รายงานวิจัยฉบับสมบูรณ์

โครงการ แมโครฝาจที่ผลิต IL-10: สัญญาณควบคุม ความยืดหยุ่นและศักยภาพในการประยุกต์ใช้บำบัด

IL-10 Producing Macrophages: Regulatory Signaling, Plasticity and Their Potential Therapeutic Application

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ชื่อโครงการ

ภาษาไทย	แมโครฝาจที่ผลิต IL-10: สัญญาณควบคุม ความยืดหยุ่นและศักยภาพในการประยุกต์ใช้บำบัด
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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
จุฬาลงกรณ์มหาวิทยาลัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ได้สนับสนุนโครงการวิจัยนี้มาโดยตลอด และยังให้โอกาสผู้วิจัยไปร่วมเสนอผลงานวิจัยในการประชุมประจำปีของ สกว. ผู้วิจัยขอขอบคุณจุฬาลงกรณ์มหาวิทยาลัยและคณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่ได้สนับสนุนโครงการวิจัยนี้ภายใต้ทุนเสริมรากฐานของกองทุนรัชดาภิเษกสมโภช

ผู้วิจัยขอขอบคุณ Professor Barbara A. Osborne (University of Massachusetts Amherst, USA) ที่ได้ให้ความอนุเคราะห์ด้านสัตว์ทดลอง แอนติบอดี พลาสมิดและเคมีภัณฑ์ที่จำเป็นต่าง ๆ ในการทำการวิจัยนี้ ตลอดจนรับนิสิตเพื่อทำวิจัย ผู้วิจัยขอขอบคุณ ผู้ช่วยศาสตราจารย์ ดร. นพ. อัษฎาศ์ ลีฬหวนิชกุล ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยในความช่วยเหลือเกี่ยวกับการทดลอง Sepsis model

สุดท้ายนี้ ผู้วิจัยขอขอบคุณผู้ร่วมวิจัย นิสิตและผู้ช่วยวิจัยทุกคนในห้องปฏิบัติการภูมิคุ้มกันวิทยาวิทยาระดับโมเลกุลและเซลล์ (ห้องปฏิบัติการ 2015) ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย (นางสาวนวลพรรณ แสงเพชร นางสาวพัชราวดี บุตรทา ดร. พรรัตน์ คงคาวิฑูร ดร. สุพัตรา ชวลิตพงษ์) สำหรับความช่วยเหลือในการทดลองต่าง ๆ ซึ่งทำให้งานวิจัยนี้สำเร็จบรรลุตามวัตถุประสงค์ไปได้ด้วยดี

คณะผู้วิจัย

สารบัญ

หน้าที่

บทคัดย่อ

การกระตุ้นแมโครฝาจมีการควบคุมโดยโมเลกุลสัญญาณปลายน้ำของ pattern recognition receptor (PRRs) เช่น TLR และ RLR ทรานสคริปชันเฟคเตอร์และการควบคุมเชิงอิพิเจเนติก ในงานวิจัยนี้ ผู้วิจัยศึกษาแมโครฝาจที่ถูกกระตุ้นกลุ่มหนึ่ง ซึ่งมีการกระตุ้นโดยไลโปพอลิแซ็กคาไรด์ (LPS) ภายใต้การมีสารประกอบอิมมูน (immune complex; IC) ที่เรียกว่า M(IC) แมโครฝาจที่กระตุ้นโดย LPS (M(LPS)) ผลิตไซโตไคน์กระตุ้นการอักเสบในปริมาณสูง เช่น IL-12 และผลิตไซโตไคน์ต้านการอับเสบ IL-10 ต่ำ M(IC) ผลิต IL-10 สูงและ IL-12 ต่ำ วัตถุประสงค์ของการวิจัยนี้ คือ การพิสูจน์กลไกระดับโมเลกุลที่เกี่ยวข้องกับฟีโนไทป์ของ M(IC) ความยืดหยุ่นและผลกระทบเชิงรวมของ M(IC) ใน *in vivo* คณะผู้วิจัยได้ศึกษาความเกี่ยวข้องของวิถีสัญญาณ Notch ต่อการกระตุ้นของ M(IC) M(IC) เพิ่มระดับของ Notch1 ที่ถูกตัด ในขณะที่การกระตุ้นเพียง IC ไม่ทำให้เกิดขึ้น การกระตุ้นวิถีสัญญาณ Notch ใน M(IC) ขึ้นกับการกระตุ้นวิถี NF-κB และ MEK/Erk M(IC) ที่เตรียมได้จากแมโครฝาจของหนูเม้าส์ที่ตัดยีน *Rbpj* ซึ่งประมวลรหัสโปรตีนจับดีเอ็นเอที่เป็นศูนย์กลางของสัญญาณ Notch ผลิต IL-10 ลดต่ำลงอย่างมีนัยสำคัญ เมื่อเปรียบเทียบกับ Wild type การลดต่ำลงของปริมาณ IL-10 ที่ผลิตพบได้เช่นกันเมื่อวิถีสัญญาณ Notch ถูกกดโดยสารกดเอนไซม์ gamma-secretase (GSI) ที่ทำหน้าที่ในการตัดโปรตีน Notch โดยพบว่ามีความผิดปกติของการเคลื่อนที่เข้าสู่นิวเคลียสของ NF-κB p50 ใน M(IC) ที่ได้รับ GSI หรือ *Rbpj*$^{-/-}$ M(IC) ซึ่งบ่งชี้ว่ามีการครอสทอล์คระหว่างสัญญาณทั้งสองใน M(IC) การวิเคราะห์เชิงทรานสคริปโตมพบว่าสัญญาณ Notch ควบคุมการถอดรหัสของยีนที่มีหน้าที่เกี่ยวกับวัฏจักรของเซลล์ การกระตุ้นแมโครฝาจ การเคลื่อนที่ของเซลล์เม็ดเลือดขาวและการผลิตไซโตไคน์ใน M(IC) การแบ่งขั้วย้อนกลับจาก M(LPS) ไปเป็น M(IC) จำเป็นต้องมีระยะเวลาพัก 3 วันระหว่าง LPS และ LPS/IC ซึ่งแสดงว่าความยืดหยุ่นของแมโครฝาจถูกควบคุมด้วยกลไกควบคุมที่มีผลระยะยาว คล้ายกับที่พบในภาวะ LPS tolerance เมื่อศึกษาเครื่องหมายฮิสโตนที่มีความสัมพันธ์กับการมีแอกทิวิตีเชิงทรานสคริปชัน คือ ฮิสโตน H3 ที่มีการเติมหมู่ไตรเมทิลที่ไลซีนลำดับที่ 4 (H3K4me3) โดยวิธี chromatin immunoprecipitation (ChIP)-sequencing (ChIP-seq) พบว่า M(IC) และ M(LPS) มีรูปแบบของ H3K4me3 ที่แตกต่างกันอย่างชัดเจน โดยในโปรโมเตอร์และ cis-regulatory element ของยีนที่เป็นเครื่องหมายของ M(IC) มีการเพิ่มสูงขึ้น โดยเฉพาะโปรโมเตอร์ของยีน *Il10* ใน M(IC) มีการเพิ่มระดับของ H3K4me3 สูงกว่าของ M(LPS) ซึ่งบ่งชี้ว่าอย่างน้อย H3K4me3 ทำหน้าที่สำคัญในการควบคุมการแสดงออกของ IL-10 ใน M(IC) เมื่อทำการโอนถ่าย M(IC) เข้าสู่หนูเม้าส์ที่เป็นแบบจำลองภาวะ ภาวะติดเชื้อในกระแสเลือด ก่อนการให้ LPS และวิเคราะห์ผลกระทบเชิงระบบของ M(IC) ใน *in vivo* พบว่ามีการลดลงอย่างมีนัยสำคัญของ IL-12 และ IL-1β ในกลุ่มสัตว์ทดลองที่ได้รับ M(IC) โดยไม่พบการเปลี่ยนแปลงในไซโตไคน์อื่น ๆ ผลทั้งหมดข้างต้นทำให้รู้ถึงกลไกควบคุมที่กำหนดการแสดงออกของ IL-10 ใน M(IC) และบ่งชี้ว่าวิถีสัญญาณ Notch ร่วมกับการดัดแปลงฮีสโตนทำหน้าที่สำคัญในการควบคุมการทำงานและความยืดหยุ่นของ M(IC) ตามลำดับ

คำสำคัญ: แมคโครฟาจ; สารประกอบอิมมูน; วิถีสัญญาณ Notch; ความยืดหยุ่น; อิพิเจเนติกส์; IL-10; ภาวะติดเชื้อในกระแสเลือด

Abstract

Activation of macrophages is regulated by immediate signaling molecules downstream of pattern recognition receptors (PRRs) such as TLRs and RLRs, specific transcription factors and epigenetic regulation. In this study, we investigated one subset of activated macrophages that are stimulated with lipopolysaccharide (LPS), in the presence of immune complex (IC) referred to as M(IC). While LPS-stimulated macrophages (M(LPS)) produce high level of inflammatory cytokines such as IL-12 and low level of anti-inflammatory IL-10, M(IC) produces high IL-10 and low IL-12. The aims of this study are to dissect the molecular mechanisms involved in specific phenotypes of M(IC), the plasticity of M(IC) and the global effect of M(IC) *in vivo*. We investigated the involvement of Notch signaling in activation of M(IC). M(IC) exhibited increased the level of cleaved Notch1, while IC stimulation alone did not. The activation of Notch signaling in M(IC) depended upon NF-κB and MEK/Erk pathway activation. M(IC) from mice the targeted deletion of *Rbpj*, which encodes a DNA-binding protein central to canonical Notch signaling, produced significantly less IL-10 when compared with wild type. A similar impact on IL-10 production was observed when Notch signaling was inhibited with a gamma-secretase inhibitor (GSI) which inhibits Notch receptor cleavage. Defects in NF-κB p50 nuclear localization were observed in GSI-treated M(IC) and in *Rbpj*$^{-/-}$ M(IC), suggesting cross-regulation between the Notch and NF-κB pathways in M(IC). Transcriptomic analysis revealed that Notch signaling regulates the transcription of genes involved in the cell cycle, macrophage activation, leukocyte migration and cytokine production in M(IC). Reversing polarization from M(LPS) to M(IC) required long resting of 3 days between LPS and LPS/IC, suggesting that plasticity of macrophages is regulated by durable regulatory mechanisms, similar to that in LPS tolerance. When one of the active histone marks, trimethylated lysine 4 on histone H3 (H3K4me3), was investigated by chromatin immunoprecipitation (ChIP)-sequencing (ChIP-seq) approach, M(IC) and M(LPS) exhibited clear distinct H3K4me3 global profiles. Promoter and cis-regulatory elements of M(IC) signature genes were found to be highly enriched. In particular, the promoter of *Il10* in M(IC) exhibited higher level of H3K4me3 than that of M(LPS), indicating that at least H3K4me3 plays a role in regulating IL-10 expression in M(IC). Finally, an adoptive transfer of M(IC) in endotoxemia mouse model before LPS administration were performed to examine the systemic impact of M(IC) *in vivo*. The cytokine profiles showed decreased systemic level of IL-12 and IL-1β while no effect was observed with other cytokines. Taken together, these results reveal the regulatory mechanism governing IL-10 expression in M(IC) and suggest that the Notch signaling pathway together with histone modification plays an important role in regulating the functions and plasticity of M(IC), respectively.

Keywords: macrophage; immune complex; Notch signaling; plasticity; epigenetics; IL-10; sepsis

หน้าสรุปโครงการ (Executive Summary)

ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนาประจำปี 2558

ชื่อโครงการ

(ภาษาไทย) แมโครฝาจที่ผลิต IL-10: สัญญาณควบคุม ความยืดหยุ่นและศักยภาพในการประยุกต์ใช้บำบัด

(ภาษาอังกฤษ) IL-10 Producing Macrophages: Regulatory Signaling, Plasticity and Their Potential Therapeutic Application

Activation of macrophages is regulated by immediate signaling molecules downstream of pattern recognition receptors (PRRs) such as TLRs and RLRs, specific transcription factors and epigenetic regulation. In this study, we investigated one subset of activated macrophages that are stimulated with lipopolysaccharide (LPS), in the presence of immune complex (IC) referred to as M(IC). While LPS-stimulated macrophages (M(LPS)) produce high level of inflammatory cytokines such as IL-12 and low level of anti-inflammatory IL-10, M(IC) produces high IL-10 and low IL-12. The aims of this study are to dissect the molecular mechanisms involved in specific phenotypes of M(IC), the plasticity of M(IC) and the global effect of M(IC) *in vivo*. We investigated the involvement of Notch signaling in activation of M(IC). M(IC) exhibited increased the level of cleaved Notch1, while IC stimulation alone did not. The activation of Notch signaling in M(IC) depended upon NF-κB and MEK/Erk pathway activation. M(IC) from mice the targeted deletion of *Rbpj*, which encodes a DNA-binding protein central to canonical Notch signaling, produced significantly less IL-10 when compared with wild type. A similar impact on IL-10 production was observed when Notch signaling was inhibited with a gamma-secretase inhibitor (GSI) which inhibits Notch receptor cleavage. Defects in NF-κB p50 nuclear localization were observed in GSI-treated M(IC) and in *Rbpj*$^{-/-}$ M(IC), suggesting cross-regulation between the Notch and NF-κB pathways in M(IC). Transcriptomic analysis revealed that Notch signaling regulates the transcription of genes involved in the cell cycle, macrophage activation, leukocyte migration and cytokine production in M(IC). Reversing polarization from M(LPS) to M(IC) required long resting of 3 days between LPS and LPS/IC, suggesting that plasticity of macrophages is regulated by durable regulatory mechanisms, similar to that in LPS tolerance. When one of the active histone marks, trimethylated lysine 4 on histone H3 (H3K4me3), was investigated by chromatin immunoprecipitation (ChIP)-sequencing (ChIP-seq) approach, M(IC) and M(LPS) exhibited clear distinct H3K4me3 global profiles. Promoters and cis-regulatory elements of M(IC) signature genes were found to be highly enriched with H3K4me3. In particular, the promoter of *Il10* in M(IC) exhibited higher level of H3K4me3 than that of M(LPS), indicating that at least H3K4me3 plays a role in regulating IL-10 expression in M(IC). Finally, an adoptive transfer of M(IC) in endotoxemia mouse model before LPS administration were performed to examine the systemic impact of M(IC) *in vivo*. The cytokine profiles showed decreased systemic level of IL-12 and IL-1β while no effect was observed with other cytokines. Taken together, these results reveal the regulatory mechanism governing IL-10 expression in M(IC) and suggest that the Notch signaling pathway together with histone modification plays an important role in regulating the functions and plasticity of M(IC), respectively. Our findings provide insight into the molecular mechanisms regulating IL-10 expression in M(IC) and its systemic impact in vivo that may lead to application of M(IC) in therapeutic against inflammatory conditions or autoimmune diseases in the future.

เนื้อหางานวิจัย

Introduction

Macrophages are innate immune cells with highly plastic nature and can frequently change phenotypes based on the microenvironment. At least *in vitro*, macrophages can be divided into several subtypes, based on the stimuli used, the expression profiles of cytokines and cell surface markers, a process termed “macrophage polarization” (1,2). To avoid confusions over different conditions used for macrophage polarization in different labs, recent suggestions by a group of immunologists working on biology of macrophages proposed the nomenclature based on the stimuli used to generate each subtype (3). Originally, a simple pro-inflammatory and anti-inflammatory or alternatively activated macrophages were used. Priming with interferon-gamma (IFNγ) and stimulating with pathogen-derived molecules such as lipopolysaccharide (LPS) induces inflammatory macrophages indicated as M(LPS). In contrast, IL-4/IL-13 treatment of macrophages indicated as M(IL-4) results in macrophages involved in wound healing and resolution of inflammation. M(LPS) are pro-inflammatory in nature and mainly produce pro-inflammatory cytokines such as TNF-α, IL-6, IFN type I, IL-12 and IL-1β. In contrast, M(IL-4) expresses arginase1 as signature marker and plays a role in resolving inflammation and tissue repair and (4,5). In addition to these classical activated macrophages, macrophages with more diverse phenotypes have also been described which do not fit into this oversimplified dichotomy. Each subset of macrophages has unique characteristic markers, some of which are common and some are different between humans and mice. The involvement or significance of each polarized macrophage *in vivo* during homeostasis and diseases are still in its infancy in most cases (6). Intracellular signaling pathways, transcription factors and epigenetics involved in macrophage polarization have been reported but it is still controversial (7).

A unique subset of activated macrophages was reported which is generated by LPS treatment in the presence of the immune complex (M(IC)). This type of macrophages produces high level of IL-10 while decreasing the production of IL-12, compared to M(LPS) (8). Transcriptomic analysis identified this subset of activated macrophages as a distinct subset with unique gene expression patterns (9). IL-10 is a founding member of a structurally related cytokine family which includes IL-19, IL-20 and IL-22. IL-10 is produced by various immune cells, including monocytes, macrophages, dendritic cells and T lymphocytes (10). Because of its central role in regulating immune response, signaling governing its expression has been extensively studied, mainly in the helper T cell type 2 (Th2) and regulatory T cells (10). High level of IL-10 is associated with depressed immune response while dysregulation of IL-10 is reported in autoimmune diseases (11). Therefore, manipulating IL-10 is a valuable tool to control or enhance immune responses.

Attempts have been made in manipulating subsets of activated macrophages to control the pathological conditions in certain diseases (12). Transfer of M(IC) is reported to decrease severity of experimental autoimmune encephalomyelitis (EAE), an autoimmune disease model of multiple sclerosis (13). Although, the potential for therapeutic application of IL-10 producing macrophages is appealing, the highly plastic nature of macrophages may present an obstacle for their use in clinical settings. Therefore, understanding the regulatory mechanism leading to IL-10 production in macrophages and the plasticity of M(IC) and M(LPS) may help in designing a better strategy in applying this subset of macrophages for treatment.

In macrophages, IL-10 is regulated mainly by p38 MAPK and NF-κB pathways (10). These signaling cascades further activate downstream transcription factors to directly regulate transcription of *Il10* mRNA such as Sp1, STAT3, C/EBP and NF-κBp50 (14,15). Although, LPS stimulation alone in M(LPS) can induce IL-10 expression in a late phase via TLR4 signaling pathway, the level produced in this setting is relatively low. In M(IC), signaling downstream of FcγR such as PI3K/Akt pathway together with p38 MAPK and Erk from TLR pathways crosstalk and regulate *Il10* transcription (14).

In addition to the regulation by transcription factors, the chromatin remodeling of *Il10* promoter and its vicinity such as *cis*-regulatory element is also reported. A specific chromatin signature defined as a DNaseI hypersensitivity site in the *Il10* locus not found in T lymphocytes, was identified in macrophages upon stimulation. This study suggested a distinct regulatory mechanism for transcription of *Il10* between macrophages and T lymphocytes exist (16). This specific site locates approximately 4.5

kb upstream of the transcription start site and contains NF-κB binding motif. Furthermore, a dynamic and transient remodeling of *Il10* promoter was reported in M(IC), suggesting a reversible regulation via chromatin remodeling (17). As described above, macrophages are highly plastic in the activated phenotypes. Besides the regulation by transcription factors, regulation of chromatin environment may also play important roles in the plasticity of macrophages (7,18). In fact, the phenotype of M(IL-4) is partially regulated by epigenetic regulation such as histone methylation in the promoter of signature gene *ArgI* (19,20). Detailed analysis of other histone modifications in M(IC) has not been documented.

Previously, our group has reported the involvement of an evolutionarily conserved signaling pathway called Notch signaling in macrophage activation via TLR ligation (21). We also investigated the involvement at the molecular level of Notch signaling in IL-6 and IL-12p40 expression in macrophages upon LPS stimulation (22,23). Crosstalk between Notch signaling and MAPK, NF-κB and IRF pathways were uncovered in activated macrophages (24). Because Notch signaling also regulates IL-10 expression in helper T cells (25,26), it is intriguing to speculate the role Notch signaling plays in IL-10 production in M(IC). Furthermore, Notch signaling, in some instances, can also regulate chromatin modification and may play a critical role in polarization of macrophages (27).

In this study, we propose to study the detailed mechanism regulating IL-10 production in M(IC) in the context of Notch signaling and its cross talk with other signaling pathways. Furthermore, the plasticity of M(IC) opposing to M(LPS) will be investigated at the transcriptional and epigenetic levels. Finally, the application of M(IC) in disease setting of LPS-induced endotoxemia will be tested *in vivo*. Insights gained from this study will not only shed the light on the complex interaction between conserved signaling pathways in innate immune cells for IL-10 production, but may also lead to new approaches in regulating uncontrolled immune responses by manipulating macrophage subsets such as M(IC) in some disease settings.

Materials and Methods

Generation of Bone Marrow Derived Macrophages (BMM)

Female C57BL/6 (Nomura Siam International Co. Ltd., Thailand) (8 weeks old) were sacrificed, and bone marrow was obtained from their femurs. The cells flushed from femur cavities were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), 5% horse serum, HEPES with sodium pyruvate and 20% (v/v) L929-conditioned media for 9 days. Fresh medium was added to the culture at day 4. The cells were harvested at the end of the culture period using cold PBS and were subjected to cell surface staining with anti-F4/80 and CD11c antibodies (BioLegend, CA) to confirm the macrophage phenotype. Cells were maintained in DMEM media (HyClone, UT, USA) supplemented with 10% (v/v) FBS (HyClone), 100 U/ml penicillin (General Drugs House Co. Ltd., Thailand), 0.4 mg/ml streptomycin (M & H Manufacturing Co. Ltd., Thailand), 1% (w/v) sodium pyruvate (HyClone) and 1% (w/v) HEPES (HyClone) at 37 °C and incubated in a humidified 5% (v/v) CO_2 incubator. For *Rbpj* knock out mice, the conditional *Rbpj* KO mice were generated as described previously (28). All procedures involving laboratory animals were approved by the Institutional Animal Care and Use Committees (IACUCs) at and carried out according to the guidelines issued by Chulalongkorn University, the University of Massachusetts at Amherst (Protocol Reviews No. 024/2558 and Protocol Review No. 1323007).

Preparation of the ICs

The ICs were prepared as described previously (29). Briefly, a 10-fold molar excess of purified rabbit anti-OVA IgG (GeneTex, USA) or rabbit anti-OVA IgG (Sigma-Aldrich, USA) was mixed with OVA (Sigma-Aldrich, USA) and incubated for 30 min at room temperature. To activate the macrophages, a 1:100 volume ratio of the immune complexes to media was used for culture.

Activation of Macrophages

BMM were polarized to M(LPS) or M(IC) following the protocol described by Edwards and Mosser (29) with some modifications. BMM generated as described above were primed overnight with recombinant IFNγ (10 ng/mL) (BioLegend) and washed twice with cold PBS. Pre-warmed media and E. coli LPS (100 ng/mL; Sigma Aldrich) were added to generate M(LPS) macrophages. Alternatively, LPS together with immune complexes (OVA + anti-OVA rabbit IgG complexes) (Sigma Aldrich) were added to generate M(IC). The immune complex was prepared by adding 10 molar excess of OVA to purified anti-OVA antibody and left to form complex at room temperature for at least 30 min. The phenotypes of M(LPS) and M(IC) were confirmed by measuring the level of IL-12p70 and IL-10 by ELISA and for *Il12b* and *Il10* by quantitative realtime PCR (qPCR).

Macrophage plasticity between M(LPS) and M(IC)

BMMs were plated in tissue culture plates for 24 hr before use. Cells were polarized to M(LPS) as described above. After 24 hr, media were removed and cells were washed with warm media and further cultured for 3 hr (short term rest) or 72 hr (long term rest). Cells were re-stimulated with LPS in the presence of immune complex for 24 hr to re-polarize from M(LPS) to M(IC). Cells culture supernatants were collected to measure the level of IL-10 and IL-12 by ELISA.

Western Blotting

BMMs were activated as indicated, and the protein lysates were subjected to Western blots. The primary antibodies used in this study were as follows: rabbit anti-Notch1 (1:2000) (Santa Cruz Biotechnology, USA), rabbit anti-cleaved Notch1 (Val1744) (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) (all from Cell Signaling Technology, USA), mouse anti β-actin (1:1000) (Chemicon-Millipore, USA) and rabbit anti-GAPDH (1:4000) (Santa Cruz Biotechnology, USA). The secondary reagents conjugated with horse-radish peroxidase (HRP) were as follows: donkey anti-rabbit IgG (1:2000-1:4000) and sheep anti-mouse IgG (1:5000) (Amersham Biosciences, UK). The signals were detected by chemiluminescence on X-ray films.

RNA Extraction and Quantitative Real-time RT-PCR (qPCR)

BMMs were activated as indicated, and total RNA was isolated by using TriZol reagent (Invitrogen, UK) or an RNeasy Mini Kit (Qiagen, Germany). cDNA was synthesized, and the transcripts were amplified by using CFX Connect™ real-time PCR detection system (Bio-Rad, USA). The primer sequences used are provided in Table 1. The expression of each gene was normalized to the expression of β-actin by the $2^{-\Delta\Delta CT}$ method (30).

Table 1. List of primers used in this study.

Primer	Sequence (5' to 3')		Product Size (bp)
Il10	Forward	TCAAACAAAGGACCAGCTGGACAACATACTGC	421
	Reverse	CTGTCTAGGTCCTGGAGTCCAGCAGACTCAA	
Il12b	Forward	AACCTCACCTGTGACACGCC	309
	Reverse	CAAGTCCATGTTTCTTTGCACC	

ELISA

Cells stimulated using the procedure described in (2.2) were collected cells culture supernatants to measure level of IL-12p70 and IL-10 using LEGEND MAX$^{\text{TM}}$ mouse IL-12p70 and IL-10 ELISA kits following to the manufacturer's protocol (BioLegend, USA). Briefly, anti-IL-12p70 and anti-IL-10 antibodies were used as capture antibodies and biotinylated anti-IL-12p40 and anti-IL-10 antibodies were used as detection antibodies. Strepavidin-conjugated HRP was added to develop color. The signals were detected by microplate reader at 450 nm (Biochrom Anthos, UK).

Immunofluorescent staining

Cells were cultured in an 8-well slide chamber and activated as indicated. After washing with PBS, the cells were fixed in 4% paraformaldehyde and blocked with Fc blocker (0.5 μg). The cells were washed and incubated with an anti-NF-κB p50 monoclonal antibody (1:100) (Santa Cruz Biotechnology, USA) and then an anti-mouse

IgG (H+L) $(Fab)_2$ fragment)-conjugated with Alexa Fluor® 488 (Cell Signaling Technology, MA, USA) (1:500) secondary antibody. Nuclei were stained by DAPI (Cell Signaling Technology, USA). The cells were observed under an inverted fluorescence microscope or a confocal microscope (Olympus, Japan).

RNA sequencing and data analysis

Total RNA samples were extracted from LPS/IC-activated BMMs in the presence of DMSO or GSI for 1 hr using an RNeasy Mini Kit (Qiagen, USA). RNA samples were assessed for quality using an Agilent 2100 Bioanalyzer (Agilent, USA) and quantified using a Qubit 3.0 fluorometer (Life Technologies, USA). Strand-specific cDNA library preparation was performed using a TruSeq stranded mRNA library prep kit (Illumina, USA). The cDNAs were sequenced on a NextSeq 500 system (Illumina, USA) at the Omics Sciences and Bioinformatics Center (Chulalongkorn University). The obtained data were trimmed of Illumina adapters using Trimmomatic and aligned using STAR software. Raw reads were counted using HTSeq, and the reads were mapped against the mm10 reference genome and annotated with Entrez Gene. Differential gene expression (DE) was determined using edgeR software, and the statistics were calculated according to a quasi-negative binomial distribution. Statistical significance was indicated by an FDR cut-off of < 0.05. Heat-maps were generated by MultiExperiment Viewer (MeV 4.9). Functional annotations were performed using ToppGene Suite (http://toppgene.cchmc.org).

Chromatin Immunoprecipitation and Sequencing (ChIP-seq)

BMMs were polarized to M(LPS) or M(IC) as described above for 4 hr. Untreated cells were used as baseline control. ChIP was performed using ChIP grade anti- Anti-H3K4me3 antibody (Cell Signaling Technology, USA) using SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology) according to the manufacturer's instruction. Briefly, 37% formaldehyde was used to cross link proteins and DNA molecules located in close proximity. Crosslinked chromatins were digested with micrococcal nuclease into optimal genomic DNA lengths 150-900 bp DNA/protein fragments. Anti-H3K4me3 rabbit monoclonal antibody (C42D8) and protein G-conjugated magnetic beads were added and the mixtures were incubated at 4°C overnight. Magnetic beads were separated and washed. ChIP Elution buffer were added, and the beads were incubated at 65°C for 30 minutes to reverse cross linking of protein/DNA complex. The supernatants were collected and purified using DNA purification kit. The ChIP DNA fragments were sequenced on by Next Generation Sequencing method at Beijing Genome Institute (Beijing, China)

Data Analysis of ChIP-seq

ChIP-seq data were generated and obtained in a fastq format. Overall ChIP-seq data analysis was depicted in Figure 1. In brief, trimmed sequence was examined for its quality by FastQC and aligned to reference genome by Bowtie2 (more than 97% mapped). Both MACS and MACS2 were used to identify regions of enrichment (peak-calling). IGV or Circos were employed to visualize the designated ChIP-enrichment within the target loci or globally, respectively. Epigenomic correlation or differentiation between control and treatment were evaluated by EpiMINE. In order to reveal the possible molecular mechanism that causes enrichment bias, CEAS was used to probe the enrichment differences within designated *cis*-regulatory regions. MEME-ChIP was used to identify the novel enrichment motifs and possible associated regulators/transcription factors. At the end, the possible regulatory signaling pathways and networks were revealed by DAVID analysis.

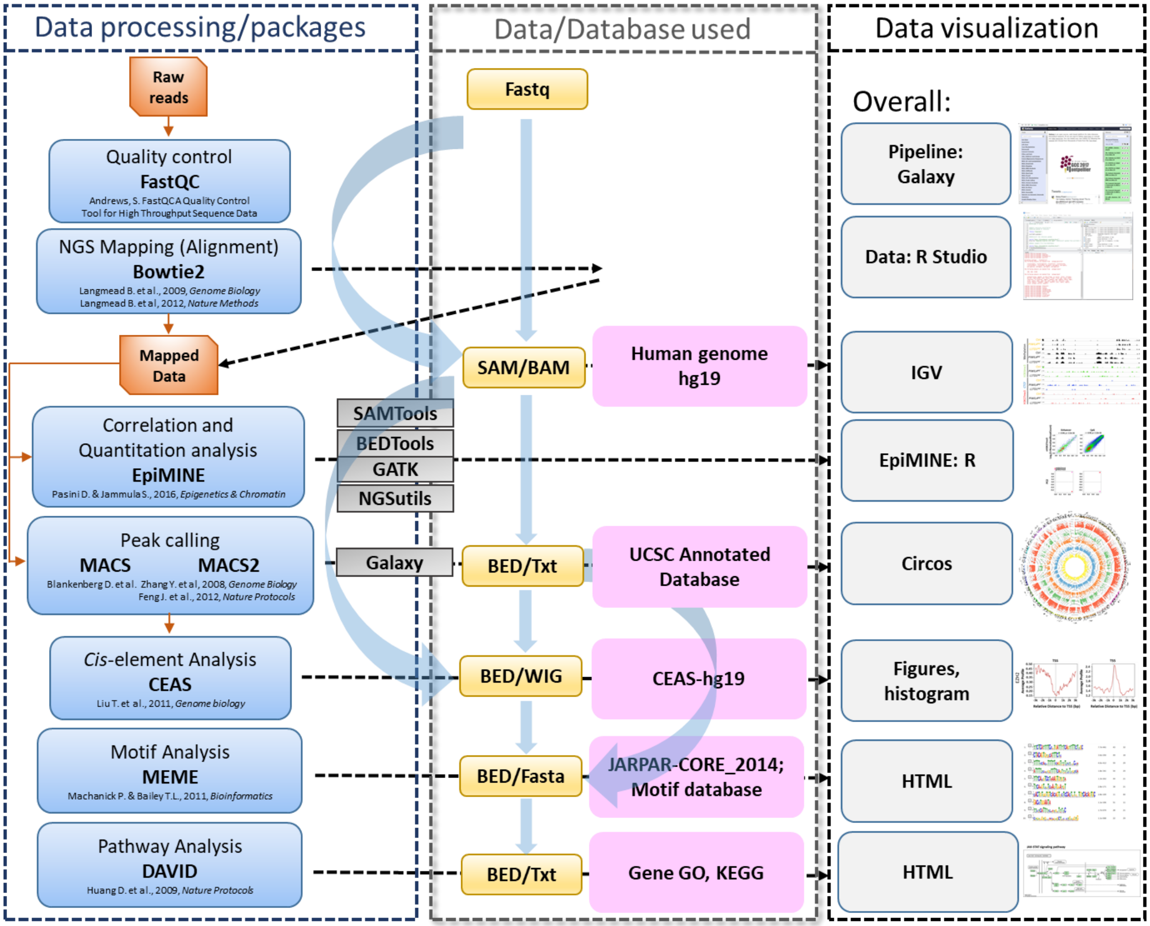


Figure 1: ChIP-seq data analysis pipeline. Used Bioinformatics packages (left panel), database (center) and visualization tools (right) were listed.

Adoptive Transfer of Macrophages in LPS-induced Sepsis Model

BMMs were polarized to M(IC) as described above. Eight to ten weeks old female C57BL/6 mice were randomly divided in to two groups (n=4 for each group). The treatment group received an adoptive transfer of 1x10^6 cells of M(IC) while the control group was adoptive transferred with 1x10^6 cells of unstimulated macrophages for 3 hr by intraperitoneal route before LPS challenge. Following the transfer, each group was treated with LPS at a sub-lethal dose (1 mg/kg body weight) to induce sepsis by intraperitoneal route. Blood samples were collected at 1 and 6 hr after LPS challenge (Figure 2). Serum were used to measure the level of IL-1β, IL-4, IL-7, TNFα, IL-6, IL-12p40 and IL-10 using Bio-plex Pro^{TM} assay kits (BioRad, USA), following to the manufacturer's protocol. Anti-IL-1β, anti-IL-4, anti-IL-7, anti-TNFα, anti-IL-6, anti-IL-12p40 and anti-IL-10 antibodies coupled on beads were used as capture antibodies and biotinylated anti-IL-1β, anti-IL-4, anti-IL-7, anti-TNFα, anti-IL-6, anti-IL-12p40 and anti-IL-10 antibodies were used as detection antibodies. Strepavidin-conjugated PE were added to develop the signals. The signals were detected by Bio-Plex system or similar Luminex-based reader.

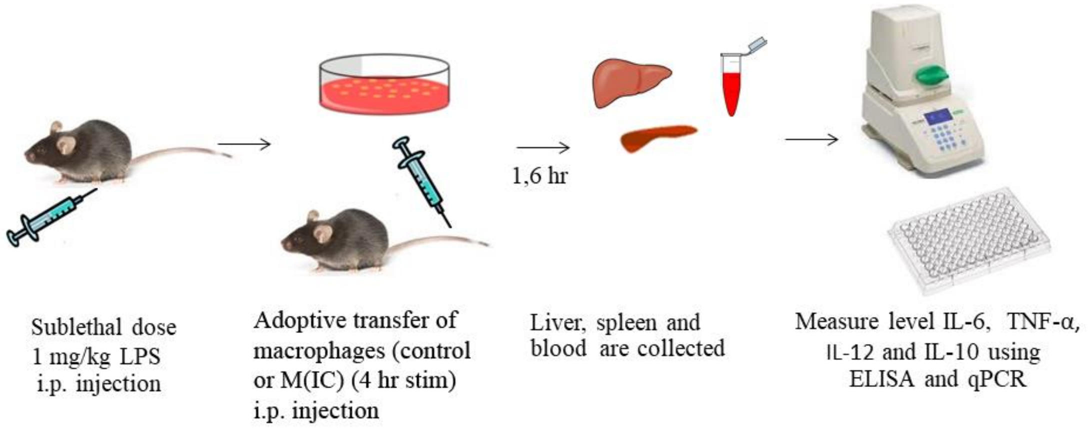


Figure 2. Adoptive transfer of M(IC) in mice with LPS-induced sepsis

Statistical Analysis

Statistical analyses were performed using SPSS version 15.0 and GraphPad Prism version 5.0. Student's t-test (paired or unpaired) and one-way ANOVA (α=0.05) with Tukey's multiple comparison test were used when comparing different conditions.

Results

1) Optimization of M(IC) Polarization

In this study, an immune complex of ovalbumin (OVA) and anti-OVA IgG was used to polarize macrophages to become M(IC). BMMs from C57BL/6 mice were primed overnight with recombinant interferon gamma (IFNγ) and washed with PBS the following day. Cells were subsequently stimulated with lipopolysaccharide (LPS) and immune complex to induce M(IC) while the control cells were stimulated with LPS alone (referred to here as M(LPS). The mRNA level of *Il10* and *Il12b* were measured by quantitative RT-PCR at 4 hr. As shown in Figure 3, M(LPS) expressed higher *Il12b* but lower *Il10* than M(IC). Secreted cytokines were measured by ELISA at 6 hr and M(IC) produced significantly higher IL-10 than M(LPS). As a negative control, IFNγ-primed BMMs stimulated with anti-OVA IgG alone in the presence of LPS produced comparable level of IL-10 as M(LPS). Furthermore, M(IC) produced less IL-6 than M(LPS) but the level of TNFα was comparable between M(IC) and M(LPS). These results were consistent with previous reports indicating that M(Ic) produces more IL-10 and less IL-12 than M(LPS+ IFNγ) while the level of some other pro-inflammatory cytokines such as TNFα remains unaltered.

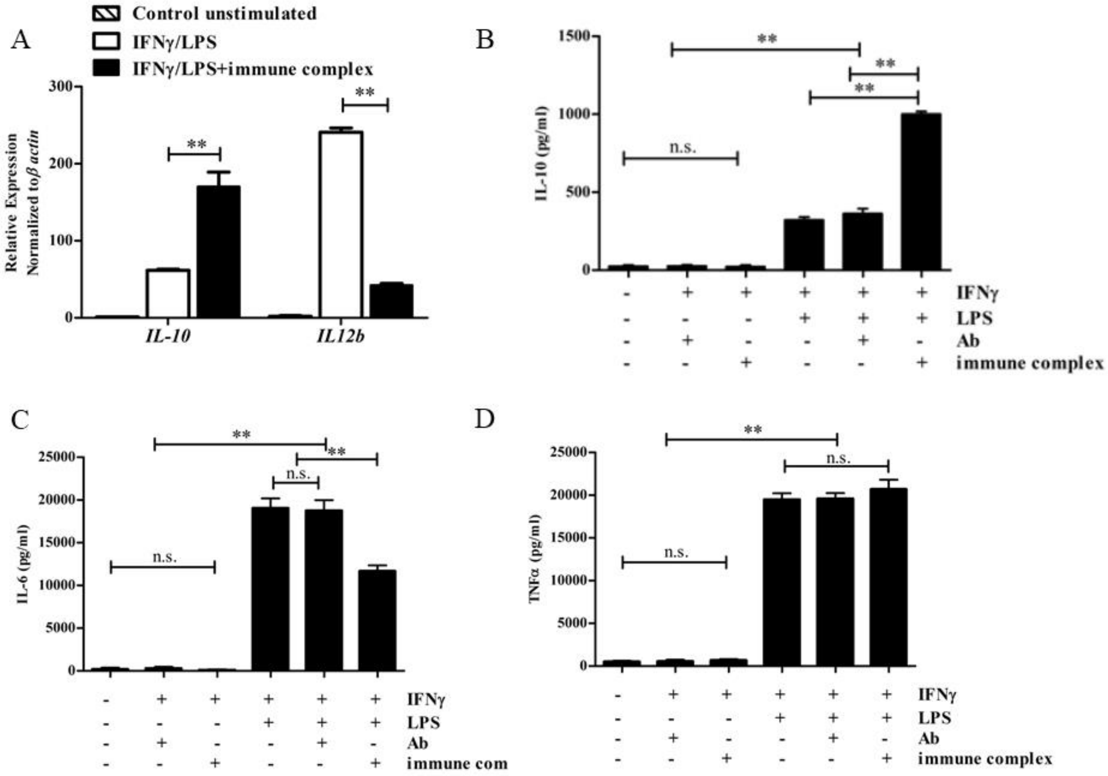


Figure 3. The mRNA levels and secreted levels of key cytokines in M(IC) and M(LPS) BMMs were stimulated as indicated for 4 hr (A) or 6 hr (B-D). Total RNA was harvested and the mRNA level of *Il10* or *Il12b* were measured by qPCR (A). Culture supernatants were subjected to ELISA to measure the amount of IL-10 (B), IL-6 (C) or TNFα (D). ** indicates statistical significance with $p \leq 0.05$.

2) Expression of Notch receptors and Notch ligands and activation of Notch signaling in M(IC)

Next, to investigate the involvement of Notch signaling in IL-10 production in M(IC), we investigated the expression profiles of two major Notch receptors mainly expressed in macrophages, Notch1 and Notch2. BMMs were stimulated to become M(IC) as described above and the expressions of Notch1 and Notch2 were detected by Western blot. As shown in Figure 4A, the levels of both Notch1 and Notch2 increased at 1, 3 and 6 hr after stimulation with LPS and immune complex. More importantly, the cleaved Notch1 (val 1744) was readily detectable at 1 hr after stimulation, suggesting that Notch signaling was activated in M(IC). To narrow down the ligand(s) responsible for activation of Notch signaling in M(IC), M(IC) were subjected for cell surface staining for Notch ligands in comparison with M(LPS) at 3 hr after stimulation. As shown in Figure 4B, M(LPS) increased the level of Jagged1, Jagged2 and Delta-like1 (Dll1), but not Dll4. In M(IC), the level of Jagged1 and Jagged2, but not Dll1 and Dll4, were increased. Compared to M(LPS), the level of Jagged2 and Dll1 decreased slightly. These results suggest that M(IC) showed distinct expression patterns of Notch receptors and ligands.

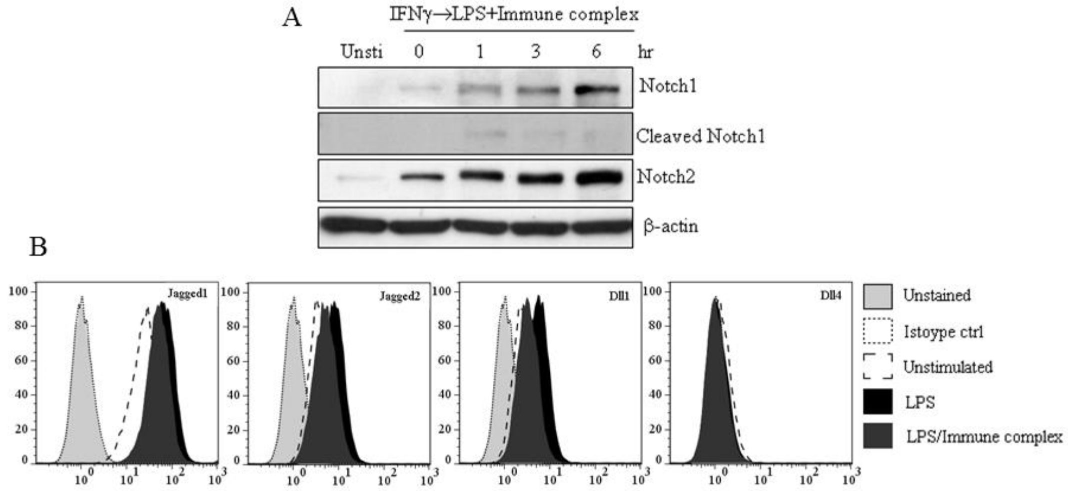


Figure 4. Expression of Notch receptors and Notch ligands in M(IC)

(A) BMMs were stimulated to become M(IC) as described above at different time points. The expression of Notch1, Notch2 and cleaved Notch1 (val1744) were detected by Western blot. β-actin was used as loading control.

