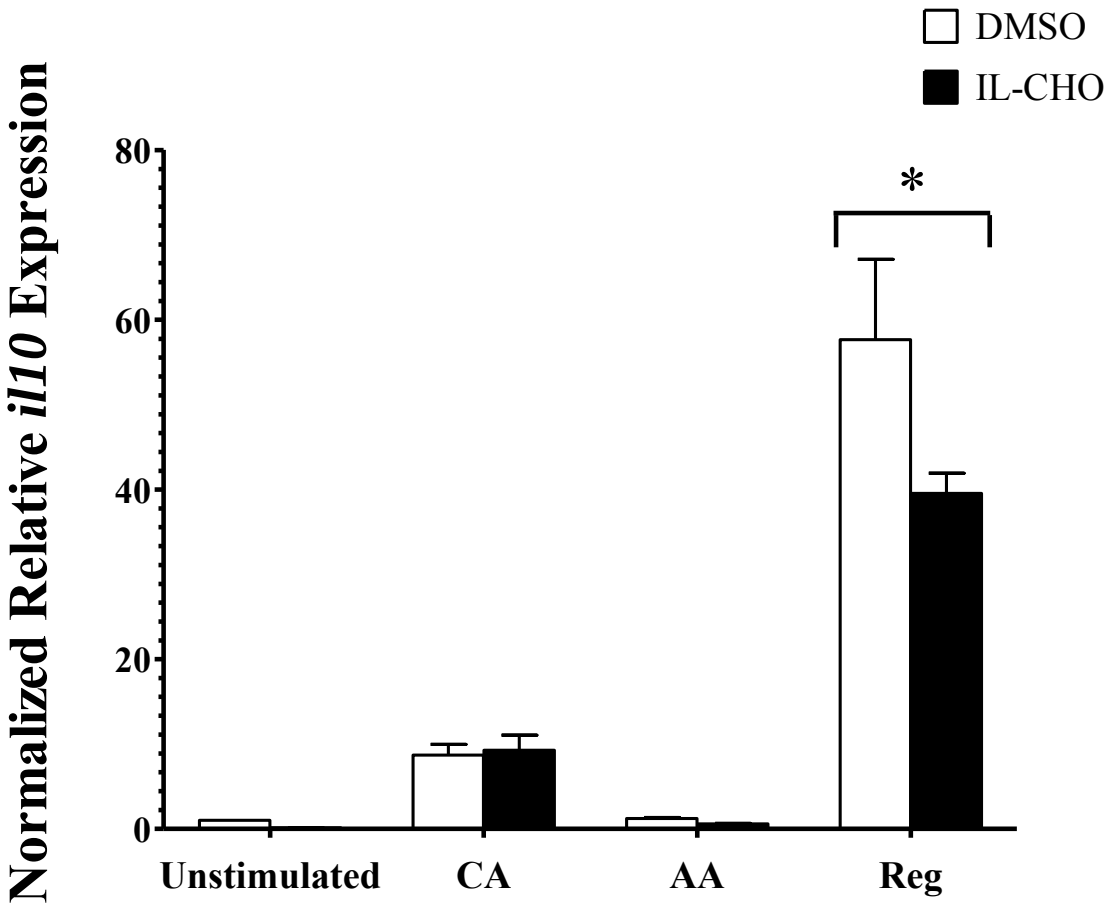

Figure 4.11 Effected of GSI treatment on *il10* expression in polarized macrophages. Macrophages were pretreated with IL-CHO (25 μM) or vehicle control DMSO as described in materials and methods before subjecting to polarization. Quantitative RT-PCR of *il10* was examined. The statistical analysis was carried out using parametric 1-tail of Student's t-test for comparing the effect of treatments. (*) indicated where statistical significance, if $p < 0.05$.