(B) BMMs were stimulated to become M(IC) or M(LPS) as described above for 3 hr and subjected for cell surface staining using antibody against Jagged1, Jagged2, Dll1, Dll4. Cells were analyzed by flow cytometry.

To identify the stimuli responsible for activation of Notch signaling in M(IC), BMMs were primed with IFNγ and stimulated with LPS alone, anti-OVA IgG alone, LPS with anti-OVA IgG, immune complex alone or LPS with immune complex. The activation of Notch signaling was determined by Western blot to detect the presence of cleaved Notch1 (val1744). As shown in Figure 5, anti-OVA IgG or immune complex alone did not induce the cleavage of Notch1 whereas the addition of LPS alone or in the presence of Ab or immune complex triggered the cleavage of Notch1. This result strongly indicates that the signaling leading to activation of Notch signaling (cleavage of Notch receptor) mainly depends on the signaling downstream of TLR4, but not that of FcγR after antigen crosslinking.

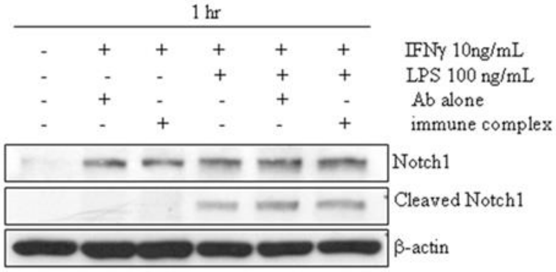


Figure 5. The effect of stimulus on activation of Notch signaling in activated macrophages

BMMs were primed with IFNγ overnight and activated by indicated stimuli for 1 hr. Cell lysates were analyzed for the cleavage of Notch1 (val1744). β-actin was used as loading control.

3) Effect of inhibitors of p38MAPK, MEK1/2, PI3K/Akt, NF-κB signaling pathways on activation of Notch signaling pathways in M(IC)

To investigate the signaling pathway(s) responsible for activation of Notch signaling downstream of TLR/FcγR ligation in M(IC), BMMs primed with IFNγ were pretreated with pathway specific inhibitor of MEK1/2, p38MAPK, PI3K/Akt and NF-κB pathways or vehicle control DMSO for 30 min. Cells were subsequently stimulated with LPS and immune complex for 1 hr. Cell lysates were analyzed to detect the appearance of cleaved Notch1 (val1744). As shown in Figure 6A, all inhibitors reduced the level of cleaved Notch1 at different degree. Inhibitors of MEK1/2 (U0126) and NF-κB (BAY-11) were most effective in reducing the level of cleaved Notch1 while inhibitors of p38MAPK (SB203580) and PI3K/Akt (LY294002) showed only minimal effect. Therefore, NF-κB and MEK1/2 pathways are responsible for inducing Notch activation downstream of TLR4/FcγR activation.

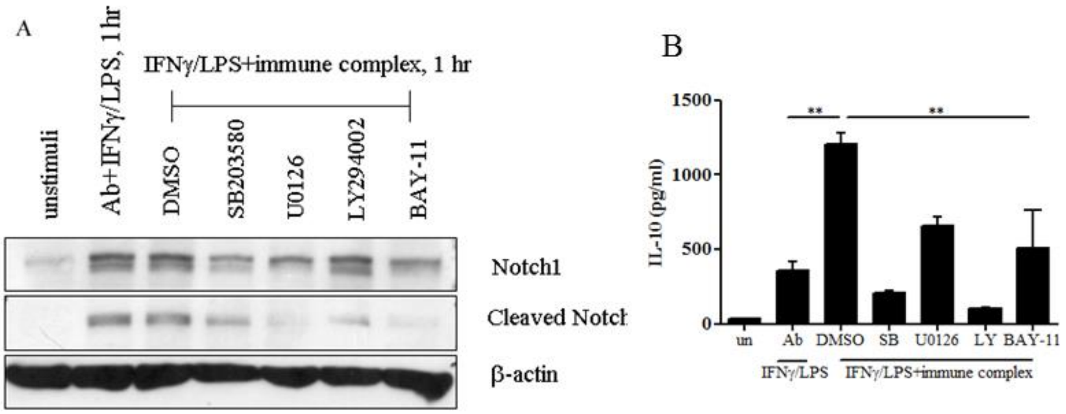


Figure 6. The effect of specific inhibitors of p38MAPK, MEK1/2, PI3K/Akt, NF-κB pathways on activation of Notch signaling in M(IC)

(A) BMMs were primed with IFNγ overnight and pre-treated with indicated inhibitor or vehicle control DMSO. Subsequently, cells were stimulated with LPS and immune complex. Cell lysates were analyzed for the cleavage of Notch1 (val1744). β-actin was used as loading control.

(B) BMMs were treated and stimulated as described above. IL-10 in the culture supernatant was measured by ELISA. ** indicates statistical significance with $p \leq 0.05$.

To examine whether treatment using specific inhibitor affects the production of IL-10, the amount of IL-10 in the culture supernatants from M(IC) treated with each specific inhibitor were measured by ELISA. As shown in Figure 6B, p38 MAPK and PI3K/Akt inhibitors strongly suppressed IL-10. Interestingly, NF-κB and MEK1/2 inhibitors significantly but partially reduced the level of IL-10 in M(IC). These results suggested that p38 MAPK and PI3K/Akt pathways are predominant signaling while NF-κB and MEK/Erk pathways is partially responsible for regulating IL-10 production in M(IC).

4) Effect of inhibiting Notch signaling on IL-10 production in M(IC)

Because Notch signaling is activated in M(IC) at early time point, the effect of inhibiting Notch signaling on IL-10 production was examined. First, gamma secretase inhibitor (GSI) was used to inhibit Notch receptor cleavage by enzyme gamma secretase. As shown in Figure 7A, cleaved Notch1 was completely disappeared in the GSI treatment, compared with that of the DMSO control, suggesting that GSI treatment effectively inhibited the Notch signaling pathway (Figure 7A). The production of IL-10 was decreased by almost 50% in GSI treated cells, compared with the mock control as measured by intracellular staining assay and ELISA (Figure 7B, C). GSI treatment also reduced the level of IL-10 in LPS-activated macrophages. The amount of IL-10 in GSI-treated LPS/IC-activated macrophages was comparable to that of LPS-activated macrophages, suggesting that Notch signaling may be responsible for inducing higher IL-10 production in M(IC).

To confirm this observation, macrophages from mice targeted deletion of *CSL/Rbpj* mice were used (Figure 7D). As expected, the production of IL-10 was reduced in *CSL/Rbpj* deletion macrophages which were activated by LPS+IC compared with their control (Figure 7D). However, IL-10 production was not decreased in LPS-activated macrophages, in contrast to GSI treatment. These data implied that canonical Notch signaling which required the activity of gamma secretase and CSL/RBP-jκ is critical for IL-10 production in M(IC), but not in M(LPS).

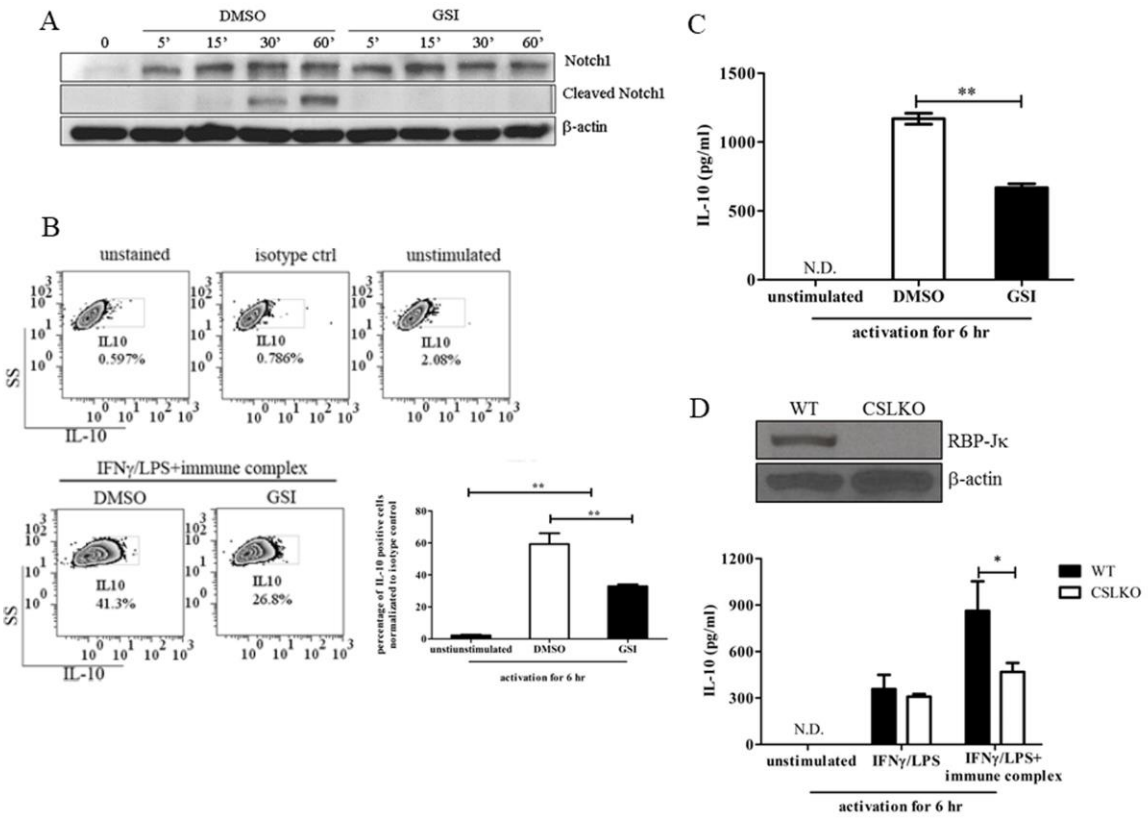


Figure 7 The effect of GSI and deletion of *CSL/Rbpj* on IL-10 production in M(IC)

(A) BMMs were activated by LPS/IC for 5, 15,30 and 60 min in the presence of vehicle control DMSO or GSI (25 μM). Cleaved Notch1 (Val 1744) and Notch1 were detected by Western blot.

(B) BMMs were activated by LPS/IC for 6 hr in the presence of vehicle control DMSO or GSI (25 μM). Expression of IL-10 was detected by intracellular cytokine staining. The results were summarized as graph from three independent experiments.

(C) IL-10 in the culture supernatants collected from BMMs treated as in (B) were measured by ELISA.

(D) The expression of CSL/RBP-Jκ in BMMs from WT (ctrl) or *CSL/Rbpj* KO mice was measured by Western blot. BMMs from WT (ctrl) or *CSL/Rbpj* KO mice were activated by LPS or ILPS+IC for 6 hr. The level of IL-10 was detected by ELISA. * and ** indicate statistical significance (*p*<0.05) according to one-way ANOVA with Tukey's multiple comparison test. The results represent the mean±SD of triplicate determinations from one of two independent experiments.

5) Effect of GSI treatment on activation of MAPK PI3K/Akt pathways in M(IC)

Next, we examined whether Notch signaling affected MAPK and PI3K signaling using GSI treatment to suppress the activation of Notch signaling pathway. BMMs 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 were not affected by the GSI treatment. Furthermore, the activation of SAPK/JNK and PI3K remained intact (Figure 8 A, B). Therefore, GSI did not affect IL-10 production in M(IC) via these signaling pathways. When the activation of NF-κB signaling pathway was investigated by observing the nuclear localization of p50 subunit, GSI treatment clearly suppressed the activation of p50 (Figure 8C). Therefore, the Notch signaling pathway affects IL-10 production in M(IC) by interfering with NF-κB p50 nuclear accumulation and activation of NF-κB pathway.

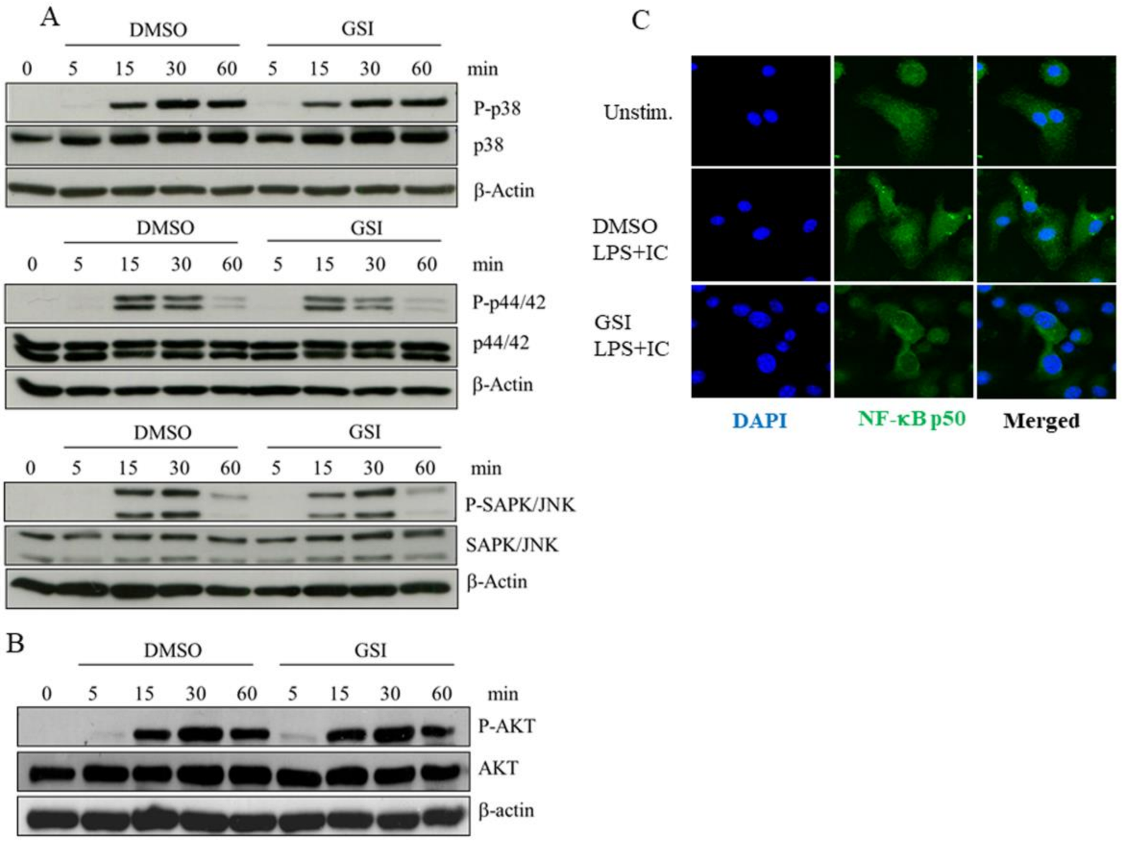


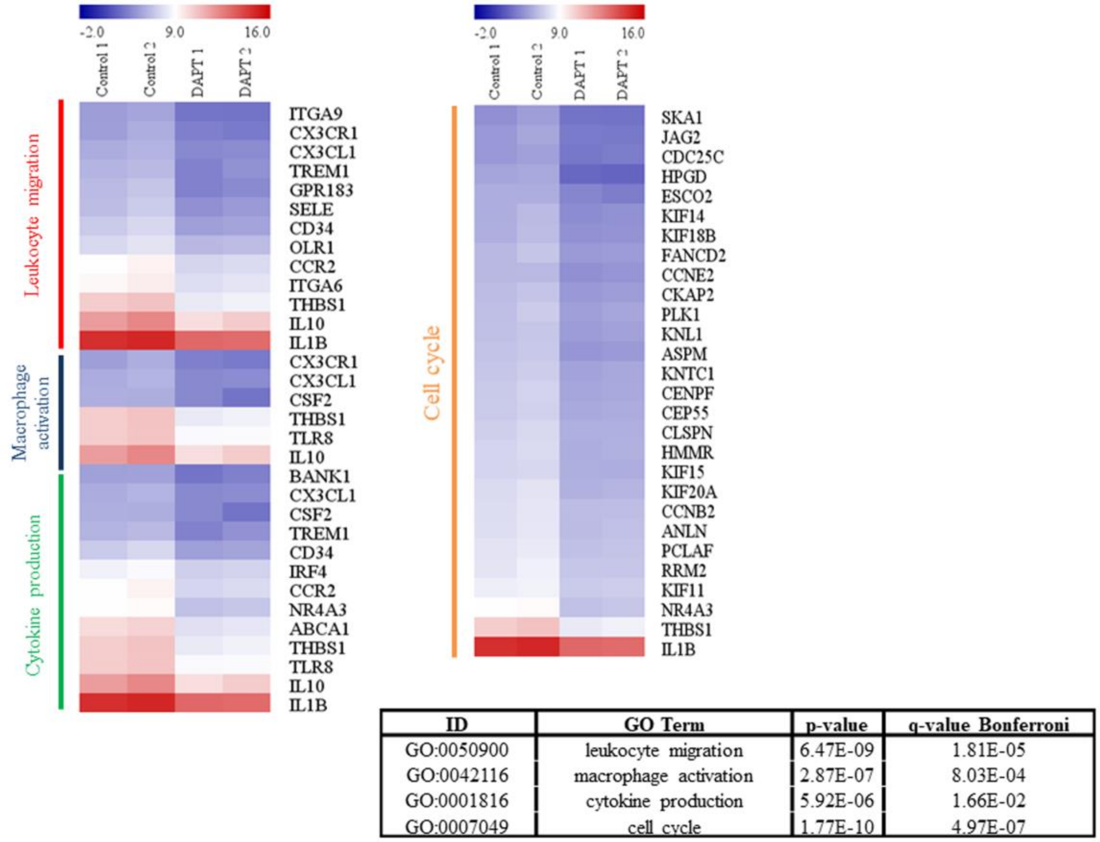
Figure 8. GSI treatment did not affect the activation of MAPK, PI3K/AKT and NF-κB signaling pathways

(A-B) BMMs 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-p38, p38 Phospho-p44-42, p44-42, Phospho-SAPK/JNK, SAPK/JNK, Phospho-AKT, AKT and loading control (β-actin) were detected by Western blot.

(C) BMMs were activated by LPS+IC for 4 hr in the presence of vehicle control DMSO or GSI (25 μM). NF-κB p50 was detected by immunofluorescence staining (green). Nuclei were stained with DAPI. Cells were observed under confocal laser scanning microscope.

6) GSI treatment changes the gene expression profiles of LPS/IC-activated macrophages

To investigate the global effect of inhibiting the activation of Notch signaling in LPS/IC-stimulated macrophages, a transcriptomic analysis by RNA-seq was performed to determine differential gene expression. When comparing BMMs stimulated with LPS/ICs in the presence of GSI and BMMs treated with vehicle control, 147 genes were found to be differentially expressed with log2 fold changes of greater than 1.5. More genes were downregulated in GSI-treated macrophages, suggesting that Notch signaling is positively involved in regulating gene expression (data not shown). The gene ontology (GO) enrichment analysis of the biological processes involving the upregulated and downregulated genes revealed that leukocyte migration, macrophage activation, cytokine production and cell cycle were significantly affected by GSI treatment in LPS/IC-stimulated macrophages (Figure 9 and data not shown). Interestingly, the biological processes associated with cell cycle were profoundly affected by GSI treatment in LPS/IC-stimulated macrophages (Figure 9).



ID	GO Term	p-value	q-value Bonferroni
GO:0050900	leukocyte migration	6.47E-09	1.81E-05
GO:0042116	macrophage activation	2.87E-07	8.03E-04
GO:0001816	cytokine production	5.92E-06	1.66E-02
GO:0007049	cell cycle	1.77E-10	4.97E-07

Figure 9. Heatmap of differentially expressed genes in M(IC) treated with DMSO *vs*. GSI Total RNA isolated from LPS/IC-activated macrophages treated with vehicle DMSO or GSI (25 μM) was subjected to RNA-seq. Differentially expressed genes were subjected to a GO enrichment analysis. Heatmaps of genes associated with the GO terms leukocyte migration, macrophage activation, cytokine production and cell cycle are shown.

Among the genes with reduced mRNA levels, *Il10*, *Il12b*, and *Il1beta* and the Notch ligand *Jag2* and nuclear hormone receptor *Nr4a3* were validated (data not shown). These genes are reported to be partially regulated by the NF-κB pathway. Furthermore, the pro-inflammatory genes *Il23r*, *Saa3*, *Ptges* and *Nos2* were validated as genes upregulated by GSI treatment (data not shown). Taken together, the transcriptomic data indicated that Notch signaling in M(IC) regulates expression of key genes partially through NF-κB.

7) Examine the plasticity between M(LPS) and M(Ic)

To address how plastic macrophages are after being stimulated to become M(LPS) or M(IC), cells were first treated as depicted in Figure 10. After 4 hr of primary stimulation with LPS or LPS/immune complex, culture supernatants were collected to measure IL-10 and IL-12p70. After washing out stimuli and resting for 2 hr, cells were re-stimulated with opposing stimuli and the culture supernatants were collected at 4 hr and The levels of secreted IL-10 and IL-12p70 were subjected to ELISA. As shown in Figure 10, in both direction of reverse polarization, IL-10 was completely suppressed by in the second round of stimulation by LPS or LPS+IC. The level of IL-12p70 was induced when M(LPS) were redirected to M(IC) while it was reduced when M(IC) were redirected to M(LPS). These results indicated that expression of IL-10 was strongly affected by the first round of LPS stimulation and the level did not recover in the second round of stimulation regardless of the stimuli. Therefore, in this successive stimulation scheme, macrophages showed no plasticity in changing the phenotypes.

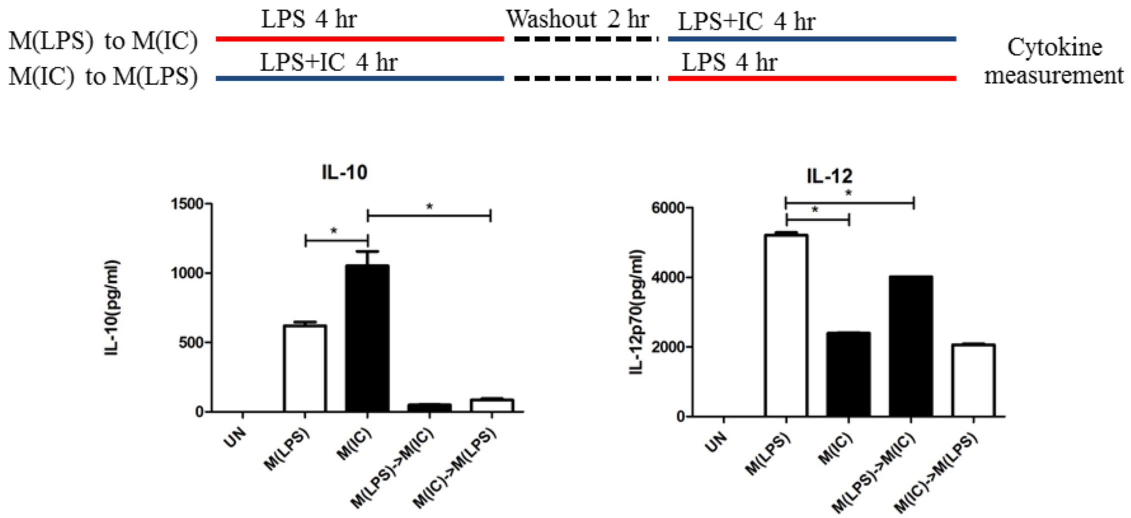


Figure 10. The effect of type switching of macrophages on cytokines production

The protocol used for macrophage plasticity test (short resting period) is shown. The culture supernatants collected from the first round of stimulation or the second rounds of stimulation were subjected to ELISA for measuring IL-10 and IL-12p70. The results represent the mean±SD of triplicate determinations from two independent experiments. * indicated statistical significance ($p<0.05$).

Next, the duration between the two rounds of stimuli were extended into 3 days, and the plasticity was tested by measuring the level of IL-10 and IL-12p70 by ELISA. As shown in Figure 11, the level of IL-10 was upregulated much higher than those in M(LPS) or M(IC) when M(LPS) were re-stimulated with LPS+IC while that of IL-12p70 was severely reduced. This result indicated that the resting period of 3 days restores the response of BMMs to the second round of stimulation and LPS together with IC successfully induce M(LPS) to become IL-10^{hi}IL-12^{lo} M(IC).

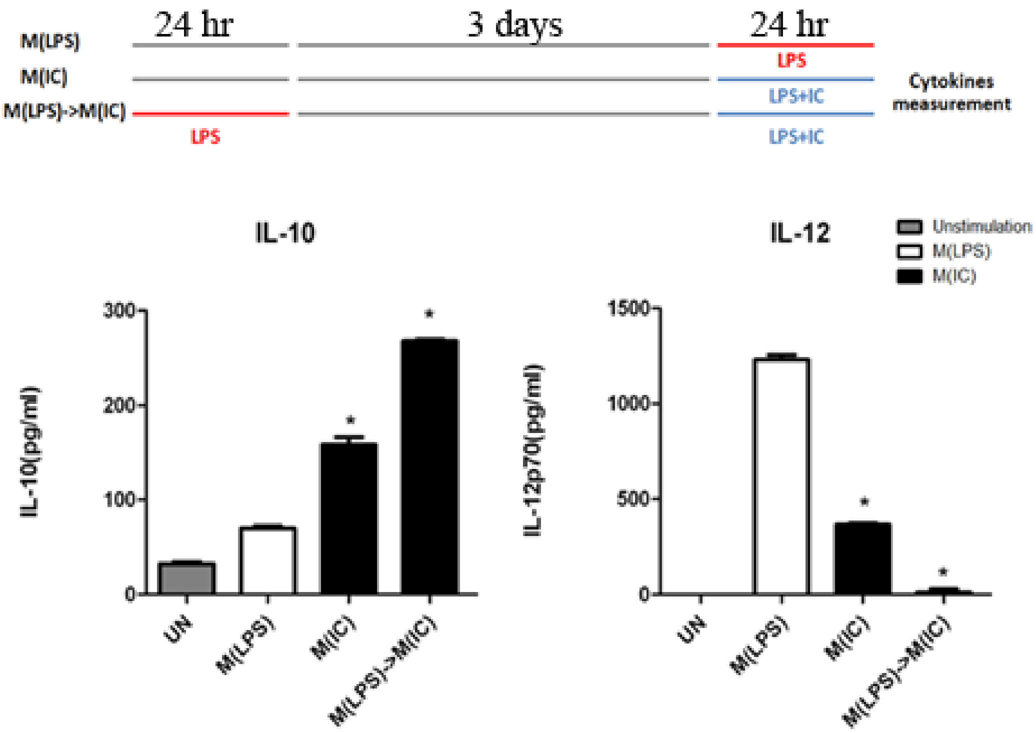


Figure 11. The effect of type switching of macrophages on cytokines production

The protocol used for macrophage plasticity test (long resting period) is shown. The culture supernatants collected from the first round of stimulation or the second rounds of stimulation were subjected to ELISA for measuring IL-10 and IL-12p70. The results represent the mean±SD of triplicate determinations from two independent experiments. statistical significance ($p<0.05$).

8) The promoter accessibility of *Il10* in M(IC)

Because high IL-10 expression during re-polarization from M(LPS) to M(IC) could be achieved after at least 48 hr of resting period between the first and second round of stimulation, we hypothesized that epigenetic regulation plays a role in IL-10 expression in M(IC). The promoter accessibility at the transcription factor SP1 binding site (-294/-73 bp) was investigated because it was previously reported that SP1 binding sites in the Il10 promoter plays important role in IL-10 production in macrophages (31).

To monitor the accessibility of *Il10* promoter during polarization of macrophages, chromatin accessibility kit was used. BMMs were activated by LPS or LPS+IC for various times (15, 30, 45, 60 min). Chromatins were extracted and digested by micrococcal nuclease. Using this chromatin as a template, the primer sets corresponding to SP1 binding site in the promoter region of *Il10* were amplified. The results were calculated as a fold enrichment (FE) according to the manufacturer’s instruction. As shown in Figure 12, SP1 binding site in the *Il10* promoter in M(LPS) were readily accessible at 15 min and peaked at 30 min after stimulation. In contrast, in M(IC), the opening of the promoter region was delayed and the level was lower than that in M(LPS). This result suggested that in the SP1 binding region in Il10 promoter, LPS stimulation enable chromatin accessibility while the presence of IC delays this event. This result is in contrast of what it is expected for M(IC). It is possible that other regions in the *Il10* promoter or enhancers plays more important roles in the transcription of *Il10* expression in M(IC) which is different from that in M(LPS).

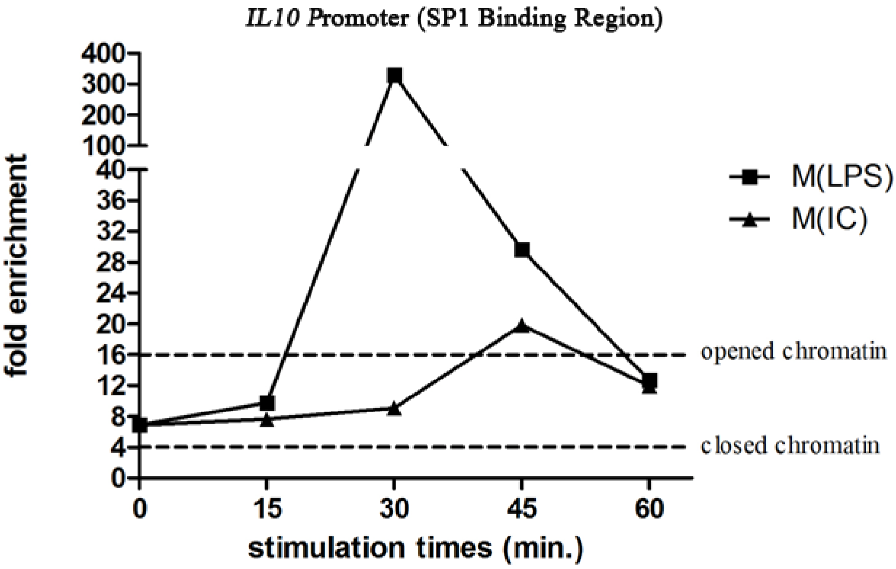


Figure 12. Chromatin accessibility of *Il10* promoter in M(LPS) or M(Ic)

BMMs were stimulated with LPS or LPS+IC for indicated time. Chromatins were extracted and subjected for qPCR using primers spanning the SP1 binding site in the *Il10* promoter. Fold enrichment (FE) was calculated by $2^{(\text{Nse CT - noNse CT})}$. When the FE% is more than 16, it indicates that the target region is in the opened chromatin status while the FE% less than 4 implies that the target gene region is in the closed chromatin status.

9) Active histone mark (trimethylation of H3K4) in M(IC)

To understand the plasticity of M(IC) at the epigenomic level, the global histone mark was investigated in M(IC) compared to M(LPS). Trimethylation at lysine 4 on subunit histone 3 (H3K4me3) is one of the well-defined active histone marks often associated with the promoter regions upstream of the transcriptional start sites. In resting macrophages, H3K4me3 marks the promoters of genes that are constitutively expressed and also the TLR4-responsive promoters even before LPS stimulation (7). Therefore, we performed ChIP-seq analysis to investigate the profiles of H3K4me3 in M(LPS) and M(IC).

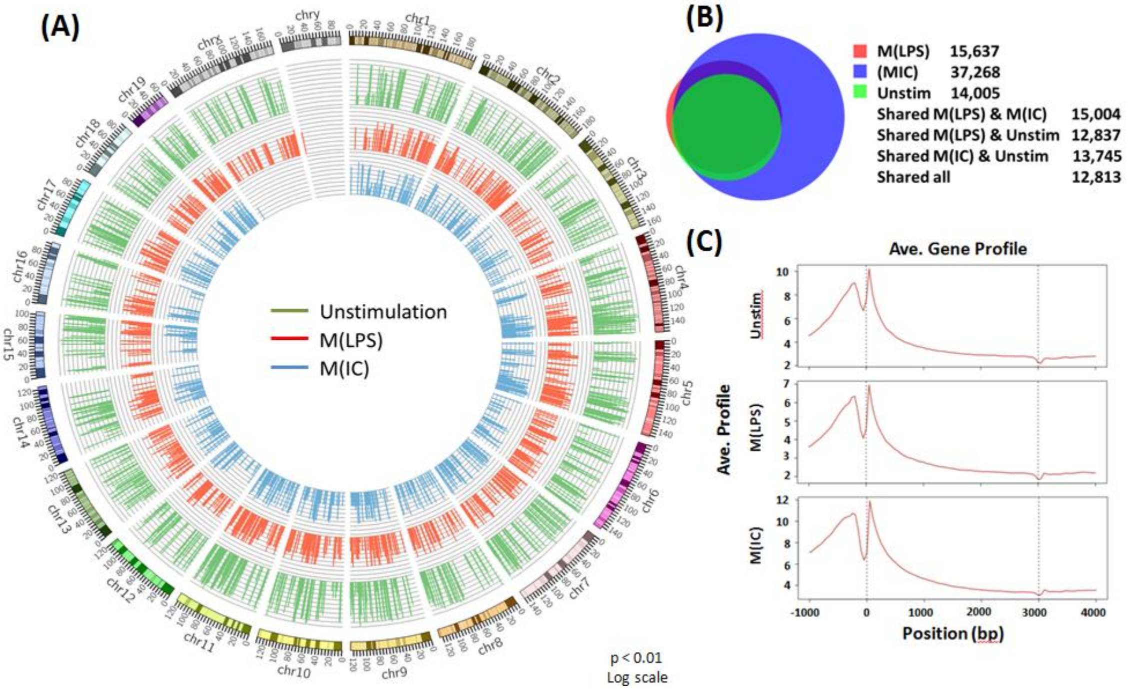


Figure 13. Genome-wide H3K4me3 enrichment in M(LPS) and M(IC)

BMMs were stimulated with LPS or LPS+IC for 4 hr and subjected to ChIP using anti-H3K4me3 antibody. ChIP DNA fragments were sequenced and analyzed.

(A) Global H3K4me3 enriched peaks in unstimulated BMMs, M(LPS) and M(IC) were shown in a Circos plot.

(B) Peaks in unstimulated BMMs, M(LPS) and M(IC) were depicted in Venn diagrams.

(C) Average gene profiles of globally H3K4me3 enrichment between unstimulated BMMs, M(LPS) and M(IC).

Circos plot displayed global H3K4me3 enrichment in 3 types of macrophages. The result showed clear differences in genome-wide enrichment of peaks among the 3 types of macrophages (Figure 13A). The Venn diagrams were used to compare H3K4me3 enrichment overlapping between unstimulated macrophages, M(LPS) and M(IC). From this result, M(IC) showed unique peaks that were not found in the other two types of macrophages while unstimulated and M(LPS) showed much more overlapping peaks (Figure 13B). For average gene profiles, the results showed that all three types of macrophages have the same pattern (Figure 13C).

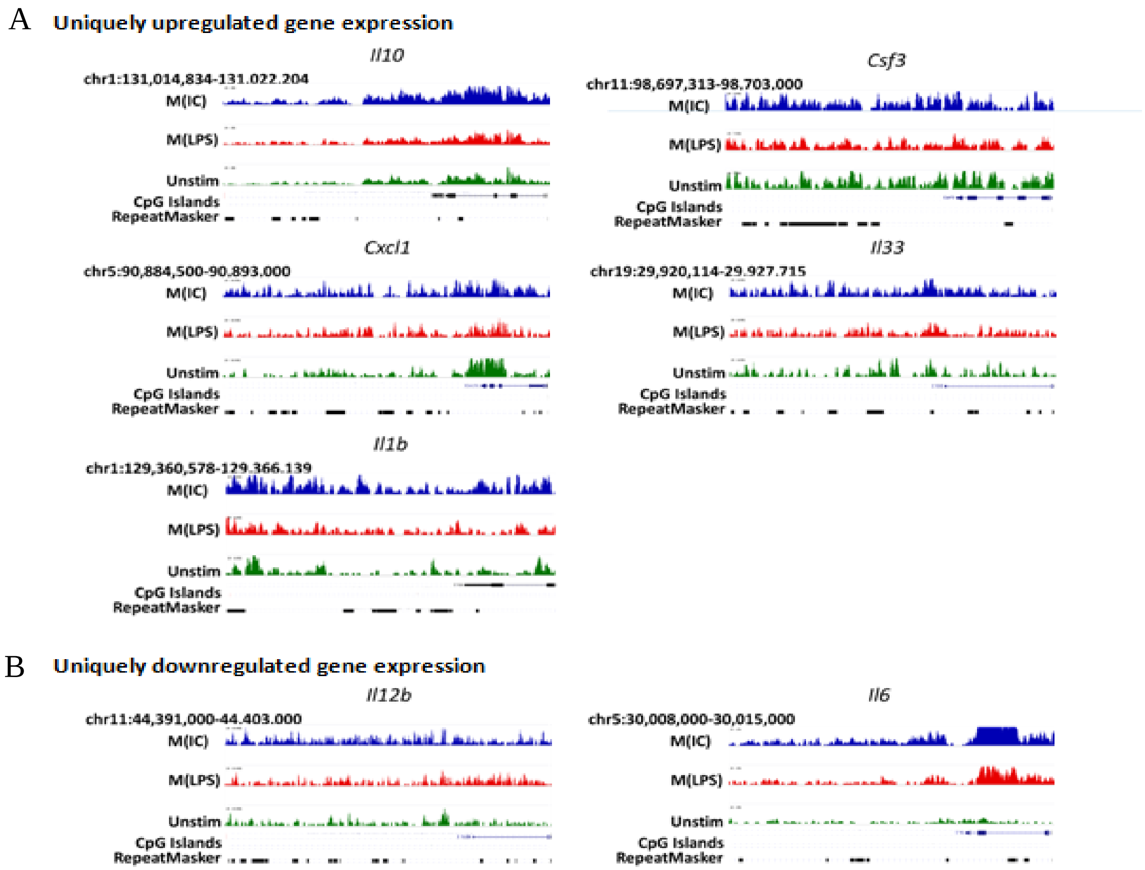


Figure 14. H3K4me3 enrichment at some target loci in unstimulated macrophages, M(LPS) and M(IC)

IGV is used to compare H3K4me3 enrichment in the target loci unique to M(IC). Based on the study by Flemming *et al*. (9), *Il10*, *Cfs3*, *Cxcl1*, *Il33* and *Il1b* were chosen as the uniquely upregulated genes in M(IC) (A) while *Il12b* and *Il6* were chosen as the uniquely downregulated genes in M(IC) (B).

Flemming *et al*. used RNA-seq to characterize the transcriptomes of M(IC) and identified unique genes that are either upregulated or downregulated in M(C) (9). Some of these genes were chosen and the enrichment of H3K4me3 were individually analyzed. As shown in Figure 14, IGV showed some differences in H3K4me3 enrichment in the targeted loci. Increasing H3K4me3 enrichments were observed mainly in the promoter and encoding regions of *Il10, Cxcl1, Csf3, Il33* and *Il1b* in M(IC) when compared with M(LPS). Most robust increase was seen in *Il10*, consistent with increase

IL-10 expression. Some genes such as *Csf3* and *Il33*, the H3K4me3 enrichment was observed even in unstimulated cells. In contrast, the H3K4me3 enrichment in uniquely downregulated gene, *Il12b* and *Il6*, was not observed between MIC) and M(LPS) (Figure 14).

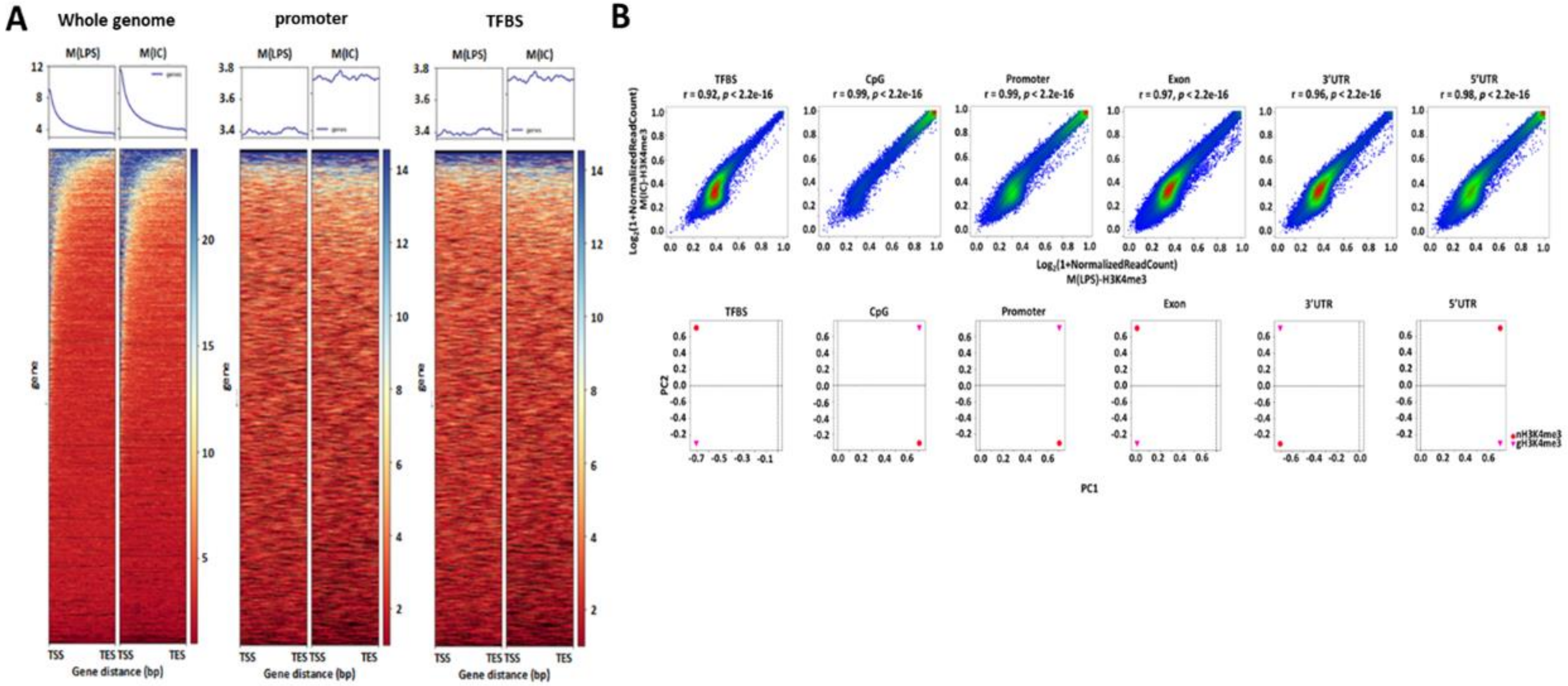
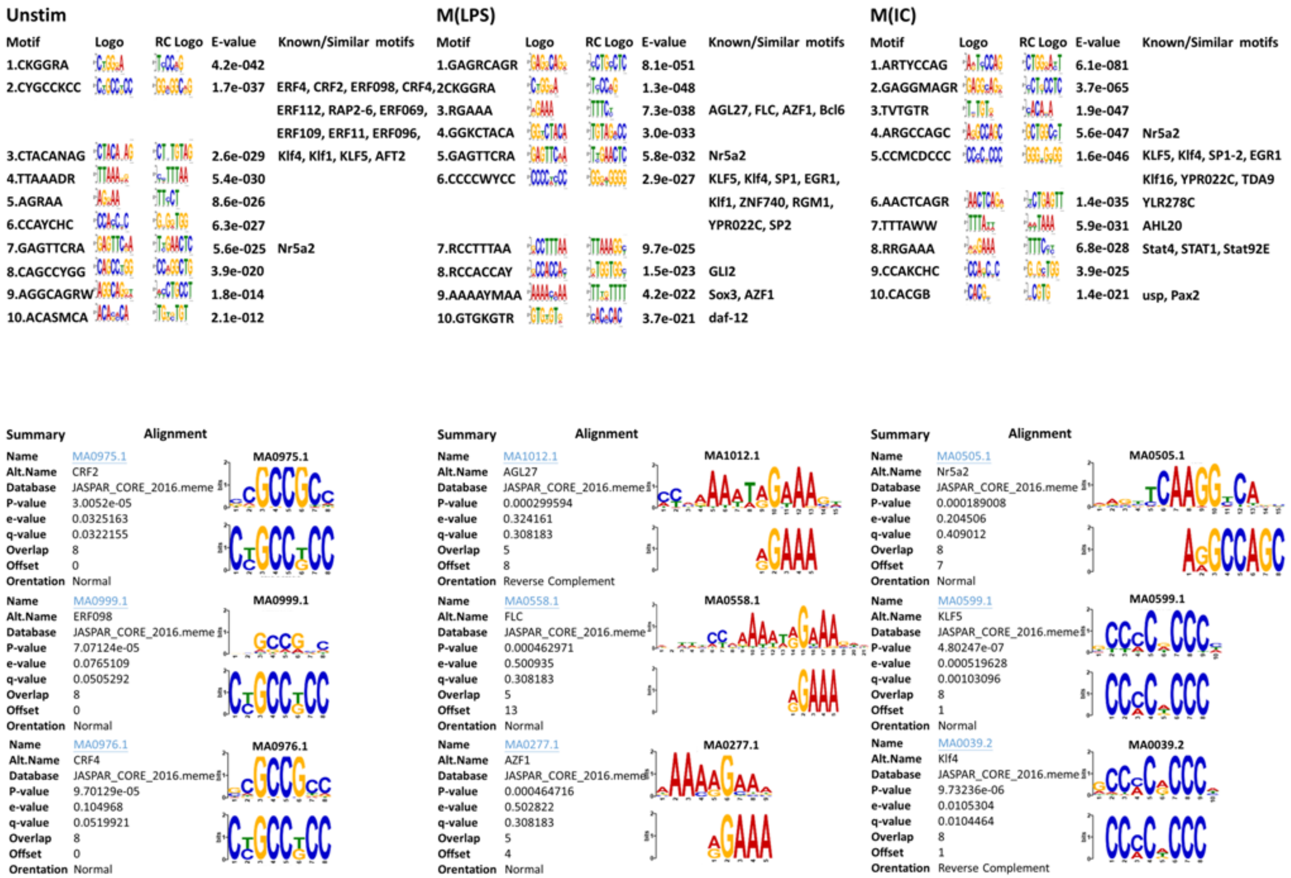


Figure 15 The enrichment of H3K4me3 in *cis*-regulatory regions and the correlation between M(LPS) and M(IC)

(A) A heatmap depicting differential enriched peaks in whole genome, promoter regions and transcription factor binding sites between M(LPS) and M(IC).