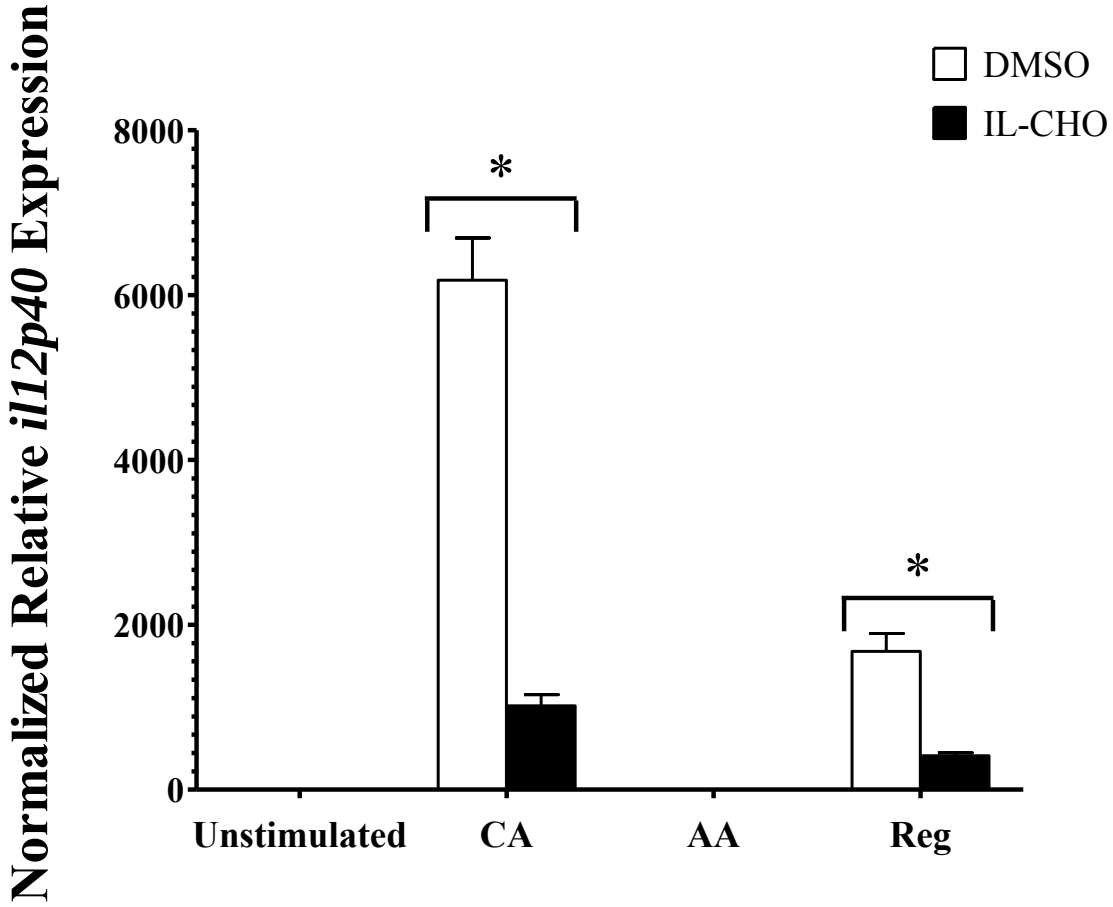

Figure 4.12 Effected of GSI treatment on *il12p40* expression in macrophage polarization. Macrophages were pretreated with IL-CHO (25 μM) or vehicle control DMSO as described in materials and methods before subjecting to polarization. Quantitative RT-PCR of *il12p40* was examined. The statistical analysis was carried out using parametric 1-tail of Student's t-test for comparing the effect of treatments. (*) indicated where statistical significance, if $p < 0.05$.

4.7 Effects of inhibition of Notch signaling on phosphorylation of ERK1/2

The effect of inhibition of Notch signaling on activation of MAPK, specifically focusing on ERK1 and ERK2 in CA and regulatory macrophages (Figure 4.13). GSI treatment resulted in decreased ERK1/2 phosphorylation in CA macrophages but not in regulatory macrophages. On the other hand, GSI treatment did not have any effect on MAPK in AA macrophages. These results help explain the phenomenon of decresing *il10* , when Notch signaling is inhibited in both CA and Reg MΦ. Because activated-ERK is associated with the transcription of *il10* mRNA, if there is a decrease phosphorelated - ERK would result in reduction of levels of *il10* mRNA.