(B) PCA plots showing the H3K4me3 enrichments in the *cis*-regulatory regions and the scatter plots generated from EpiMINE showing the correlation between M(LPS) and M(IC).

The results of the H3K4me3 enrichments within the TSS and 2000 kb near the TSS were shown in a heatmap (Figure 15A). The results showed that M(IC) had higher global H3K4me3 enrichment, and higher H3K4me3 enrichments at the promoter regions and transcription factors binding site (TFBS) when compared with M(LPS) (Figure 15A). For the epigenomic correlation, the scatter plots showed that M(LPS) and M(IC) had high correlation with the Pearson correlation coefficient of more than 0.95 in the TFBS, the CpG island, the promoters, exons, 3'UTR and 5'UTR, (Figure 15B). However, the principal component analysis (PCA) plot revealed the two macrophage types, M(LPS) and M(IC), are well separated in all *cis*-regulatory regions (Figure 15B).



Unstim

Motif	Logo	RC Logo	E-value	Known/Similar motifs
1.CKGGRA			4.2e-042	
2.CYGCCKCC			1.7e-037	ERF4, CRF2, ERF098, CRF4, ERF112, RAP2-6, ERF069, ERF109, ERF11, ERF096, Klf4, Klf1, KLF5, AFT2
3.CTACANAG			2.6e-029	
4.TTAAADR			5.4e-030	
5.AGRAA			8.6e-026	
6.CCAYCHC			6.3e-027	
7.GAGTTCRA			5.6e-025	Nr5a2
8.CAGCCYGG			3.9e-020	
9.AGGCAGRW			1.8e-014	
10.ACASMCA			2.1e-012	

M(LPS)

Motif	Logo	RC Logo	E-value	Known/Similar motifs
1.GAGRCAGR			8.1e-051	
2CKGGRA			1.3e-048	
3.RGAAA			7.3e-038	AGL27, FLC, AZF1, Bcl6
4.GGKCTACA			3.0e-033	
5.GAGTTCRA			5.8e-032	Nr5a2
6.CCCCWYCC			2.9e-027	KLF5, Klf4, SP1, EGR1, Klf1, ZNF740, RGM1, YPR022C, SP2
7.RCCTTTAA			9.7e-025	
8.RCCACCAY			1.5e-023	GLI2
9.AAAAYMAA			4.2e-022	Sox3, AZF1
10.GTGKGTR			3.7e-021	daf-12

M(IC)

Motif	Logo	RC Logo	E-value	Known/Similar motifs
1.ARTYCCAG			6.1e-081	
2.GAGGMAGR			3.7e-065	
3.TVTGTR			1.9e-047	
4.ARGCCAGC			5.6e-047	Nr5a2
5.CCMCDCCC			1.6e-046	KLF5, Klf4, SP1-2, EGR1 Klf16, YPR022C, TDA9
6.AACTCAGR			1.4e-035	YLR278C
7.TTTAWW			5.9e-031	AHL20
8.RRGAAA			6.8e-028	Stat4, STAT1, Stat92E
9.CCAKCHC			3.9e-025	
10.CACGB			1.4e-021	usp, Pax2

Summary **Alignment**

Name	MA0975.1	Name	MA1012.1
Alt.Name	CRF2	Alt.Name	AGL27
Database	JASPAR_CORE_2016.meme	Database	JASPAR_CORE_2016.meme
P-value	3.0052e-05	P-value	0.000299594
e-value	0.0325163	e-value	0.324161
q-value	0.0322155	q-value	0.308183
Overlap	8	Overlap	5
Offset	0	Offset	8
Orentation	Normal	Orentation	Reverse Complement
Name	MA0999.1	Name	MA0558.1
Alt.Name	ERF098	Alt.Name	FLC
Database	JASPAR_CORE_2016.meme	Database	JASPAR_CORE_2016.meme
P-value	7.07124e-05	P-value	0.000462971
e-value	0.0765109	e-value	0.500935
q-value	0.0505292	q-value	0.308183
Overlap	8	Overlap	5
Offset	0	Offset	13
Orentation	Normal	Orentation	Normal
Name	MA0976.1	Name	MA0277.1
Alt.Name	CRF4	Alt.Name	AZF1
Database	JASPAR_CORE_2016.meme	Database	JASPAR_CORE_2016.meme
P-value	9.70129e-05	P-value	0.000464716
e-value	0.104968	e-value	0.502822
q-value	0.0519921	q-value	0.308183
Overlap	8	Overlap	5
Offset	0	Offset	4
Orentation	Normal	Orentation	Normal

Summary **Alignment**

Name	MA0505.1
Alt.Name	Nr5a2
Database	JASPAR_CORE_2016.meme
P-value	0.000189008
e-value	0.204506
q-value	0.409012
Overlap	8
Offset	7
Orentation	Normal
Name	MA0599.1
Alt.Name	KLF5
Database	JASPAR_CORE_2016.meme
P-value	4.80247e-07
e-value	0.000519628
q-value	0.00103096
Overlap	8
Offset	1
Orentation	Normal
Name	MA0039.2
Alt.Name	Klf4
Database	JASPAR_CORE_2016.meme
P-value	9.73236e-06
e-value	0.0105304
q-value	0.0104464
Overlap	8
Offset	1
Orentation	Reverse Complement

Figure 16. The novel enrichment motifs and possible associated regulators/transcription factors in M(IC)

MEME-ChIP was used to identify the novel enrichment motifs and possible associated regulators/transcription factors in unstimulated macrophages, M(LPS) and M(IC).

The results analyzed by MEME-ChIP showed some of common known motifs in both M(LPS) and M(IC) (Figure 16). However, some known and unknown motif elements are found highly enriched only in M(IC) but not in M(LPS). Among these motifs, some are novel motifs for transcription factors or epigenetic modifiers binding sites that is important in M(IC) polarization. Some motifs that are unique to M(LPS) are Stat4, Stat1, Stat92E, Pax2 and Usp.

10) The application of M(IC) in sepsis mouse model

To test whether the adoptive transfer of M(IC) has any impact on the pro-inflammatory cytokine production in LPS-induced endotoxemia model, polarized M(IC) were prepared for 4 hr *in vitro*. They were adoptive transferred into naïve mice ($1x10^6$ cells/mouse). M(IC) as described in materials and methods, followed by an *i.p.* administration of sublethal dose of LPS as described above (Figure 2). Blood, livers and spleens were collected at 1 and 6 hr and subjected to ELISA or qPCR to measure the level of inflammatory cytokines.

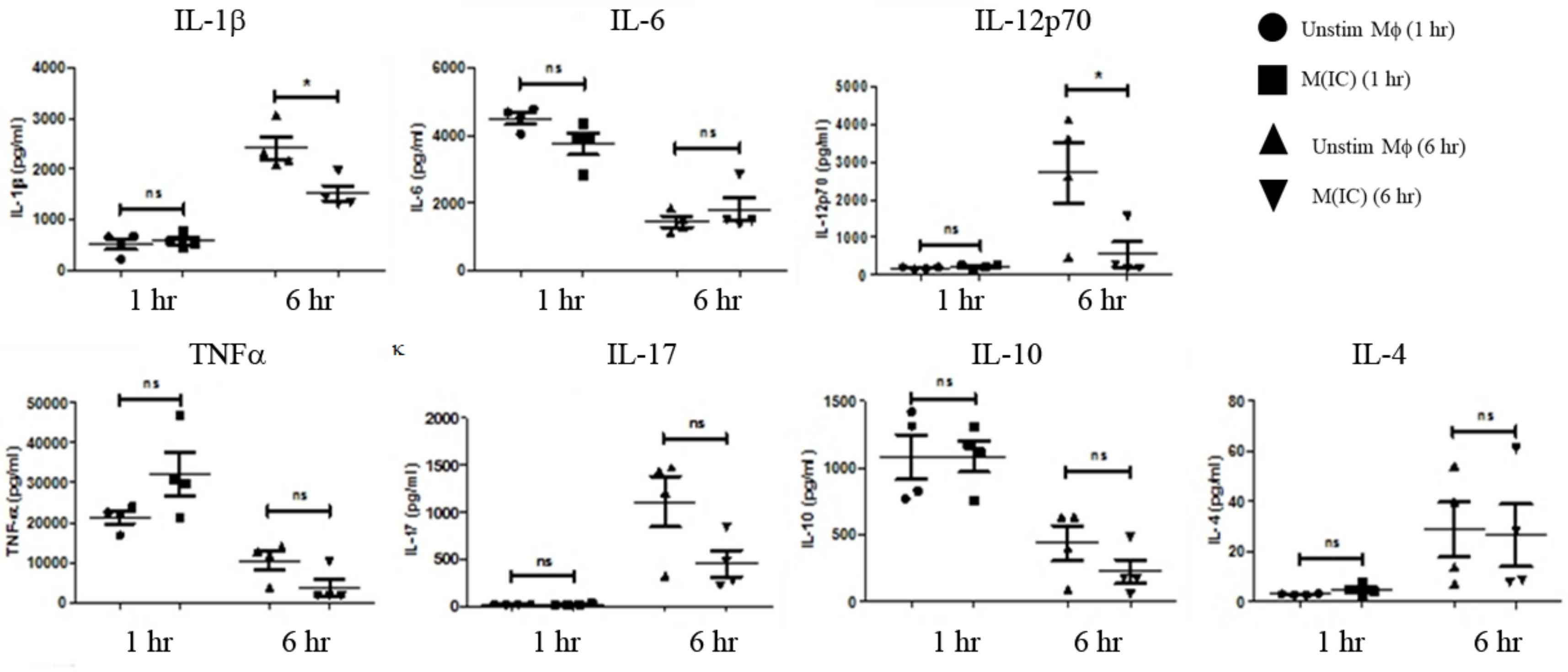


Figure 17. Serum cytokines in mice receiving adoptive transfer of M(IC)

Mice (C56BL/6) received adoptive transfer of M(IC) or control unstimulated macrophages by *i.p.* administration, followed by sublethal dose of LPS by *i.p.* administration. Blood were collected at 1 and 6 hr after LPS injection (n=4) and the sera were subjected to Bioplex for cytokine measurement. *p<0.05, n.s.=no statistical significance.

The results showed that in the group of mice receiving adoptive transfer of M(IC), the levels of IL-1β and IL-12p70 were significantly decreased at 6 hr after LPS challenge while the levels of IL-6, TNFα, IL-17, IL-10, and IL-4 were not significantly different between the two groups (Figure 17). The cytokines profiles revealed that adoptive transfer with M(IC) in sepsis mouse model helped decrease the production of pro-inflammatory cytokine IL-1β and IL-12p70. Because the level of IL-10 is not significantly different between the two groups, it is possible that adoptive transfer of M(IC) may interfere with LPS-induced systematic response by other mechanisms besides the production of IL-10.

Discussion

Activation of macrophages is regulated by immediate signaling molecules downstream of PRRs such as TLRs and RLRs, specific transcription factors and epigenetics regulation. At the same time, they are also known to exhibit phenotypic plasticity depending on the microenvironments and stimuli (6). In this study, we investigated one subset of activated macrophages that are stimulated with PRR ligand, LPS, in the presence of immune complex. Transcriptomic evidences clearly demonstrated that they express distinctive gene profiles that are different from inflammatory macrophages and may play an important role in pathological conditions such as in autoimmune diseases and infectious diseases (2,9,32). We aimed to understand the signaling pathway(s) governing their activation, their plasticity and the involvement of epigenetic regulation. Finally, we tested their biological effects in vivo by an adoptive transfer in endotoxemia model.

In this study, we confirmed the involvement of Notch signaling in M(IC). Signaling through LPS/TLR4 is essential for Notch activation and Dll4, but not Jagged1, was identified as the main ligand that activates Notch1. An antibody or ICs alone was not sufficient to initiate Notch signaling activation as determined by the cleavage of Notch receptors. Previously, Foldi *et al* reported that the activation of Notch signaling was initiated through the downstream signaling of TLR4 in LPS-stimulated macrophages, mainly through the MAPK and NF-κB pathways and autoamplified by Jagged1 (33). The differences in ligand usage to initiate Notch activation between LPS-stimulated *vs*. LPS/IC-stimulated macrophages may result in transduction of difference signals that affect difference cellular responses. Recent report confirmed that difference Notch ligands (Dll1 and Dll4) sends pulsatile or sustained signaling dynamics via the same Notch receptor and induces opposing cell fate during embryogenesis (34). The surface expression of Dll4 was reduced in LPS/IC-stimulated macrophages, possibly due to the internalization of the protein after ligand/receptor ligation. In this study, Notch1 was investigated, but we found that the level of Notch2 also increased upon stimulation (Fig 2A). Therefore, multiple Notch receptors may be involved in the regulation of M(IC).

The production of IL-10 in macrophages stimulated with TLR was regulated primarily by the MAPK and NF-κB signaling pathways (10). In the presence of ICs, which trigger the signaling downstream of FcγRs, signals generated by ICs and LPS cooperate to enhance the production of IL-10 (35). The level of IL-10 produced was affected to varying degrees when specific inhibitors (MEK1/2 MAPKs, p38 MAPK, NF-κB IκB kinase, PI3K and Notch signaling were used, indicating that each signal contributes differentially to controlling IL-10 production in macrophages activated by LPS with ICs. The limitation of this study using inhibitors is the undesirable off-target effect. Detailed characterization of the contribution of each pathway may be required to confirm this observation.

We further investigated the role of NF-κB in macrophages that were activated by LPS and ICs because NF-κB signaling was reported to control IL-10 production in macrophages stimulated with LPS (10,36). A previous study demonstrated the role of NF-κB in supporting MAPK signaling in LPS-induced IL-10 production in macrophages. Furthermore, various DNaseI hypersensitive sites (HSs) containing NF-κB binding motifs were identified in the *Il10* locus (-55/-46), emphasizing the important role of NF-κB in the direct regulation of IL-10 production in various cell types, including macrophages (16,36). Among the NF-κB subunits, NF-κB1/p50 is the most abundantly found subunit in tumor-associated macrophages that plays a critical role in suppressing anti-tumor responses by decreasing IL-12 production and increasing IL-10 level (37). In contrast to other NF-κB subunits, p50/p50 homodimer often functions as transcriptional repressor. In LPS-activated macrophages, however, p50 homodimer forms a complex with CREB-binding protein and activates transcription of *Il10* (36). In line with this report, macrophages from $p50^{-/-}$ mice is highly susceptible to LPS-induced sepsis (35). These data all indicate that NF-κB p50 plays a critical role in regulating IL-10 production in macrophages. Our results added another piece of evidences that Notch signaling acts together with NF-κB p50 to regulate *il10* expression in M(IC).

The transcriptomic analysis revealed that genes involved in the cell cycle were consistently downregulated upon GSI treatment. Previously, it was reported that FcγR crosslinking induces cell cycle progression through the ERK pathway (38). In addition, the transcriptomic study by Fleming *et al*. revealed that in the cluster of genes in regulatory macrophages stimulated with LPS and ICs, prostaglandin E2 (PGE2) and adenosine are associated with increased cell growth and proliferation. Accordingly, it was postulated that regulatory macrophages may contribute to homeostasis and promote cellular repair (9). Therefore, in M(IC), Notch signaling may play an important role in regulating cell growth and proliferation.

When plasticity of M(IC) was tested, we discovered that the duration of the first and the second stimulation is crucial for reversing activated phenotypes. Shorter duration was unable to yield the phenotypic changes while longer duration (3 days) allows cells to recover and changes the phenotype from M(LPS) to M(IC). Upon LPS exposure, macrophages acutely express genes related to inflammation and the responses is turned off after the removal of the stimuli. Repeated LPS exposure renders cells unresponsive to LPS, the so called LPS tolerance which is part of innate memory (39). The short duration of resting resulted in decreasing activation of immediate signaling cascade, in particular MAPK p38 and NF-κB p65 while longer duration recovered the activation. Epigenetic modifications including histone modifications play crucial role in regulating the LPS tolerance phenotypes (40). Using ChIP-seq approach, we compared genome wide profiles of active histone mark, H3K4me3. H3K4me3 is strictly associated with promoters of actively transcribed genes. Modifications are mediated by histone methyltransferases and histone demethylases (41). In resting macrophages, promoters of the TLR responsive genes are readily modified by H3K4me3, consistent with the results obtained in our ChIP-seq data from unstimulated macrophages (7). Interestingly, M(IC) showed distinct H3K4me3 profile, compared to that of resting or M(LPS). When unique upregulated or downregulated genes in M(IC) were examined, clear increased H3K4me3 was visible in Il10 promoters and cis-regulatory elements, while other genes showed similar H3K4me3 pattern as M(LPS). This result indicated that the expression of IL-10 in M(IC) is mainly regulated by epigenetic modification, especially H3K4me3. This evidence added to the previous report that histone phosphorylation is involved in *Il10* mRNA transcription in M(IC) (31).

Finally, in an attempt to understand the impact of M(IC) in vivo, we performed an adoptive transfer of M(IC) in systemic inflammatory condition of LPS-induced endotoxemia. Adoptive transfer of M(IC) reduced serum level of IL-12p70 and IL-1β at 1 or 6 hr after LPS administration. The levels of IL-10 and other cytokines were not altered. This result indicated that M(IC) may interfere with systemic LPS response beyond the production of IL-10. Adoptive transfer of M(IC) reduced sepsis-associated lethality in mice (9). How M(IC) globally reduced some inflammatory cytokines is not known.

In conclusion, we demonstrated that Notch signaling plays a key role in regulating the phenotypes of M(IC). In particular, Notch signaling regulates IL-10 production in M(IC) by regulating NF-κB p50 nuclear localization. Plasticity of M(LPS) to M(IC) was observed only when the duration between the two stimulation were longer than 3 days. When active histone mark, H3K4me3, was examined, the promoter and the cis-regulatory elements of *Il10* was clearly enriched in M(IC), compared to M(LPS). Adoptive transfer of M(IC) in endotoxemia model reduced inflammatory cytokines, IL-12p70 and IL-1β. The results obtained in this study helps to further understand the molecular mechanisms of macrophage activation and epigenomic regulation in the presence of immune complexes and may shed new light on the systemic impact of M(IC) *in vivo*.

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Output ที่ได้จากโครงการ

1. ผลงานตีพิมพ์ในวารสารวิชาการระดับนานาชาติจำนวน 3 เรื่อง ได้แก่

   (1) Wongchana W, Lawlor RG, Osborne BA, **Palaga, T***. Impact of Notch1 Deletion in Macrophages on Proinflammatory Cytokine Production and the Outcome of Experimental Autoimmune Encephalomyelitis. (2015) *J Immunol*. 195, 5337-5346. (IF=4.539)

   (2) **Palaga T***, Wongchana W, Kueanjinda P. Notch Signaling in Macrophages in the Context of Cancer Immunity. (2018) *Front Immunol*. 9, 652 (58). (IF=5.551)

   (3) Wongchana W, Kongkavitoon P, Tangtanatakul P, Sittplangkoon C, Butta P, Chawalitpong S, Pattarakankul T, Osborne BA, **Palaga T***. Notch signaling regulates the responses of lipopolysaccharide-stimulated macrophages in the presence of immune complexes. (2018) *PLoS One*, 13(6), e0198609. (IF=2.766)

   ผลงานตีพิมพ์ที่ได้จัดส่งและระหว่างรอผลการพิจารณาและผลงานที่กำลังเตรียมเพื่อส่งตีพิมพ์จำนวน 2 เรื่อง ได้แก่

   (1) Sangphech N, Srisawasdi S, **Palaga T***. Notch signaling modulates peroxisome proliferator-activated receptor gamma level in Interleukin 4-stimulated human macrophages through AKT and NEDD4L. (Submitted to *J. Biol. Chem*.)

   (2) Ruenjaiman V, Leu YW, Pongpanich M, Butta P, **Palaga T***. Epigenetic modification in LPS/immune complex-stimulated macrophages. (in preparation)

2. เป็นวิทยากรรับเชิญเพื่อบรรยายในงานประชุมวิชาการ Research Innovation Symposium in Life Science and Technology ในหัวข้อ IL-10 Production in Immune Complex-Stimulated Macrophages จัดโดย Tokyo Institute of Technology ณ กรุงโตเกียว ประเทศญี่ปุ่น ระหว่างวันที่ 8 มีนาคม พ.ศ. 2560 ถึงวันที่ 11 มีนาคม พ.ศ. 2560
3. เป็นวิทยากรรับเชิญเพื่อบรรยายแก่ National Cheng Kung University Medical College ในหัวข้อ Epigenomics in Immune Cells and Cancer Cells ณ เมืองไทนัน ประเทศไต้หวัน ระหว่างวันที่ 15 มีนาคม พ.ศ. 2561 ถึงวันที่ 16 มีนาคม พ.ศ. 2561

4. เข้าร่วมเสนอผลงานใน Gordon Research Conference การประชุม Notch Signaling in Development, Regeneration and Disease ณ เมือง Lewiston มลรัฐ Maine ประเทศสหรัฐอเมริกา ตั้งแต่วันที่ 31 เดือน กรกฎาคม พ.ศ. 2559 ถึงวันที่ 5 เดือน สิงหาคม พ.ศ. 2559 โดยนำเสนอในรูปแบบปากเปล่า หัวข้อ Notch Signaling Plays a Role in Regulating IL-10 Production in Immune Complex-Stimulated Macrophages
5. เข้าร่วมเสนอผลงานในการประชุม International Congress of Immunology 2016 (ICI2016) ณ เมือง Melbourne ประเทศออสเตรเลีย ระหว่าง 21 สิงหาคม พ.ศ. 2559 ถึงวันที่ 26 สิงหาคม พ.ศ. 2559 โดยนำเสนอในรูปแบบปากเปล่า หัวข้อ Notch Signaling Plays a Role in Regulating IL-10 Production in Immune Complex-Stimulated Macrophages
6. เข้าร่วมเสนอผลงานในการประชุม Japanese Society of Immunology The 46th Annual Meeting ณ เมือง Sendai จังหวัด Miyagi ประเทศ ญี่ปุ่น ระหว่าง 12 ธันวาคม พ.ศ. 2560 ถึงวันที่ 14 ธันวาคม พ.ศ. 2560 โดยนำเสนอในรูปแบบปากเปล่า หัวข้อ Notch Signaling Regulates Macrophages Activation by LPS in the Presence of Immune Complex
7. นิสิตที่สำเร็จการศึกษาระดับปริญญาเอกจำนวน 3 ราย

   7.1) นายปฏิภาค เขื่อนจินดา (หลักสูตรจุลชีววิทยาทางการแพทย์ บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย ปีการศึกษา 2557)

   7.2) นางสาววิภาวี วงศ์ชนะ (หลักสูตรเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปีการศึกษา 2558)

   7.3) นางสาวสุพัตรา ชวลิตพงษ์ (หลักสูตรเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปีการศึกษา 2560)
8. นิสิตที่สำเร็จการศึกษาระดับปริญญาโทจำนวน 3 ราย

   8.1) นางสาวศิรดา อารมณ์ชื่น (หลักสูตรจุลชีววิทยาและเทคโนโลยีจุลินทรีย์ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปีการศึกษา 2559)

   8.2) นางสาวปรียาพรรณ ศรีพลอย (หลักสูตรจุลชีววิทยาและเทคโนโลยีจุลินทรีย์ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปีการศึกษา 2559)

   8.3) นางสาวอรพรรณ ตั้งวิเชียร (หลักสูตรจุลชีววิทยาและเทคโนโลยีจุลินทรีย์ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปีการศึกษา 2559)

ภาคผนวก

1. Reprint จำนวน 3 เรื่อง
2. Submitted manuscript จำนวน 1 เรื่อง
3. บทคัดย่อการไปเสนอผลงานในงานประชุมวิชาการ Gordon Research Conference
4. บทคัดย่อการไปเสนอผลงานในงานประชุมวิชาการ International Congress of Immunology 2016 (ICI2016)
5. บทคัดย่อการไปเสนอผลงานในงานประชุมวิชาการ Japanese Society of Immunology The 46th Annual Meeting

The Journal of Immunology

Impact of Notch1 Deletion in Macrophages on Proinflammatory Cytokine Production and the Outcome of Experimental Autoimmune Encephalomyelitis

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Impact of Notch1 Deletion in Macrophages on Proinflammatory Cytokine Production and the Outcome of Experimental Autoimmune Encephalomyelitis

Wipawee Wongchana,[*] Rebecca G. Lawlor,[†] Barbara A. Osborne,[†,‡] and Tanapat Palaga[§,¶]

Notch signaling is involved in regulating TLR-mediated responses in activated macrophages. In this study, we investigated the impact of Notch signaling in macrophages in an experimental autoimmune encephalomyelitis (EAE) model. To examine the impact of deficiency in Notch signaling in activated macrophages in EAE, an adoptive transfer of activated macrophages derived from *Notch1*$^{fl/fl}$ × *Mx1cre*$^{+/-}$ (Notch1 knockout [N1KO]) or *CSL/Rbp-jκ*$^{fl/fl}$ × *Mx1cre*$^{+/-}$ (CSL/RBP-Jκ KO) mice was performed prior to induction of EAE. Mice receiving activated N1KO macrophages showed decreased severity of EAE compared with mice receiving wild-type or CSL/RBP-Jκ KO macrophages. In vitro restimulation of splenocytes by myelin oligodendrocyte glycoprotein 35–55 peptide from these mice revealed that cells from mice receiving N1KO macrophages produced significantly less IL-17 compared with the control mice, whereas IFN-γ production was similar in both groups. We found that activated N1KO, but not CSL/RBP-Jκ KO, macrophages produced less IL-6 and had lower CD80 expression compared with wild-type and did not exhibit any defect in IL-12p40/70 production, whereas activated macrophages from CSL/RBP-Jκ KO mice phenocopied γ-secretase inhibitor treatment for reduced IL-12p40/70 production. Furthermore, the nuclear translocation of the NF-κB subunit c-Rel was compromised in γ-secretase inhibitor–treated and CSL/RBP-Jκ KO but not N1KO macrophages. These results suggest that Notch1 and CSL/RBP-Jκ in macrophages may affect the severity of EAE differently, possibly through modulating IL-6 and CD80 expression, which is involved in the Th17 but not Th1 response. *The Journal of Immunology*, 2015, 195: 5337–5346.

Macrophages are innate immune cells that bridge innate and adaptive immune responses. Signaling through IFN-γ receptors, together with TLRs, activates macrophages, resulting in enhanced phagocytic activity and the production of cytokines. This activation also leads to increased expression of costimulatory molecules that enables macrophages to present peptide Ags to activate Th cells. Furthermore, activated macrophages produce various types of pro- and anti-inflammatory cytokines, such as TNF-α, IL-6, IL-10, and IL-12, that are involved in the activation and differentiation of many cell types, including CD4$^+$ Th cells.

The Notch signaling pathway regulates differentiation, proliferation, survival, and cell fate decisions in both myeloid and lymphoid lineage cells (1). There are four mammalian Notch receptors (Notch1–4) and five ligands (Delta-like 1, 3, and 4 and Jagged 1 and 2). The interaction between Notch ligands and receptors induces the enzymatic cleavage of the Notch receptors, first by an ADAM protease and subsequently by γ-secretase, resulting in the release of the Notch intracellular domain. The intracellular domain of Notch then translocates to the nucleus and forms a complex with the DNA-binding protein CSL/RBP-Jκ, which initiates the transcription of the Notch target genes (2). Notch1 is the best studied Notch receptor, and it has been shown to play critical roles in regulating the effector function of immune cells and to be involved in diseases such as cancer and autoimmune disease (3–6).

The dysregulation of cytokine production and hyperactivation of macrophages are linked to many inflammation-related diseases such as sepsis. Additionally, autoimmune diseases such as rheumatoid arthritis (7) are also associated with TLR-activated macrophages. Moreover, it has been shown that the Notch signaling pathway cooperates with TLR signaling in macrophages under pathological conditions that can lead to autoimmune diseases, systemic lupus erythematosus in particular (6).

The progression and severity of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, is well known to be mediated by both Th1 and Th17 CD4$^+$ T cells. Recently, several studies have shown that not only autoreactive T cells but also other factors such as pro- and anti-inflammatory cytokines, chemokines, and costimulatory molecules, which are

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; EAE, experimental autoimmune encephalomyelitis; GSI, γ-secretase inhibitor; KO, knockout; MFI, mean fluorescence intensity; MOG, myelin oligodendrocyte glycoprotein; N1KO, Notch1 knockout; WT, wild-type.

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produced by other cells types, play crucial roles in sustaining the disease (8–12).

Several reports have elucidated the roles of Notch signaling in the EAE model, especially in T cells. Using a γ-secretase inhibitor (GSI) incorporated into rodent chow (LY chow) to inhibit Notch signaling in vivo, it was demonstrated clearly that Notch signaling regulates Th1 and Th17 responses in the EAE model. A reduction in the severity of EAE-induced inflammation in GSI-treated animals was observed, as were decreases in the signature Th1 (IFN-γ) and Th17 (IL-17) cytokines in restimulated cultures of activated T cells in vitro (13, 14). Additionally, the Notch ligand, Delta-like 4, present on APCs, including macrophages, interacts with Notch receptors on Ag-specific T cells and regulates the trafficking and accumulation of T cells in the CNS (15). These results are similar to a recent study that showed that the induction of EAE in mice overexpressing a dominant-negative form of Mastermind-like 1 in CD4$^+$ T cells or in mice with a targeted deletion of CSL/RBP-Jκ in myelin-reactive T cells was prevented in >95% of the animals when compared with the controls (16).

Macrophages also play important roles in EAE. Observed EAE severity was reduced upon removal of infiltrating macrophages in the CNS (17) by deleting *IL-4R* in macrophages (18) and by transferring immune complex/LPS-activated macrophages into mice in which EAE had been induced (19). Furthermore, it has been shown that the expression of Delta-like 4 on macrophages is important in the development of EAE (15). However, the impact on EAE of signaling initiated by Notch1 in activated macrophages has not been documented.

Recently, several studies have reported on the involvement of Notch signaling in the regulation of cytokines such as IL-10, IL-6, and IL-12 in TLR-activated macrophages (20, 21). We previously reported that Notch1 directly regulates the *Il6* promoter and its expression in IFN-γ/LPS–activated macrophages (22). Furthermore, using GSI to inhibit the activation of Notch signaling in macrophages, we observed decreased IL-12p40/70 protein, correlating with defects in activation of the MAPK signaling pathway and c-Rel nuclear translocation (23). IL-12, a proinflammatory cytokine, is produced mainly by dendritic cells and activated macrophages. IL-12p70, a biologically active form of IL-12, is composed of two subunits (p40 and p35) and is essential for driving type I immune response (24, 25). IL-12 plays an essential role in Th type 1 cell differentiation (26). The regulation of *Il12p40* expression requires various transcription factors, including the NF-κB–, c-Rel-, and p50-containing complex (27). The NF-κB/Rel family promotes the transcriptional induction of *Il12p40* during the activation of macrophages but is not involved in nucleosome remodeling of the *Il12p40* promoter (28). The regulation of the *Il12p40* promoter by histone deacetylation (HDAC3) during transcription in macrophages has been observed. Furthermore, the anti-inflammatory cytokine IL-10 plays an antagonistic role by controlling the hyperinflammatory conditions (29). However, the role of Notch1 and CSL/RBP-Jκ in regulating IL-12p40/70 production in macrophages has not been clearly investigated.

In this study, we first investigated the impact of adoptive transfer of Notch1 or CSL/RBP-Jκ–deficient macrophages in an EAE model and determined the requirement of Notch1 and CSL/RBP-Jκ in TLR-activated macrophages for the regulation of IL-12p40/70 expression.

Materials and Methods

Animals

Wild-type (WT) C57BL/6 mice were purchased from Harlan Laboratories (South Easton, MA) or the National Laboratory Animal Center, Mahidol University (Salaya, Thailand). All transgenic mice used in this study were purchased from The Jackson Laboratory (Bar Harbor, ME). Notch1 knockout (N1KO) and CSL/RBP-Jκ KO mice were generated by breeding *Notch1*$^{fl/fl}$ (*Notch1*$^{\mathrm{tm2Rko/GridJ}}$) or *Rbp-jκ*$^{fl/fl}$ (*Rbpj*$^{\mathrm{tm1Hon}}$) mice to *mx1Cre*$^{+/-}$ [B6.Cg-Tg(Mx1cre)1Cgn/J] mice. To conditionally delete *Notch1* or *CSL/RBP-Jκ*, female mice with the genotype of *Notch1*$^{fl/fl}$ × *Mx1 cre*$^{+/-}$ (N1KO), *Notch1*$^{fl/fl}$ × *Mx1 cre*$^{-/-}$ mice (control), *Rbp-jκ*$^{fl/fl}$ × *Mx1 cre*$^{+/-}$ mice (CSL/RBP-Jκ KO), and *Rbp-jκ*$^{fl/fl}$ X *Mx1 cre*$^{-/-}$ mice (control) were injected with 12–15 μg/g body weight of poly(I:C) (Imgenex, San Diego, CA) every other day for 5 d. Animals were rested for 3 wk prior to sacrifice and use in experiments. 2D2 TCR transgenic mice [C57BL/6-Tg(Tcra2D2, Tcrb2D2)1Kuch/J] were maintained by breeding hemizygous mice to WT C57BL/6 mice. Female mice aged 7–12 wk were used for all experiments. Ten-week-old animals were used for EAE induction. All animals were housed in animal facilities according to the guidelines approved by the Institutional Animal Care and Use Committees at the University of Massachusetts at Amherst and Chulalongkorn University.

Generation of bone marrow–derived macrophages

Bone marrow cells from femur cavities were flushed and incubated in DMEM (Lonza, Walkersville, MD, USA) supplemented with 10% FBS (Life Technologies, Grand Island, NY), HEPES (Lonza), sodium pyruvate (Lonza), streptomycin/penicillin G (Lonza), 5% (v/v) horse serum (Thermo Scientific), and 20% (v/v) L929-conditioned media. Fresh medium was added to the culture at day 4. Cells were harvested on day 7 using cold PBS. Cell surface staining with anti-F4/80– Alexa Fluor 488 and CD11b–Alexa fluro 647 Abs (BioLegend, San Diego, CA) was used to confirm a macrophage phenotype (Supplemental Figs. 1A, 2B). The obtained bone marrow–derived macrophages (BMDMs) were cultured in DMEM complete media without horse serum and L929-conditioned media before activation.

Cell culture and activation of BMDMs

BMDMs were primed overnight with recombinant murine IFN-γ (10 ng/ml) (BioLegend) and washed twice with medium and PBS. *Salmonella* LPS (100 ng/ml) (Sigma-Aldrich, St. Louis, MO) was added to activate the macrophages for the indicated times. In some experiments, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (25 μM) or DMSO (0.01%) (Calbiochem, San Diego, CA) was used to pretreat the macrophages before activation.

Intracellular staining and cell surface staining

The BMDMs were activated as described above. For intracellular staining, brefeldin A (for IL-12p40/70 and IL-6 detection) or monensin (for IL-10 and GM-CSF detection) was added at the beginning of the activation by LPS. The cells were pretreated with Fc Block (BD Biosciences), followed by surface staining and fixation/permeabilization using a BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Anti-mouse F4/80–Alexa Fluor 488 (BioLegend), anti-mouse IL-12p40/70–PE (BD Biosciences and BioLgend), anti-mouse IL-10–allophycocyanin (BioLegend), anti-mouse CD206-PE (BioLegend), and anti-mouse GM-CSF–PE (BioLegend) were used. Anti-mouse Notch1-PE (clone N1A) was used to stain for Notch1 using the FoxP3 Staining Buffer Set (eBioscience, San Diego, CA) according to the manufacturer's instructions. In some experiments, anti-mouse CD80, anti-mouse CD86, anti-mouse MHC class II (BD Pharmingen), anti-mouse PD-L1 (BioLegend), and anti-mouse CD4 (BD Biosciences) Abs were used for cell surface staining. The cells were acquired on a FACS LSR II (Becton Dickinson) or Cytomics FC 500 MPL (Beckman Coulter) and analyzed with FlowJo software (FlowJo, Ashland, OR).

ELISA

Culture supernatants from the BMDMs treated as described were harvested at 6 or 24 h after stimulation. Secreted IL-12p70 levels were detected using an IL-12p40/70 ELISA (BD Pharmingen). For some experiments, supernatants were collected and subjected to detection for IL-17 (BD Biosciences), IFN-γ (BD Biosciences), IL-10 (BD Biosciences), IL-2 (BD Biosciences), and GM-CSF (BioLegend) by ELISA according to the manufacturers' instructions.

Immunofluorescent staining

Cells were cultured in eight-well chamber slides and activated as indicated. After washing with PBS, the cells were fixed with 4% paraformaldehyde and incubated with a rabbit anti–c-Rel polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA), followed by an anti-rabbit IgG [H+L (Fab′)$_2$

fragment]–PE (Cell Signaling Technology, Danvers, MA). The cells were observed under an inverted fluorescent microscope or a confocal microscope.

Real-time PCR

BMDMs were activated as above for 4 h. Total RNA was isolated using an RNAqueous kit (Ambion, Austin, TX). cDNA was synthesized, and transcripts were amplified by a quantitative PCR Stratagene Mx3000P. Primer sequences were as follows: *Il23p19*, 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*, forward, 5′-AAC CTC ACC TGT GAC ACG CC-3′, reverse, 5′-CAA GTC CAT GTT TCT TTG CAC C-3′; *arginase 1*, forward, 5′-CAG AAG AAT GGA AGA GTC AG-3′, reverse, 5′-CAG ATA TGC AGG GAG TCA CC-3′; and *β-actin*, forward, 5′-ACC AAC TGG GAC GAC ATG GAG AA-3′, 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.

Adoptive transfer of activated macrophages and EAE disease score evaluation

BMDMs obtained from control, N1KO, and CSL/RBP-Jκ mice were activated as described above for 30 min. The cells were washed three times using warm PBS and the cell number was adjusted to 2×10^6 cells in 200 μl PBS. Two hundred microliters activated BMDMs or PBS was injected i.p. into naive C57BL/6 mice. After 4 h, EAE induction emulsion Hooke Kits (Hooke Laboratories, Lawrence, MA) were administered into the flanks of the animals according to the manufacturer's instructions. Pertussis toxin was injected i.p. at both 2 and 24 h after immunization. The progression and the severity of EAE were monitored and scored from 0 to 5 as follows: 0, no disease; 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hind- and forelimb paralysis; 5, morbidity and death. The data are reported as the mean daily clinical score (14). The mice that received WT and N1KO macrophages were euthanized during the peak of the disease (days 15–16 postimmunization) and their spleens were collected. Splenocytes were cultured at 37°C with medium alone or with different concentrations of myelin oligodendrocyte glycoprotein (MOG)$_{35–55}$ Ag (Hooke Laboratories) for 3 d. The culture supernatants from the restimulated splenocytes were evaluated by ELISA for the detection of IL-17 and IFN-γ as described above. In some experiments, the EAE-induced mice were monitored for 27–28 d. GM-CSF was detected from restimulated splenocytes by ELISA and intracellular cytokine staining as described above.

Coculture of T cells and macrophages

CD4$^+$ T cells were negatively isolated from the spleen of 2D2 TCR transgenic mice by a mouse CD4 T lymphocyte enrichment set–DM (BD Biosciences) according to the manufacturer's instructions. WT and N1KO BMDMs (2×10^5 cells) were plated in 48-well plates and activated as described above for 2 h. CD4$^+$ T cells (5×10^5 cells) were added to each well in RPMI 1640 (Hyclone) supplemented with 10% FCS (Life Technologies), HEPES (Lonza), penicillin/streptomycin (Lonza), and 2-ME (Sigma-Aldrich) in the presence of 150 μg/ml MOG$_{35–55}$ peptide. Five days following the primary stimulation, cells were washed and 5×10^5 CD4$^+$ T cells were stimulated in 48-well plates coated with 1 μg/ml anti-CD3 Ab and incubated for 24 h. Culture supernatants from secondary stimulation conditions were collected and IL-17, IFN-γ, IL-10, and IL-12 were detected by ELISA.

Tracking macrophages after adoptive transfer

To observe trafficking of BMDMs after adoptive transfer, 2×10^6 BMDMs from WT and N1KO mice were labeled using a CellTrace CFSE cell proliferation kit (Invitrogen) according to the manufacturer's instructions for adherent cells overnight before activation and following EAE induction. Four hours after the first pertussis toxin injection, cells from fluids of peritoneal cavity, spleens, omentum, and lymph nodes were collected. Omentum and lymph nodes were digested by collagenase type IV (Invitrogen). Cells were stained and gated on the CD11b$^+$ population.

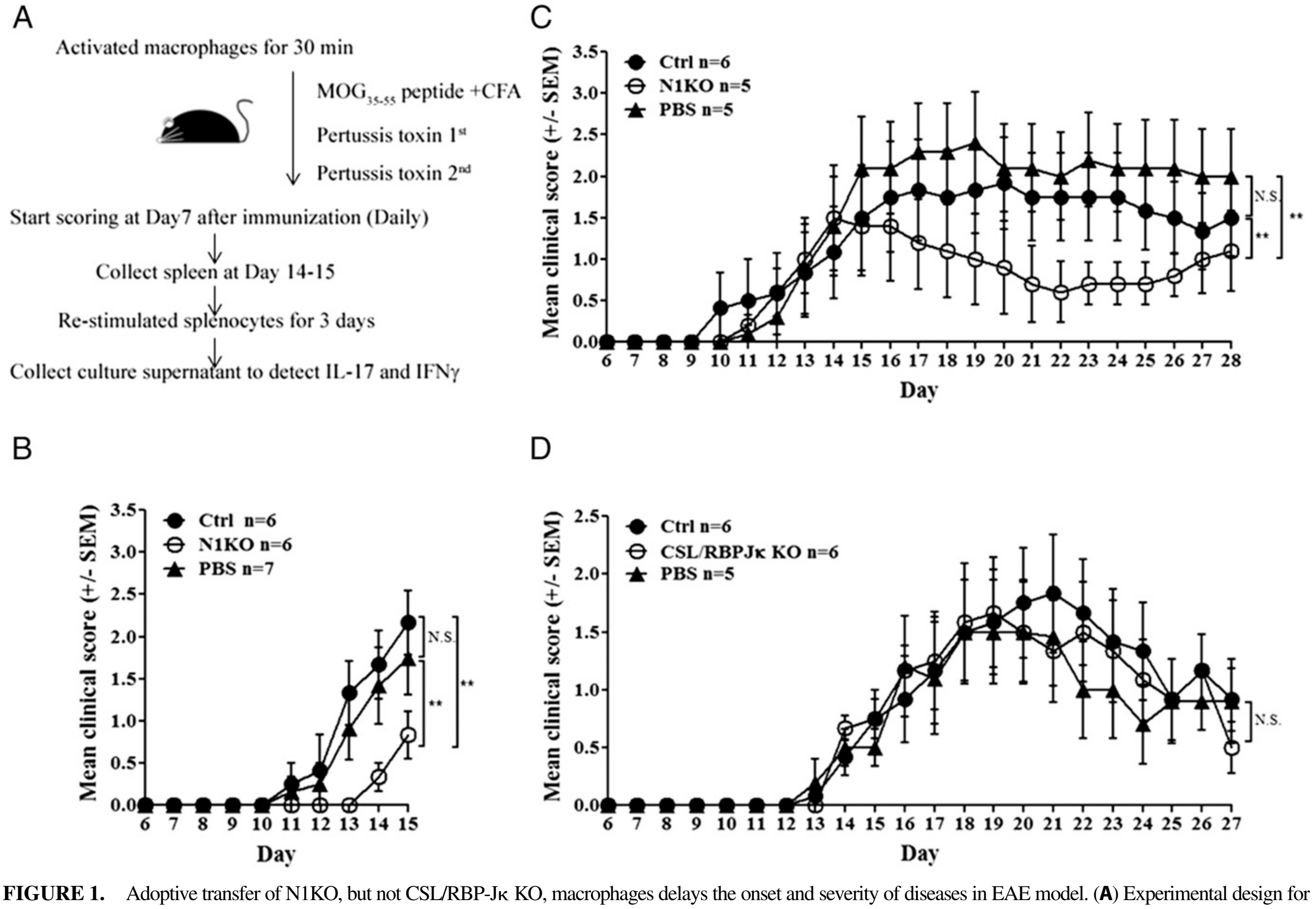

FIGURE 1. Adoptive transfer of N1KO, but not CSL/RBP-Jκ KO, macrophages delays the onset and severity of diseases in EAE model. (**A**) Experimental design for BMDM transfer in EAE mice. (**B**) Mean clinical scores in EAE mice receiving WT BMDMs, N1KO BMDMs, or PBS for 15 d. $^{**}p < 0.05$. (**C** and **D**) Mean clinical scores in EAE mice receiving WT BMDMs, N1KO BMDMs, CSL/RBP-Jκ KO BMDMs, or PBS. The clinical scores were monitored for 27–28 d. $^{**}p < 0.05$.

Statistical analysis

Statistical analyses were performed using SPSS version 15.0 and GraphPad Prism version 5.0. A one-way ANOVA ($\alpha = 0.05$) was used when comparing two conditions.

Results

Adoptive transfer of activated macrophages lacking Notch1, but not CSL/RBP-Jκ, decreases severity and delays onset of diseases in the EAE model

Macrophages have been identified as one of the effector cells that play a critical role in EAE (17). The adoptive transfer of macrophages activated with LPS with an immune complex that predominantly produces IL-10 in an EAE model resulted in delaying onset of disease and decreasing disease severity (19). Notch signaling is involved in the activation of macrophages and regulates the production of cytokines such as IL-6, IL-12p70, and IL-10 (20–23, 30), which are produced during EAE inflammation. Furthermore, the involvement of Notch signaling in regulating disease severity in the EAE model by using GSI was demonstrated (13, 14). For these reasons, we hypothesized that Notch1 in macrophages may play a crucial role in regulating disease outcome in the EAE model. BMDMs that were generated from conditional N1KO and control WT mice were used in this study. *CSL/RBP-Jκ*, a gene encoding a DNA-binding protein that plays a central role in the canonical Notch signaling pathway, was also deleted in macrophages and used in the transfer experiment. The expression of two macrophage markers, CD11b and F4/80, were equivalent in the N1KO, CSL/RBP-Jκ, and WT control BMDMs, suggesting that the loss of *Notch1* or *CSL/RBP-Jκ* did not interfere with macrophage differentiation (Supplemental Figs. 1A, 2B). The loss of expression of Notch1 or CSL/RBP-Jκ in the N1KO or CSL/RBP-Jκ KO macrophages was confirmed by Western blot (Supplemental Figs. 1B, 2C) and flow cytometry (Supplemental Fig. 1C, 1D). BMDMs from the WT or conditional KO mice were activated by IFN-γ/LPS for 30 min in vitro before adoptive transfer by i.p. injection into the WT recipients 4 h prior to EAE induction (Fig. 1A). We observed a significant delay in the onset of the disease and a decrease in disease severity in the animals that received activated N1KO macrophages, compared with those receiving activated WT macrophages or PBS control (Fig. 1B, 1C). In contrast, no difference in the onset or the severity of diseases was found between animals receiving CSL/RBP-Jκ KO macrophages and those of the control (Fig. 1D). These data indicated that Notch1 expression in transferred macrophages influences EAE outcome but the expression of CSL/RBP-Jκ in macrophages is dispensable for this effect.

Because Notch signaling in macrophages is required for the optimal production of IL-12p40/70 and IL-6 and *Il23p19* mRNA, which are involved in Th cell polarization, and because EAE is a Th1/Th17-driven autoimmune disease, we hypothesized that Notch1 in macrophages could influence the response of Th1 and/or Th17 in EAE by affecting IFN-γ and IL-17 production. Splenocytes from mice that received an adoptive transfer of N1KO macrophages and EAE induction were restimulated with the $\mathrm{MOG}_{35–55}$ peptides in vitro and the levels of IL-17 and IFN-γ were measured in the culture supernatant by ELISA. We found that the level of IL-17 decreased significantly in the $\mathrm{MOG}_{35–55}$ peptide–stimulated splenocytes from animals that received activated N1KO macrophages, compared with those from the control mice (Fig. 2A). Surprisingly, IFN-γ levels were not different between the two groups (Fig. 2B). Recent evidence pointed to the pathogenic role of GM-CSF–producing $\mathrm{CD4}^+$ T cells in autoimmunity (31, 32). In the restimulation assay with $\mathrm{MOG}_{35–55}$ peptides of splenocytes from mice receiving control or N1KO macrophages, no differences were found in the percentages of $\mathrm{CD4}^+$ T cells with GM-CSF or secreted GM-CSF (Supplemental Fig. 3A, 3B).

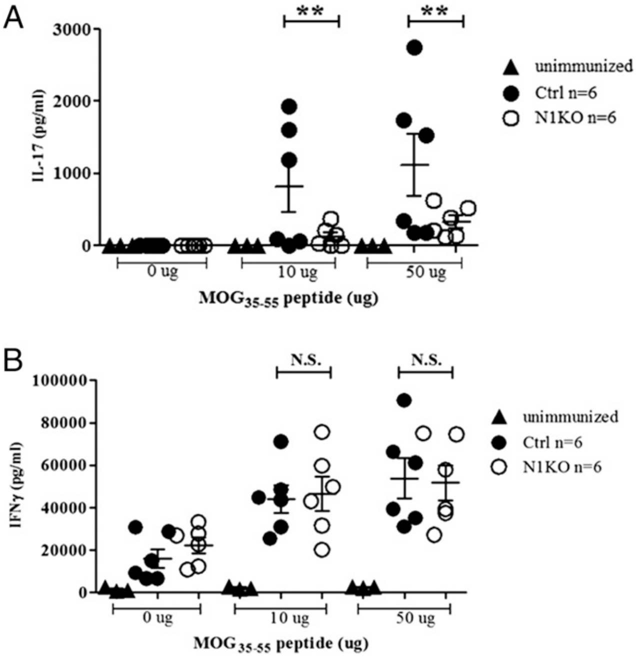

FIGURE 2. Adoptive transfer of N1KO macrophages affects Th cell response in restimulation assay. Splenocytes from animals ($n = 6$/group) were collected after 15 d of EAE induction and restimulated by using $\mathrm{MOG}_{35–55}$ peptide (10 and 50 μg/ml) and cultured for 3 d. The supernatants were then measured for IL-17 (**A**) and IFN-γ (**B**) by ELISA. $^{**}p < 0.05$.

To address whether transferred macrophages trafficked differently, we detected the appearance of macrophages labeled with fluorescent dye upon transfer for 10 h. As shown in Supplemental Fig. 3C, most transferred macrophages were found to migrate to omentum or to remain in the peritoneal fluids. More importantly, no significant differences in the appearance of transferred macrophages in tested tissues were found between control and N1KO macrophages. These data imply that the transfer of Notch1-deficient activated macrophages affects the onset and progression of EAE, possibly through its influence on the activation of a Th17-type but not a Th1-type response, and CSL/RBP-Jκ in activated macrophages is dispensable for this effect.

Notch1 is dispensable for IL-12p40/70 production in IFN-γ/LPS–activated BMDMs

Notch signaling has been shown to regulate IL-12p40/70 production, and Notch1 is especially important for the regulation *Il12p35* (*Il12a*) and *p40* (*Il12b*) mRNA expression in LPS-activated macrophages (30). Based on the results obtained in this study in the EAE model, we asked specifically whether Notch1 is important for IL-12p40/70 production in IFN-γ/LPS–activated macrophages. BMDMs that were generated from conditional N1KO and WT mice were activated with IFN-γ and LPS, and IL-12p40/70 was detected by intracellular cytokine staining. Surprisingly, there was no significant difference in either the percentage of IL-12p40/70^+ cells or the mean fluorescence intensity (MFI) between N1KO BMDMs and the control BMDMs upon activation with IFN-γ/LPS (Fig. 3A, 3B). Moreover, there was no significant difference in the level of IL-12p40/70 production detected in the culture supernatants by ELISA from IFN-γ/LPS–activated N1KO BMDMs compared with those from the control BMDMs at any time points tested (Fig. 3C). Next, we examined whether activity of the γ-secretase in N1KO macrophages could be involved in IL-12p40/70

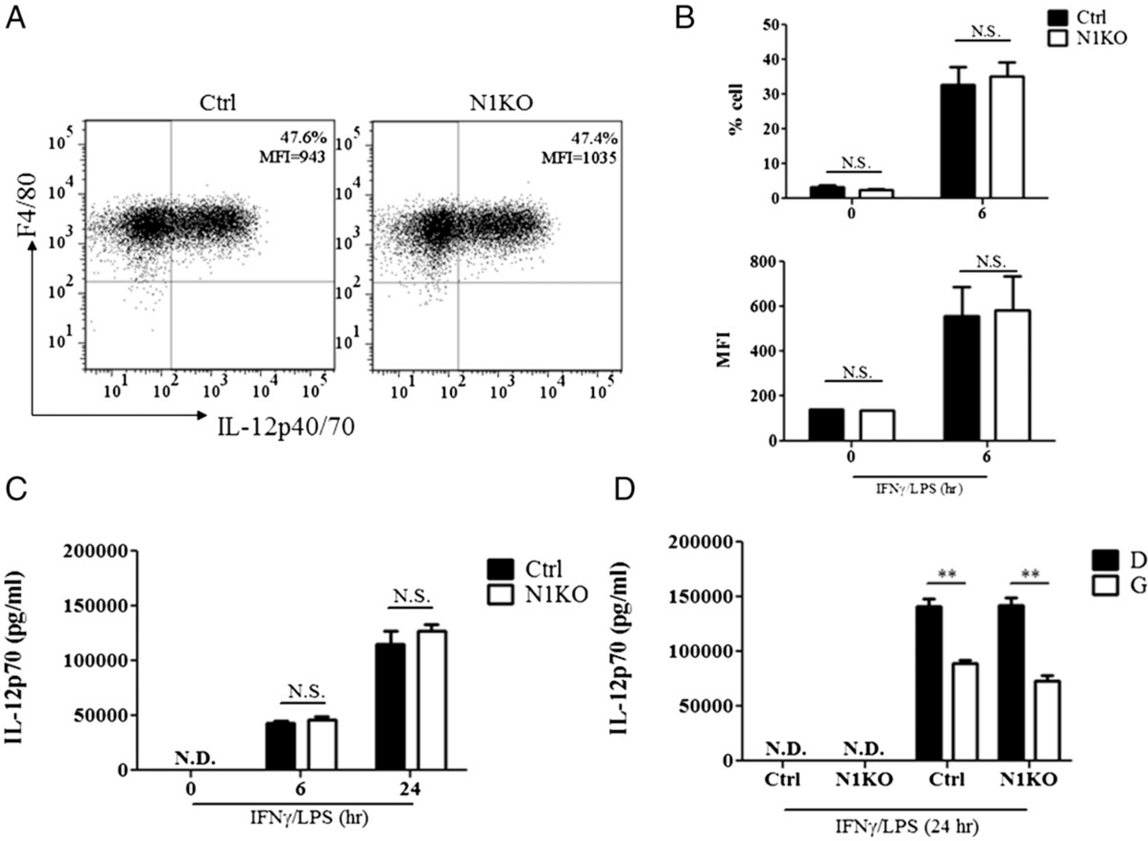

FIGURE 3. Notch1 is dispensable for IL-12p40/70 production in IFN-γ/LPS–activated BMDMs. (**A**) BMDMs from WT control or N1KO mice were activated with IFN-γ/LPS for 6 h, and the level of IL-12p40/70 was measured by intracellular cytokine staining. (**B**) The percentages of F4/80$^+$/IL-12p40/70$^+$ cells and MFI for IL-12p40/70 in F4/80$^+$/IL-12p40/70$^+$ population in each condition described in (A) is shown ($n = 3$–4 animals/group). (**C**) The amount of IL-12p40/70 production was determined in the culture supernatants at the indicated time points by ELISA. (**D**) WT and N1KO BMDMs were stimulated with IFN-γ/LPS for 24 h in the presence of vehicle control DMSO or GSI (25 μM). The amount of IL-12p40/70 in the culture supernatants was measured by ELISA. All data are representative of three independent experiments. $^{**}p < 0.05$. N.D., not detectable.

production in IFN-γ/LPS–activated N1KO BMDMs using GSI treatment. The reduction of cleaved Notch1 in GSI-treated WT BMDMs was confirmed by Western blot (Supplemental Fig. 2A). We found that the levels of IL-12p40/70 were similarly reduced upon GSI treatment in both N1KO and the control BMDMs, consistent with our previous results (Fig. 3D). These results implied that Notch1 is dispensable for optimal IL-12p40/70 production in IFN-γ/LPS–activated macrophages, and other γ-secretase substrates, perhaps other Notch receptors, may be essential for this regulation.

Production of IL-12p40/70 in macrophages partially depends on the canonical Notch signaling pathway

Because γ-secretase has other target substrates besides the Notch receptors, the involvement of Notch in regulating IL-12p40 production cannot be addressed by the use of GSI. To address whether canonical Notch signaling is important for IL-12p40/70 production elicited by IFN-γ and LPS stimulation, BMDMs from conditional CSL/RBP-Jκ KO mice and control mice were used and the results were compared with GSI treatment in WT BMDMs. The production of IL-12p40/70 in the WT BMDMs that were treated with GSI showed a significant reduction in both the MFI and the percentage of IL-12p40/70$^+$ cells (Fig. 4A), compared with the vehicle control–treated BMDMs, consistent with the previous report (23). A similar trend in the reduction in IL-12p40/70 was observed in IFN-γ/LPS–activated CSL/RBP-Jκ KO BMDMs (Fig. 4B–D). These results strongly suggest that the activity of γ-secretase and the canonical Notch signaling via CSL/RBP-Jκ are required for the optimal production of IL-12p40/70 in macrophages.

Defect in c-Rel nuclear translocation in IFN-γ/LPS–activated macrophages requires CSL/RBP-Jκ but not Notch1

The production of IL-12p40/70 in activated BMDMs was shown to be partially c-Rel–dependent (33). In our previous study, we found that GSI treatment in WT BMDMs affected the nuclear translocation of c-Rel upon stimulation with IFN-γ/LPS (23). The activation of BMDMs from CSL/RBP-Jκ KO mice showed a reduction in c-Rel nuclear accumulation as detected by immunofluorescence staining whereas BMDMs from N1KO mice showed intense nuclear localization of c-Rel upon activation similar to those from the WT mice (Fig. 5). The pattern of c-Rel nuclear localization correlated well with the level of IL-12p40/70 production, which implied that the production of IL-12p40/70 in IFN-γ/LPS–activated BMDMs requires canonical Notch signaling, but not Notch1, in a c-Rel–dependent manner.

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

Based on the phenotypic results from the mice in the EAE experiments described above and from in vitro restimulation with the MOG peptides, we hypothesized that other cytokines or costimulatory molecules that were produced or expressed by activated N1KO macrophages could be compromised, resulting in decreased IL-17 production in an EAE setting. We investigated IL-6 and IL-10 production and *Il23p19 mRNA* expression in N1KO

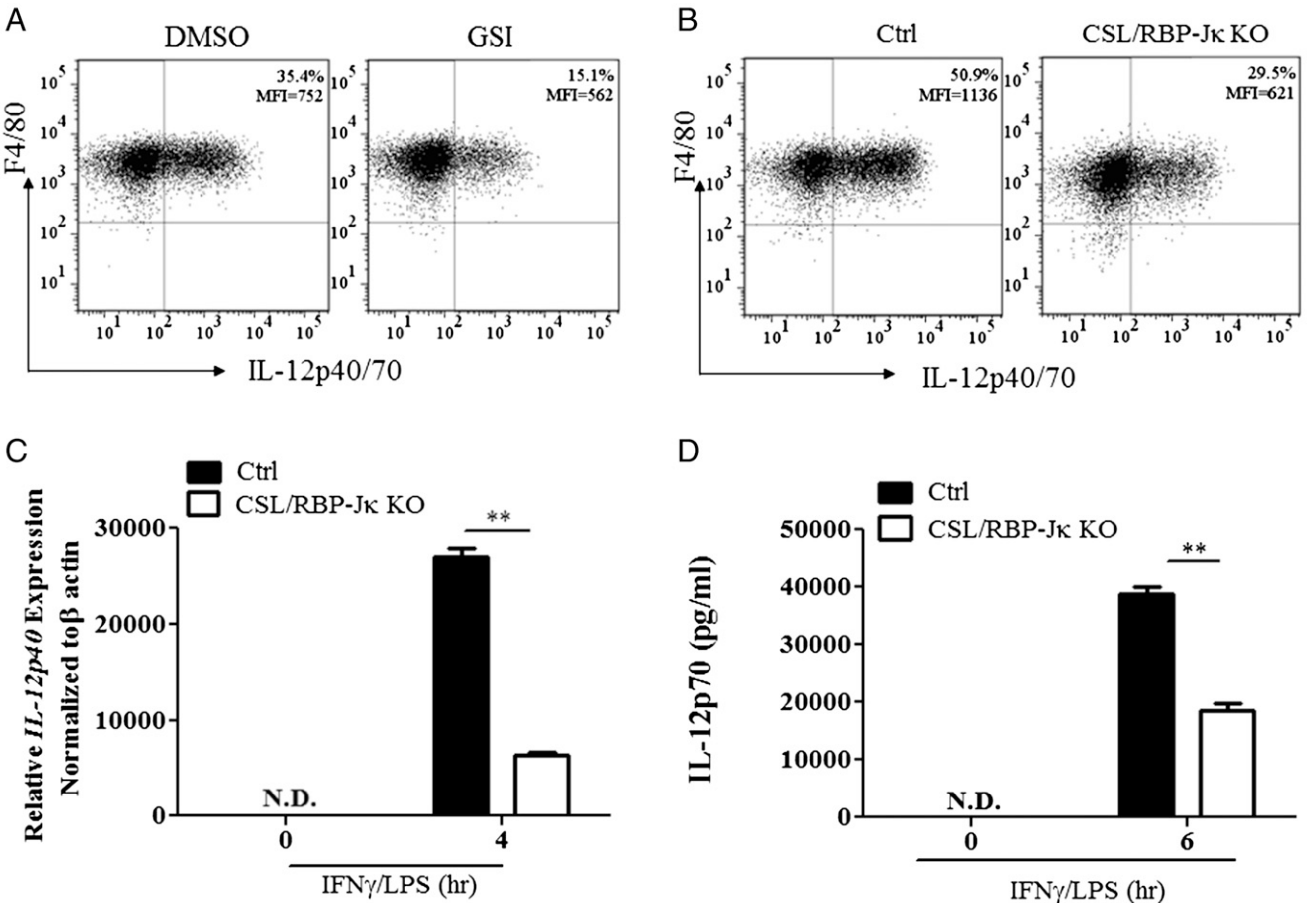

FIGURE 4. Effects of GSI treatment and targeted deletion of *CSL/RBP-Jκ* on IL-12p40/70 production in IFN-γ/LPS–activated BMDMs. (**A**) BMDMs were stimulated with IFN-γ/LPS for 6 h in the presence of vehicle control, DMSO, or GSI (25 μM). Expression of IL-12p40/70 was measured by intracellular cytokine staining. (**B**) BMDMs from WT control or CSL/RBP-Jκ KO mice were activated with IFN-γ/LPS for 6 h. The level of IL-12p40/70 was measured by intracellular cytokine staining. (**C**) BMDMs from WT control or N1KO mice were stimulated with IFN-γ/LPS for 4 h, and the expression *Il12p40* mRNA was determined by quantitative PCR. (**D**) The level of IL-12p40/70 production was detected in the culture supernatants of BMDMs treated as described in (B) for 6 h by ELISA. $^{**}p < 0.05$. N.D., not detectable.

BMDMs upon activation, all of which have been shown to play roles in EAE and to be involved in regulatory T cell/Th17 polarization (34, 35). As shown in Fig. 6A, a significant reduction in the percentages of IL-6–producing cells was observed upon activation, which is consistent with our previous report that Notch signaling partially regulates IL-6 (21, 22). However, no difference in IL-10 production and *Il23p19* mRNA expression was found between the N1KO and WT BMDMs (Fig. 6B, 6C). We further determined the expression of costimulatory molecules that had been reported to be important in the EAE model and that are involved in T cell activation (19). Among the cell surface molecules tested, the level of a costimulatory molecule, CD80, was found to be reduced in the N1KO BMDMs (Fig. 6D).

Because transferring of RBP-Jκ KO macrophages did not affect the severity of EAE as observed in transfer of N1KO macrophages, we hypothesized that those molecules that are affected by Notch1 deficiency and play a key role in EAE severity should remain unaffected in RBP-Jκ KO macrophages. Indeed, as shown in Fig. 6E, the percentages of IL-6$^+$ cells are similar in WT and RBP-Jκ KO macrophages. Additionally, the expression of CD86 and MHC class II increased whereas the level of CD80 was lower in RBP-Jκ KO macrophages (Fig. 6F).

To further investigate whether N1KO macrophages dictate immune response of Th cells, an in vitro coculture of activated macrophages and MOG$_{35–55}$-specific TCR transgenic CD4$^+$ T cells was performed. As shown in Supplemental Fig. 4A–C, coculture of naive CD4$^+$ T cells with IFN-γ/LPS–activated N1KO macrophages in the presence of MOG peptide resulted in reduced IFN-γ and IL-10 production from T cells in the secondary stimulation with plate-bound anti-CD3 Ab. IL-17 was undetectable in the culture supernatant of stimulated T cells in all conditions (data not shown). Because polarization of the Th17 response requires several cytokines, including TGF-β, IL-23, together with IL-6, it is likely that an in vitro condition may be insufficient to polarize CD4$^+$ T cells to Th17. This result indicates that Notch1 expression in activated macrophages plays an important role in dictating the optimal CD4$^+$ T cell response. It remains to be investigated whether N1KO macrophages affect other aspects of T cell response such as migration.

Taken together, these data suggest that a reduction in IL-17 in MOG$_{35–55}$-restimulated splenocytes from the mice that received activated N1KO macrophages together with the reduction in the costimulatory molecule, CD80, and IL-6 in N1KO macrophages may interfere with the optimal immune response overall and culminate in reducing the onset and progression of EAE.

Discussion

We used an animal model of EAE to evaluate the impact of Notch1 and CSL/RBP-Jκ deficiency in macrophages on the severity of this autoimmune inflammatory condition and showed that CSL/RBP-Jκ

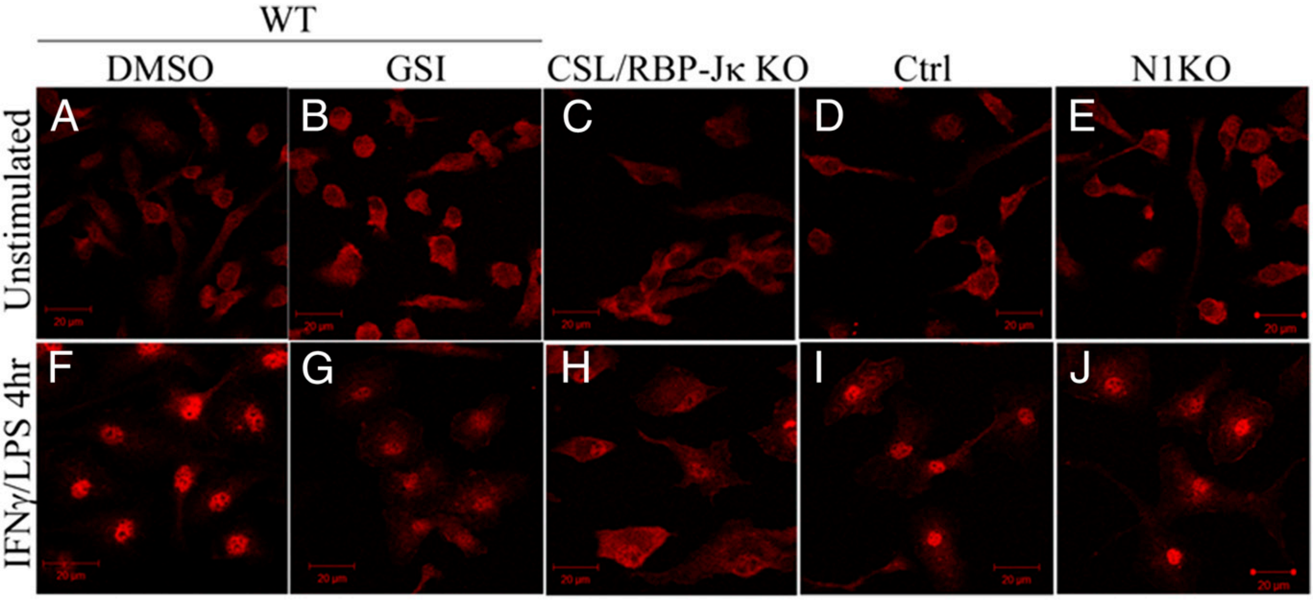

FIGURE 5. Reduction of c-Rel nuclear translocation was detected in GSI treatment and CSL/RBP-Jκ KO but not in N1KO macrophages. BMDMs obtained from WT mice were pretreated with vehicle control DMSO (**A** and **F**) or GSI (25 μM) (**B** and **G**) before stimulation with IFN-γ/LPS for 4 h. BMDMs from CSL/RBP-Jκ KO mice (**C** and **H**), WT control (**D** and **I**), and Notch1 KO mice (**E** and **J**) were left untreated or stimulated with IFN-γ/LPS for 4 h. Localization of c-Rel was detected using immunofluorescence staining. The data are representative of at least two independent experiments. Scale bars, 20 μm.

and the activity of γ-secretase, but not Notch1, are partially involved in the production of the proinflammatory cytokine IL-12p40/70 in IFN-γ/LPS–stimulated macrophages. In contrast, IL-6 production was compromised only in N1KO macrophages. Mice that were adoptively transferred with activated N1KO, but not CSL/RBP-Jκ KO, macrophages showed a partial reduction in the onset and progression of EAE. We also found that IL-17 but not IFN-γ production by MOG peptide–stimulated splenocytes was compromised in mice receiving activated N1KO macrophages.

Many reports have shown that Th1 and Th17 play important but distinct roles in this disease model. IL-12 is important for Th1 differentiation whereas IL-23 is essential for maintaining the Th17 phenotype (35). In our in vitro experiments, a deficiency in Notch1 did not have any effect on the expression of IL-12p70 and *Il23p19* mRNA in macrophages, but a reduction in IL-6 and CD80 was detected. Previous data have shown that IL-23 is not essential for Th17 differentiation, whereas IL-6 and TGF-β are involved in this process (36), and the blockade of IL-6 can impair Th17 differentiation (37). In the EAE model, it has been demonstrated that IL-6 is important in this autoinflammatory disease; for example, mice that are defective in IL-6 production show a resistance phenotype to MOG peptide–induced EAE. Signaling through the A2B adenosine receptor in APCs such as dendritic cells enhances IL-6 production and its blockade helps improve the EAE phenotype (12, 38). Taken together, these data strongly implicate that IL-6 is important for Th17 cell differentiation and plays pivotal roles in EAE. We and others have previously shown that Notch1 is involved in IL-6 production in activated macrophages (20, 22). These observations, together with the findings in this study, suggest that Notch1 in activated macrophages might play an important role in regulating the outcome of T cell responses by regulating IL-6 (Th17) but not IL-12 (Th1) in the EAE model.

Coculture of activated macrophages with $CD4^+$ T cells in the presence of specific Ags in vitro can skew $CD4^+$ T cells toward Th1 or Th2, depending on the macrophage stimuli (39). In humans, LPS or LPS/IFN-γ–activated macrophages skew Th cells toward Th17 or Th1, respectively (40). In our study, coculture of LPS/IFN-γ–activated N1KO macrophages with MOG-specific TCR transgenic $CD4^+$ T cells resulted in decreased IFN-γ and IL-10 production whereas the similar level of IL-2 was detected. More importantly, IL-17 was undetectable in this setting. Because we observed decreased IL-17 but not IFN-γ production in a MOG-restimulation assay of splenocytes from mice receiving N1KO macrophages, it is possible that in an in vivo system, transferred N1KO macrophages may influence Th cell responses by more complicated mechanisms. Therefore, it remains to be determined how Notch1 in activated macrophages is involved in determining Th1 or Th17 responses.

In the present study, endogenous macrophages were not depleted before the transfer of activated macrophages for the EAE study. Previously, a similar adoptive transfer strategy using macrophages activated by LPS and an immune complex were reported in an EAE model. This type of macrophage produced high levels of IL-10, and the decrease in disease severity was attributed to production of this anti-inflammatory cytokine (19). Because the level of IL-10 produced by N1KO macrophages was similar to that of the WT macrophages, it is unlikely that IL-10 is responsible for the decreased disease severity in our study.

Activated N1KO macrophages expressed less of the costimulatory molecule CD80. It is not clear at present how Notch signaling regulates CD80 expression. Costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) interact with CD28 on T cells; together with signaling through MHC–TCR, such interactions are important for T cell activation. Blockade of CD80 has been reported to suppress EAE (11, 41). Furthermore, blocking CD28 results in a reduction of the CD80/CD86 ratio in APCs and decreases EAE severity (10). In another autoinflammatory disease such as rheumatoid arthritis, CD80 is more important for Th1 cell differentiation than Th17 cells (42), whereas our data indicate that Notch1 in macrophages seems to have no impact on the Th1-type response in the EAE model. It is possible that macrophage and T cell crosstalk was interrupted during disease induction, in addition to the roles of cytokines that were produced by transferred macrophages on T cell polarization. Because N1KO macrophages express less IL-6 and CD80, these defects together may have led to decreased T cell activation/differentiation and may have resulted

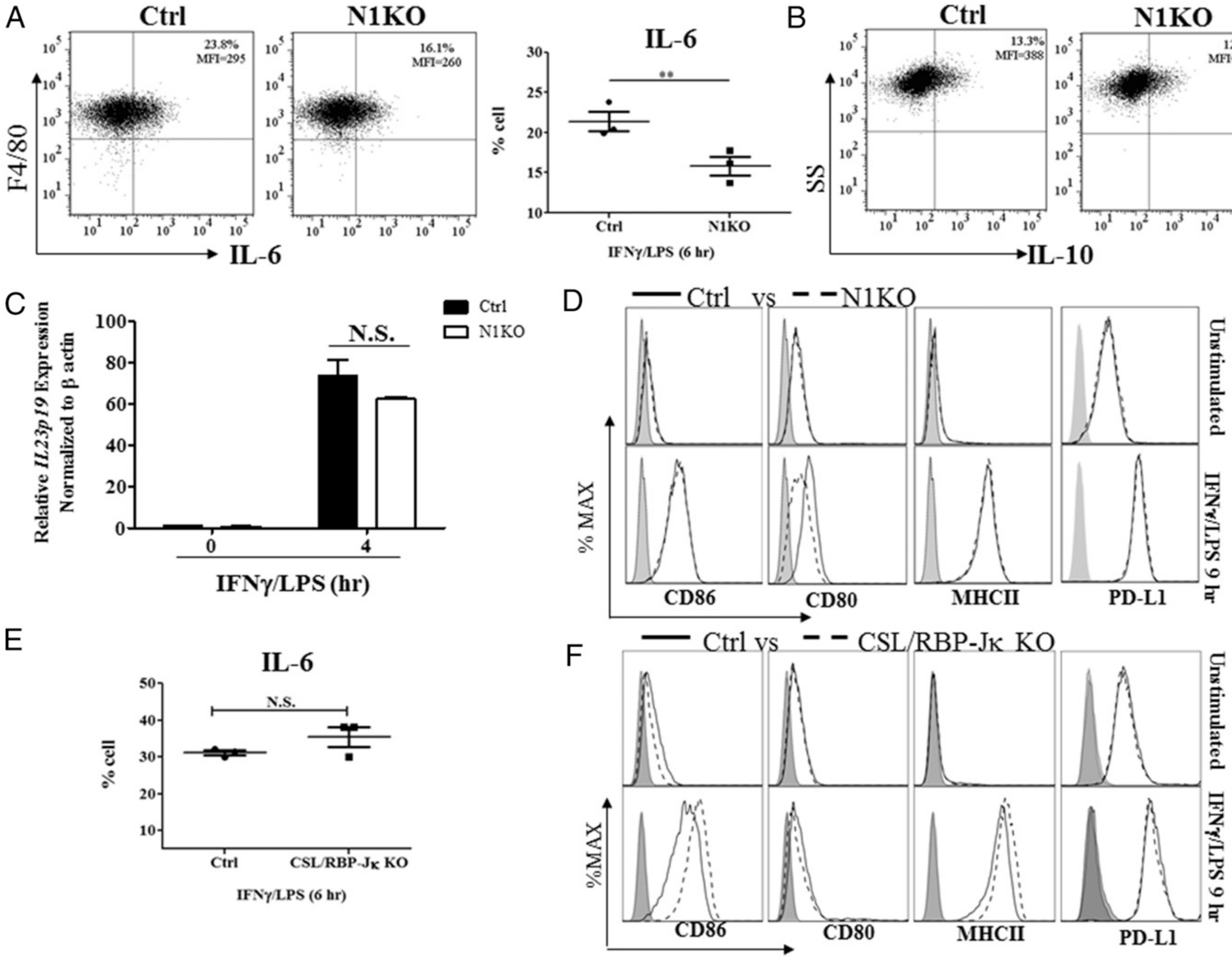

FIGURE 6. Reduced IL-6 production and CD80 expression in activated N1KO macrophages. (**A**) BMDMs from WT control or N1KO mice were stimulated with IFN-γ/LPS for 6 h, and the production of IL-6 was detected by intracellular cytokine staining. (**B**) BMDMs from WT control or N1KO mice were stimulated with IFN-γ/LPS for 6 h, and the production of IL-10 was detected by intracellular cytokine staining. (**C**) BMDMs from WT control or N1KO mice were stimulated with IFN-γ/LPS for 4 h, and the expression *Il23p19* mRNA was determined by quantitative PCR. (**D**) BMDMs treated as described in (A) for 9 h were assayed for CD86, CD80, MHC class II, and PD-L1 by flow cytometry. All data are representative of at least two independent experiments. (**E**) BMDMs from WT control or CSL/RBP-Jκ KO mice were stimulated with IFN-γ/LPS for 6 h, and the production of IL-6 was detected by intracellular cytokine staining. The percentages of IL-6^+ cells are shown (n = 3 animals/group). (**F**) BMDMs from WT control and CSL/RBP-Jκ KO mice treated as described in (A) for 9 h were assayed for CD86, CD80, MHC class II, and PD-L1 by flow cytometry. All data are representative of at least two independent experiments. $^{**}p < 0.05$. MHCII, MHC class II.

in decreased disease severity in our study. Additionally, N1KO macrophages may interfere with the activity of the endogenous macrophages, which may influence the outcome of the disease.

Understanding behavior of transferred macrophages in vivo will be a key piece of the puzzle to gain how adoptive transfer of N1KO macrophages reduces EAE severity. To this end, we performed a preliminary study tracking transferred macrophages in lymph nodes, omentum, peritoneal exudates, and spleen (Supplemental Fig. 3C). Transferred macrophages were found mainly in the omentum at 10 h after transferring but no difference was found between WT and N1KO macrophages. Further detailed analysis to pinpoint where the transferred macrophages migrate needs further investigation.

Previous in vitro studies have demonstrated that activation of macrophages by IFN-γ and LPS triggers cleavage of Notch receptors and the activation of Notch signaling; Notch signaling, in turn, directly or indirectly regulates production of proinflammatory cytokines such as IL-6 and IL-12p40/70. Inhibition of Notch signaling with a pharmacological inhibitor, GSI, which blocks γ-secretase activity, resulted in a reduction in IL-12p40/70. Our in vitro study revealed that the deletion of *Notch1* in macrophages did not show any detectable effect on IL-12p40/70 production after activation by IFN-γ and LPS. These results imply that other Notch receptors such as Notch2, which is also highly expressed on macrophages, may play a redundant role in regulating IL-12p40 expression when *Notch1* is deleted (21). In fact, we found a reduction in IL-12p40 in GSI-treated N1KO macrophages, which implies that other Notch receptors may compensate for the loss of Notch1. Xu et al. (30), however, reported that macrophages from *Notch1*$^{+/-}$ mice expressed less *Il12p40* than did the control WT macrophages upon LPS stimulation, and silencing *Notch2* in these macrophages did not further reduce the level of *Il12p40* mRNA, suggesting a dispensable role for Notch2. The discrepancy on the effect of *Notch1* deletion on IL-12p40 expression between our results from N1KO macrophages and Xu et al., who used macrophages from *Notch1* haploinsufficient mice, may be due to different approaches in generating the *Notch1* deletion and in the use of LPS in their study versus LPS with IFN-γ in our system.

Unexpectedly, adoptive transfer of activated CSL/RBP-Jκ KO macrophages did not have any impact on the onset or severity of EAE. By comparing the cytokine production (IL-6 and IL-12p40/70) or costimulatory molecule (CD80, CD86) expression between N1KO and CSL/RBP-Jκ KO macrophages, it was interesting to note that only the amount of IL-6 produced by macrophages upon in vitro activation correlates with the disease severity.

CSL/RBP-Jκ can act as a transcriptional repressor in the absence of cleaved Notch receptors or as a transcriptional activator upon forming a multiple protein complex with Notch receptors (43). The transcriptional activation places this protein at the center of "canonical" Notch signaling where Notch receptors are cleaved upon ligand engagement. Recent evidence also emerged that suggests a noncanonical and nonnuclear mechanisms of Notch signaling, both of which are independent of the transcriptional activity of CSL/RBP-Jκ (44). Interestingly, targeted deletion of CSL/RBP-Jκ in some instances spontaneously increases the expression of genes that are actively repressed by CSL/RBP-Jκ (45). In tumor-associated macrophages, loss of canonical Notch signaling confers M2-like phenotypes (46). In our study, we found that unstimulated CSL/RBP-Jκ KO macrophages showed mixed phenotypes, as higher mRNA level of *arginase 1* was observed, but not CD206 (Supplemental Fig. 4D, 4E). Because transfer of CSL/RBP-Jκ KO macrophages did not protect animals from EAE, it is unlikely that the switch from M1 to M2 plays a key role in our EAE study.

We observed a reduction in IL-12p40/70 upon the deletion of *CSL/RBP-Jκ* in macrophages, similar to the GSI treatment. However, the level of IL-12p40/70 production upon GSI treatment and deletion of CSL/RBP-Jκ showed only a partial reduction compared with the control, suggesting that the Notch signaling pathway may coordinate with other pathways such as NF-κB, C/EBP-β, and AP-1 in controlling the optimal production of IL-12p40/70 in IFN-γ/LPS–activated BMDMs (24). Because GSI treatment blocks both canonical and noncanonical Notch signaling, and because deletion of CSL/RBP-Jκ affects only canonical Notch signaling, it is possible that GSI treatment and deletion of CSL/RBP-Jκ may yield different outcomes. For IL-12p40/70 expression, a similar reduction was obtained from both approaches, suggesting that canonical Notch signaling may be responsible for IL-12p40/70 expression, whereas Notch1 may have a redundant role in this function. Furthermore, the expression of IL-6 may be regulated by noncanonical Notch signaling because only Notch1 deletion and GSI treatment, but not deletion of CSL/RBP-Jκ, affect its production (22).

NF-κB signaling and c-Rel, in particular, possibly as a dimer with p50 subunit, is required for *Il12p40* transcription in macrophages (27). Indeed, we observed that *CSL/RBP*-Jκ KO macrophages stimulated with IFN-γ/LPS exhibited reduced c-Rel nuclear accumulation, compared with the control WT macrophages. This result is consistent with that observed in the GSI-treated macrophages (23). In contrast, the activated N1KO macrophages showed a similar c-Rel pattern as the control macrophages. How CSL/RBP-Jκ and the activity of γ-secretase regulate the nuclear translocation of c-Rel upon LPS/ IFN-γ treatment needs further investigation. In contrast, Xu et al. (30) reported in their system that activation of *CSL/RBP*-Jκ KO macrophages by LPS alone did not have any effect on the activation of the NF-κB signaling pathway. In their study, canonical Notch/RBP-Jκ signaling induced the expression of a transcription factor, IFN regulatory factor 8, which acts as a regulator of genes involved in the polarization of proinflammatory macrophages. A detailed mechanism is proposed in which CSL/RBP-Jκ selectively enhances IL-1R–associated kinase 2–dependent signaling via TLR4 to the MNK kinase. This event leads to downstream translation/initiation of control through eIF4E. However, IFN regulatory factor 8 can be regulated by IFN-γ (47), and the discrepancy between the effect of CSL/RBP-Jκ deletion on the activation of NF-κB between our study and that by Xu et al. is possibly due to the difference in stimuli used, that is, LPS versus LPS/IFN-γ.