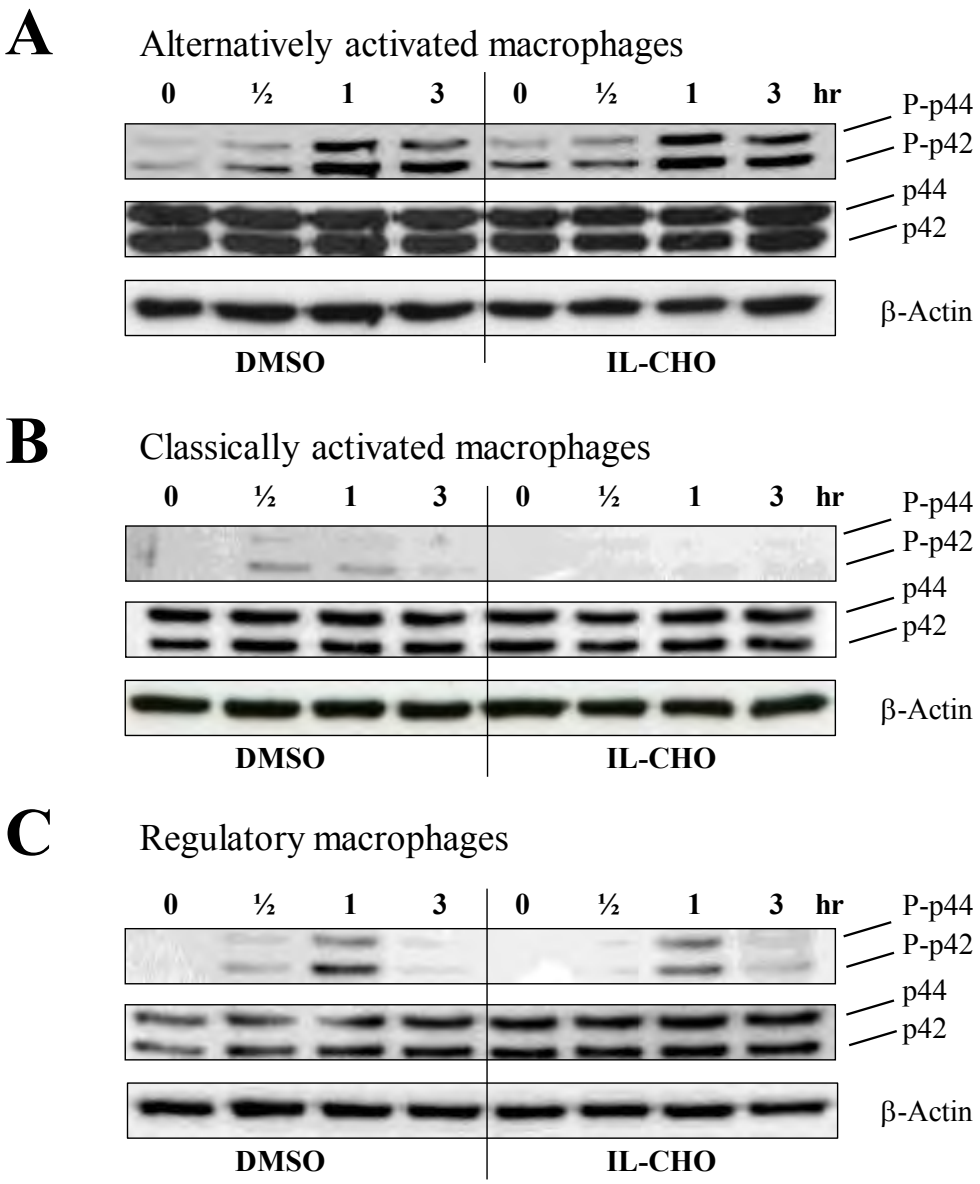

Figure 4.13 Effects of GSI treatment on phosphorylation of MAP kinases. Macrophages were treated with IL-CHO or vehicle control DMSO as described in materials and methods before subjecting to polarization. Protein lysates from each time point were analyzed for both phosphor and total forms of ERK1/2 by Western blot.

4.8 *In vivo* effect of GSI treatment on macrophage functions

To study the effect of GSI treatment on macrophage function *in vivo*, adoptive transfer of activated macrophages into BABL/c mice was conducted as described in materials and methods. The titers of sera antibodies against HEL were measured by ELISA. As shown in Figure 4.14 and 4.15, CA MΦ have stronger ability to induce total IgG production than regulatory macrophages. This is likely the result from the activation of CA MΦ which causes inflammation and a better response to HEL antigens. On the other hand, the ability to induce IgG1 and IgG2a by regulatory macrophages were lower than CA MΦ in immunized mice. Sera titers from mice receiving IL-CHO treated macrophages indicated that suppresion Notch signaling significantly increased production of total HEL specific IgG, IgG1 and IgG2a isotypes in CA MΦ when compared with mice receiving macrophages treated with the control DMSO. In regulatory MΦ, the trend of increasing in Ig production in IL-CHO condition was observed but did not reach statistical significance. Therefore, the productions of both IgG1 and IgG2a increased (Figure 4.14 and 4.15) after inhibition of the Notch signaling.

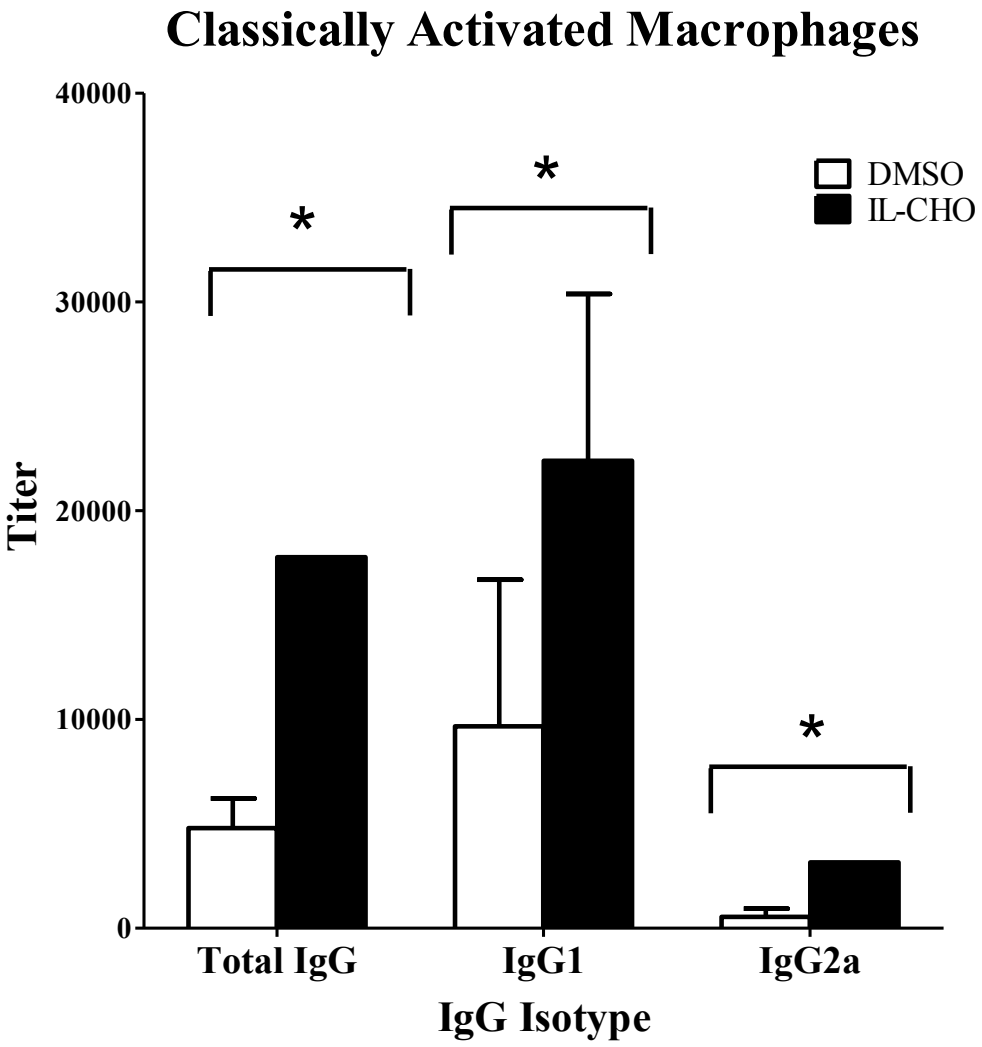

Figure 4.14 Antibody titers following passive CA macrophage transfer. CA MΦ from BALB/c mice were generated as described in materials and methods (3.13). One hour after activation, macrophages were transferred i.p. into mice (n=3) along with HEL antigens in absence of adjuvant. These procedures were repeated at 10-20 days later. Five days after the third immunization, mice were bled, and HEL-specific Ig, IgG1, and IgG2a were measured by ELISA. The titer is defined as the final dilution of sera at the cut off of absorbance at450 nm of 0.1 OD units (*, $p < 0.05$)