In T cells, Notch signaling functions to augment NF-κB signaling by facilitating 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 (48). In our study, a similar mechanism may operate in driving c-Rel nuclear retention in macrophages. This possibility needs further investigation.

Our study has reported a novel role for Notch1 in macrophages in EAE upon transfer into WT mice and for the Notch signaling pathway in regulating c-Rel activation and IL-12p40/70 expression in macrophages upon LPS/IFN-γ treatment. These findings indicate that Notch signaling in macrophages is important for the development of EAE and may have therapeutic implication for autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.

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REVIEW
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Notch Signaling in Macrophages in the Context of Cancer Immunity

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Macrophages play both tumor-suppressing and tumor-promoting roles depending on the microenvironment. Tumor-associated macrophages (TAMs) are often associated with poor prognosis in most, but not all cancer. Understanding how macrophages become TAMs and how TAMs interact with tumor cells and shape the outcome of cancer is one of the key areas of interest in cancer therapy research. Notch signaling is involved in macrophage activation and its effector functions. Notch signaling has been indicated to play roles in the regulation of macrophage activation in pro-inflammatory and wound-healing processes. Recent evidence points to the involvement of canonical Notch signaling in the differentiation of TAMs in a breast cancer model. On the other hand, hyperactivation of Notch signaling specifically in macrophages in tumors mass has been shown to suppress tumor growth in an animal model of cancer. Investigations into how Notch signaling is regulated in TAMs and translates into pro- or anti-tumor functions are still largely in their infancy. Therefore, in this review, we summarize the current understanding of the conflicting roles of Notch signaling in regulating the effector function of macrophages and the involvement of Notch signaling in TAM differentiation and function. Furthermore, how Notch signaling in TAMs affects the tumor microenvironment is reviewed. Finally, the direct or indirect cross-talk among TAMs, tumor cells and other cells in the tumor microenvironment *via* Notch signaling is discussed along with the possibility of its clinical application. Investigations into Notch signaling in macrophages may lead to a more effective way for immune intervention in the treatment of cancer in the future.

Keywords: Notch signaling, macrophages, tumor-associated macrophages, metastasis, tumor immunity

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INTRODUCTION

The biological functions of macrophages are diverse and not only limited to their role as the first line of defense during innate immune response. In addition to their protective role against infections, the known roles of macrophages have expanded in recent years, and their involvement in organ development, tissue homeostasis, and metabolic dysfunctions, such as diabetes and obesity, are increasingly appreciated. Cancer is another area in which macrophages have emerged as a crucial player in the creation of a tumor microenvironment that supports tumor growth and metastasis, in opposition to their traditional role as an innate immune cell, whose function is to eliminate cancer cells (1). Therefore, understanding the signaling pathway(s) governing the development, differentiation, activation, deactivation, proliferation, and cell death of macrophages in the context of tumorigenesis is expected to reveal novel strategies for targeting cancer growth more effectively.

The critical functions of the evolutionarily well-conserved Notch signaling pathway in myeloid lineage cell development and, in particular, monocyte/macrophage development are well recognized and have been reviewed extensively elsewhere (2, 3). Recent evidence, using state of the art technologies, revealed better defined subsets of circulating monocytes and the uniqueness and the origin of tissue-resident macrophages (TRMs). This new insight reignited the excitement in the field of macrophage biology. In addition, these studies cast new light and controversy over the origin of macrophages found in tumors, called tumor-associated macrophages (TAMs), and the involvement of TAMs in cancer progression and suppression (4, 5). Within tumors of various origins, macrophages have been observed to accumulate in large numbers and exhibit unique combinations of activated phenotypes (6). In general, TAMs in large quantities are associated with poor disease prognosis, partly by promoting tumor growth, dampening immune responses, and inducing angiogenesis and metastasis (7, 8). Together with the recent advances in the understanding of the roles, Notch signaling plays in the activation and regulation of the immune effector functions of macrophages and in TAMs, these observations have led to the conclusion that Notch signaling is one of the candidate pathways to be manipulated to enhance the host anti-tumor response. In this review, we summarize the current knowledge of the involvement of Notch signaling in macrophage activation, with an emphasis on its role(s) in TAMs. We also discuss the cross-talk among macrophages, tumor cells, and other cells associated with the tumor microenvironment and the potential utility and challenges in manipulating Notch signaling in TAMs for tumor suppression in ways that are beneficial to the host.

Notch Signaling in Macrophage Activation and Function

The biological functions of macrophages are multi-faceted depending on the external microenvironment, and some functions may be contradictory or opposing to others. For example, during infection or tissue injury, macrophages sense danger *via* various receptors, actively eliminate the source of danger by phagocytosis and chemical mediators, and trigger inflammation by producing inflammatory cytokines to alert other immune cells. After the elimination phase, wounds are healed mainly by anti-inflammatory wound-healing macrophages (9). The contradictory inflammatory and anti-inflammatory microenvironments are conducive to driving macrophage activation into two opposite functional spectra. The most simplistic view of macrophage effector functions divides activated macrophages into pro-inflammatory macrophages, in which macrophages are activated by pathogen-associated molecular patterns (PAMPs) and/or inflammatory cytokines. In contrast, anti-inflammatory macrophages, activated by IL-4/IL-13, represent a wound-healing and immunosuppressive phenotype (10). However, more detailed characterization and studies in various *in vivo* models have revealed a more complicated view of macrophage effector phenotypes that are often observed in an *in vivo* setting (11). Thus, the narrow concept of pro- vs. anti-inflammatory macrophages may be oversimplified, and the presence of various hybrid phenotypes of macrophages has been described (11). Some of the genes uniquely expressed in pro- or anti-inflammatory macrophages are summarized in **Table 1** (12, 13).

To avoid oversimplification and confusion over macrophage effector phenotypes, this review will adopt the macrophage nomenclatures proposed by Murray et al. to describe specific macrophage subsets based on the stimuli and effector functions described in each referred study (19). In some instances, where the stimuli were not identified, the microenvironments in which macrophages were described will be used.

Initial reports generally found that Notch signaling primarily operates in macrophages that are activated toward inflammatory functions such as in lipopolysaccharide (LPS)-activated macrophages M(LPS) or LPS in combination with IFNγ M(LPS + IFNγ) (15, 20, 21). Subsequent findings in various pathophysiological conditions also indicated the involvement of Notch signaling in activation and effector functions of pro-inflammatory macrophages (3). Notch signaling, therefore, favors inflammatory macrophages, and when the Notch signaling pathway is pharmacologically or genetically blocked, some of the key pro-inflammatory functions are compromised, including the decrease in the production of pro-inflammatory cytokines, such as IL-6, and the reduction in nitric oxide production (15, 22). To this end, Notch signaling is reported to directly or indirectly influence pro-inflammatory effector functions. Notch signaling can directly regulate transcription of some of the inflammation-induced signature genes, such as *il6*, *il12b,* and *nos2* (23–25). Using *Rbpj*-deficient mice, Xu et al. demonstrated that canonical Notch signaling tips the effector phenotypes toward inflammatory ones by directly influencing the transcription of a transcription factor IRF8 (22). In addition, Notch signaling also indirectly regulates pro-inflammatory phenotypes through a cross-talk with other signaling pathways, such as NF-κB and mitogen-activated protein kinases (15, 20). Interestingly, metabolic analysis found that Notch signaling supports inflammatory macrophage phenotypes by reprograming mitochondrial metabolism toward oxidative phosphorylation (25). Abrogating Notch signaling in myeloid lineage cells attenuated inflammation in a mouse model of alcoholic steatohepatitis and reduced the severity of endotoxin-induced hepatitis (25). All evidence, therefore, points to a critical role of Notch signaling in macrophage activation toward pro-inflammatory phenotypes in a canonical Notch signaling-dependent (intracellular Notch and CSL/RBP-Jκ-dependent) manner. The question remains whether inhibition of Notch signaling under an inflammatory microenvironment can switch macrophages toward the opposite phenotype, such as anti-inflammatory functions, or whether a lack of Notch signaling only dampens the inflammatory response without directing the macrophages toward other effector phenotypes.

Is Notch signaling dispensable for other types of macrophage effector functions? In macrophages treated with IL-4/IL-13 M(IL-4/IL-13), which normally induces anti-inflammatory macrophages. Notch signaling was long considered to be irrelevant; however, an indicator that Notch signaling is activated in the form of cleaved Notch1 was observed in this condition, albeit

TABLE 1 | Expression profiles of Notch ligands and receptors and some stage-specific makers in tumor-associated macrophages (TAMs).

Notch receptors/ligands or surface markers related to TAMs	Pro-inflammatory macrophages	Anti-inflammatory macrophages	Differentiation stages of TAMs based on study by Franklin et al. (14)				
			Stage 1	Stage 2	Stage 3	Stage 4	Stage 5 (TAM)
CCR2	+	−	+	+	+	+	+
Ly6C	+	−	+	−	−	−	−
CD11c			−	+	+	+	+
MHCII	+	+	−	−	+	+	+
CD11b	+	+[a]	high	high	high	low	low
Vascular cell adhesion molecule1			−	−	−	−	+[b]
CD38	+	−					
Erg2	−	+[c]					
Notch receptors							
Notch1	+	+		+	+	+	+
Notch2	+[d]	+		+	+	+[e]	+
Notch3	+						
Notch4							
Notch ligands							
Jagged1	+						
Jagged2							
Dll1	+	+					+
Dll3							
Dll4	+	+[g]					+[f]

[a] Italiani and Boraschi (12) provide reviews on murine blood monocyte subsets based on Ly6C expression and their functions in inflammation and tissue repair.

[b] Franklin et al. (14) propose TAM markers found in a breast cancer mouse model.

[c] Jablonski et al. (13) propose novel markers of M(LPS + IFNγ) and M(IL-4) (CD38 and Erg2) based on gene expression profiles that can exclusively distinguish M(LPS + IFNγ) from M(IL-4).

[d] Palaga et al. (15) report the gene expression profile of LPS-stimulated RAW264.7 macrophages.

[e] Ishifune et al. (16) report that Notch receptors are required for CD11c$^+$ CX3CR1$^+$ macrophage (found in the luminal bed of the small intestine) differentiation, thereby suggesting that Notch1 and Notch2, but not Notch3 may be required for TAM differentiation as TAM is also CD11c$^+$.

[f] Wang et al. (17) report the Notch gene expression profile in anti-inflammatory-like macrophages isolated from tumors.

[g] Bansal et al. (18) report Notch profiles in RAW264.7 M(LPS + IFNγ) and M(IL-4 + IL-13).

with different kinetics than those reported in M(LPS + IFNγ) (26). More importantly, in macrophages with targeted deletion of *Rbpj*, CSL/RBP-Jκ, possibly through canonical Notch signaling, was found to be required for activation of M(IL-4) or M(chitin), including the expression of the gene signature associated with M(IL-4), such as *Arg1* expression (27). This involvement was independent of STAT6, C/EBPβ, and IRF8. In addition, our observation revealed that Notch signaling functions in macrophages activated by PAMPs in the presence of immune complexes and LPS M(LPS + Ic), which predominantly produce high amounts of IL-10 and low levels of IL-12 to function in dampening the immune response (28, 29). Together, these data indicate the need for re-thinking the roles that Notch signaling plays in macrophage activation. Notch signaling may be involved in various types of macrophage activation in a context-dependent manner. Whether Notch signaling functions as an instructor or a signal amplifier during macrophage activation remains to be determined, but this feature is similar to what has been postulated for the involvement of Notch signaling in the polarization of helper T cells (30).

Notch Receptors and Ligands During Macrophage Activation

Four Notch receptors and five Notch ligands have been identified thus far. Differences in signals sent *via* different combinations of ligand–receptor interactions have long been suspected. For example, two ligands, Dll1 and Dll4, send different signals through the same receptor, Notch1, that are either pulsatile or sustained, thereby inducing different cell fates (31). During macrophage activation, various Notch receptors and ligands have been detected (**Table 1**). All Notch receptors, except for Notch4, are expressed in pro-inflammatory M(LPS) or M(LPS + IFNγ) (15). Notch3 is selectively upregulated in pro-inflammatory macrophages, such as in M(LPS) and M(LDL) (21). Notch1 and Notch2 are required for differentiation of CD11c$^+$ CX3CR1$^+$ macrophage subset in the small intestine (16). Similarly, Jagged1, Dll1, and Dll4 are detected in pro-inflammatory macrophages (18). In M(LPS), Foldi et al. reported that Jagged1 is the ligand responsible for autoamplification of Notch signaling in pro-inflammatory macrophages (32). The importance of the Notch-Dll4 axis in pro-inflammatory macrophages was highlighted in a study using blocking antibodies against Dll4. The results revealed that the anti-Dll4 antibody reduced pro-inflammatory macrophage accumulation in inflammatory lesions and attenuated atherosclerosis and metabolic disease (33). Furthermore, during influenza infection, Dll1 expression on macrophages is crucial for dictating the effective anti-viral responses of CD4 and CD8 T cells (34). Nevertheless, knowledge of the effect of specific combinations of Notch receptors and ligands on macrophage activation is still limited, and requires each receptor and ligand to be specifically blocked to evaluate the relevance of different interaction pairs.

Origins and Functions of TAMs

In solid tumors, TAMs are a dominant cell type in tumor tissues of various origins, often second to the tumor cells themselves (35). This observation leads to the obvious questions of where these TAMs originate and what are their functions in tumors. There are two potential sources of TAMs. TAMs can develop from newly recruited monocytes from circulation or be derived from TRMs. These sources are not mutually exclusive and depend mainly on the tumor type (5). In a breast cancer model, newly recruited monocytes differentiated to become TAMs, while in brain tumors, both blood-derived monocytes and resident microglia cells contributed to the TAM population (14, 36). When TAMs arise from monocytes recruited from circulation, tumor cells need to secrete factor(s) that trigger the migration of monocytes to the tumor sites (**Figure 1**).

Macrophage phenotypes, in general, are considered highly plastic and can change depending on the microenvironment, and this may also be true for the phenotypes of TAMs in the tumor microenvironment (10). In one study, human breast cancer cells skewed TAMs toward an anti-inflammatory phenotype partly by secretion of M-CSF (39). In an *in vivo* model of BALB/c 4T1 mammary carcinoma, the tumor microenvironment condition encouraged monocyte precursors to differentiate into diverse TAM subsets with either pro- or anti-inflammatory phenotypes (40). Furthermore, studies in renal cell carcinoma have shown mixed pro- and anti-inflammatory phenotypes of TAMs (41). These observations indicate that there are variations in TAM phenotype that depend on the type of tumors and that the activation of TAMs is highly complex and context-dependent.

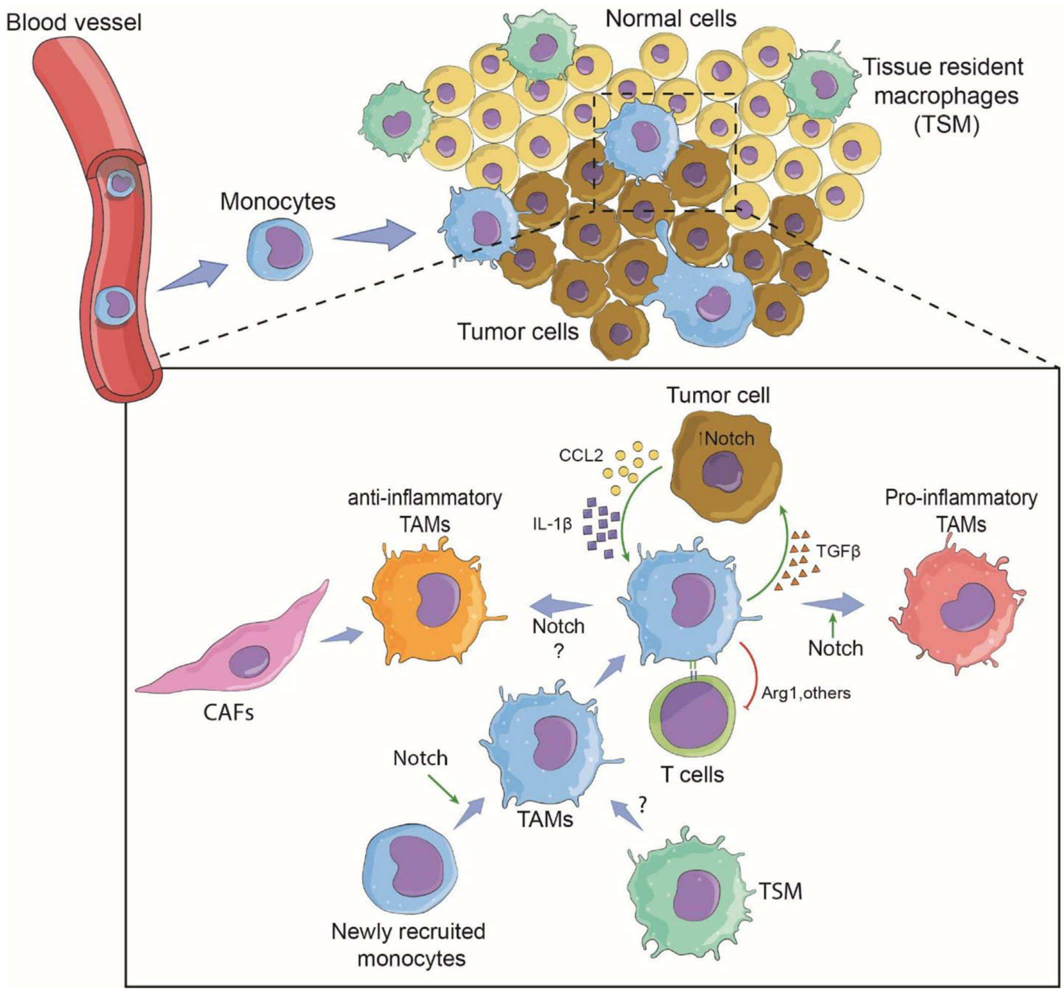

FIGURE 1 | Involvement of Notch signaling during tumor-associated macrophage (TAM) differentiation and tumor growth. Tumor cells recruit monocytes from circulation by secreting chemotactic factors and inflammatory cytokines. Notch signaling may be required for terminally differentiated TAMs. Within the tumor microenvironment, newly recruited monocytes are conditioned to become pro-inflammatory or anti-inflammatory TAMs *via* the cytokine milieu and possibly the canonical Notch signaling (14). Tissue-resident macrophages may also contribute to tumor growth by changing to TAMs. TAMs support tumor growth directly by secreted cytokines and growth factors, and indirectly by affecting T-cell response against the tumor (37). The pro-tumoral function can be Notch signaling dependent or independent. Cancer-associated fibroblasts (CAFs) may also cross-talk with TAMs *via* Notch signaling (38).

Notch Signaling and TAMs

In TAMs, Notch1 and 2 have been detected in breast cancer model, while Dll1 and Dll4 have been detected in a lung cancer model (**Table 1**) (14, 17). Jagged1 expression in a breast cancer cell line was shown to modulate TAM differentiation resulting in anti-inflammatory and IL-10-producing TAMs (42). In human cancer, evidence is still lacking regarding the expression profiles of Notch receptors and ligands in TAMs associated with different types of cancer. Recent study of head and neck head and neck squamous cell carcinoma, increasing Notch1 level is associated with CD68$^+$/CD163$^+$ TAMs, indirectly suggest the link between Notch signaling and TAMs (43). Knowing the expression profiles of Notch receptors and ligands in TAMs and the importance of the signals that they send will provide better targets for intervention.

Notch Signaling and Migrations of Monocytes and Differentiation Into TAMs

For monocyte-derived TAMs, the presence of TAMs begins with the recruitment of blood monocytes/macrophages to the tumor microenvironment through newly formed blood vessels around the solid tumor (14, 44). Diverse chemokines, i.e., CCL2 (MCP-1), CCL5 (RANTES), CCL7 (MCP-3), CXCL8 (IL-8), and CXCL12 (SDF1), released by tumor cells induce migration, differentiation, and survival of tumor-infiltrating myeloid cells (45, 46). The chemokine receptor CCR2 has been a subject of intense study as a key molecule of monocyte recruitment into tumors. An *in vitro* study revealed that GM-CSF-induced macrophages M(GC) showed higher CCR2 expression than their M-CSF-induced counterparts M(MC). After CCL2 stimulation, M(GC) exhibited enhanced LPS-mediated IL-10 production, indicating an anti-inflammatory role. These phenomena were confirmed by an *in vivo* study in which *Ccr2*-deficient bone marrow-derived macrophages displayed profiles indicative of inflammatory macrophages (47). In the MMTV-PyMT mammary tumor model, a decrease in the number of TAMs in the tumor site was observed in *Ccr2*-null background animals, suggesting the importance of CCR2/CCL2 signaling in the recruitment of TAMs to tumor sites (14). Further investigation revealed that the deletion of *Rbpj* in macrophages results in loss of CCR2 and TAM markers, suggesting a cross-talk between canonical Notch signaling and the CCR2/CCL2 signaling pathway in TAMs in the tumor microenvironment. One can speculate that in the early phase, monocytes are recruited to the tumor site in a CCR2-dependent manner and perhaps begin to encourage activation toward an inflammatory phenotype, but tumor cells educate these cells by creating a tumor microenvironment that re-directs them toward a tumor-friendly phenotype in a later phase of tumor growth (**Figure 1**). In fact, a gradual increase in M(IL-4)-associated markers such as a high level of CD206 expression and low or no MHC Class II molecule expression has been reported in TAMs in a mouse colon cancer model and in human cancer samples (37). Interestingly, expression of the immune checkpoint receptor, programmed cell death protein 1 (PD1), was significantly increased in CD206$^+$ TAMs compared to the expression in TAMs negative for CD206.

In basal-like breast cancer, tumor cells secrete both CCL2 and IL-1β in a Notch-dependent manner, and the secreted cytokine/chemokines, in turn, recruit monocytes to the tumor site (48). In this case, canonical Notch signaling directly regulates the expression of CCL2 and IL-1β, leading to the adhesion of monocytes to blood vessel and extravasation to migrate toward tumor tissue. CCL2 can be produced by bone marrow-derived stromal cells or tumor cells, while tumor cells produce IL-1β (49). Once monocytes are recruited, tumor microenvironments train/educate monocytes to differentiate to become TAMs with a pro-tumor phenotype that can function to support tumor growth and metastasis (5). In this breast cancer model, TAMs interact with cancer cells *via* TGFβ to potentiate the expression of Jagged1, one of the Notch ligands (48). The Notch/Jagged1 positive feedback loop amplifies cytokine/chemokine secretion leading to more TAM recruitment. In an animal model of breast cancer using MMTV-PyMT mice, Franklin et al. showed conclusively that TAMs are recruited from blood inflammatory monocytes and exhibit phenotypes and functions that are distinct from mammary TRMs. Importantly, the terminal differentiation of these TAMs from monocytes is CSL/RBP-jκ-dependent, indicating that the canonical Notch signaling pathway plays a vital role in TAM differentiation (14). Therefore, at least for TAMs in this breast cancer model, Notch signaling plays both an extrinsic role, i.e., regulating the production of recruiting factors by tumor cells, and an intrinsic role, i.e., regulating the differentiation of TAMs. Whether TAMs associated with other tumor types also require CSL/RBP-jκ for their differentiation or function is still an open question.

Notch Signaling in Anti-Tumor Responses of TAMs

Forced activation of the Notch receptor in TAMs in a Lewis lung carcinoma cell (LCC) model of cancer was shown to repress tumor-promoting activity by enhancing the anti-tumor phenotype and suppressing the pro-tumor phenotype. The mechanism of anti-tumor activity is reported to be mediated in part by microRNAs (miRNAs) (50). miRNAs are small regulatory non-coding RNAs of 21–22 nt that play important roles in regulating gene expression through post-transcriptional silencing of targets mRNAs. miRNAs play important roles in the activation and effector function of macrophages in TAMs by regulating their target genes and signaling pathway (51). In the LCC model, miR-152a, which is under regulation by Notch signaling, targets factor-inhibiting hypoxia 1 and IRF4, a transcription factor involved in M(IL-4) activation, to enhance the anti-tumor phenotype (52). In addition, another miRNA downstream of Notch signaling, miR-148a-3p, also helps to skew the activation of macrophages toward the anti-tumor phenotype by targeting the PTEN/Akt pathway and activation of the NF-κB pathway (53). This observation is consistent with the role of Notch signaling in favoring anti-tumor macrophage activation, and by forced activation of the Notch signaling pathway, these processes can result in the suppression of tumor growth.

Targeted deletion of *Rbpj* in macrophages resulted in reduced activity of CD8$^+$ T cells by diminishing the cytotoxic activity against tumor cells in a B16 cell melanoma model (17), suggesting that the cross-talk between TAMs and CTLs is crucial for the anti-tumor immune response, and Notch signaling plays an important role in eliciting the anti-tumor activity of CTL. Moreover, activation of Notch signaling in macrophages was demonstrated to increase the CD8$^+$ T cell population infiltrating the tumor site in the LCC model (50). These data indicate the ability of Notch signaling in TAMs to increase anti-tumor activity directly as pro-inflammatory macrophages or indirectly *via* cytotoxic T cells.

With the use of the opposite approach, manipulating canonical Notch signaling in TAMs in a mouse model of cancer was clearly demonstrated to be able to control tumor growth. Targeted deletion of *Rbpj* in macrophages resulted in anti-inflammatory phenotypes under pro-inflammatory inducers (such as LPS), and these macrophages lost the ability to control tumor growth (17). Therefore, if the Notch signaling pathway is dampened in TAMs, this dampening probably results in TAMs shifting toward an anti-inflammatory-like phenotype and helping tumor growth. One caveat is that this study employed *in vitro*-activated macrophages mixed with a tumor cell line that was administered to mice. Whether switching the Notch signaling on or off in TAMs after differentiation in the tumor influences the anti-tumor immunity remains an open question.

Contradictory to the studies described above, several reports have indicated that activation of Notch signaling supports anti-inflammatory phenotypes of macrophages and possibly favors TAMs (27, 54). A study in breast cancer patients who exhibited resistance to aromatase inhibitor treatment showed higher expression of Jagged1 in the tumor and an increasing density of anti-inflammatory TAM infiltration in breast cancer tissue compared to that in control (42). This study indirectly suggests that Jagged1 on cancer cells may drive TAMs into pro-tumor phenotype by activating Notch signaling in TAMs. These contradictory reports on Notch signaling in TAMs imply that the difference in TAM phenotype possibly depends on the tumor microenvironment and types of tumor, and this need to be taken into consideration. In addition, different Notch ligands may activate Notch signaling in different ways, and this may impact the phenotypes of TAMs.

TAMs, Tumor Angiogenesis, and Notch Signaling

Angiogenesis requires contact between macrophages and endothelial cells together with cytokines and angiogenic molecules. Inflammatory macrophages, including TAMs, are involved in angiogenesis based on the expression of cytokines, such as TNF-α and IL-6, and angiogenic factors, such as vascular endothelial growth factor (VEGF) (5). Because Notch signaling, directly or indirectly, regulates the expression of genes involved in angiogenesis, such as VEGFR and EphrinB2 (55), Notch signaling in TAMs may regulate tumor angiogenesis. In retinal choroidal neovascularization (CNV), the deletion of *Rbpj* in myeloid cells results in the inhibition of the inflammatory response in the retina and choroid after injury. This inhibited inflammatory response is accompanied by suppression of VEGF and TNF-α production and CNV development in the choroid (56). Moreover, Notch1-expressing macrophages interact with two Dll4-expressing sprouts of endothelial cells, leading to the activation of Notch signaling in macrophages. This interaction regulates the function of macrophages during vessel anastomosis in retina angiogenesis (57). Loss of Notch1 in myeloid lineage cells reduces microglia recruitment and results in abnormal angiogenesis (58).

Vascular cell adhesion molecule (VCAM) 1 is highly expressed in TAMs, whereas loss of VCAM1 in macrophages reduces the number of hematopoietic stem cells in the spleen and the inflammation in atherosclerosis due to an inability of macrophages to attach to vascular endothelial cells (59). Although little is known about the role of Notch signaling in the regulation of VCAM1 expression in macrophages, lung endothelial cells express high levels of VCAM1, and increased numbers of TAMs have been observed in lung cancer tissue compared to that in control. Endothelial cells were reported to undergo cellular senescence after implantation of tumor cells expressing Notch ligands (Dll4 and Jagged1), suggesting that VCAM1 expression in endothelial cells is under the regulation by Notch signaling and, together with Notch activation, required for TAM localization (60). VCAM1 expression in endothelial cells is under regulation of the Notch signaling pathway even in the absence of inflammatory cytokines. However, in the presence of IL-1β, VCAM1 expression in endothelial cells is greatly enhanced in a Notch-dependent manner (61). These studies suggest that endothelial VCAM1 is important for the survival of TAMs in the tumor microenvironment. However, this interaction through VCAM1 may be bidirectional because VCAM1 is also highly expressed in TAMs, suggesting that it may play an important role in the survival of endothelial cells as well. Blood vessel endothelial cells have also been found to play a role in TAM differentiation. A recent study demonstrated that Dll1 expressed by endothelial cells lining the blood vessels in mice induced conversion of Ly6C^{hi} to Ly6C^{lo} monocytes in a Notch2-dependent manner (62). This study was the first to demonstrate that the Notch ligand Dll1 in the blood vessel can induce phenotypic changes in monocytes through the Notch2 receptor under steady-state conditions.

The Role of Notch-Dependent TAMs in Supporting Tumor Growth and Immune Suppression

As described above, TAMs can directly support tumor growth by secreting factors, such as TGFβ (48). TAMs also affect the overall anti-tumor immunity mounted by other immune cells, such as T lymphocytes, in tumor sites by dampening the immune functions. Arginase 1, an arginine-degrading enzyme produced by M(IL-4), can suppress CTL activity (63). Recently, anti-inflammatory macrophage-like (CD206$^+$ MHC II^{low} or ^{negative}), but not pro-inflammatory macrophage-like (CD206$^-$ MHCII^{hi}) TAMs have been reported to express PD1 in both a mouse model and in human cancers over time with disease progression (37). The so-called immune checkpoint inhibitor is used to block this PD1-PD-L1 interaction and trigger a vigorous host immune response against the tumor. Interestingly, blocking this interaction results in increasing phagocytosis by macrophages and a reduction in tumor growth in mouse models of cancer (37). Although there is no evidence linking Notch signaling and PD1 in TAMs, there is a report indicating that canonical Notch signaling regulates the expression of PD1 in activated $CD8^+$ T cells (64). Cancer-associated fibroblasts (CAFs) are indicated as accomplices in malignant cancers (38). Because CAFs and TAMs are reported to collaborate *via* cell–cell interaction in promoting tumor progression (65), it is possible that Notch signaling may contribute in the cross-talk between the two cell types. Taken together, these observations suggest that Notch signaling may be involved in regulating this immune suppression mechanism in TAMs *via* an immune checkpoint inhibitor.

Challenges and Potential for Manipulating Notch Signaling in TAMs for Therapy

Notch signaling clearly plays important roles in TAMs, either to promote or suppress tumor growth. Therefore, Notch signaling in TAMs can be a drug target for manipulating host anti-cancer immunity. If Notch signaling in TAMs is pro-tumoral, suppressing it would benefit the host. In contrast, if TAMs require Notch signaling to become more inflammatory anti-tumor macrophages, it needs to be stimulated. Various types of gamma-secretase inhibitor that is a pan-Notch signaling inhibitor are often used to suppress Notch signaling in cancer clinical trials (66). Unfortunately, this inhibitor has off-target effect and is highly toxic if applied systemically. Therefore, designing a method that specifically inhibits Notch signaling in TAMs is desirable. One approach is to use a stapled peptide derived from part of mastermind-like protein that interferes with canonical Notch signaling. If coupled with a TAM-specific delivery system, this peptide could specifically inhibit Notch signaling in TAMs (67, 68). Antibody-based specific antibody blocking has also been investigated for targeting the ligand-binding domain or the negative regulatory region of Notch receptors (69). To activate Notch signaling to favor inflammatory macrophages, an activating antibody that mimics ligand binding may be used. In any case, an intelligent method that targets TAMs is required to minimize the side effects.

Remaining Unresolved Questions and Future Directions

Notch signaling in macrophages clearly affects their biological functions both directly and indirectly. Notch signaling also affects TAMs and functions in monocyte recruitment, tumor-mediated training, and angiogenesis. Notch signaling in TAMs is, therefore, an attractive signal to manipulate to promote anti-tumor immunity. Macrophages have been reported to be epigenetically modified by stimuli that contribute to "trained immunity" and "tolerance," at least *in vitro* (70). If the manipulation of macrophage polarization of TAMs through Notch signaling is to be considered as an alternative for cancer treatment, we must ask whether the epigenetic marks on TAMs imprinted by the tumor microenvironment, created by cancer cells, can be reversed or erased so that TAMs could act to benefit the host.

AUTHOR CONTRIBUTIONS

TP is responsible for designing the article concept and scope, reviewing 50% of the content, and conceptualizing the figure. WW is responsible for reviewing 20% of the content. PK is responsible for reviewing 30% of the content and designing the table and part of the scope of the article.

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PLOS ONE

RESEARCH ARTICLE

Notch signaling regulates the responses of lipopolysaccharide-stimulated macrophages in the presence of immune complexes

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Abstract

Macrophages exhibit diverse effector phenotypes depending on the stimuli and their microenvironment. Classically activated macrophages are primed with interferon (IFN)γ and stimulated with pathogen-associated molecular patterns. They produce inflammatory mediators and inflammatory cytokines, such as IL-12. In the presence of immune complexes (ICs), activated macrophages have decreased IL-12 production and increased IL-10 production and presumably act as regulatory macrophages. Notch signaling has been shown to regulate the effector functions of classically activated macrophages. In this study, we investigated whether Notch signaling is active in lipopolysaccharide (LPS)-stimulated macrophages in the presence of ICs. LPS/IC stimulation increased the level of cleaved Notch1 in murine macrophages, while IC stimulation alone did not. Delta-like 4, but not Jagged1, was responsible for generating cleaved Notch1. The activation of Notch signaling by LPS/ICs depended upon NF-κB and MEK/Erk pathway activation. Macrophages with the targeted deletion of *Rbpj*, which encodes a DNA-binding protein central to canonical Notch signaling, produced significantly less IL-10 upon LPS/IC stimulation. A similar impact on IL-10 production was observed when Notch signaling was inhibited with a gamma-secretase inhibitor (GSI). Defects in NF-κB p50 nuclear localization were observed in GSI-treated macrophages and in *Rbpj*$^{-/-}$ macrophages, suggesting cross-regulation between the Notch and NF-κB pathways. Transcriptomic analysis revealed that Notch signaling regulates the transcription of genes involved in the cell cycle, macrophage activation, leukocyte migration and cytokine production in LPS/IC-stimulated macrophages. Taken together, these results suggest that the Notch signaling pathway plays an important role in regulating the functions of macrophages activated by LPS and ICs.

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Introduction

Macrophages mediate both innate and adaptive immune responses. Signaling through lipopolysaccharide (LPS)/TLR4 results in the execution of host defense functions, such as phagocytosis and killing activities, by macrophages [1], and the cascade of downstream signaling molecules that are induced by LPS facilitates the transcriptional activation of inflammatory-associated cytokines, such as TNFα, IL-1β, IL-6, IL-12, and type I interferon, as well as the production of relatively low amounts of IL-10. Additionally, the priming of macrophages with IFNγ enhances TLR-induced cytokine gene expression, partly by facilitating the remodeling of chromatin to increase chromatin accessibility and the recruitment of TLR-induced transcription factors to the regulatory promoter regions [2]. These macrophages are well-characterized as classically activated macrophages [3].

Alternatively, macrophages can be activated by signaling through Fc gamma receptor (FcγRs) via antigen-antibody complexes. Immune complexes (ICs) and IgG-opsonized pathogens or particles bind to FcγRs expressed on the surfaces of macrophages; FcγRs are functionally characterized as activation or inhibitory receptors [4]. Mosser *et al.* reported a unique phenotype of activated macrophages that are stimulated by IFNγ/LPS in the presence of ICs. This stimulation leads to macrophage activation that yields high levels of IL-10 and low levels of IL-12 while maintaining the levels of other innate cytokines, such as TNFα. In addition to these signature cytokines, LPS/IC-activated macrophages also express unique gene expression profiles that are different from classically activated macrophages or IL-4-stimulated macrophages, the so-called M2 macrophages [5, 6]. Because of the cytokine profiles opposite those of classically activated macrophages, at least those for IL-12 and IL-10, these macrophages are considered to be distinct from classically activated macrophages and are called type II or regulatory (also called M2b or M(IC)) macrophages [3, 7, 8]. The adoptive transfer of these regulatory macrophages alleviates the severity of autoimmune disease in a mouse model of experimental autoimmune encephalomyelitis (EAE), suggesting that they have a systemic impact *in vivo* [9].

IL-10 is one of the key signature cytokines of LPS/IC-activated macrophages; IL-10 causes these macrophages to function as regulatory cells during the immune activation state. The role of IL-10 produced by IC-stimulated macrophages is indicated by the worsening outcomes of some infectious diseases caused by intracellular pathogens [10]. Furthermore, macrophages activated by TLR ligands in the presence of ICs are linked to some autoimmune diseases, particularly systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [11, 12]. Because IL-10 functions as a regulatory cytokine that is important for controlling the inflammatory process, the regulatory mechanism of IL-10 expression has been extensively studied in immune cells, including macrophages [13, 14]. In macrophages, the transcription of *Il10* mRNA is selectively regulated by various transcription factors, including Erk, Sp1 and NF-κB. The production of IL-10 is induced in TLR-dependent and TLR-independent manners in macrophages. In LPS-activated macrophages, IL-10 is produced at relatively low levels, and its transcription is controlled mainly by the NF- κB pathway (p50 and p65) and the MAPK and STAT pathways [15–17]. Signaling through FcγRs in LPS/IC-stimulated macrophages amplifies the activation of Erk and p38 MAPK signaling, thus augmenting chromatin remodeling and the binding of Sp1 to the *Il10* promoter [18]. Furthermore, PI3K/AKT signaling downstream of FcγRs is also responsible for optimal IL-10 expression [19]. Although detailed signaling pathways involving TLRs and FcγRs have been reported in the regulation of IL-10 production, the involvement of other signaling pathways, including Notch signaling, remains largely unexplored.

The Notch signaling pathway regulates multiple cellular processes, including differentiation, proliferation and survival [20]. Notch signaling comprises four Notch receptors (Notch1-4), five ligands (Delta-like (Dll) 1, 3 & 4 and Jagged 1 & 2) and the DNA binding protein CSL/RBP-Jκ. The interactions between Notch ligands and receptors induce the sequential enzymatic cleavage of Notch receptors by ADAM metalloprotease and gamma-secretase, resulting in the release of the intracellular domain of the Notch receptor. The cleaved Notch receptor forms a complex with CSL/RBP-Jκ in the nuclei, and together, they regulate the transcription of Notch target genes [21]. We and others demonstrated that TLR-activated macrophages induced the expression of the full-length Notch1 receptor as well as the production of cleaved Notch receptors [22, 23]. Signaling downstream of TLRs induces expression of Jagged1 in NF-κB and MAPK dependent manner. Jagged1/Notch create an autoamplification loop of Notch signaling that can be enhanced by IFNγ [24]. TLR and Notch together induces expression of the Notch target genes, *Hes1* and *Hey1*. These two proteins attenuate the expression of IL-6 and IL-12, the effect that can be surpassed by IFNγ treatment [23]. The activation of Notch signaling through TLR activation is important for the production of pro-inflammatory cytokines, including TNFα, IL-6, IL-10 and IL-12, in TLR-activated macrophages [22, 25, 26]. Thus, Notch signaling plays important roles in the polarization and activation of pro-inflammatory macrophages [23, 26–29].

There are reports that Notch signaling regulates IL-10 production in other immune cells. For example, Notch signaling promotes *Il10* mRNA expression via STAT4 in Th1 cells [30]. 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 be involved in the production of cytokines that are targets of NF-κB signaling [22]. However, whether Notch signaling is active in LPS/IC-stimulated macrophages and/or plays a role in regulating cellular functions, such as IL-10 production, have not been elucidated. Here, we showed that in LPS/IC-stimulated macrophages, Notch signaling is activated, and the crosstalk among Notch signaling, signaling downstream of FcγRs and TLR signaling cooperates to regulate gene expression, including IL-10, in LPS/IC-stimulated macrophages. This work highlights the complex role that Notch signaling plays in inflammatory and regulatory macrophages.

Materials and methods

Animals

Wild type C57BL/6 mice were purchased from the National Laboratory Animal Center (Salaya, Thailand). Conditional *Rbpj* KO mice were generated as described previously [31]. All animal procedures were approved by the Institutional Animal Care and Use Committees (IACUCs) of Chulalongkorn University and the University of Massachusetts at Amherst and performed according to the guidelines approved by the IACUCs (Protocol Review No. 1323007). Mice were humanely sacrificed by CO_2 inhalation in the euthanasia chamber.

Generation of bone marrow-derived macrophages (BMMs)

Bone marrow cells were flushed from the femur cavities of mice and used for generating BMMs as described elsewhere [22]. In brief, 5×10^6 bone marrow cells were plated in non-tissue culture treated plates (Hycon, Thailand) and cultured at 37˚C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, HEPES, sodium pyruvate, streptomycin/penicillin G (all from LONZA, USA, or HyClone, UK), 5% (v/v) horse serum (Thermo Scientific, USA) and 20% (v/v) L929-conditioned media. Fresh DMEM supplemented with 20% L929-conditioned media and 5% horse serum was added to the cultures on day 4. The cells were harvested on day 7 and used for all the experiments.

Activation of BMMs

BMMs were primed overnight with recombinant murine IFNγ (10 ng/mL) (BioLegend, USA) and washed twice with media and warm PBS. *Salmonella* LPS (100 ng/mL) (Sigma-Aldrich, USA), purified rabbit IgG against ovalbumin (OVA) (GeneTex, USA) and IgG-opsonized OVA (immune complex) were added to activate the macrophages as indicated. GSI, DAPT (25 μM) (Calbiochem, USA) and vehicle control DMSO (0.01%) (Sigma-Aldrich, USA) were incubated with the macrophages overnight before activation. BAY-11(10 μM), SB203580 (10 μM), U0126 (10μM), Y294002 (50 μM) (all inhibitors were purchased from Calbiochem, USA) or DMSO (0.01%) (Sigma-Aldrich, USA) was used to pretreat the macrophages for 30 min before activation. Macrophage activation was confirmed by measuring *Il12b* and *Il10* mRNA expression by quantitative RT-PCR (qPCR).

Preparation of the ICs

The ICs were prepared as described previously [5]. Briefly, a 10-fold molar excess of purified rabbit anti-OVA IgG (GeneTex, USA) or rabbit anti-OVA IgG (Sigma-Aldrich, USA) was mixed with OVA (Sigma-Aldrich, USA) and incubated for 30 min at room temperature. To activate the macrophages, a 1:100 volume ratio of the immune complexes to media was used for culture, in addition to LPS.