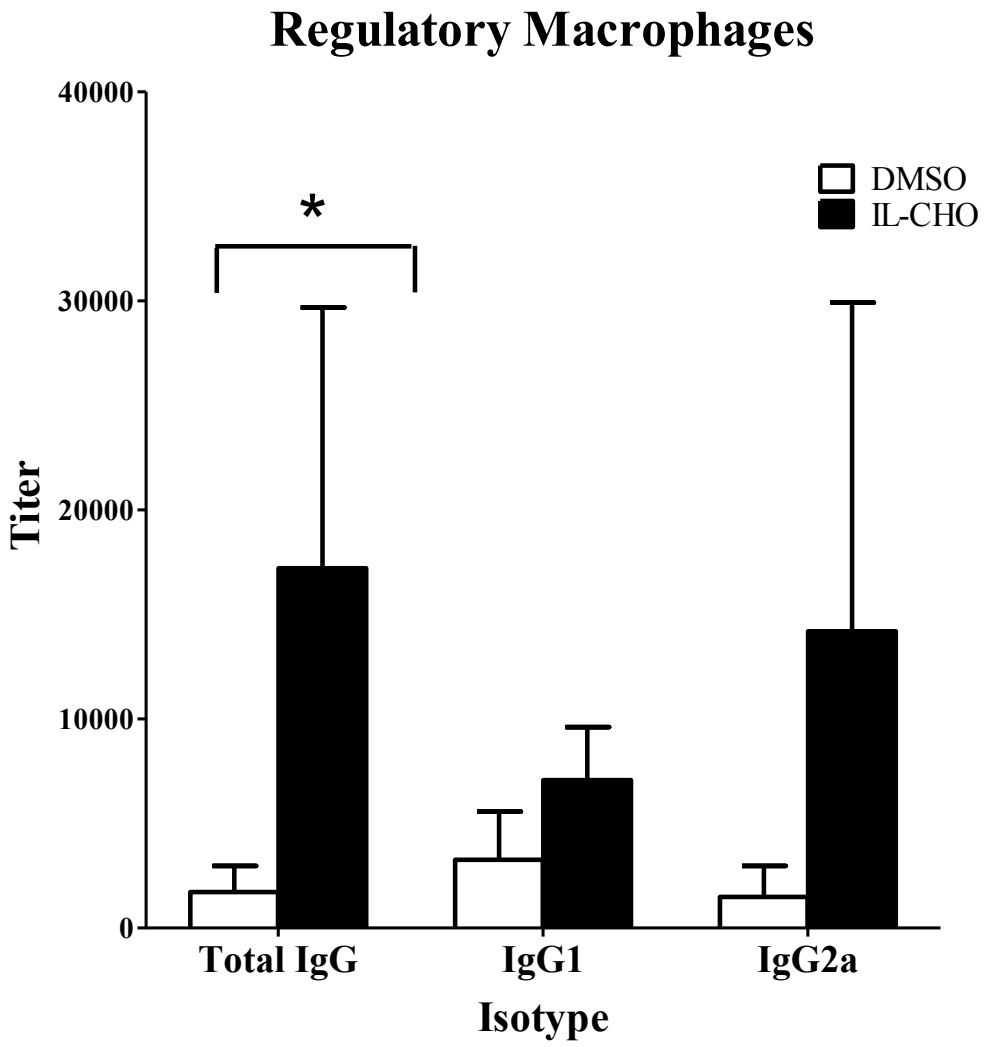

Figure 4.15 Antibody titers following passive regulatory macrophage transfer. Regulatory MΦ from BALB/c mice were generated as described in materials and methods (3.13). One hour after activation, macrophages were transferred i.p. into mice (n=3) along with HEL antigens in absence of adjuvant. These procedures were repeated at 10-20 days later. Five days after the third immunization, mice were bled, and HEL-specific Ig, IgG1, and IgG2a were measured by ELISA. The titer is defined as the final dilution of sera at the cut off of absorbance at450 nm of 0.1 OD units (*, $p < 0.05$)

CHAPTER V

DISCUSSIONS

Macrophages were polarized to three subsets of effector macrophages, and mRNA expression of *arg1, il10* and *il12p40* were used as makers of AA, CA and Reg MΦ, respectively. All markers were detected by qPCR which confirmed previous report by Edwards and Mosser [4]. The level expressions of MHC class II is another characteristics signature of effectors macrophages [4, 81]. The results obtained in this study differed from previous studies which reported that the expression of MHC class II in regulatory MΦ was higher than CA MΦ [4]. The discrepancies may be due to slightly different methods for polarization of macrophages.

During investigation of Notch signaling in macrophages polarization, we founded that Notch1 was highly expressed in CA and regulatory MΦ and cleaved Notch1 (Val 1744) appeared at the beginning of polarization in both CA and regulatory MΦ. The appearance of cleaved Notch1 (Val1744) was slower kinetics in AA MΦ. This result is consistent with previous studies indicating that Notch1 is important for pro-inflammatory processes [82]. Expression of *dll1* and *jagged1* were found to be at the highest in CA and regulatory MΦ, consistent with previous study which showd that in murine and human monocyte-derived macrophages expressed Jagged1 and Dll1 which were induced by TLR4 ligation and regulated by IFNγ [43]. Expression of *dll4* was found to be highest in AA and regulatory MΦ, suggesting that AA-MΦ may utilize dll4 as ligand to activate Notch receptor in other cells. Moreover, we found that expression of *jagged2* was significantly increased in AA-MΦ.

Expression of *hes1*, a well defined target of Notch activation increaseed after stimulation in CA and regulatory MΦ but not AA-MΦ, which similar to previous studies that show hes1 was detected after stimulation via TLR4 [53]. In AA macrophgaes,expression of *hes1* decreased after stimulation while the appearance of cleaved Notch1 (Val 1744) was detected. Therefore, hes1 expression may not be solely dependent on the activation of Notch signaling.

We found that GSI treatment has differential effects on marker gene expression (Figure 3B). GSI treatment caused decreased *il10*, *il12p40 and il23p19* (data not show) mRNA expression both in CA and regulatory macrophages, respectively. Moreover, GSI treatment did not affect *arg1* mRNA level in AA macrophages. Previous reported by Wang *et al*.[55], show that GSI treatment of macrophages affected level of IL-12 expression in M1 or classically activated macrophages, which are consistent with these results. Therefore, we concluded that Notch signaling may function in controlling polarization of CA-like effector macrophages. The effect of GSI treatment could be seen in the function as antigen presenting cells in macrophages, in CA and regulatory MΦ, but not AA-MΦ (Figure 4.14-4.15). These results are in agreement with previous report, which showed that suppression of Notch signaling affected MHC class II expression in M1 macrophages [55]. More importantly, we demonstrated that Notch signaling play a critical role in antigen presentation in regulatory macrophages. Futuremore, we found that inhibition of Notch signaling affects phosphorylation of MAP kinase pathway. The level of phospho ERK1/2 decreased in CA but not in other types. This effect of GSI treatement on ERK supported previous reports that demonstrated that ERK1/2 decreased after GSI treatment in PIM2 inducing Notch activation though TLR2 [83]. Moreover, GSI treatment resulted in increased SAPK/JNK in all macrophages

subtypes (data not show). This result is consistent with previous report which found that γ-secretase component presinilin1 negatively regulates phosphorylation of SAPK/JNK [84]. We suggest that, when suppressed Notch signaling by GSI, it might reduce some of MAP kinase phosphatase such as MKP1,-3,-5 and MKP7 which are responsible for dephosphorylation at Thr183-Tyr185 position on the loop motive of SAPK/JNK [85]. Taken together, the model of the involvement of Notch signaling in polarization of macrophages is proposed in Figure 5.1.

For investigation into the effect of GSI treatment on macrophage function *in vivo*, adoptive transfer of activated macrophages into mice were carried out. We demonstrated that CA MΦ have the ability to induce total IgG production than regulatory macrophages. These results were inconsistent with previous studies which found that, the regulatory macrophages which induced with LPS plus E-IgG induced stronger T_H2 type immune responses by promoting immunoglobulin production more than CA MΦ *in vivo* [86]. This discrepancy may be due to different immune complex used and/or immunization strategy. In our case, because of high IL-10 secreted by regulatory macrophages, this cytokine may suppress immune responses [87], and decreased response to HEL. In this experiment, αOVA-OVA were used as immune complex to generate regulatory macrophages. Taken together, these results imply that Notch signaling may be involved in the process of macrophages polarizations and inhibition of its activation results in incomplete polarization of the phenotype into CA or regulatory MΦ. Therefore, blocking Notch signaling affecting macrophage functions, which affects T cell response *in vivo*.