Intracellular staining and cell surface staining

For intracellular staining, monensin was added at the start of the activation. Fc receptors were blocked with a FACs staining buffer containing Fc blocker (0.5 μg) (BD Bioscience, USA), followed by cell surface staining and fixation/permeabilization using a BD Cytofix/Cytoperm kit (BD Biosciences, USA) according to the manufacturer's instructions. For IL-10, an anti-mouse IL-10-PE antibody (0.4 μg) (BioLegend, USA) was used. For detecting Notch ligands, anti-hamster Jagged1-PE, anti-hamster Jagged2-PE, anti-hamster Dll1-PE and anti-hamster Dll4-PE (BioLegend, USA) were used for cell surface staining. The cells were sorted on a Cytomics FC 500 MPL cytometer (Beckman Coulter, USA) and analyzed using FlowJo software (Tree Star, CA, USA).

Preparation of cytosolic and nuclear extracts

BMMs (wildtype or *Rbpj* KO mice) were activated and treated as indicated. Cytosolic and nuclear extracts were prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instruction (Thermo Fisher Scientific, USA). Protein concentrations were measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Equal amount of proteins were analyzed by Western blots.

Blocking of Notch ligands

BMMs were primed with IFNγ overnight in the presence of Jagged 1-specific blocking antibody (BioLegend, USA) or Dll4-specific blocking antibody (BioXcell, USA) or isotype controls. After washing in PBS, cells were stimulated with LPS and OVA-IgG IC as indicated above. Cell lysates were analyzed by Western blot and total RNA was extracted and used for determine mRNA expression.

Western blots

BMMs were activated as indicated, and the protein lysates were subjected to Western blots. The primary antibodies used in this study were as follows: rabbit anti-Notch1 (1:2000) (Santa

Cruz Biotechnology, USA), rabbit anti-cleaved Notch1 (Val1744) (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) (all from Cell Signaling Technology, USA), mouse anti β-actin (1:1000) (Chemicon-Millipore, USA) and rabbit anti-GAPDH (1:4000) (Santa Cruz Biotechnology, USA). The secondary reagents conjugated with horse-radish peroxidase (HRP) were as follows: donkey anti-rabbit IgG (1:2000–1:4000) and sheep anti-mouse IgG (1:5000) (Amersham Biosciences, UK). The signals were detected by chemiluminescence on X-ray films.

RNA extraction and qRT-PCR

BMMs were activated as indicated, and total RNA was isolated by using TriZol reagent (Invitrogen, UK) or an RNeasy Mini Kit (Qiagen, Germany). cDNA was synthesized, and the transcripts were amplified by using a Mini-Opticon or CFX Connect™ real-time PCR detection system (Bio-Rad, USA). The primer sequences used are provided in S1 Table. The expression of each gene was normalized to the expression of β-actin by the $2^{-\Delta\Delta CT}$ method.

ELISA

Culture supernatants from BMMs treated as indicated were harvested at the indicated times after stimulation. Secreted IL-12p70 and IL-10 levels were detected by using an IL-12p40/70 ELISA (BD Pharmingen, USA) and IL-10 ELISA (BioLegend, USA). ELISAs was performed according to the manufacturer's instructions.

Immunofluorescent staining

Cells were cultured in an 8-well slide chamber and activated as indicated. After washing with PBS, the cells were fixed in 4% paraformaldehyde and blocked with Fc blocker (0.5 μg). The cells were washed and incubated with an anti-NF-κB p50 monoclonal antibody (1:100) (Santa Cruz Biotechnology, USA) and then an anti-mouse IgG (H+L) $(Fab)_2$ fragment)-conjugated Alexa Fluor® 488 (Cell Signaling Technology, MA, USA) (1:500) secondary antibody. Nuclei were stained by DAPI (Cell Signaling Technology, USA). The cells were observed under an inverted fluorescence microscope or a confocal microscope (Olympus, Japan).

RNA sequencing and data analysis

Total RNA samples were extracted from LPS/IC-activated BMMs in the presence of DMSO or GSI for 1 hr using an RNeasy Mini Kit (Qiagen, USA). RNA samples were assessed for quality using an Agilent 2100 Bioanalyzer (Agilent, USA) and quantified using a Qubit 3.0 fluorometer (Life Technologies, USA). Strand-specific cDNA library preparation was performed using a TruSeq stranded mRNA library prep kit (Illumina, USA). The cDNAs were sequenced on a NextSeq 500 system (Illumina, USA) at the Omics Sciences and Bioinformatics Center (Chulalongkorn University). The obtained data were trimmed of Illumina adapters using Trimmomatic and aligned using STAR software. Raw reads were counted using HTSeq, and the reads were mapped against the mm10 reference genome and annotated with Entrez Gene. Differential gene expression (DE) was determined using edgeR software, and the statistics were calculated according to a quasi-negative binomial distribution. Statistical significance was indicated by an FDR cut-off of < 0.05. Heat-maps were generated by MultiExperiment Viewer (MeV 4.9). Functional annotations were performed using ToppGene Suite (http://toppgene.cchmc.org).

Data has been deposited in NCBI Gene Expression Omnibus (GEO) and is accessible through GEO accession number GSE114020.

Statistical analysis

Statistical analyses were performed using SPSS version 15.0 and GraphPad Prism version 5.0. Student's t-test (paired or unpaired) and one-way ANOVA ($\alpha = 0.05$) with Tukey's multiple comparison test were used when comparing different conditions.

Results

Expression of IL-10 and IL-12p40 in LPS/IC-activated macrophages

To confirm the cytokine profiles in macrophages activated by LPS or LPS with ICs, the relative levels of *Il10* and *Il12b* mRNA were determined 4 hrs after activation. Consistent with previous reports, compared with those activated by LPS, macrophages activated by LPS and ICs showed significantly higher levels of *Il10* mRNA, whereas the levels of *Il12b* mRNA were significantly lower (Fig 1A) [5]. To determine the effect of IC treatment on cytokine production, IL-10 levels were measured in macrophages activated with various stimuli. As shown in Fig 1B, IL-10

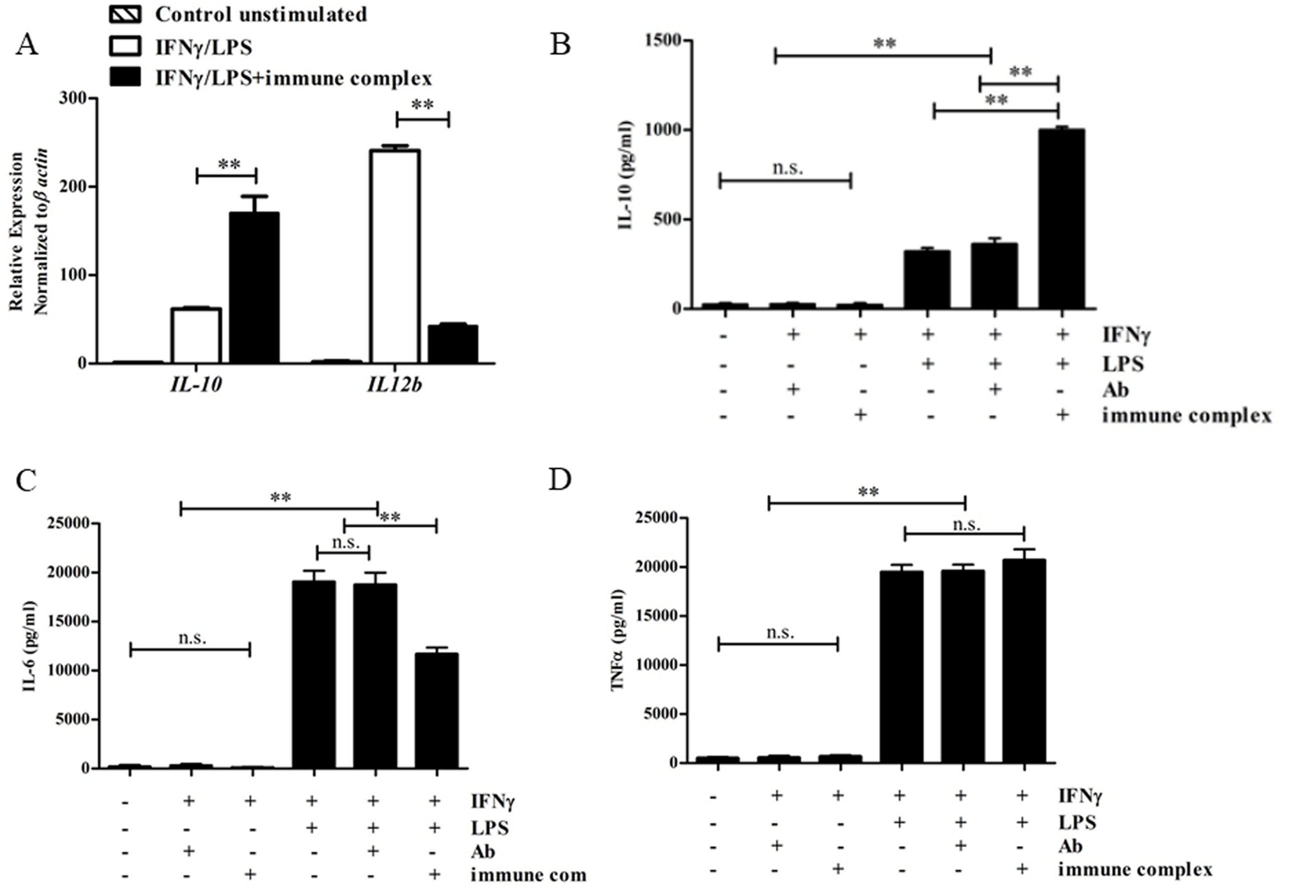

Fig 1. Cytokine expression in macrophages stimulated with LPS or LPS/ICs. (A) IFNγ-primed BMMs were activated by LPS (100 ng/mL) or LPS/ICs for 4 hrs. The expression levels of *Il10* and *Il12b* mRNA were measured by qPCR. (B-D) IFNγ-primed BMMs were activated by ICs (anti-OVA IgG) in the presence or absence of LPS (100 ng/mL) for 6 hrs. The levels of IL-10 (B), IL-6 (C) and TNFα (D) in the culture supernatant were measured by ELISA. ** indicates statistical significance ($p<0.05$) according to one-way ANOVA with Tukey's multiple comparison test. n.s. indicates no statistical significance. The results represent the mean±SD of triplicate determinations from one representative experiment of two independent experiments.

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production was detected in all LPS-stimulated conditions, while priming with IFNγ alone or ICs alone did not result in detectable IL-10 levels. As expected, compared to that stimulated by other conditions, IL-10 production reached the highest level upon activation by LPS and ICs (Fig 1B). Furthermore, stimulation with LPS in the presence of free antibody alone did not increase the amount of IL-10, suggesting that IC crosslinking is essential for enhancing IL-10 production.

We next measured the levels of pro-inflammatory cytokines, *i.e.*, TNFα and IL-6, in LPS-stimulated macrophages the presence of ICs [12, 32, 33]. We found that TNFα was produced at comparable levels in the presence or absence of ICs, whereas the production of IL-6 was dampened in the presence of ICs, compared with that in the presence of LPS alone (Fig 1C and 1D). These data are consistent with previous reports and indicate that the crosslinking of FcγRs by ICs is essential for tipping the balance of cytokine production, which is accomplished mainly by decreasing IL-6 and IL-12 levels and increasing IL-10 levels [5].

Activation of Notch signaling in LPS/IC-activated macrophages

Next, we determined whether Notch receptors and their ligands are expressed and whether the signaling is activated in LPS/IC-activated macrophages. First, the presence of cleaved Notch1 as an indicator of active Notch signaling was determined. The appearance of cleaved Notch1 was readily detectable at 1 hr after stimulation and persisted for at least 6 hrs. In parallel, the levels of Notch1 and 2 were also upregulated (Fig 2A). To investigate the stimuli contributing to the activation of Notch signaling in this setting, the cells were activated by LPS, anti-OVA antibody alone, ICs alone or the combination of LPS and ICs or anti-OVA antibody. As shown in Fig 2B, the activation of Notch signaling, as determined by the appearance of cleaved Notch1, was detected in only the condition where LPS was used, while anti-OVA antibody alone or ICs alone did not result in the appearance of cleaved Notch1. Therefore, signaling through TLR4 is crucial for the activation of Notch signaling. At 3 hrs after activation, higher levels of surface expression of the Notch ligand Jagged1 were observed compared to the other ligands while the level of Dll4 decreased (Fig 2C). Therefore, macrophages activated by LPS/ICs had upregulated expression levels of Notch1 and Notch2 and at least one Notch ligand. More importantly, the activation of Notch signaling was initiated after stimulation with LPS/ICs. These data indicated that the cleavage of Notch1 in LPS/IC-activated macrophages depends solely upon TLR4 signaling induced by LPS stimulation.

To investigate which Notch ligand(s) is responsible for Notch1 cleavage in LPS/IC-activated macrophages, specific antibody blocking was performed. As shown in Fig 2D, blocking Jagged1 did not show any effect on the level of cleaved Notch1 while blocking Dll4 clearly reduced the level of cleaved Notch1. These results indicated that Dll4 is likely the ligand responsible for Notch1 activation in LPS/IC-activated macrophages.

LPS/IC stimulation initiated the activation of Notch signaling in a NF-κB and MAPK-dependent manner

Foldi *et al.* reported that the activation of Notch signaling in TLR agonist-stimulated macrophages was initiated by Jagged1 auto-amplification through the NF-κB and MAPK signaling pathways [24]. Therefore, we asked whether the activation of Notch signaling, as determined by the presence of cleaved Notch1, with LPS/ICs is dependent on the NF-κB and/or MAPK pathways, similar to what was observed in LPS-activated macrophages. We used pharmacological inhibitors to specifically inhibit the NF-κB pathway (Bay-11), Erk/MEK1/2 pathway (U0126), p38 MAPK pathway (SB203580) and PI3K/Akt pathway (LY94002) and detected the appearance of cleaved Notch1. As shown in Fig 3A, cleaved Notch1 almost completely

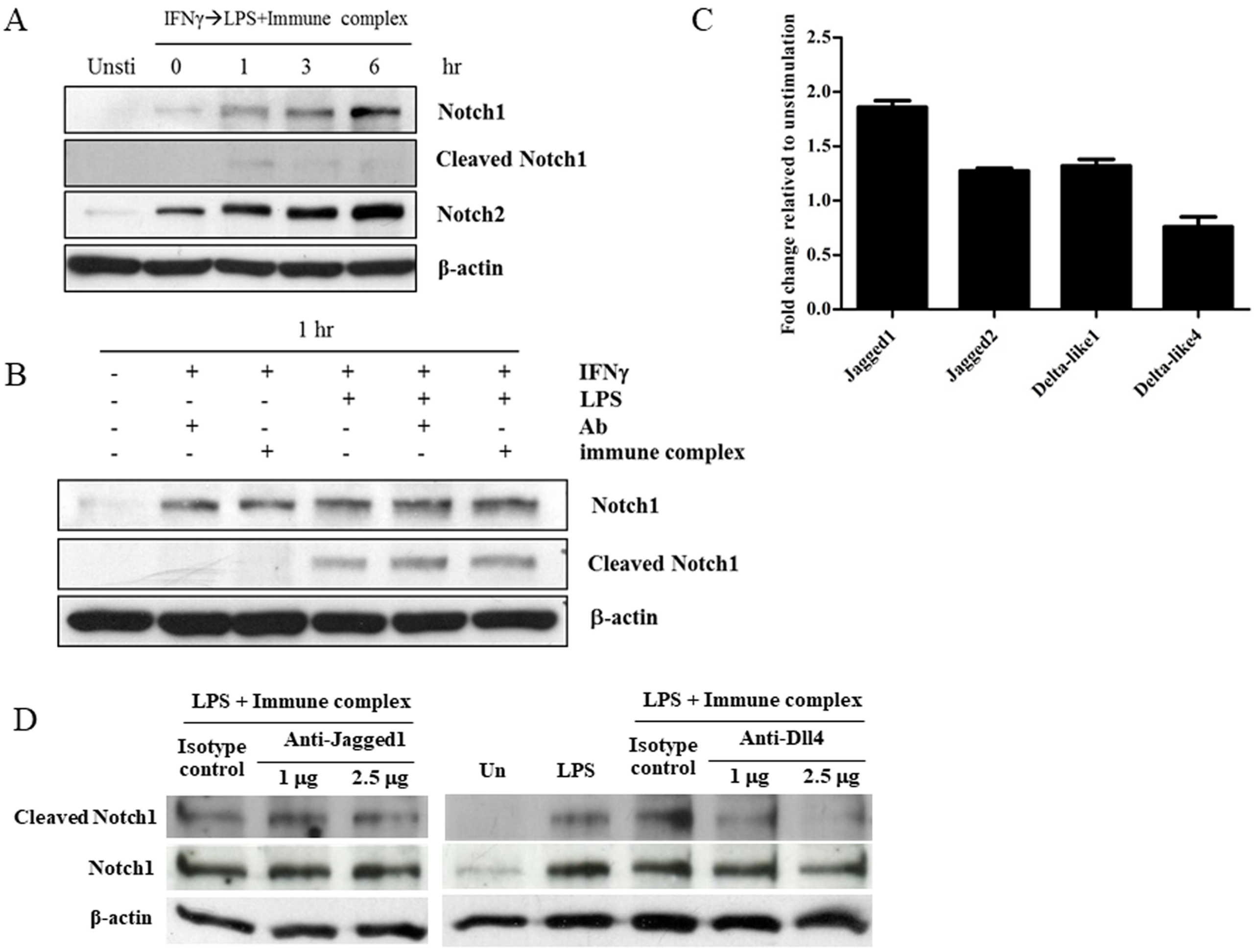

Fig 2. Stimulation by LPS is responsible for the activation of Notch signaling in LPS/IC-activated macrophages. (A) IFNγ-primed BMMs were activated by LPS/ICs for 0, 1, 3 and 6 hrs. Notch1, Notch2 and cleaved Notch1 (Val1744) were detected in whole cell lysates by Western blotting. β-actin was used for the loading control. Representative data from 1 of 2 independent experiments are shown. (B) BMMs were activated by anti-OVA IgG or ICs in the presence or absence of LPS (100 ng/mL) for 1 hr. Notch1 and cleaved Notch1 (Val 1744) were detected by Western blotting. β-actin was used as the loading control. Representative data from 1 of 2 independent experiments are shown. (C) Expression levels of Jagged1, Jagged2, Dll1 and Dll4 were detected by flow cytometry, and the relative levels were normalized to those from the unstimulated conditions. The results represent the mean±SD of triplicate determinations from one representative experiment of two independent experiments. (D) Cell lysates prepared from anti-Jagged1, anti-Dll4 antibody blocking or isotype control were analyzed for Notch1 and cleaved Notch1 (Val 1744) by Western blotting. Representative data from one of two independent experiments are shown. β-actin was used as the loading control.

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disappeared after macrophages were pretreated with either an NF-κB inhibitor or an Erk inhibitor, whereas Notch1 activation was only partially reduced after pretreatment with a p38 MAPK inhibitor or a PI3K inhibitor (Fig 3A). Therefore, the activation of Notch signaling was NF-κB-dependent and/or Erk/MEK-dependent in LPS/IC-activated macrophages, while the requirement of p38 MAPK and PI3K for Notch signaling was minimal.

To confirm the effect of these inhibitors on IL-10 production in LPS/IC-activated macrophages, IL-10 levels were quantitated by ELISA. As shown in Fig 3B, all inhibitors affected the expression of IL-10 to varying degrees. Treatment with specific inhibitors of p38 MAPK and PI3K resulted in a profound reduction in IL-10, whereas inhibitors of the Erk/MEK1/2 and NF-κB pathways resulted in partial reductions in IL-10; these findings indicate that p38 and PI3K are the main signaling pathways regulating IL-10 production, while the Erk/MEK1/2 and

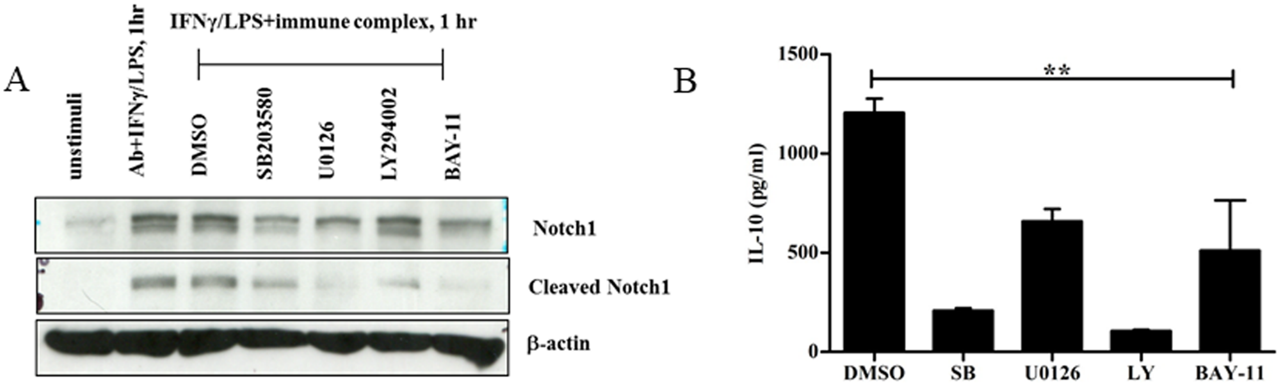

Fig 3. Effect of specific inhibitors of the MAPK, NF-κB and PI3K signaling pathways on Notch signaling activation and IL-10 production in LPS/IC-activated macrophages. (A) IFNγ-primed BMMs were pre-treated with Bay-11 (10 μM), SB203580 (10 μM), U0126 (10 μM), LY94002 (50 μM) or vehicle control DMSO for 30 min. The cells were subsequently activated by LPS/ICs for 1 hr, and protein lysates were created. Notch1 and cleaved Notch1 (Val 1744) were detected by Western blot. β-actin was used as the loading control. Representative data from one representative experiment of 3 independent experiments are shown. (B) Culture supernatants from the cells treated as in (A) were collected 6 hrs after stimulation, and the IL-10 levels were measured by ELISA. ** indicates statistical significance ($p<0.05$) according to one-way ANOVA with Tukey's multiple comparison test. The results represent the mean±SD of triplicate determinations from one representative experiment of 2 independent experiments.

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NF-κB pathways partially contribute to this regulation (Fig 3B). Taken together, the NF-κB and Erk/MEK pathways play an important role in the activation of Notch signaling and partially regulate IL-10 production in LPS/IC-activated macrophages.

Effect of GSI treatment and CSL/RBP-Jκ deletion on IL-10 production in LPS/IC-activated macrophages

To study the role that Notch signaling plays in regulating IL-10 production, GSI was used to inhibit the cleavage of Notch receptors. The appearance of cleaved Notch1 was examined to validate the efficacy of GSI in inhibiting Notch signaling activation. Cleaved Notch1 was not produced upon GSI treatment, indicating that GSI treatment effectively inhibited the activation of the Notch signaling pathway (Fig 4A). Furthermore, the level of *hes1*, one of Notch target genes, was significantly reduced (S1A Fig). The percentage of cells producing IL-10 in LPS/IC-stimulated macrophages decreased by almost 50% for GSI-treated cells, compared with the mock control-treated cells, as measured by intracellular IL-10 staining (Fig 4B). This reduction at the cellular level reflected the *Il10* mRNA level and secreted IL-10 in the supernatant (S1B Fig and Fig 4C). GSI treatment also reduced the level of IL-10 in LPS-activated macrophages but to a lesser extent than that observed in LPS/IC-stimulated macrophages. Interestingly, the percentages of cells expressing IL-10 in LPS/IC-activated macrophages in the presence of GSI was comparable to that of IFNγ/LPS-activated macrophages (data not shown).

To confirm the impact of canonical Notch signaling on IL-10 production, bone marrow-derived macrophages from mice with targeted *Rbpj* deletion were used (Fig 4D). The level of IL-10 was significantly reduced in *Rbpj* knockout (KO) macrophages that were activated by LPS/ICs compared with that in the wildtype control macrophages (Fig 4D). However, IL-10 production was not decreased in *Rbpj* KO LPS-activated macrophages, in contrast to the findings observed for GSI treatment. The effect of Dll4 blocking on *Il10* expression was examined and the results show that blocking of Dll4, but not Jagged1, reduced the *i110* mRNA level in LPS/IC stimulated macrophages (S1C Fig. These data implied that the activation of canonical Notch signaling, which requires Dll4, the activity of gamma-secretase and the presence of

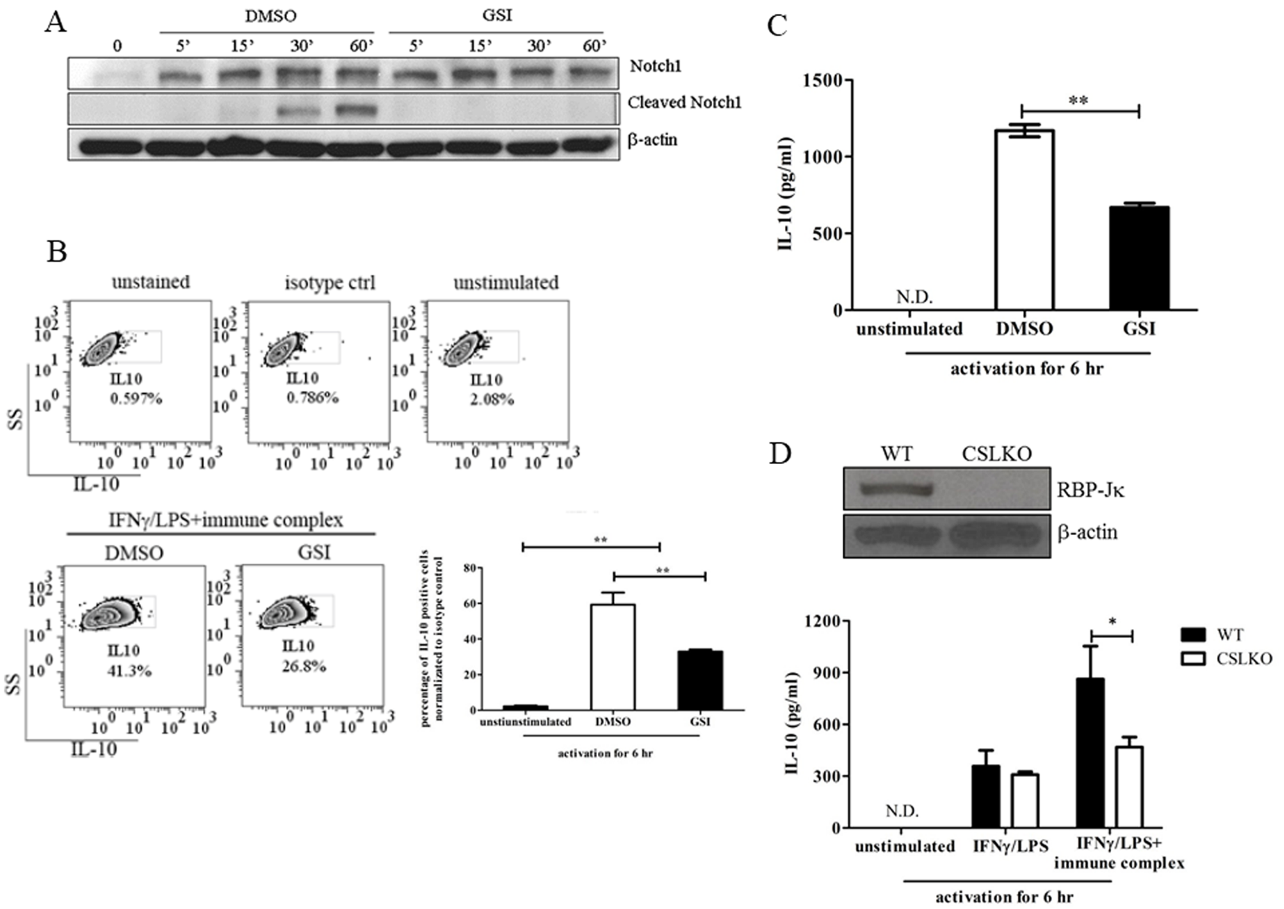

Fig 4. Effects of inhibiting Notch signaling by GSI or targeted *Rbpj* deletion on IL-10 production in LPS/IC-activated macrophages. (A) IFNγ-primed BMMs were pre-treated with vehicle control DMSO or GSI (25 μM) for 30 min. The cells were subsequently activated by LPS/ICs for 5, 15, 30 and 60 min. Cleaved Notch1 (Val 1744) and Notch1 were measured in whole cell lysates by Western blotting. Representative data from one of two independent experiments are shown. (B) IFNγ-primed BMMs were pre-treated with vehicle control DMSO or GSI (25 μM) and subsequently activated by LPS/ICs for 6 hrs. IL-10 expression was detected by intracellular cytokine staining. Representative data from one of two independent experiments are shown. (C) Culture supernatants were collected from BMMs stimulated for 6 hrs as described in (B), and the IL-10 levels were measured by ELISA. (D) CSL/RBP-Jκ expression in BMMs from wildtype (ctrl) or *Rbpj* KO (CSL KO) mice was detected by Western blotting. Representative data from one of two independent experiments are shown. BMMs from wildtype (ctrl) or *Rbpj* KO mice were activated by LPS or LPS/ICs for 6 hrs. IL-10 levels were detected by ELISA. * and ** indicate statistical significance ($p<0.05$) according to one-way ANOVA with Tukey's multiple comparison test. The results represent the mean±SD of triplicate determinations from one of two independent experiments.

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CSL/RBP-jκ, is critical for IL-10 production in macrophages activated by LPS/ICs but not in macrophages activated by LPS alone.

Effects of GSI treatment and deletion of CSL/RBP-Jκ on signaling downstream of TLR and FcγR

Next, we asked whether Notch signaling affects signaling downstream of TLR and FcγR by using GSI treatment. Macrophages were activated by LPS/ICs for 5, 15, 30 and 60 min in the presence of GSI or vehicle control DMSO, and the phosphorylation status of p38, Erk (p44/42), SAPK/JNK and Akt was detected. As shown in Fig 5A and 5B, the phosphorylation of p38, Erk and SAPK/JNK, which are downstream of MAPK signaling, were not affected by GSI treatment. Furthermore, the activation of PI3K also remained intact for the duration of the

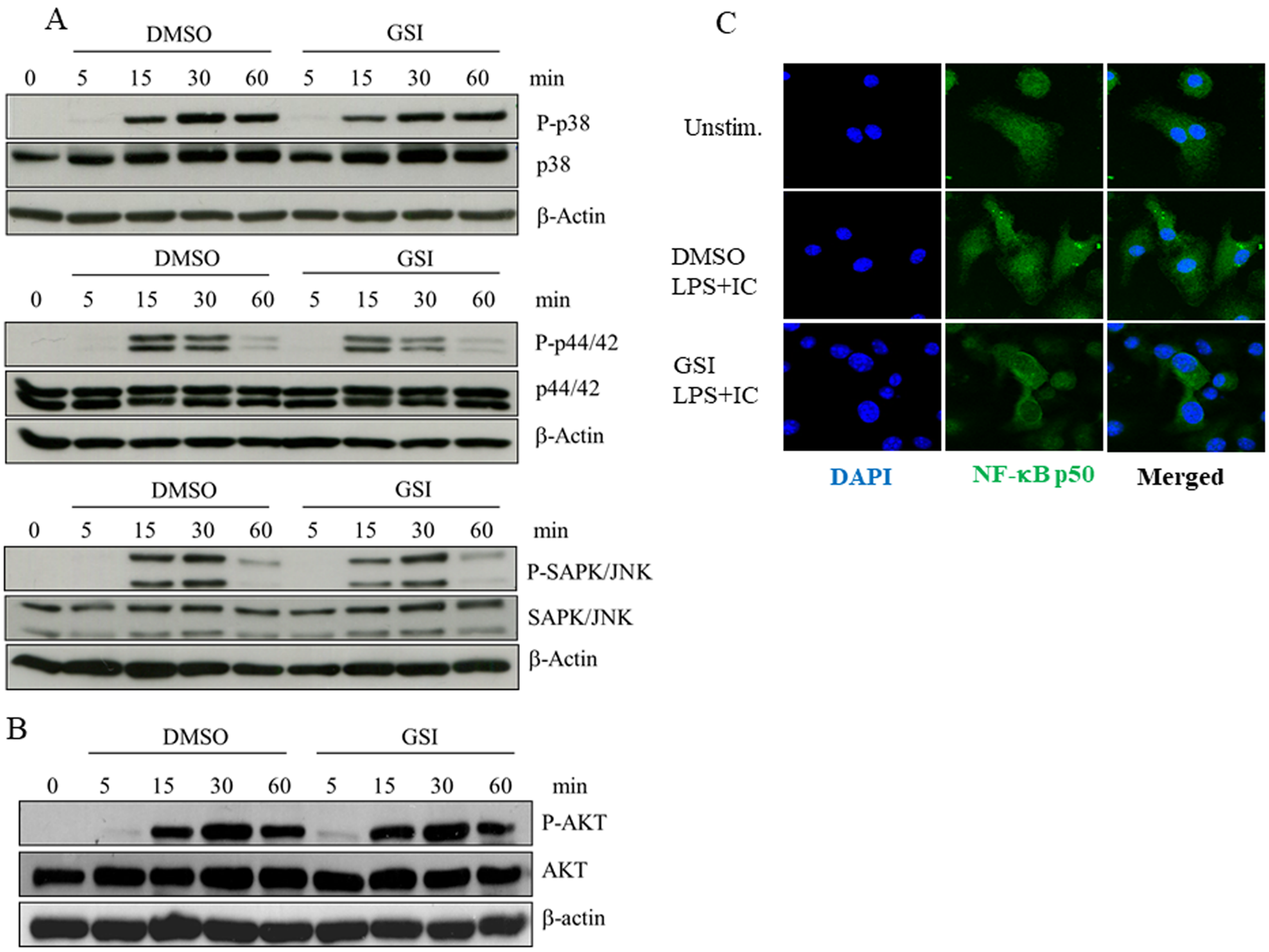

Fig 5. Effects of GSI treatment on MAPK, PI3K/AKT and NF-κB pathway activation in LPS/IC-activated macrophages. (A-B) IFNγ-primed BMMs were pre-treated with vehicle control DMSO or GSI (25 μM) for 30 min and activated by LPS/ICs for 5, 15, 30 and 60 min. Phospho-p38, p38 phospho-p44-42, p44-42, phospho-SAPK/JNK, SAPK/JNK, phospho-Akt, Akt and the loading control β-actin were detected by Western blotting. Representative data from 1 of 3 independent experiments are shown. (C) IFNγ-primed BMMs were activated by LPS/ICs for 4 hrs in the presence of vehicle control DMSO or GSI (25 μM). NF-κB p50 was detected by immunofluorescence staining. The arrows indicate cells with decreased or no p50 nuclear translocation (green). Representative data from 1 of 2 independent experiments are shown.

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experiment, even when Notch signaling was inhibited. These results suggest that GSI treatment did not affect the immediate downstream signaling pathways in LPS/IC-stimulated macrophages.

When NF-κB signaling pathway activation was determined according to the nuclear localization of the p50 subunit, GSI treatment clearly suppressed the activation of p50 (Fig 5C and S2 Fig). To confirm this observation, nuclear accumulation of NF-κB p50 were examined by Western blot after separation into the cytosolic and nuclear fractions. Consistent with the results obtained from immunofluorescent staining, GSI decreased the level of nuclear NF-κB p50 at 4 hr after stimulation (Fig 6A). To examine whether similar defect occurs in *Rbpj* KO macrophages, nuclear and cytosolic fractions were prepared from wildtype or *Rbpj* KO macrophages. As shown in Fig 6B, NF-κB p50 was readily accumulated in the nuclei obtained from wild type macrophages at 1 and 4 hr after stimulation. In contrast, delay in NF-κB p50 nuclei accumulation in *Rbpj* KO macrophages was observed at 1 hr. The level of nuclei NF-κB p50 in

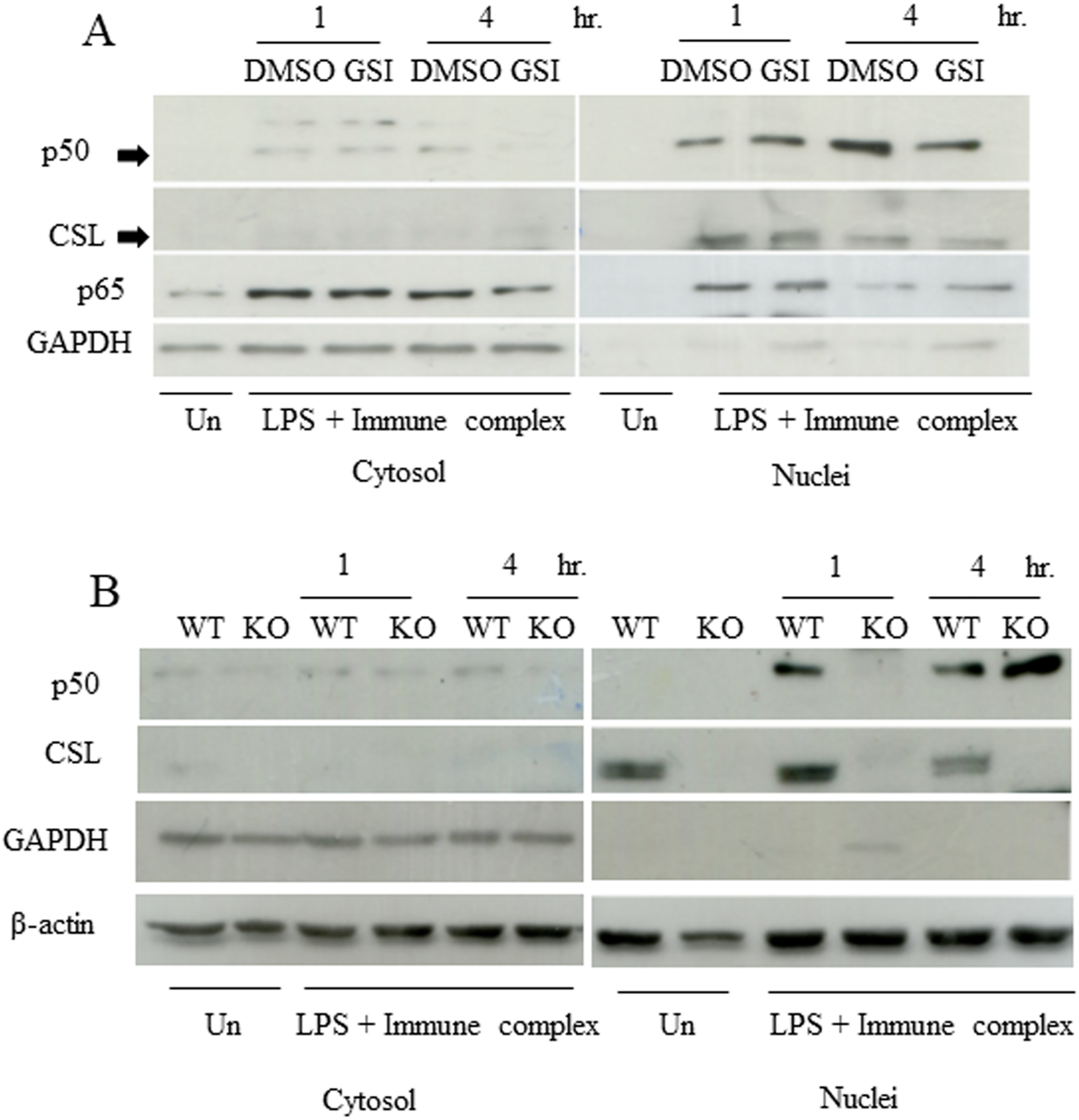

Fig 6. Defects in NF-κB p50 nuclei accumulation by GSI treatment or deletion of CSL/RBP-Jκ in LPS/IC-activated macrophages. (A) BMMs were pretreated with DMSO or GSI for 1 and 4 hr and the cytosolic and nuclei fractions were analyzed for NF-κB p50 by Western blotting. GAPDH and CSL/RBP-Jκ were used as cytosolic and nuclei markers, respectively. (B) BMMs from wildtype or *Rbpj* KO mice were stimulated with LPS/IC for 1 and 4 hr and the cytosolic and nuclei fractions were analyzed for NF-κB p50 as described for (A). Representative data from 1 of 2 independent experiments are shown.

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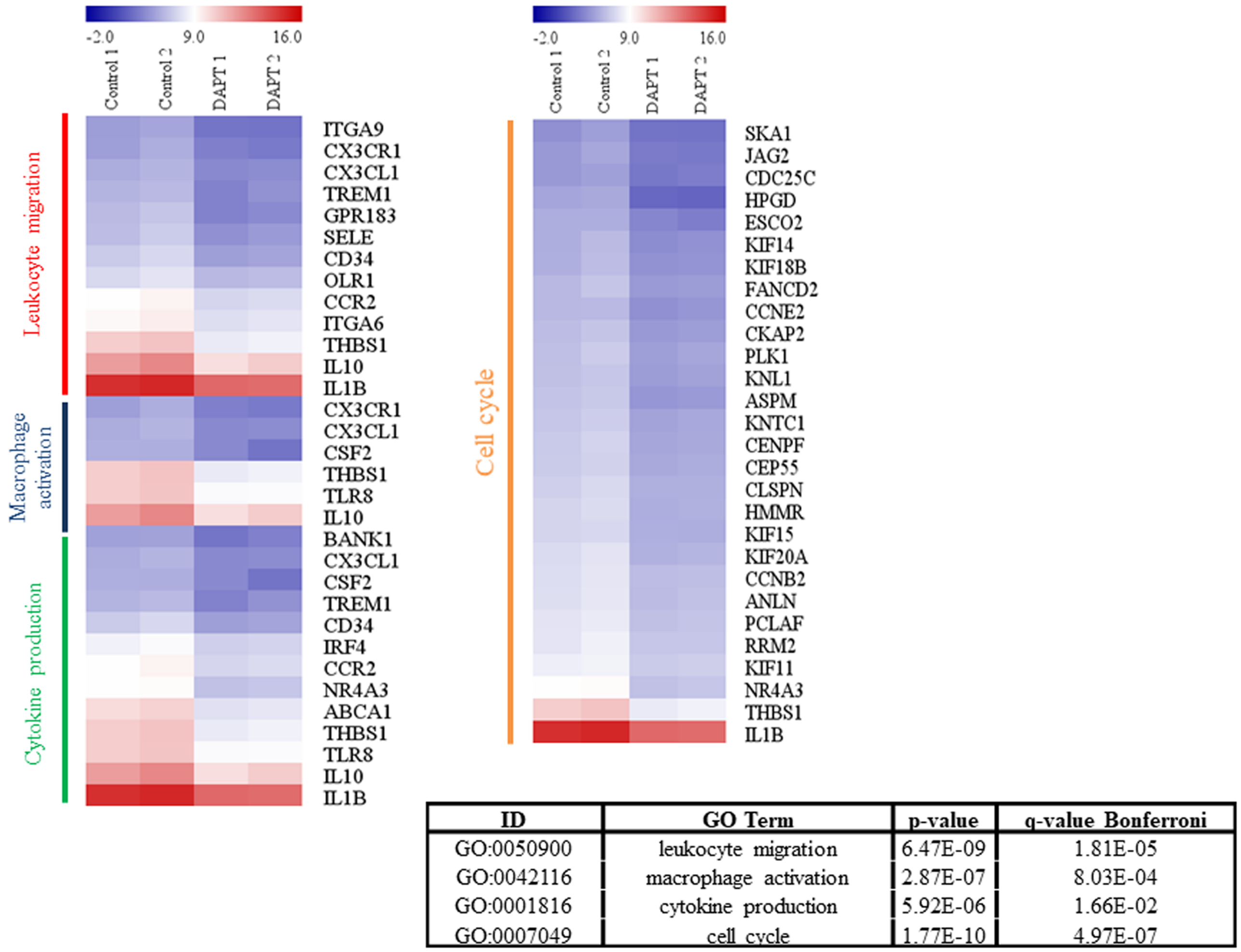
Rbpj KO macrophages recovered later at 4 hr. Taken together, these data suggest that the Notch signaling pathway positively regulates NF-κB activation, at least through inhibiting the p50 subunit, and this, in turn, affects the phenotypes of LPS/IC-activated macrophages, such as IL-10 production.