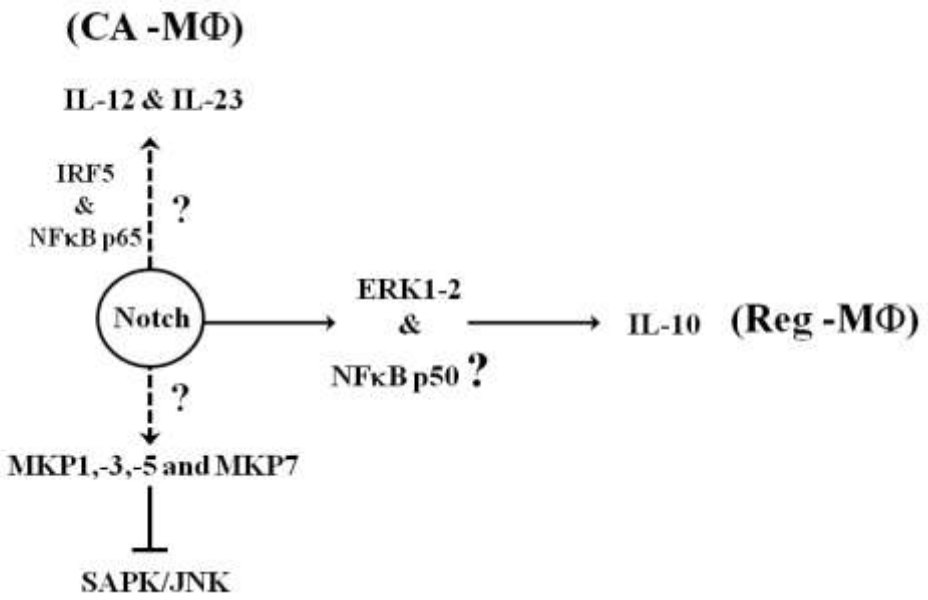

Figure 5.1 Model of Notch signaling regulates macrophages polarization. Notch signaling may regulate the expression of IL-12 and IL-23 though NF-κB p65 plus IRF5 in CA macrophages. In regulatory MΦ, Notch signaling may regulate IL-10 expression though activation of ERK1/2 or plus NF-κB p50. Moreover, Notch signaling may inhibit SAPK/JNK through induction of MAPK phosphatase such as MKP1,-3,-5 and MKP7 caused suppression of SAPK/JNK phosphorylation.

CHAPTER VI

CONCLUSION

1) Notch signaling molecules are differentially expressed in 3 types of polarizaed macrophages, including Notch receptors, Notch ligands, and one of Notch target gene, *hes1*.

2) Notch1/cleaved Notch1 are detected strongly in CA and regulatory macrophages while basal level was detected in AA and unstimulated macrophaegs.

3) GSI treatment affected marker gene expression in CA and regulatory macrophages, but not in AA macrophages.

4) Inhibition of Notch signaling affected MAP kinase pathway macrophages by reducing phosphorylation of ERK1/2 in CA macrophages

5) Notch signaling is essential for complete polarization of CA and regulatory macrophages in vivo as its inhibition resulted in bias of T cell responses *in vivo*.

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APPENDICES

APPENDIX A

SOLUTIONS AND BUFFERS PREPARATIONS

1. Completed DMEM 100 ml

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

2. BMMϕ Freezing media solution A, B

Solution A

Completed DMEM	80%
FBS	20%

Solution B

Completed DMEM	60%
DMSO	20%
FBS	20%

Preparation will be mixed 1:1 v/v of solution A and B

3. FBS inactivation

Commercial FBS which were kept at -20°C was thawed at 4°C for overnight and heat inactivated at 56°C for 30 min.

4. Penicillin and streptomycin

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

5. Buffer A for protein extraction

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

6. Buffer B for protein extraction

Buffer A	990 µl
Nonidet P-40	10 µl

7. 10% SDS-polyacrylamide gel (8 ml)

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

8. 5% stacking gel (2 ml)

Sterile water	1.204 ml
40% Acrylamide and Bis-acrylamide solution	0.25 ml
1 M Tris-HCl pH 6.8	0.504 ml
10% SDS	0.02 ml
10% APS	0.02 ml
TEMED	0.002 ml

9. 2×Laemmli buffer (SDS-dye) (10 ml)

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

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

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

11. Transfer buffer for Western blot

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

12. 1×PBS pH 7.4 (1000 ml)

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

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

13. 1xPBSTween (washing buffer for Western blot and ELISA)

1×PBS	500 ml
Tween20	0.05%

14. 3% Blocking solution for Western blot and ELISA (200 ml)

1xPBSTween	200 ml
Non-fat dry milk	6 g

15. ECL substrate of HRP

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

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

16. Solution A 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

17. Solution B ECL

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

18. Film developer and fixer

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

19. 0.01% DEPC water for RNA (100 ml)

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

20. 75% Ethanol in DEPC (100 ml)

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

21. 0.2M Citric acid (500 ml)

19.69 g of Citric Acid dissolved in add just volume to 500 ml by DW

22. 0.2M TMB buffer (500 ml)

Firstly, 33.25g of Tripotassium Citrate Monohydrate and 19.69 g of Citric acid were dissolved in 350 ml of DW. After that, add just pH near 4 by 0.2M Citric acid and add just volume to 500 ml by DW for final.

23. TMB substrate total 250 µl/ 10 ml TMB buffer

TMB (3,3,5,5-tetramethylbenzidine) 2.5 mg in 250 µl of DMSO and keep at dark.