GSI treatment changes the gene expression profiles of LPS/IC-activated macrophages

To investigate the global effect of inhibiting the activation of Notch signaling in LPS/IC-stimulated macrophages, a transcriptomic analysis by RNA-seq was performed to determine differential gene expression. When comparing BMMs stimulated with LPS/ICs in the presence of GSI and BMMs treated with vehicle control, 147 genes were found to be differentially expressed with $\log_2$ fold changes of greater than 1.5. More genes were downregulated in GSI-treated macrophages, suggesting that Notch signaling is positively involved in regulating gene expression (S3 and S4 Figs). The gene ontology (GO) enrichment analysis of the biological processes involving the upregulated and downregulated genes revealed that leukocyte migration, macrophage activation, cytokine production and cell cycle were significantly affected by GSI treatment in LPS/IC-stimulated macrophages (Fig 6 and S3 and S4 Figs). Interestingly, the biological processes associated with cell cycle were profoundly affected by GSI treatment in LPS/IC-stimulated macrophages (Fig 7).



ID	GO Term	p-value	q-value Bonferroni
GO:0050900	leukocyte migration	6.47E-09	1.81E-05
GO:0042116	macrophage activation	2.87E-07	8.03E-04
GO:0001816	cytokine production	5.92E-06	1.66E-02
GO:0007049	cell cycle	1.77E-10	4.97E-07

Fig 7. Heatmap of differentially expressed genes in LPS/IC-activated macrophages treated with DMSO *vs*. GSI. Total RNA isolated from LPS/IC-activated macrophages treated with vehicle DMSO or GSI (25 μM) was subjected to RNA-seq. Differentially expressed genes were subjected to a GO enrichment analysis. Heatmaps of genes associated with the GO terms leukocyte migration, macrophage activation, cytokine production and cell cycle are shown.

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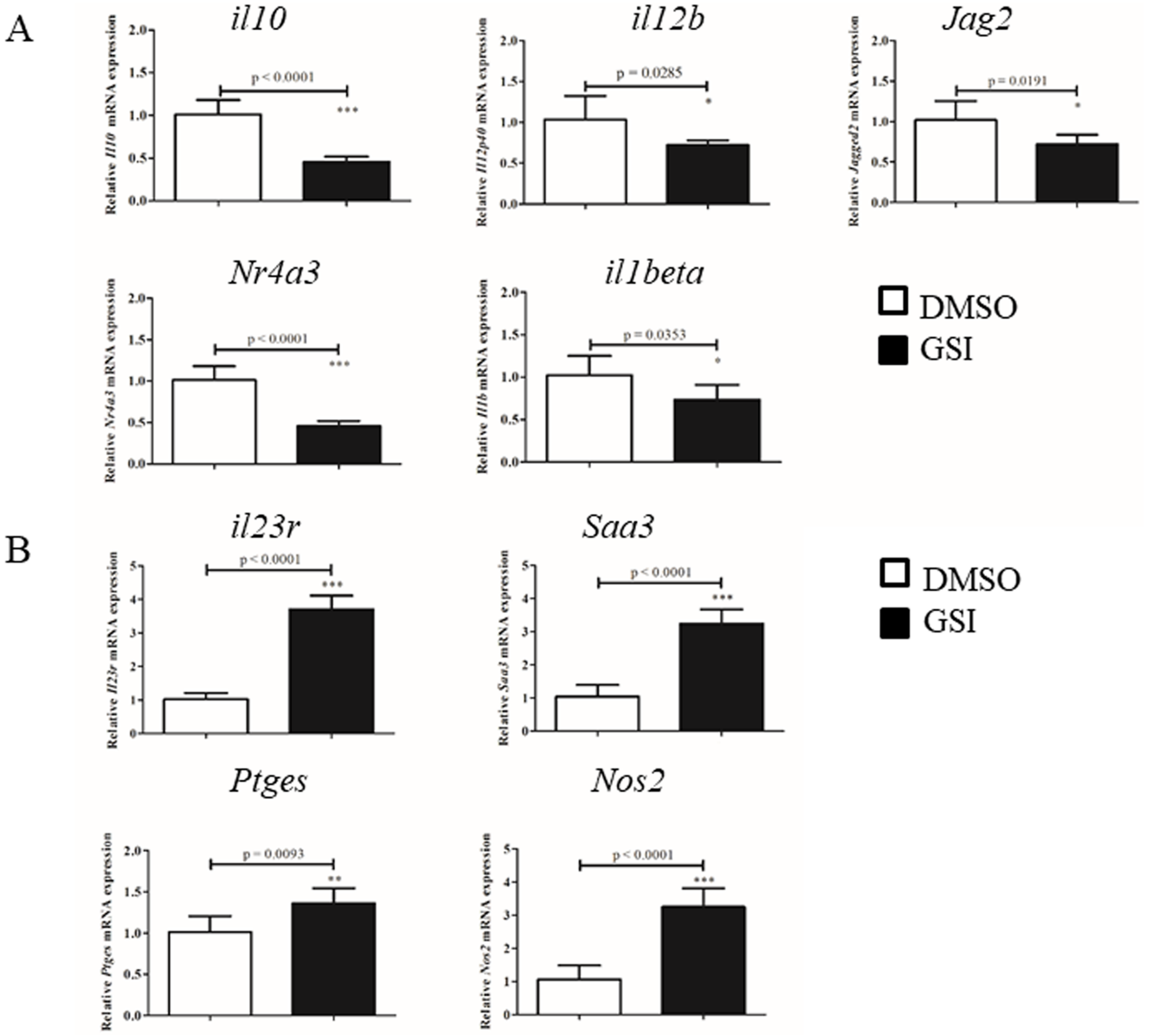

Fig 8. qPCR validation of differential gene expression induced by GSI treatment. Representative genes whose levels of expression were downregulated (A) or upregulated (B) by GSI treatment were validated in IFNγ-primed BMMs activated by LPS/ICs and treated with vehicle control DMSO or GSI. *, ** and *** indicate statistical significance ($p<0.05$) according to unpaired t-tests. The data represent the mean±SD of triplicate determinations from 1 of 2 representative independent experiments.

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Among the genes with reduced mRNA levels, *Il10*, *Il12b*, and *Il1beta* and the Notch ligand *Jag2* and nuclear hormone receptor *Nr4a3* were validated (Fig 8A). These genes are reported to be partially regulated by the NF-κB pathway. Furthermore, the pro-inflammatory genes *Il23r*, *Saa3*, *Ptges* and *Nos2* were validated as genes upregulated by GSI treatment (Fig 8B).

To confirm whether deletion of CSL/RBP-Jκ yields similar effect on gene expression in LPS/IC-stimulated macrophages, the same set of genes validated in Fig 8 were examined in BMMs from wildtype or *Rbpj* KO mice. As shown in Fig 9, only *Jag2* and *il1beta* mRNA showed reduced level in *Rbpj* KO macrophages, consistent with GSI treatment. In contrast, other genes showed no differences (*Nr4a3*, *il23r*, *Nos2*) or decreasing level (*Saa3*, *Ptges*).

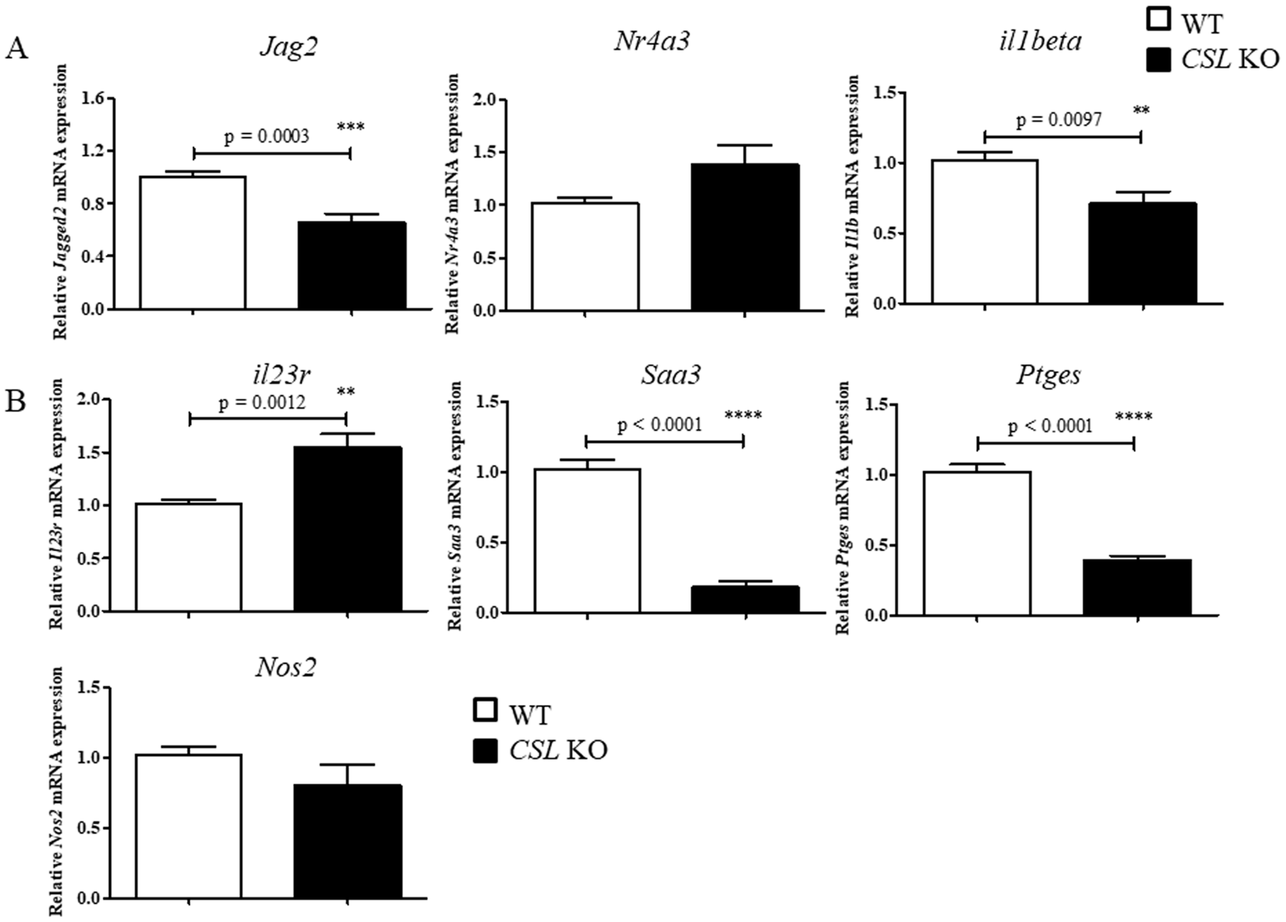

Fig 9. qPCR validation of differential gene expression in wildtype or *Rbpj* KO macrophages. Representative genes whose levels of expression were downregulated (A) or upregulated (B) by GSI treatment were validated in BMMs from wildtype or *Rbpj* KO mice upon activation by LPS/ICs. *, ** and *** indicate statistical significance ($p<0.05$) according to unpaired t-tests. The data represent the mean±SD of triplicate determinations from one of two representative independent experiments.

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Therefore, in LPS/IC-stimulated macrophages, Notch signaling directly or indirectly suppresses inflammation via regulating the expression of inflammation-related genes. Taken together, the data indicate that Notch activation plays a complex role in regulating gene expression in LPS/IC-stimulated macrophages and may regulate inflammatory functions in this regulatory macrophage type via crosstalk with NF-κB pathways.

Discussion

In this study, we confirmed the involvement of Notch signaling in LPS/IC-stimulated macrophages. Signaling through LPS/TLR4 is essential for Notch activation and Dll4, but not Jagged1, was identified as the main ligand that activates Notch1. An antibody or ICs alone was not sufficient to initiate Notch signaling activation as determined by the cleavage of Notch receptors. Previously, Foldi *et al* reported that the activation of Notch signaling was initiated through the downstream signaling of TLR4 in LPS-stimulated macrophages, mainly through the MAPK and NF-κB pathways and autoamplified by Jagged1 [24]. The differences in ligand usage to initiate Notch activation between LPS-stimulated *vs*. LPS/IC-stimulated macrophages may result in transduction of difference signals that affect difference cellular responses. Recent report confirmed that difference Notch ligands (Dll1 and Dll4) sends pulsatile or sustained signaling dynamics via the same Notch receptor and induces opposing cell fate during embryogenesis [34]. The surface expression of Dll4 was reduced in LPS/IC-stimulated macrophages, possibly due to the internalization of the protein after ligand/receptor ligation. In this study, Notch1 was investigated, but we found that the level of Notch2 also increased upon stimulation (Fig 2A). Therefore, multiple Notch receptors may be involved in the regulation of LPS/IC-stimulated macrophages.

The production of IL-10 in macrophages stimulated with TLR was regulated primarily by the MAPK and NF-κB signaling pathways [13]. In the presence of ICs, which trigger the signaling downstream of FcγRs, signals generated by ICs and LPS cooperate to enhance the production of IL-10 [7]. The level of IL-10 produced was affected to varying degrees when specific inhibitors (MEK1/2 MAPKs, p38 MAPK, NF-κB IκBα, PI3K and Notch) were used, indicating that each signal contributes differentially to controlling IL-10 production in macrophages activated by LPS with ICs. The limitation of this study using inhibitors is the undesirable off-target effect. Detailed characterization of the contribution of each pathway may be required to confirm this observation.

We further investigated the role of NF-κB in macrophages that were activated by LPS and ICs because NF-κB signaling was reported to control IL-10 production in macrophages stimulated with LPS [13, 17]. A previous study demonstrated the role of NF-κB in supporting MAPK signaling in LPS-induced IL-10 production in macrophages [35]. Furthermore, various DNaseI hypersensitive sites (HSs) containing NF-κB binding motifs were identified in the *Il10* locus (-55/-46), emphasizing the important role of NF-κB in the direct regulation of IL-10 production in various cell types, including macrophages [15, 17]. Among the NF-κB subunits, NF-κB1/p50 is the most abundantly found subunit in tumor-associated macrophages that plays a critical role in suppressing anti-tumor responses by decreasing IL-12 production and increasing IL-10 level [36]. In contrast to other NF-κB subunits, p50/p50 homodimer often functions as transcriptional repressor. In LPS-activated macrophages, however, p50 homodimer forms a complex with CREB-binding protein and activates transcription of *Il10* [17]. In line with this report, macrophages from $p50^{-/-}$ mice is highly susceptible to LPS-induced sepsis [7]. These data all indicate that NF-κB p50 plays a critical role in regulating IL-10 production in macrophages. Our results added another piece of evidences that Notch signaling acts together with NF-κB p50 to regulate *Il10* expression in LPS/IC-stimulated macrophages.

How does Notch signaling cross regulate NF-κB pathway and prime macrophages for the effect of IC for optimal IL-10 production? The mechanism can be direct (physical interaction) or indirect (via other proteins). In T cell activation, Shin *et al.* showed that the intracellular domain of Notch1 in the nuclei interacts directly with NF-κB (p50) and sustains NF-κB activation to maintain T cell activation. Decreased NF-κB activation was observed in the absence of the nuclear localization of the Notch1 protein, indicating that the Notch protein regulates the activation of NF-κB, at least the p50 subunit [37]. In T cell leukemia, Notch signaling through one of its target gene, *Hes1*, sustains NF-κB activation by repressing expression of deubiquitinase CYLD. CYLD is a negative regulator of IKK complex [38]. In our study, GSI treatment and *Rbpj* KO yielded similar results of the defects in nuclear localization of NF-κB p50. Furthermore, the level of *Hes1* decreased upon GSI treatment. These data suggested that Notch/RBP-Jκ affects NF-κB p50 nuclear localization in a canonical Notch-dependent manner, possibly through Hes1 or direct association. While the effect of *Rbpj* deletion on NF-κB p50 was observed only at early time point (1 hr post stimulation), GSI treatment exhibited longer effect up to 4 hr.

The transcriptomic analysis revealed that genes involved in the cell cycle were consistently downregulated upon GSI treatment (Fig 7). Previously, it was reported that FcγR crosslinking induces cell cycle progression through the ERK pathway [39]. In addition, the transcriptomic study by Fleming *et al*. revealed that in the cluster of genes in regulatory macrophages stimulated with LPS and ICs, prostaglandin E2 (PGE2) and adenosine are associated with increased cell growth and proliferation. Accordingly, it was postulated that regulatory macrophages may contribute to homeostasis and promote cellular repair [6]. Therefore, in LPS/IC-stimulated macrophages, Notch signaling may play an important role in regulating cell growth and proliferation.

Among the genes identified to be differentially expressed by GSI treatment, the transcript levels of genes associated with pro-inflammatory functions, *Il23r*, *Saa3*, *Ptges* and *Nos2*, were increased. In contrast, only the level of *Il23r* was reduced in *Rbpj* KO macrophages, while those of *Saa3*, *Ptges* increased. These results highlighted the differences in the cellular phenotypes obtained by GSI treatment *vs*. *Rbpj* KO. The discrepancies between the two approaches may reflect the requirement for canonical vs. non-canonical Notch signaling for LPS/IC-stimulated macrophages [40]. GSI affects both pathways while *Rbpj* KO affects only the former. Furthermore, γ-secretase has various type I transmembrane substrates besides Notch receptors and some of the impacts observed by GSI treatment may be the results of inhibition of other substrates [41]. Further experiments are needed for this verification.

Macrophages function as antigen presenting cells. During antigen presentation, cytokine milieu created by macrophages can dictate helper T cell response. IL-10 producing macrophages generated by stimulation with LPS/IC decreases disease severity of EAE upon adoptive transfer, suggesting an in vivo impact on adaptive immune responses [9]. Furthermore, adoptive transfer of Notch1 deficient LPS-activated macrophages reduced IL-17 producing helper T cells in an EAE model [31]. These observations together with the findings reported here strongly suggest that modulation of Notch signaling in LPS/IC-stimulated macrophages may alter the outcome of adaptive immune responses.

We propose a model of how Notch signaling is involved in the regulation of IL-10 in LPS/IC-activated macrophages based on the results obtained in this study (see S5 Fig). In this model, Notch signaling is activated via the signaling downstream of TLR4 (signal 1), mainly by the NF-κB and Erk pathways. Notch signaling, in turn, regulates genes directly or indirectly involved in the production of IL-10 and other genes by crosstalk with the NF-κB pathway, mainly p50 subunit. Both the NF-κB and Erk pathways, in turn, activate and sustain the activation of Notch signaling, possibly through the upregulation of the Notch ligands. Therefore, the production of IL-10 in IC-activated macrophages is regulated by the NF-κB and Erk pathways in a Notch-dependent manner via TLR4 signaling. p38 and PI3K signalings via TLR4 and FcγR (signal 2) also contributes in regulating IL-10 production, possibly, in a Notch-independent manner.

In this report, we demonstrated the role that Notch signaling plays in regulating the phenotypes of LPS/IC-stimulated macrophages. In particular, Notch/Dll4 axis regulates IL-10 production in LPS/IC-stimulated macrophages by regulating NF-κB p50 nuclear localization. The results obtained in this study may help to further understand the molecular mechanisms of macrophage activation in the presence of immune complexes and may shed new light on inflammatory-related diseases, including autoimmune disorders.

Supporting information

S1 Table. List of primers used in this study.
(DOCX)

S1 Fig. Effect of GSI and ligand blocking antibody on *Il10* and *hes1* mRNA level in LPS/IC-activated BMDMs. (A-B) IFNγ-primed BMMs activated by LPS/ICs and treated with vehicle

control DMSO or GSI for 1 or 4 hr. The relative mRNA level of *hes1* or *Il10* were measured by qPCR. ** and *** indicate statistical significance ($p<0.05$ and 0.01) according to two way ANOVA with Post Tests. The data represent the mean±SD of triplicate determinations. (C) BMMs were treated with indicated antibodies (isotype control, anti-Jagged1 antibody or anti-Dll4 antibody) during IFNγ priming. After the priming, LPS/IC were added to BMMs for 1 hr and the level of *Il10* mRNA was evaluated by qPCR. ** indicated statistical significance ($p<0.005$) according to unpaired t-tests. The data represent the mean±SD of triplicate determinations.
(TIF)

S2 Fig. Images of macrophages after immunofluorescent staining of NF-κB p50 with lower magnification. BMMs from wildtype or *Rbpj* KO mice were stimulated with LPS/IC for 1 and 4 hr and the cytosolic and nuclei fractions were analyzed for NF-κB p50. Representative data from 1 of 2 independent experiments are shown.
(TIF)

S3 Fig. Heat map of downregulated gene set in LPS/IC-activated BMDMs treated with GSI.
(TIF)

S4 Fig. Heat map of upregulated gene set in LPS/IC-activated BMDMs treated with GSI.
(TIF)

S5 Fig. A proposed model how Notch signaling is involved in regulating gene expression in LPS/IC-stimulated macrophages (see text for details). Red arrows indicated the links observed in this study.
(TIF)

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Writing – original draft: Wipawee Wongchana, Pornrat Kongkavitoon, Tanapat Palaga.

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Notch signaling modulates Peroxisome proliferator-activated receptor gamma level in Interleukin 4-stimulated human macrophages through Akt and NEDD4L

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Running Title: *Notch signaling modulates PPARγ expression in IL-4-stimulated macrophages*

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ABSTRACT

Macrophages in various diseases exhibit distinct phenotypes, depending on the stimuli and microenvironment. Macrophage phenotypes are classified at least into 2 types that are pro-inflammatory type induced by pathogen associated molecular patterns and wound healing type induced by interleukin (IL)-4. These phenotypic polarizations strongly associate with severity of some chronic diseases such as atherosclerosis and cancer. Notch signaling and a nuclear receptor, peroxisome proliferator-activated receptor (PPAR) γ, are known to regulate macrophage polarization and their crosstalk was reported in some cell types such as adipocytes. Whether Notch signaling and PPARγ interact during macrophage polarization have not been documented. In this study, human monocytic cell line THP-1 and primary human monocytes derived macrophages activated Notch signaling upon IL-4 stimulation. Inhibition of Notch signaling reduced phosphorylation of Akt downstream of IL-4/IL-4R and also one of its target *PPARG* mRNA. Hyperactive Notch signaling by overexpression of Notch1 intracellular domain (NIC1) increased PPARγ protein stability by decreasing proteasome degradation, but did not change the level or stability of mRNA. Transcriptomic analysis uncovered the links between Notch and PPARγ through NEDD4L, an E3 ubiquitin ligase. Deletion of *NEDD4L* in the presence of NIC1 in THP-1 significantly reduced Akt phosphorylation and *PPARG* mRNA transcription upon IL-4 stimulation. Activation of Notch signaling affected lipid metabolism in IL-4-stimulated macrophages by increasing lipid accumulation via CD36, one of the targets of PPARγ. Collectively, we present evidences linking Notch signaling and PPARγ via Akt and NEDD4L in IL-4-stimulated macrophages that play a critical role in macrophage lipid uptake.

Introduction

Macrophages have multifaceted functions in immune system depending on microenvironments and stimuli they received. Their functions can be described as spectrum of activation which one end is inflammatory phenotype and another end is inflammation dampening would healing phenotype (1,2). In a simplified *in vitro* system, macrophages are polarized to two opposing phenotypes, that are pro-inflammatory such as LPS-stimulated macrophages (referred to as M(LPS)) and pro-healing such as IL-4-stimulated macrophages (referred to as M(IL-4)). Both types of macrophages could be found in normal and disease tissues, highlighting the important roles they play in tissue homeostasis and disease pathogenesis (2). For example, pro-inflammatory macrophages are found in atherosclerotic plaque that promotes plaque formation. The reversing phenotype of pro-inflammatory to pro-healing phenotype reduced plaque size, resulting in good disease prognosis (3). In tumor, however, tumor-associated macrophages show pro-healing-like phenotype and support tumor progression and metastasis (4). Therefore, macrophage activation is a double-edged sword and it is expected that controlling its activation can be powerful therapeutic choices for such chronic conditions (3,5).

Interleukin-4 (IL-4) is a well-known multi-functional cytokine that can activate macrophages and induce pro-healing phenotypes of M(IL-4) (6). Interaction between IL-4 and IL-4α receptor triggers receptor heterodimerization with IL-2Rγ or IL-13Rα1 to activate STAT6 and AKT1. This early event leads to a second wave of activation of transcription factors such as PPARγ, EGR2 (7-10). M(IL-4) upregulates genes involved in anti-inflammation, lipid metabolism, apoptotic cell clearance and cellular metabolism (1,11-13). Potential anti-inflammatory proteins from M(IL-4) include 15-lipooxygenase, CD36, monoamine oxidase A (MAO-A) (14,15).

PPARγ is a ligand dependent nuclear hormone receptor in a PPAR family which differentially expresses in various cell types and development stages (16). Ligand binding to PPARγ leads to the heterodimer formation with retinoid-X-receptor (RXR) or liver-X-receptor (LXR), and recruitment of other co-activators to the conserved PPARγ binding site (peroxisome proliferator response element, PPRE) (11,17). The PPARγ target genes, including CD36, lipoprotein lipase (LPL), fatty acid binding protein P4, are necessary for metabolic regulation in macrophages (16,18). Moreover, PPARγ agonist alone can stimulate macrophages to anti-inflammatory phenotype (13,19). Macrophages from PPARγ knockout mice, showed impaired phagocytic activity to clear apoptotic cell in wounds, resulting in increased TNFα production (12). Although PPARγ is expressed in macrophages in atherosclerotic lesions, there are two conflicting roles for this protein in this disease (20). One role is to decrease inflammation and plaque formation (21). In contrast, it also function to enhance plaque formation by induction lipid uptake via CD36 (20). The contradictory roles of PPARγ in macrophages associated with atherosclerotic lesions may reflect the complicated interacting signaling network in these cells.

PPARγ stability is regulated mainly by proteasome degradation. For instance, IFNγ-induced PPARγ phosphorylation at Ser112 by ERK1/2 targets PPARγ for ubiquitination and proteasome degradation in adipocytes (22). However, phosphorylation of PPARγ at Ser112 by MEK/ERK in hepatocellular carcinoma increases PPARγ activity, promotes glycolysis and tumor cell proliferation (23). A ubiquitin-like protein, NEDD 8 is covalently bound to PPARγ and competes with ubiquitin to reduce PPARγ degradation and increased its activity in adipocytes (24). In addition, NEDD4 , a ubiquitin ligase, ubiquitinate PPARγ but prevent it from degradation (25). Therefore, multiple layers of mechanisms regulate PPARγ protein stability and appear to be a cell type and context dependent.

NEDD4L, a human homolog of murine NEDD4, is an evolutionary conserved E3 protein which belongs to the NEDD4 family of HECT domain E3 ubiquitin ligases (26). It functions by controlling ion channel in lung and kidney of which the functions were severely affected in *NEDD4-2* knockout mice (27,28). Recent report in IL-4-activated monocytic cell line U937 found that NEDD4L is directly associated with IL-4Rα, IL-4Rγ and GRB10, and subsequently ubiquitinates and degrades IL-4 receptor by proteasome to limit Il-4 signaling (29). Additionally, NEDD4L also controls TGFβ signaling in keratinocytes by target phosphorylated SMAD2/3 for degradation (30).

The conserved Notch signaling pathway was reported as one of regulator for macrophages activation (31,32). Initiation of Notch signaling occurs when Notch ligands (Jagged1-2, Delta-like1,3-4) binds to their counterparts, Notch receptor (Notch1-4). This interaction induces ADAM metalloprotease and γ-secretase to cleave Notch receptor (33). This process releases the intracellular domain of Notch receptor and allows it into nucleus to bind with CSL/RBP-Jκ and Mastermind-like protein (MAML) to form a transcriptional complex that activates the Notch target genes (such as *HES1*, *HEY1*) (34-36). Notch activation was observed in many inflammation related diseases such as atherosclerosis and diabetes (37,38). Blockade of Notch activation improved severity of some of these diseases (37,38). Therefore, it was proposes that Notch activation is responsible for activation of pro-inflammatory macrophages (31,39). Forced Notch activation in macrophages with Notch ligand, DLL4, switched macrophages to pro-inflammatory phenotype with increased tumoricidal activity in a mouse tumor model (40). Moreover, IL-4-stimulated macrophages in the presence of stimulation via DLL4 interferes with M(IL-4) and causes cells to undergo apoptosis (41). However, it was found in a breast cancer model that CSL/RBP-Jκ is important for TAM differentiation (42). Therefore, the role of Notch signaling in IL-4-stimulated macrophages is still controversial and required more investigation.

The crosstalk between Notch signaling and PPARγ have been reported. During keratinocyte differentiation, Jagged1, possibly through Notch activation, increases PPARγ expression and, in turn, inhibits NF-κB by physical association between NF-κBp65 and PPARγ. This association caused keratinocytes to undergo terminal differentiation (43). In 3T3-L1, a pre-adipocyte cell line, Notch1 upregulates PPARγ and PPARδ that are necessary for adipocyte differentiation. In this study, we uncovered the link between Notch and PPARγ in M(IL-4) with the involvement of NEDD4L and the impact of this crosstalk on lipid uptake in M(IL-4) are presented.

Results

IL-4 activates Notch signaling in human macrophages

To verify the phenotype of IL-4 activated macrophages, THP-1 was activated by IL-4 at various time points and the protein lysate was subjected to analysis of the IL-4 downstream signaling cascade and its target genes. Consistent with previous reports, the results showed that STAT6 and AKT were phosphorylated upon IL-4 stimulation as well as some M(IL-4) signature proteins, TGM2 and PPARγ, were increased (Supplementary Figure S1A, Figure 1 A-B). Next, the activation of Notch signaling was examined by detection of cleaved Notch1 and mRNA of one of Notch target genes, *HEY1*. Rapid induction of cleaved Notch1 (Val1744) was detected within 15 min after IL-4 stimulation (Figure 1 C-D). Moreover, *HEY1* mRNA level was increased at 3 h post stimulation (Figure1 E). Similar results were obtained from primary human monocyte derived macrophages (HMDMs) that IL-4 also activated Notch signaling (Supplementary Figure S1B, Figure 1F). Furthermore, treatment with γ-secretase inhibitor, DAPT, completely abrogated IL-4-induced cleaved Notch1 in this setting. These results suggested that IL-4 stimulation of macrophages activated Notch signaling in human macrophages and this activation requires the γ-secretase activity.

Notch signaling increases PPARγ expression upon IL-4 stimulation

To investigate what roles the Notch signaling may play in M(IL-4), THP-1 was subjected to retrovirally transduction with plasmids harboring Notch1 intracellular domain (NIC1) or dominant negative MAML (DNMAML) to increase or inhibit Notch signaling, respectively. NIC1 expression constitutively activates Notch signaling without the requirement of receptor/ligand binding or γ-secretase activit. DNMAML interferes with the Notch/RBP-Jκ/MAML transcriptional activating complex and functions to inhibit Notch signaling. The effect of NIC1 and DNMAML overexpression on Notch signaling was confirmed by detecting the level of *HEY1* mRNA. NIC1 overexpressing THP-1 showed increased *HEY1* in cells with or without stimulation by IL-4. In contrast, DNMAML failed to upregulate *HEY1* upon IL-4 activation (Supplementary Figure S2). Therefore, NIC1 and DNMAML overexpression in THP-1 represented hyperactivation and hypoactivation of Notch signaling phenotype, respectively. Next, we investigated these effects on PPARγ, one of the key signature proteins of M(IL-4), in these cells by Western blot. PPARγ protein expression was increased in IL-4-stimulated NIC1 overexpressing THP-1, compared with the control (Figure 2A), while DNMAML overexpressing cells exhibited reduced PPARγ expression (Figure 2A). Consistent with this result, DAPT-pretreated THP-1 (Supplementary Figure S3) or HMDMs decreased PPARγ expression upon IL-4 treatment (Figure 2B). Taken together, Notch signaling positively regulates PPARγ expression in M(IL-4).

To explore how Notch signaling increases PPARγ expression, first, the effect of NIC1 overexpression on IL-4 receptor and its activation were examined. IL-4Rα protein level was measured in IL-4-activated THP-1 by flow cytometry. Consistent results with previous reports, we found that IL-4Rα decreased after IL-4 stimulation (Figure 3A) (29). More importantly, the control THP-1 or NIC1 or DNMAML overexpressing THP-1 similarly decreased IL-4Rα expression level upon IL-4 stimulation (Figure 3A).

Next, the effect of Notch signaling on the downstream signaling of IL-4 was investigated by DAPT treatment. We pretreated THP-1 or HMDMs with vehicle control or DAPT before stimulation with IL-4 and investigated the phosphorylation level of STAT6 and AKT Cleaved Notch1 was detected to confirm the efficacy of DAPT to inhibit Notch receptor cleavage. DAPT treatment significantly reduced the level of cleaved Notch1 in THP-1 (Figure 3B) but did not change the level of phosphorylation of STAT6. Reduced the phosphorylated AKT, however, was observed both in THP-1 cell line and HMDMs (Figure 3B, C). Therefore, Notch signaling did not interfere with IL-4 receptors but appeared to positively regulate AKT phosphorylation upon IL-4 stimulation of macrophages.

Notch signaling regulates PPARγ degradation by proteasome

The results thus far indicated that Notch signaling was activated upon IL-4 stimulation in human macrophages, and one of the signature proteins of M(IL-4), PPARγ was increased by Notch signaling. Next, we examined *PPARG* mRNA expression in NIC1 or DNMAML overexpressing THP-1 by qPCR. *PPARG* mRNA expression was increased at 3 h after IL-4 activation (Figure 4A). Overexpression of NIC1 or DNMAML did not change the level of *PPARG* mRNA at 3 h (Figure 4A) or 6 h after stimulation (Supplementary Figure S4). Next, the stability of this mRNA was examined. NIC1 overexpressing THP-1 was activated with IL-4 for 3 h, and subjected to actinomycin D treatment before chasing at 0, 45 and 90 min. *PPARG* mRNA level was measured by qPCR. The result showed that the half-life of *PPARG* mRNA in control IL-4-activated THP-1 was 104.3+/-16.47 min while that of NIC1 overexpressing THP-1 was 94.91+/-7.583 min (Figure 4B). Therefore, Notch signaling did not regulate *PPARG* transcription or its mRNA stability.

Because previous reports found that PPARγ was regulated by proteasome degradation (25), we asked whether Notch signaling regulates PPARγ protein degradation by this mechanism. In this study, THP-1 was pretreated with MG132, a proteasome inhibitor, and subsequently stimulated with IL-4 for 4 h and PPARγ was detected by Western blot. As expected, the result clearly showed that PPARγ level was increased in MG132 treated cells, indicating the involvement of proteasome degradation (Figure 5A). When NIC1 overexpressing THP-1 was treated with MG132, the level of PPARγ was similar between MG132-treated and vehicle control treated cells, indicating that inhibition of proteasome did not enhance the effect of NIC1 overexpression on stabilizing PPARγ (Figure 5A). To confirm that NIC1 overexpression prolongs PPARγ protein stability, cells were activated with IL-4 for 4 h before treatment with cycloheximide, an inhibitor of *de novo* protein synthesis, and the level of PPARγ was chased every 20 min, for 100 min by Western blot. The results showed that IL-4-activated NIC1 overexpressing THP-1 cells significantly delayed degradation of PPARγ, resulting in more protein accumulation than that of the control (Figure 5B-C). Calculated protein half life in NIC1 overexpressing cells was approximately twice longer than the control. Collectively, Notch signaling in IL-4 stimulated macrophages delays proteasome-dependent degradation of PPARγ.

Transcriptomic analysis of IL-4 activated NIC1 or DNMAML overexpressing THP-1

In order to understand the global impact of activation of Notch signaling in M(IL-4), RNA-seq was performed in THP-1 overexpressing NIC1 or DNMAML in the presence or absence of IL-4 (Figure 6A). Transcriptome analysis revealed changes in transcripts of 392 genes upon IL-4 activation, 45% of which were commonly altered in IL-4 activation among control, NIC1 and DNMAML overexpressing cells. IL-4 stimulation in NIC1 overexpressing cells induced changes in 530 genes, compared with unstimulated NIC1, 58.7% of which were upregulated and 41.3% of which were downregulated. IL-4 stimulation in DNMAML overexpressing cells altered expression of 461 genes, compared with unstimulated DNMAML, 5.4% of which were upregulated and 94.6% of which were downregulated. Among these genes, 28, 143, 80 genes were uniquely expressed upon IL-4 stimulation in control, NIC1 overexpressing and DNMAML overexpressing cells, respectively, compared with each unstimulating control (Figure 6B).

Next, we applied GSEAPreranked analysis to uncover the enrichment gene sets in comparison between IL-4-activated control and NIC1 overexpressing THP-1. As expected, significant enrichment of Notch signaling pathway was observed in IL-4-activated NIC1 overexpressing cells (Supplementary Table S1). Moreover, IL-4-activated NIC1 overexpressing THP-1 showed enriched gene set of TNFα signaling via NF-κB inflammatory response and hypoxia (Supplementary Table S1, Figure S5A). Hypoxia condition or forced Notch activation in macrophages increases inflammatory cytokines and mediators including TNFα, nitric oxide and IL-12 (40,44). Furthermore, it is hypoxic microenvironment in some pathologic conditions, including infection, sterile inflammation and cancer (44,45). HIF1α, a transcription factor involved in response to hypoxia, was reported as interacting partner with Notch to control target gene transcription (46). Therefore, the hypoxia hallmark was focused for further study.

We combined the DESeq2 analysis with GSEAPreranked analysis results and found that a HECT domain E3 ubiquitin ligase, NEDD4L stood out in this combination which was observed to be upregulated only in IL-4-activated NIC1 overexpressing THP-1 (Figure S5A-C). An interacting network analysis revealed the link among NEDD4L, PPARγ and Notch1 via SGK1 in RNA-seq dataset of IL-4-activated NIC1 overexpressing cells, compared with the control (Supplementary Figure S5 D-E) (47). Therefore, the involvement of NEDD4L in PPARγ in M(IL-4) was further investigated.

NEDD4L increased PPARG expression in NIC1 overexpression

To explore whether NEDD4L functionally interacts with Notch signaling in regulating PPARγ expression, NEDD4L expression in IL-4 activated NIC1 overexpressing THP-1 cell line was investigated. Consistent with the RNA-seq data, *NEDD4L* mRNA was increased in IL-4-activated NIC1 overexpressing cells, compared with the control at 3 h (Supplementary Figure S6A), but no difference was found at the protein level between unstimulated and IL-4-stimulated cells at 4 h (Supplementary Figure S6B). In HMDMs, IL-4 stimulation increased the level of *NEDD4L* mRNA but also did not increase the level of NEDD4L. DAPT pretreatment did not affect NEDD4L expression level (Supplementary Figure S6C). Thus, activation of Notch signaling increased *NEDD4L* mRNA in M(IL-4).

Next, we used CRISPR/Cas9 system to knockout *NEDD4L* gene at two different positions (N4L#1KO and N4L#2KO) in THP-1 and obtained the stable cell lines with *NEDD4L* deletion. Complete deletion of NEDD4L was confirmed by Western blot (Figure 7A). Previous report indicated that NEDD4, a close relative of NEDD4L, functions as an E3 ubiquitin ligase to target Notch receptor for degradation. However, in our study, the levels of both cleaved Notch1 and total Notch1 in cells with deficient *NEDD4L* was not different from the control. Next, the level of PPARγ was examined in IL-4 activated *NEDD4L* deficient cells by Western blot. The result showed that deletion of *NEDD4L* did not affect the level of PPARγ in M(IL-4) (Figure 7A). Therefore, depletion of NEDD4L alone is not sufficient to alter IL-4-induced PPARγ in macrophages.

To investigate whether NIC1 overexpression increases PPARγ via NEDD4L, we used lentiviral transduction to overexpress NIC1 or control plasmid in NEDD4L-KO cell lines and observed PPARγ expression in IL-4 activated cells by Western blot. To our surprise, NIC1 overexpression has a severe consequence on the PPARγ level in the absence of NEDD4L in M(IL-4) (Figure 7B). While IL-4 stimulation in control cells clearly induced the upregulation of PPARγ, NIC1 overexpressing cells in the absence of NEDD4L failed to do so.

From the results of the network analysis, there was a link between Notch1 and NEDD4L through SGK1. SGK1 is a downstream effector of PI3K and shares approximately 45-55% homology in the catalytic domain with AKT (47,48). Therefore, we further investigated AKT phosphorylation in NIC1 overexpressing *NEDD4L* deficient THP-1. NIC1 overexpression or NEDD4L depletion alone did not alter AKT phosphorylation after IL-4 stimulation at the time point examined (Figure 7B and Figure S6B). In contrast, severe reduction in Akt phosphorylation was detected in NIC1 overexpression in *NEDD4L* deficient background (Figure 7B). To summarize this finding, the band densities of PPARγ, NEDD4L and the band density ratio of phosphor Akt/Total Akt were measured and plotted as a heatmap in Figure 7C. This result clearly showed a severe impact of NIC1 overexpression in *NEDD4L* deficient background on phosphorylation of Akt and the expression of PPARγ upon IL-4 activation in macrophages.

To investigate whether this effect of NIC1 overexpression in *NEDD4L* deficient cells on PPARγ expression is at the transcriptional level, we investigated IL-4-induced *PPARG* mRNA expression by qPCR. The level of *PPARG* mRNA expression were significantly decreased when NIC1 was overexpressed, while *NEDD4L* deletion alone had no effect (Figure 7D). These results indicated that Notch signaling directly or indirectly regulates *PPARG* transcription via NEDD4L. Taken together, these results led us to propose that NIC1 overexpression may induce suppressor(s) of AKT phosphorylation and/or PPARγ and this suppressor(s) is under the negative regulation of NEDD4L. In the absence of NEDD4L, NIC1 overexpression allows this suppressor(s) to function to inhibit Akt phosphorylation and the transcription of *PPARG* mRNA.

NIC1 overexpression upregulated CD36 expression via PPARγ

To understand the biological relevance of Notch signaling in M(IL-4), we further examined some M(IL-4) functions which are known to be mediated through PPARγ. We compared RNAseq results with the PPARγ pathway from BioCarta database to identify the PPARγ gene set (Figure 8A). *CD36* was predominantly increased in unstimulated and IL-4-stimulated NIC1 overexpressing THP-1 while DNMAML reduced *CD36* mRNA (Figure 8A). Lipid metabolism in macrophages is controlled by PPARγ which requires a scavenger receptor, CD36, for uptake of lipid. Therefore, CD36 expression in IL-4-treated THP-1 was examined by flow cytometer. Rosiglitazone, a PPARγ ligand, was used to treat macrophages as positive control for PPARγ-induced CD36 expression. The level of CD36 surface expression were similar in all conditions (Supplementary Figure S7).

During lipid uptake by macrophages, CD36 on the cell surface binds to lipid and internalizes lipid, resulting in the receptor/ligands residing within the cells (49). Therefore, CD36 on cell surface decreases during the active lipid uptake while the intracellular level increases. To examine the possibility that intracellular CD36 in NIC1 overexpressing cells are higher than the control, the intracellular CD36 level was determined by flow cytometer. As expected, NIC1 overexpressing THP-1 had higher level of intracellular CD36 without any additional stimuli and the level of CD36 expression increased further in the presence of rosiglitazone. This is possibly because NIC1 stabilized PPARγ expression and, in turn, PPARγ induces CD36 expression and internalization (Figure 8B). Neither IL-4 nor rosiglitazone treatment increased CD36 expression in the control cells (Figure 8B). In addition, cells overexpressing DNMAML failed to increase CD36 in response to IL-4 or rosiglitazone (Figure 8B). Therefore, activation of Notch alone increases PPARγ function in macrophages.

To verify that increased expression of CD36 correlates with its function, we examined lipid uptake by stimulated THP-1 with oxLDL, an inducer of and ligand for CD36. IL-4- or oxLDL-stimulated THP-1 clearly increased cellular lipid content. Correlating with CD36 level, NIC1 overexpressing THP-1 increased lipid accumulation to higher level than the control cells or DNMAML overexpressing cells in unstimulated condition (Figure 8C). In response to IL-4, lipid accumulation was higher in NIC1 overexpressing cells while oxLDL stimulation yielded similar increased lipid accumulation in all conditions.

Discussion

In this study, we reported that Notch signaling was rapidly activated in human macrophages by IL-4 and this activation affects the expression of PPARγ, one of the signature proteins induced in M(IL-4). The requirement of the so called canonical Notch signaling complex to activate transcription of the target genes was addressed by overexpressing DNMAML and the use of gamma secretase inhibitor. The results obtained from using cell line and primary human macrophages suggested that the cleavage of Notch receptor(s) by gamma secretase, but not the formation of Notch/RBP-jκ/MAML, plays important role in regulating the level of PPARγ (50). Whether the canonical Notch signaling regulates other phenotypes of M(IL-4) are currently not known. Previous studies reported that Notch signaling positively regulates pro-inflammatory macrophages such as those activated by pathogen associated molecular patterns such as LPS. In our RNA-seq results obtained from NIC1 overexpressing macrophages, the gene sets of TNFα signaling via NF-κB and inflammatory response were enriched (Supplementary Table 1). Therefore, activation of Notch signaling in macrophages in the presence of IL-4 induced mixed macrophage phenotypes of pro- and anti-inflammatory. We here proposed that Notch signaling may also participate in the alternative activation of macrophages. Recently, it was reported that RBP-Jκ is required for activation of macrophages by chitin in mice, which is consistent with our observation (51).

The first step toward investigating how overexpression of NIC1 increased PPARγ, the signaling downstream of IL-4 was examined. IL-4 signaling pathway activates phosphorylation of STAT6 and AKT. In Th2 cell, GATA3, IL-4 and STAT6 act on an autocrine loop to amplify IL-4 that is necessary for Th2 differentiation (52,53). IL-4 stimulation phosphorylates STAT6 to turn on a Th2 master regulator GATA3 to induce IL-4 production (53). In T cells, Notch signaling plays a role in GATA3 expression in STAT6-independent manner (53). When DAPT was used in our study, reduced phosphorylation of AKT was observed, suggesting that Notch signaling may also regulate IL-4 signaling pathway. In contrast, no evidence was found for the crosstalk between Notch signaling and STAT6 in M(IL-4).

Previous study reported that Notch activation was important for initial IL-4 expression in naïve CD4+ T cells by direct regulation of the enhancer regions (52). In this setting, CSL binding site was found in a conserved noncoding sequence-2 (CNS-2), which is an active enhancer of *il4* locus in naïve CD4+ T cells (52). In our study, we did not detect increasing of *PPARG* mRNA expression when Notch signaling is hyperactivated, indicating that Notch signaling alone did not regulate *PPARG* at the transcriptional level. Recent study in osteoblastic cells found that Notch increased connective tissue growth factor (*ctgf*) mRNA and protein level by regulating the transcription but destabilizing *ctgf* mRNA (54). In M(IL-4), Notch signaling did not alter *PPARG* mRNA stabilization.

In IL-4 stimulated THP-1 cell line, PPARγ was degraded by proteasome as previously reported in 3T3-L1 pre-adipocytes (25). The mechanism by which Notch signaling delays proteasome degradation of PPARγ is currently not known. In pancreas, Notch signaling controlled protein stability of Ngn3, a regulator of pancreatic endocrine formation, via HES1. In pancreatic cancer with *KRAS* mutation, inhibitor of κB kinase 2 (Ikk-2) synergized with Notch to induce HES1 expression for suppressing PPARγ (55). Because DNMAML did not yield any impact on stability of PPARγ, the role of HES1 can be excluded.

Proteasome degradation of PPARγ is mediated by various E3 ubiquitin ligases, including SIAH2, FBXO9, MKRN1 and NEDD4 (56). In our RNA-seq data, the mRNAs of these genes, except the slight increase of *SIAH2*, were not significantly changed by overexpression of NIC1. Therefore, other unknown E3 unbiquitin ligase(s) may be responsible PPARγ degradation in M(IL-4) which is under the regulation of Notch signaling.

We attempted to identify the links between Notch signaling and PPARγ in M(IL-4) using transcriptomic approaches. The differentially expressed genes from unstimulated cells or M(IL-4) with NIC1 overexpression were compared with those in the control cells using BioCarta database and network analysis. SGK1 and NEDD4L were identified as potential candidate for this purpose. SGK1 is a serine/threonine kinase that play a role in cellular stress response (47,48). SGK1 was found to be important for M2 activation via STAT3 and may be the key link between Notch and PPARγ (57).

NEDD4L regulates IL-4 signaling feedback mechanism in U937 and stabilized PPARγ in 3T3-L1 cell line. Moreover, NEDD4, a close relative of NEDD4L, had been reported in *Drosophila* to ubiquitinate Notch during endocytosis to prevent ligand independent activation (58).

IL-4 upregulated HIF-2α in mouse macrophages had been reported (59) and this protein is important for increasing arginase I (M2 marker) in mouse macrophages (59). However, how IL-4 increased HIF-2α is unclear. Previous report revealed that Notch1 was an interacting partner with HIF-1α to enhance the transcription of its target gene and vice versa (44,46,60). It might be possible that NIC1 may interact with HIF-2α to induce hypoxic genes in human M(IL-4).

In our study, we found *NEDD4L* mRNA was upregulated in IL-4 activated NIC1 overexpressing THP-1 and NEDD4L is linked with SGK1 on the network analysis. Furthermore, NEDD4L, also known as NEDD4-2, is one of the genes the mRNA level of which was increased in oxLDL-loaded stimulated THP-1, suggesting a role in activation of the foam cells (61). NEDD4L is an E3 ubiquitin ligase that mainly functions to regulate membrane proteins such as TGFβR1, ENaC (26). Deletion of *Nedd4l* results in perinatal lethality with inflammatory lung disease and progressive kidney disease (62). These conditions are explained by dysregulation of ENaC which controls Na^{2+} level and fluid balance but its function(s) in immune cells have not been reported.

We hypothesized that NEDD4L might be responsible for Notch-induced PPARγ stability in M(IL-4) but from the gene deletion results, NEDD4L is not involved in degradation of PPARγ. *NEDD4L* knockout THP-1 did not show any changes in the level of PPARγ or AKT phosphorylation upon IL-4 activation during the observed timepoints. To our surprise, NIC1 overexpression in *NEDD4L* knockout THP-1 dramatically decreased both mRNA and protein level of PPARγ. These results suggested that Notch signaling regulates PPARγ via NEDD4L by other mechanisms rather than by maintaining PPARγ protein stability. Additionally, we found AKT phosphorylation was impaired in NIC1 overexpressing M(IL-4). The decreased AKT phosphorylation may partially explain reduction in *PPARG* mRNA upon IL-4 stimulation. How deletion of *NEDD4L* leads to decrease AKT phosphorylation only when NIC1 is overexpressed is currently unknown. Interestingly, NEDD4L is responsible for ubiquitination of Tmbim1, a protein localized to late endosome and lysosome and this ubiquitination is required for Tmbim1-mediated degradation of TLR4 in a model of fatty liver disease (63).

To understand the role of Notch signaling in functions of M(IL-4) , we examined PPARγ target genes which appeared on the transcriptomic data. IL-4 stimulation alone did not increase CD36 expression but increased lipid accumulation, possibly by other receptors such as scavenger receptor A (SRA) (64). As expected, lipid accumulation and CD36 were increased in IL-4 stimulated or unstimulated NIC1 overexpressing THP-1. Previous report stated that Notch signaling increased inflammation, lipid accumulation and regulate matrix remodeling in in atherosclerosis (65). Our findings add another jigsaw to the model where Notch regulates lipid accumulation in macrophages via CD36 the target of PPARγ signaling in atherosclerosis.

In conclusion, we provide the two mechanisms to regulate PPARγ expression in M(IL-4) human macrophages by Notch signaling. First, activation of Notch1 signaling in IL-4 stimulated human macrophages produces NIC1, which stabilizes PPARγ protein expression by decreasing proteasome degradation. Second, Notch signaling regulates AKT phosphorylation in M(IL-4) and this activity requires NEDD4L to control *PPARG* mRNA level.

Experimental Procedures

Cell culture and primary human macrophages

Ethic approval for the use of healthy donor blood was granted by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB No. 055/60). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy donors by Ficoll gradient centrifugation (GE Healthcare, UK). CD14+ monocytes were separated from PBMCs by the human CD14 MicroBeads (MACS Miltenyi Biotec, Germany). To differentiate CD14+ monocytes to macrophages, monocytes were maintained in M-CSF (20 ng/ml) (Biolegend, USA) in complete medium (iMDM media supplemented with 5% human serum and antibiotics, (Hyclone, UK) for 7 days. Media were changed every 2 days. A549, a human lung carcinoma cell line (reference no. JCRB0076, Japanese Collection of Research Bioresources Cell Bank, Japan), and THP-1, human leukemia cell line cell line (reference no. JCRB0112, National institutes of biomedical innovation, health and nutrition Japanese Collection of Research Bioresources cell bank), were cultured in DMEM and RPMI-1640, respectively. Media was supplemented with 10% fetal bovine serum, sodium pyruvate, HEPES and antibiotics. THP-1 cell line was treated with phorbol 12-myristate 13-acetate (PMA, Calbiochem, Germany) (5 ng/ml) for 2 days to differentiate from monocytes to macrophages.

Retroviral and lentiviral transduction

The retroviral plasmid vector for DN-MAML [MSCVMam(12–74)-EGFP] was a kind gift from Dr. Warren Pear (University of Pennsylvania, USA). The retroviral plasmid vector for expression NIC1 (MSCV-GFP-Myc-NIC1) was a kind gift of Dr. Barbara A. Osborne (University of Massachusetts Amherst, USA). A control empty vector, MSCV-IRES-GFP (plasmid 20672), was obtained from Addgene (USA). The retroviral vectors and packaging construct pCL-Ampho (Imagenex, Canada) were co-transfected into 293 T cells using the FuGene® HD transfection reagent (Roche, USA) according to the manufacturer's instructions. Culture supernatants containing retroviruses were harvested twice at 48 and 72 h after transfection and used to transduce THP-1 cells, as described elsewhere (50). Transduction efficiency was confirmed by fluorescent microscopy and flow cytometry. The lentiviral plasmid vector for NEDD4L knockout (N4L#1KO and N4L#2KO) and a control vector, plentiCRISPR (cat no. SC1812) were purchased from GenScript (USA). The lentiviral plasmid vector NIC1 (EF1α-CMV-hN1-GFP) and empty vector (EF1α-CMV-DEST-GFP) obtained from Dr. Dilip Kumar (A*STAR, Singapore). The lentiviral vector packaging construct containing gene encoding VSVG was a kind gift from Dr. Barbara A. Osborne and psPAX2 was purchased from Addgene (USA). The lentivirus transduction protocol was similar to retrovirus transduction described above.

Western blot

After separating by SDS-PAGE, the proteins were transferred onto a PVDF membrane using the semi-dry transfer Transfer-Blot®SD (Bio-Rad, USA). The primary antibodies that were used are as follows: rabbit anti-cleaved Notch1 Ab (Val1477), rabbit anti-PPARγ Ab,rabbit anti-TGM2 ab, rabbit anti-phosphor-AKT Ab, rabbit anti-total-AKT Ab, rabbit anti-NEDD4L Ab (all were purchased from Cell signaling Technology, USA), rabbit anti-Notch1 Ab (Santa Cruz Biotech, USA) and mouse anti-β-actin Ab (Merck-Millipore, USA). The secondary antibodies that were used in this study were as follows: horseradish peroxidase conjugated sheep anti-mouse IgG Ab (GE Healthcare, UK) and horseradish peroxidase conjugated goat anti-rabbit IgG Ab (Cell signaling Technology). The signal was detected using the Amersham Hyperfilm™ECL chemiluminescent detection method (Amersham Bioscience, UK).

Real-time PCR

Cells were treated as indicated, and total RNA was extracted using TRIzol®(Invitrogen,UK). One hundred nanograms to 1 ug of RNA was used as a template to synthesize cDNA using reverse transcriptase (Fermentas, Canada). The qPCR was performed using iQ™SYBR®Green SuperMix (Bio-Rad, USA) following the manufacturer's protocol, and qPCR was performed using Bio-Rad CFX Connected Real Time System (Bio-Rad, USA). Expression of β-actin was used as a reference. The relative expressions of the mRNA levels were calculated and analyzed as previous described (66).

Flow cytometry

For cell surface staining, cells were harvested and blocked the FC receptor with human serum. After this step, cells were stained with anti-CD36-PE Ab (ImmunoTools, Germany) or anti-IL-4Rα-PE Ab (ImmunoTools) with dead-live dye 7-AAD (BD™Pharmagen, USA). For intracellular staining, after blocking the FC receptor, cells were fixed in 3% formaldehyde and permeated by cold methanol, followed by anti-CD36 Ab staining. Cells were analyzed by flow cytometer (FC500, Beckman Coulter, USA). The acquired data were analyzed using FlowJo data analysis software (Tree Star, Inc., USA)

mRNA stability assay

Cells were stimulated with IL-4 (20 ng/ml) for 3 h prior to actinomycin D (ActD, 1 μg/ml) (Merck, Germany) treatment. Total RNA was collected at indicated times after ActD treatment. RNA was extracted as described above and converted to cDNA for detecting *PPARG* and β-actin expression by qPCR. mRNA half-life was calculated using the equation: $T1/2 = \ln 2/k$, when k is the constant value of mRNA degradation (67).

Protein degradation and half-life

Cells were pretreated with MG132 (Calbiochem, Germany) (1 μM) before stimulating with recombinant human (rh) IL-4 (20 ng/ml) for 4 h. Cells were stimulated with rhIL-4 (20 ng/ml) for 4 h prior to cyclohexamide (CHX, 1 μg/ml; Sigma Aldrich, USA) treatment. Protein lysate were subjected to Western blot. Band density of proteins were quantitated using ImageJ software (NIH, USA).

Library preparation and RNA sequencing

RNA was extracted using RNeasy® Mini kit (QIAGEN, Germany). Total RNA was used to perform RNA sequencing at Omics Sciences & Bioinformatics Center, Chulalongkorn University, Thailand. In brief, sample quality and quantity were measured by Bioanalyzer (Aligent2100 Bioanalyzer System, Canada). Sample library was prepared using TruSeq mRNA library kit (Illumina, USA). RNA sequencing was performed using NextSeq500 (Illumina). Data has been deposited in NCBI Gene Expression Omnibus (GEO) and is accessible through GEO accession number GSE115914.

Differential gene expression analysis was performed on Galaxy (https://usegalaxy.org). Heatmap was generated using MORPHEUS (https://software.broadinstitute.org/morpheus), and differentially expressed genes were clustered using hierarchical clustering with complete linkage on one minus Pearson correlation metric. Vein diagram was generated using Venny2.1 (http://bioinfogp.cnb.csic.es/tools/venny).

GSEAPreranked analysis

GSEAPreranked was performed using Broad Institute GSEA software version 3.0 and Molecular Signature Database (MSigDB) version 6.1. Gene set database was h.all.v6.1.symbol.gmt [Hallmark]. Weight scoring was applied for ranking statistic of data set.

Network analysis

Interested genes target was submitted to STRING version 10.5 (https://string-db.org), Network was clustered using k-mean clustering. Connected line represented evidence base interaction (in 5 criteria; text meaning, experiments, database, gene-fusion, neighborhood and co-occurrence).

Lipid staining

Cells were stimulated as indicated, washed twice with PBS, fix in 10% formaldehyde for 10 min and 100% formaldehyde for 1 h. Fixed cells were washed twice with water, incubated with 60% isopropanol in water for 5 min before completely dry. Dry cells were stained with oil red o solution for 10 min, excessive dye was washed off with water 4 times. Cells were visualized under an inverted microscope (Olympus, Olympus Corporation, Japan). Oil red O staining lipid in cells was eluted by 100% isopropanol to measure OD at 492 nm.

Statistical analysis

All statistical analyses except RNASeq analysis was performed using GraphPad Prism software Statistical significance was determined using two-way ANOVA, one-way ANOVA or unpaired t-test. *p*-value of less than 0.05 were considered significant.

Data and materials availability

The differential gene expression data by RNA sequencing have been deposited in NCBI Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO accession number GSE115914.

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Figure Legends:

Fig. 1 *IL-4 activated Notch signaling in human macrophages.*

THP-1 cell was pretreated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for indicated time. (A) TGM2 and PPARγ was detected using Western blot. (B) The band density by Western blot were measured by ImageJ software. The result represented 5-6 independent experiments. (C) Cleaved N1 and Notch1 were determined by Western blot. (D) The band density of cleaved Notch1 were measured by ImageJ software. (E) *HEY1* mRNA expression was examined by qPCR. *β-ACTIN* was used to normalize gene expression. (F) HMDMs were pretreated with DAPT (50 μM) before stimulating with IL-4 (20 ng/ml) for 4 h. Cleaved Notch1 and Notch1 were examined by Western blot. β-actin was used as loading control. The result is representative of 3 donors. *indicated the statistically significant differences when compared with unstimulated condition at $p < 0.05$.

Fig. 2 *Notch signaling increased PPARγ expression upon IL-4 stimulation in macrophages.*

(A) Control, NIC1 or DNMAML overexpressing THP-1 were treated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for 4 and 24 h. Notch1 and PPARγ were detected by Western blot. (B) HMDMs were pretreated with DAPT (50 μM) before stimulating with IL-4 (20 ng/ml) for 6 and 24 h. PPARγ protein expression was examined by Western blot. β-actin was used as loading control. The result is representative of 3 independent experiments.

Fig.3 *Activation of Notch signaling did not interfere with IL-4 signaling in human macrophages*.

(A) Control, NIC1 and DNMAML overexpressing THP-1 were pretreated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for indicated time. IL-4Rα expression was examined by flow cytometry. (B) THP-1 cell was pretreated with PMA (5 ng/ml) for 48 h. Cell was treated with DAPT (25 μM) for 1 h before stimulating with IL-4 (20 ng/ml) for 0, 15, 30 and 60 min. Cleaved Notch1, Notch1, phosphorylation and total protein of STAT6 and AKT were detected by Western blot. β-actin was used as loading control. (C) HMDMs were pretreated with DAPT (50 μM) for 1 h before stimulation with IL-4 (20 ng/ml) for 0,15, 30 and 60 min.

Fig.4 *Notch signaling did not regulate transcription or stability of PPARG mRNA.*

(A) THP-1 cell was pretreated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for 3 h. *PPARG* mRNA expression was determined by qPCR. *β-ACTIN* was used as housekeeping gene. (B) THP-1 cell was pretreated with PMA (5 ng/ml) for 48 h. Cells were stimulated with IL-4 (20 ng/ml) for 3 h, and subsequently treated with actinomycin D (1 μg/ml) for 0, 45 and 90 min. *PPARG* mRNA expression was determined by qPCR. mRNA half-life was calculated according to the equation that is $T1/2 = \ln 2/k$, when k is the constant value of mRNA degradation (67). The experiments were performed in 3 independent experiments. NS indicated no statistically significant differences.

Fig.5 *Notch signaling regulates PPARγ degradation by proteasome.*

(A) Control, NIC1 and DNMAML overexpressing THP-1 cell was pretreated with PMA (5 ng/ml) for 48 h. Cell was pretreated with MG132 (1 μM) for 1 h, and subsequently stimulated with IL-4 (20 ng/ml) for 4 h. Expression of Notch1, PPARγ were detected by Western blot. (B) After pretreatment with PMA (5 ng/ml), cells were stimulated with IL-4 (20 ng/ml) for 4 h, prior to treatment with cycloheximide (CHX, 1 μM). Protein lysate was collected every 20 min for total 100 min. PPARγ and Notch1 protein expression was examined by Western blot. β-actin was used as loading control. (C) Decay graph of PPARγ protein in IL-4-activated control or NIC1 overexpressing THP-1. The level of PPARγ protein was normalized with β-actin. The normalized expression was calculated as % PPARγ expression relative to that at 0 min of CHX treatment. The experiments were performed in 3 independent experiments. *indicated that the statistically significant differences at $p < 0.05$.

Fig.6 *Transcriptomic analysis of IL-4 activated NIC1 or DNMAML overexpressing THP-1.*

(A) Differential gene expression is depicted as a heatmap showing all genes with significant difference based on FDR < 0.05. (B) A Venn diagram of genes with significant differentially expressed in IL-4 stimulated control, NIC1 and DNMAML overexpressing THP-1 cell.

Fig.7 *NEDD4L increased PPARG expression in NIC1 overexpressing THP-1.*

(A) *NEDD4L* knockout THP-1 cell (N4L-KO) and (B) NIC1 overexpression in NEDD4L-KO background in THP-1 cell (N4L-KO+NIC1) were pretreated with PMA (5 ng/ml) for 48 h. Cells were subsequently stimulated with IL-4 (20 ng/ml) for 4 h. NEDD4L, Notch1, PPARγ phosphor-and total AKT protein expression were examined by Western blot. β-actin was used as loading control. The result is representative of 3 independent experiments. (C) Heatmap summary of relative protein level of PPARγ, ratio of phosphor-to total AKT and NEDD4L expression in each condition under IL-4 stimulation. (D) *PPARG* mRNA expression was examined by qPCR. *β-ACTIN* was used to normalize gene expression. The result represented 3 independent experiments.

Fig.8 *NIC1 overexpression upregulated IL-4 and OxLDL-induced CD36 expression and lipid uptake.*

(A) Heatmap of genes in the PPARγ pathway from BioCarta database that showed differential expression. (B) Control, NIC1 and DNMAML overexpressing THP-1 were treated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for 18 h. Surface staining of CD36 were detected by flow cytometry. (C) Control, NIC1 and DNMAML overexpressing THP-1 were treated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) and oxLDL (50 mg/ml) for 24 h. Intracellular lipid was stained by oil red O. Lipid staining was dissolved with 100% isopropanol for measurement absorbance at 492 nm. The result represented 3 independent experiment. *, $, # indicated that the statistically significant differences at condition with contain similar symbol at $p < 0.05$.

Supplementary Figures

Fig. S1. Signaling downstream of L-4 in stimulated human macrophages. PMA-pretreated THP-1 cell (A) or HMDMs (B) were stimulated with IL-4 (20 ng/ml) for 0, 15, 30 and 60 min. Level of phosphorylation and total protein of STAT6 and AKT were detected by Western blot. β-actin was used as loading control. The result was representative of 3 independent experiments.

Fig. S2. Characteristic of hyper-or hypoactivation of Notch in THP-1 cell. Control or NIC1 and DNMAML overexpressing THP-1 cells were pretreated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for 3 h. *HEY1* mRNA expression was determined by qPCR. *β-ACTIN* was used as housekeeping gene. The experiment was performed in 3 independent experiments. *indicated that the statistically significant differences at $p < 0.05$.

Fig. S3. DAPT treatment decreased PPARγ in IL-4 stimulated macrophages. THP-1 cell was pretreated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for 6, 18 and 24 h. The result is representative of 3 independent experiments.

Fig.S4. Effect of hyper-or hypoactivation of Notch on *PPARG* mRNA level. Control or NIC1 and DNMAML overexpressing THP-1 cells were pretreated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for 6 h. *PPARG* mRNA expression was determined by qPCR. *β-ACTIN* was used as housekeeping gene. The experiment was performed in 3 independent experiments. *indicated that the statistically significant differences at $p < 0.05$.

Fig.S5. Visualization of candidate hypoxia hallmark of GSEAPreranked analysis. (A) GSEAPreanked output result of hypoxia hallmark in the comparing between control with IL-4 and NIC1 overexpressing cells with IL-4. (B) Vein diagram of the significance enrich genes in hypoxia hallmark from GSEAPreranked analysis compared with transcriptomic data of IL-4 stimulated IRES or NIC1. Vein diagram was generated using Venny2.1 software. (C) Vein diagram of the significant enrich genes in hypoxia hallmark from GSEAPreranked analysis compared with transcriptomic data of IL-4 stimulated control or NIC1 overexpressing cells (under upregulation criteria log2(fold change) greater than 1.5 and downregulation criteria log2(fold change) less than -1). (D) Network analysis. Notch1, PPARγ and NEDD4L were subjected to STRING version 10.5. Network links were generated based on evidence interaction (in 5 criteria; text meaning, experiments, database, gene-fusion, neighborhood and co-occurrence), subsequently clustered using k-mean clustering method (cluster=3). (E) Vocalno plot of transcriptomic changes in control cells with IL-4 compared with NIC1 overexpressing cells with IL-4.

Fig.S6. NEDD4L expression in NIC1 or DNMAML overexpressing THP-1. (A) Control, NIC1 or DNMAML overexpressing THP-1 were pretreated with PMA (5 ng/ml) for 48 h. Cells were stimulating with IL-4 (20 ng/ml) for 3 h. *NEDD4L* mRNA expression was detected by qPCR. β-actin was used as housekeeping gene. The experiment was performed in 5 independent experiments. *indicated that the statistically significant differences at $p < 0.05$. (B-C) (B) Control or NIC1 and DNMAML overexpressing THP-1 were pretreated with PMA (5 ng/ml) for 48 h and (C) HMDMs were pretreated with DAPT (50 μM). Both cells were stimulated with IL-4 (20 ng/ml) for 4 h. Notch1, PPARγ, NEDD4L, phosphor-and total-AKT were detected by Western blot. β-actin was used as loading control. The result is representative of 3 independent experiments in THP-1 and 3 donors in HMDMs.

Fig.S7. Surface expression of CD36 in IL-4 and rosiglitazone stimulated NIC1 or DNMAML overexpressing THP-1 cell. Control, NIC1 or DNMAML overexpressing THP-1 were treated with PMA (5 ng/ml) for 48 h before stimulating with IL-4 (20 ng/ml) for 18 h. Surface expression of CD36 were detected cy flow cytometry. The experiment was performed in 3 independent experiments.

Table S1. GSEAPreranked analysis between IL-4-treated control THP-1 compared with IL4-treated NIC1 overexpressing THP-1.

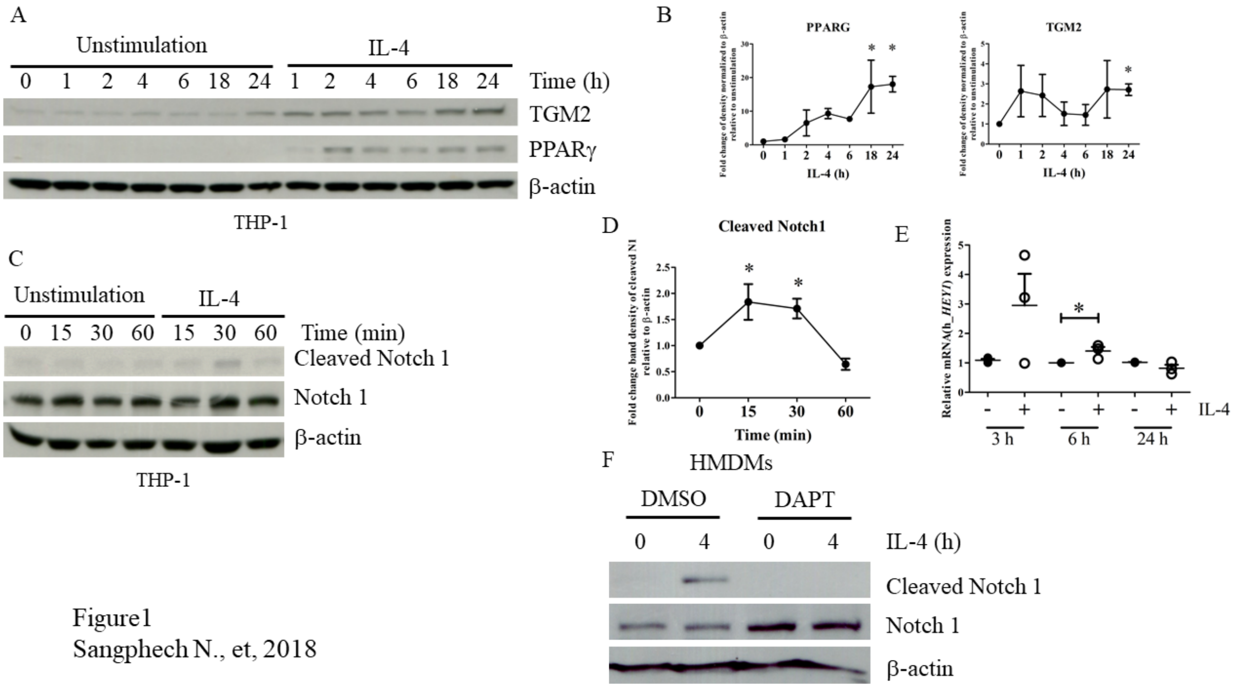

Figure1
Sangphech N., et, 2018

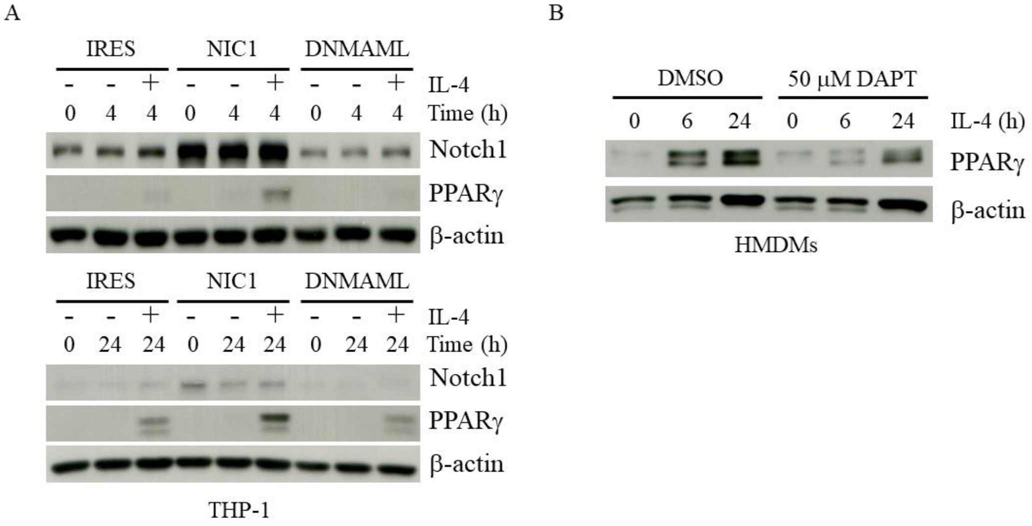

Figure 2
Sangphech N., et al, 2018

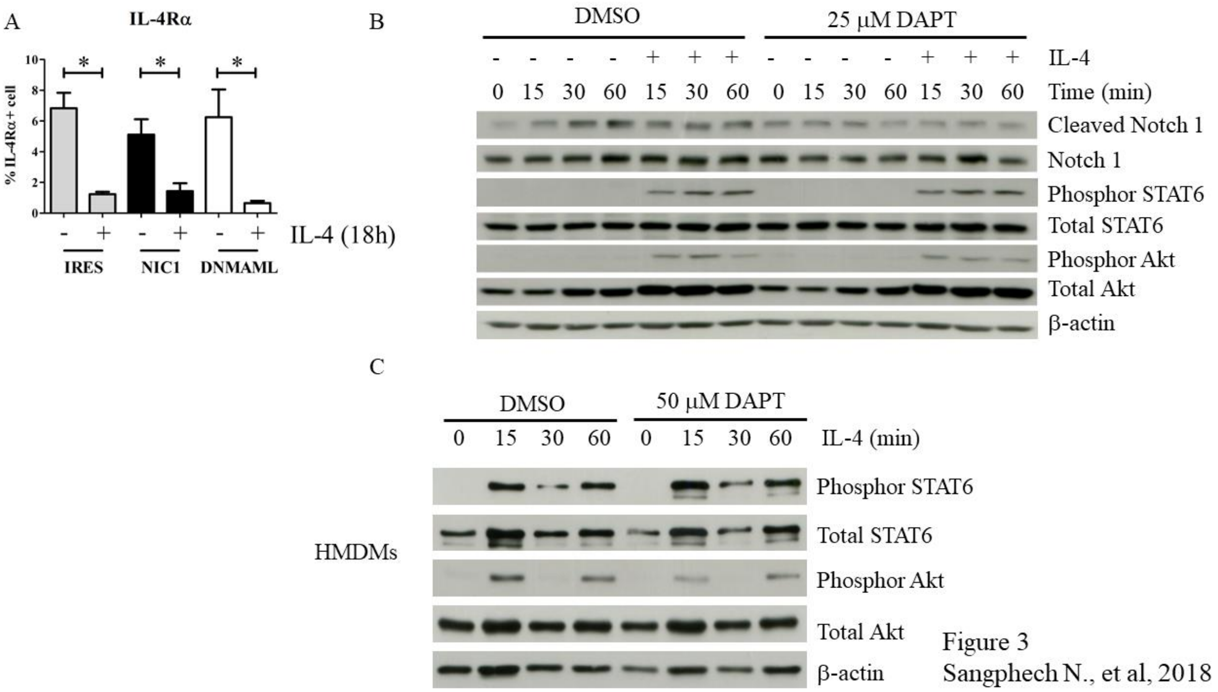

Figure 3
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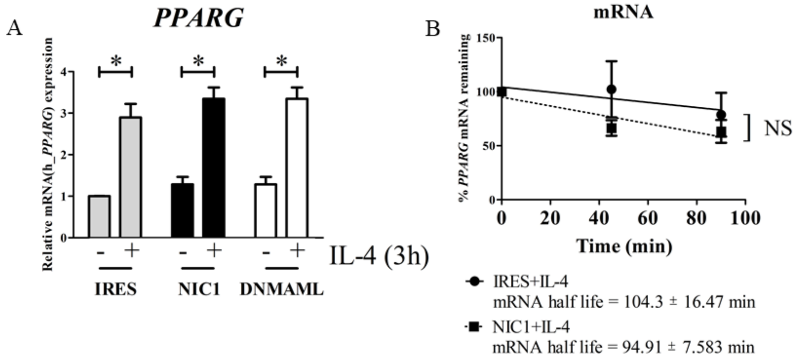

Figure 4
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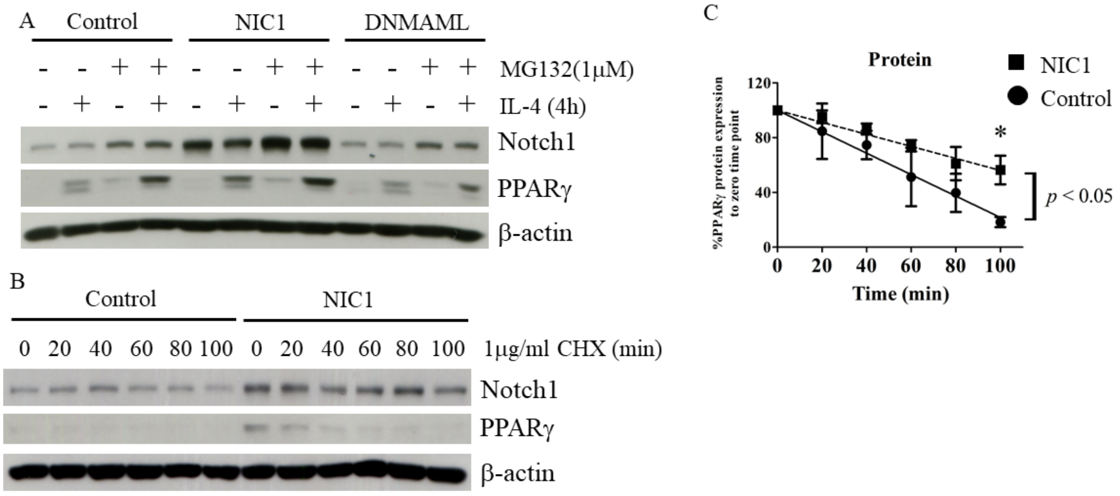

Figure 5
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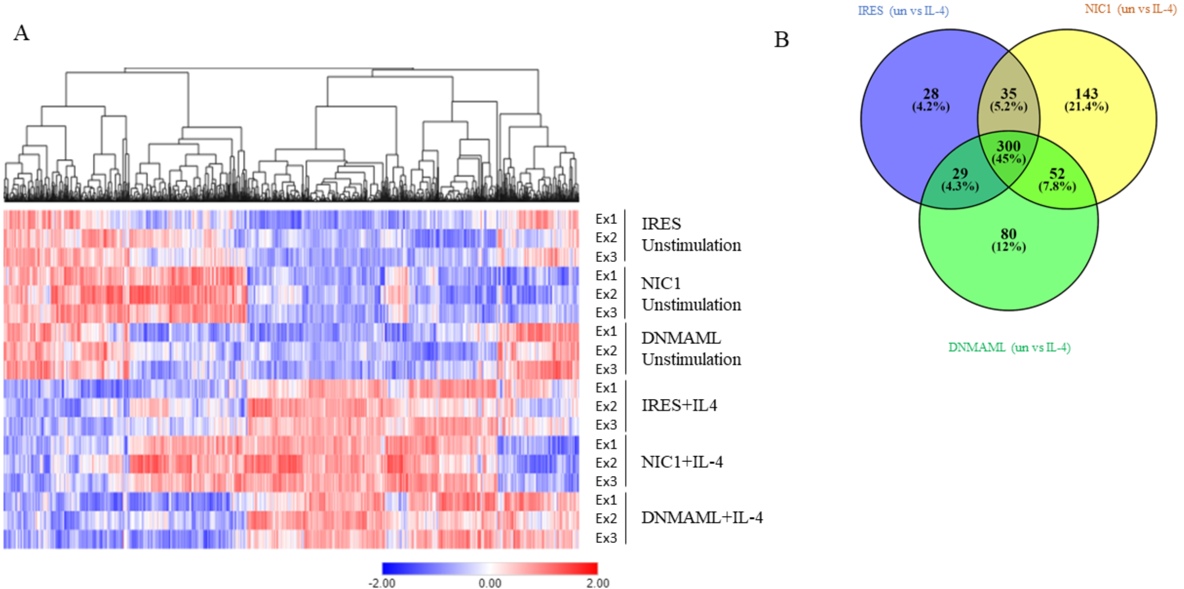

Figure 6
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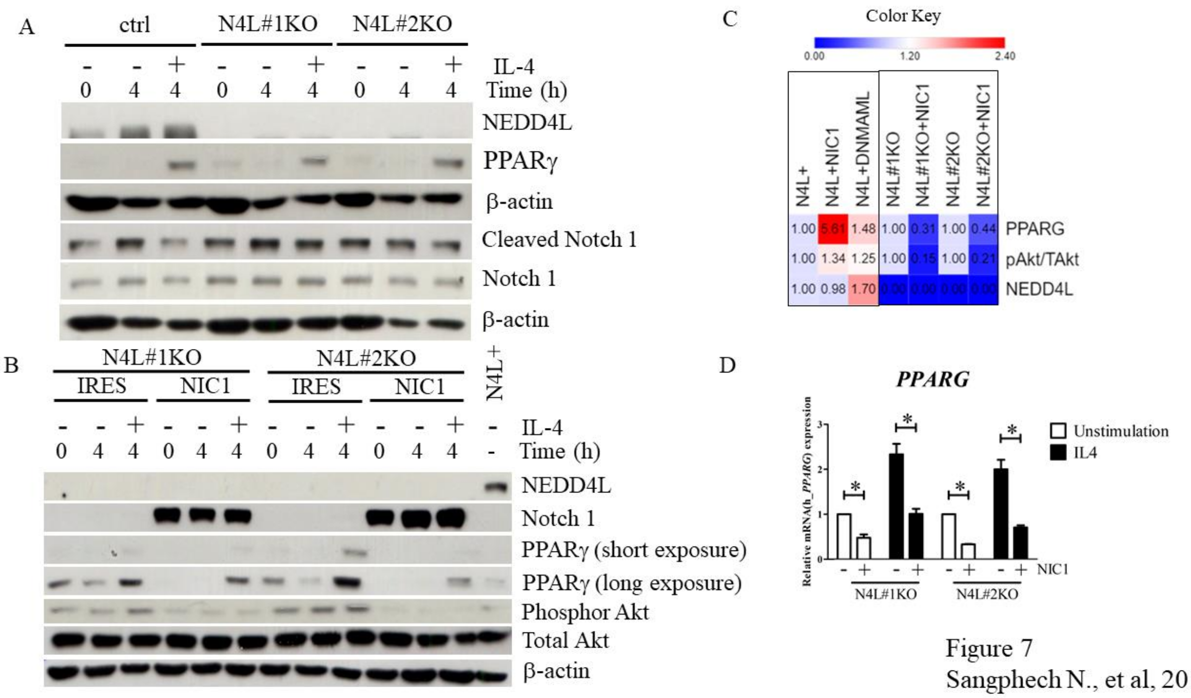

Figure 7
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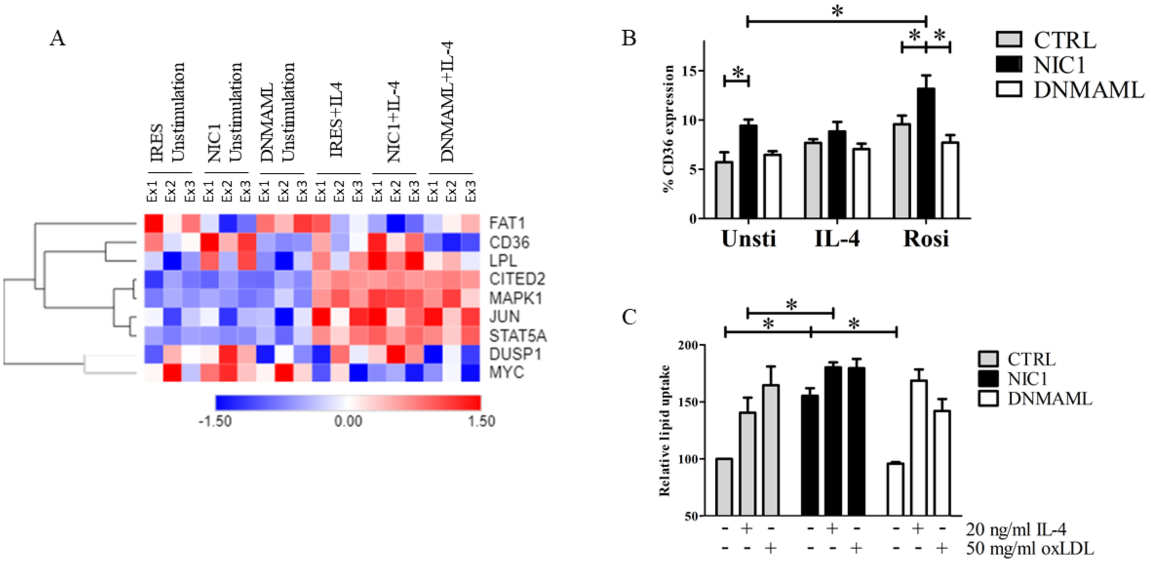

Figure 8
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