24. RIPA buffer total (10 ml)

Material	Volume (ml)	Final concentration
1M Tris-HCl pH 7.4	0.5	50 mM
0.5 M NaCl	3	150 mM
20% NP-40	0.5	1% (v/v)
10% (w/v) Sodium Deoxyclolate	0.5	0.5% (v/v)
20% (w/v) SDS	0.05	0.1% (v/v)
Add just volume to 10 ml by DW	Keep at 4°C	
1 tablet of protease inhibitor (Roach)		

25. Immune complex preparation (20 µl)

3.4 µl αOVA (3-5 mg/ml, sigma) : 6.6 µl 1xPBS : 10 µl 1µM OVA

Mixed by pipette and incubated for one hour at room temperature.

26. FACS buffers (3 ml)

1) Permeabilizing buffers 3 ml

1xPBS 2928 µl

10% Saponin in 1xPBS	30 µl
10% NaN_3	12 µl
FBS	30 µl

2) FACS staining buffer (50 ml)

1xPBS	49.4 ml
10% NaN_3	0.1 ml
FBS	0.5 ml

APPENDIX B

EQUATIONS

1. $10^{1/4}$ dilutions calculation for MTT assay and ELISA

Calculations

$100 + x = (X)10^{1/4}$

$100 = 1.778(X) - X$

$100 = 0.778(X)$

$128.5 = X$

100+x = 100+128.5 = 228.5 µl is a volume of 1st dilution.

X is a pipette volume for mixed to next dilution.

2. ΔΔCT equation

Amount of target = $2^{-\Delta\Delta T}$ [79]

3. To convert Dalton to gram for prepare 1µl OVA

Calculations

1 Dalton = 1 g/mole

OVA 43000 Daltal = 43000 g/mole

OVA 1 mg/ml = $\left[\frac{1x10^{-3}}{43000}\right]x1000$ for OVA stock

= $2.32x10^{-5}$ mol/l

= 23.2 μM

If prepared 100 μl of 1 μM OVA

23.2 μM (X) = 1μM (100μl)

X = 4.3 μl

∴ Add 4.3 μl of 1mg/ml OVA stock to 95 μl sterile DW for prepare 1 μM OVA

APPENDIX C

PRIMER SETS AND CONDITIONS FOR REAL-TIME PCR

Table of Primer sets and conditions for real-time PCR that follow by Narayana et al. (2008).

Primer		Sequence	Step	Temp (°C)	Time (sec)	Hold cycle	Product Size (bp)
mNotch1	F	**5-agaatggcatggtgcccag-3**	hot start	95	600	45	126
			heat	94	60		
	R	**5-tggtggagaggctgctgtgtag-3**	annealing	60	30		
			extension	72	60		
mNotch2	F	**5-gatggaggtgactgttccctca-3**	hot start	95	600	45	156
			heat	94	60		
	R	**5-cgtcttgctattcctctggcac-3**	annealing	60	30		
			extension	72	60		
mNotch3	F	**5-gatttcccatacccacttcgg-3**	hot start	95	600	45	190
			heat	95	30		
	R	**5-tgtgtaatgcaaaaccctcagg-3**	annealing	60	30		
			extension	72	60		
mNotch4	F	**5-gttgaagaattgatcgcagcc-3**	hot start	95	600	45	219
			heat	95	30		
	R	**5-aggaaaagcggcgtctgtt-3**	annealing	60	30		
			extension	72	60		
mDLL1	F	**5-ggacctcagtgagaggcatatgg-3**	hot start	95	600	45	203
			heat	95	30		
	R	**5-ggcaattggctaggttgttcatg-3**	annealing	60	30		
			extension	72	30		
mDLL3	F	**5-agttgcacttctcctaccgcg-3**	hot start	95	600	45	209
			heat	94	60		
	R	**5-acggcattcatcaggctcttc-3**	annealing	60	30		
			extension	72	60		
mDLL4	F	**5-gtgaactgcacatcagcgattg-3**	hot start	95	600	45	212
			heat	94	60		
	R	**5-gttgcagacgaagttgtttggg-3**	annealing	60	30		
			extension	72	60		
mJagged1	F	**5-agaagtcagagttcagaggcgtcc-3**	hot start	95	600	45	113
			heat	95	30		
	R	**5-agtagaaggctgtcaccaagcaac-3**	annealing	60	30		
			extension	72	30		

mJagged2	F	**5-tgctgtggaggtggctatgtct-3**	hot start	95	600	45	152
			heat	95	30		
	R	**5-tgtttccaccttgacctcggt-3**	annealing	60	30		
			extension	72	30		
mHes1	F	**5-gagaggctgccaaggtttttg-3**	hot start	95	600	45	168
			heat	94	60		
	R	**5-cactggaaggtgacactgcg-3**	annealing	60	30		
			extension	72	60		

∴ Hot start follows recommendation of manufactories.

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