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Involvement of Notch Signaling Pathway in Regulating IL-12 Expression via c-Rel in Activated Macrophages

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Abstract

Macrophages play an important role both in innate and adaptive immune responses. Treatment with interferon (IFN) γ together with lipopolysaccharide (LPS) activates pro-inflammatory macrophages which secrete various pro-inflammatory cytokines including IL-12. IL-12 promotes a Th1 type immune response by directly controlling the differentiation of $CD4^+$ T helper 1 cells. Activation of Notch signaling pathway was reported in activated macrophages but the involvement of this signaling pathway in IL-12 expression has not been documented. In this study, we investigated the role of Notch signaling in regulating expression of the IL-12/IL-23 subunit, IL-12p40. Using a gamma-secretase inhibitor (GSI) to inhibit Notch signaling, we observed a profound decrease in *il12p40* mRNA levels and IL-12p70 secretion upon IFNγ/LPS stimulation. On the other hand, overexpression of activated form of Notch1 in activated RAW264.7 macrophage-like cell lines significantly increased the level of *il12p40* mRNA. GSI treatment did not affect the expression of *irf5*, a master regulator of *il12p40* transcription in macrophages. Detailed analysis of the signaling cascades that were affected by this inhibition showed that c-Rel nuclear translocation was inhibited and Erk1/2 activation was compromised by GSI treatment. Addition of exogenous tumor necrosis factor (TNF) α only partially rescued the expression of *il12p40* in the presence of GSI. Unexpectedly, inhibition of Notch signaling using a dominant negative (DN) Mastermind-like (MAML) transcription co-activator, did not affect c-Rel nuclear localization upon activation or *il12p40* mRNA levels, suggesting that the transcriptional activity of Notch signaling is dispensable for the activation of c-Rel. These results strongly suggest that Notch signaling in activated macrophages is involved in regulating the expression of *il12p40* directly via c-Rel and indirectly via TNFα production.

Keywords

Notch signaling; macrophages; IL-12p40; c-Rel

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1. Introduction

Macrophages play important roles in host defense by contributing to both innate and adaptive immune responses. Their pro-inflammatory roles are well recognized; they can produce pro-inflammatory cytokines and mediators upon encountering danger signals, such as pathogen associated molecular patterns (PAMPs) (Zhang and Mosser, 2008). Macrophages also function as antigen presenting cells (APCs) by presenting peptide antigens to $CD4^+$ T helper cells. Through their critical role as APCs, macrophages create a microenvironment and express cell surface molecules that instruct $CD4^+$ T helper cells to differentiate into appropriate helper T cell lineages. In addition to their pro-inflammatory role, recent evidence suggests that macrophages also have a contrasting role in resolving inflammation during wound healing and tissue repair (Martinez et al., 2009; Mosser and Edwards, 2008).

The immune system must have the flexibility to mount the most effective and efficient responses to diverse invading pathogens. Therefore, most immune cells, including macrophages, are highly plastic as they can readily change phenotypes in response to changing environmental signals (Biswas and Mantovani, 2010; Stout and Suttles, 2004). Previous studies have identified two distinct subsets of macrophages based on their cytokine production and biological functions (Mosser, 2003). Macrophages stimulated with IFNγ are highly inflammatory and produce chemical mediators, including nitric oxide through the expression of inducible nitric oxide synthetase (iNOS). In contrast, TGFβ-treated macrophages stimulate the expression of arginase1 instead of iNOS, which generates ornithine and urea from arginine (Mills et al., 2000). Because these two macrophage subsets influence T helper cells in opposing ways, the former was originally termed “M1” and the latter was termed “M2”, which is in parallel with the Th1/Th2 paradigm (Mills et al., 2000). It is currently believed that macrophage activation results in a spectrum of phenotypes, ranging from a pro-inflammatory phenotype at one end to an anti-inflammatory phenotype at the other (Mantovani et al., 2004; Mosser and Edwards, 2008). IFNγ-primed macrophages treated with LPS are equivalent to M1 macrophages which express iNOS and produce IL-12. This type of activated macrophages promotes a Th1 type immune response.

IL-12 is a heterodimer cytokine of IL-12p40 and p35 subunit. IL-12p40 expression in macrophages is under the regulation of various signaling pathways, including NF-κB, PU.1 and c-Rel (Grazia Cappiello et al., 2001; Laderach et al., 2003; Sanjabi et al., 2000). Recently, IRF5 was shown to be a master regulator of M1-specific genes, such as *il12p40*, *il12p35* and *il23p19*, while it represses the expression of *il10*, an M2-specific marker gene (Krausgruber et al., 2011).

We and others have reported that the well-conserved Notch signaling pathway plays a critical role during the macrophage response to danger signals via TLR signaling cascades (Fung et al., 2007; Hu et al., 2008; Monsalve et al., 2006; Palaga et al., 2008). These studies have elucidated that Notch signaling via its target gene *Hes*/*Hey* regulates the macrophage inflammatory response partly via the NF-κB and/or STAT pathways. Notch signaling is involved in cell fate determination and cellular differentiation in various cell types, such as neuronal cells, muscle cells, adipocytes and hematopoietic cells (Artavanis-Tsakonas et al., 1999). During helper T cell polarization, Notch signaling has been shown to regulate Th1/Th2 differentiation likely through direct regulation of the main lineage-specific transcription factors in T cells and selective expression of Notch ligands on APCs (Amsen et al., 2009; Osborne and Minter, 2007). Furthermore, Notch signaling directly regulates cytokine production such as IL-10 in T cells and IL-6 in macrophages (Rutz et al., 2008; Wongchana and Palaga, 2011). Because Notch signaling plays a role at critical steps of various effector

cell functions and cytokine productions, we hypothesized that it might also be involved in the activation of macrophages. In this study, we show that the inhibition of Notch signaling affects the expression of *il12p40* mRNA. Furthermore, we provide evidence that Notch signaling regulates IL-12p40 expression directly via c-Rel and indirectly via TNFα production in activated macrophages.

2. Materials and Methods

2.1 Animals and Generation of Bone Marrow Derived Macrophages (BMM)

Female C57BL/6 (National Laboratory Animal Center, Mahidol University, Salaya, Thailand) 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. All procedures involving laboratory animals were carried out according to the guidelines issued by Chulalongkorn University, and all animal protocols were reviewed by the IACUC (protocol review No. 0923013). The murine macrophage-like RAW 264.7 cell line (ATCC No. TIB-71) was used in this study. 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.

2.2 Activation of Macrophages

BMMs or RAW264.7 cell line were activated by priming overnight with recombinant murine IFNγ (10 ng/mL) (R&D Systems, Minneapolis, MN, USA) and washed twice with cold PBS. Pre-warmed media and *Salmonella* LPS (100 ng/mL) (Sigma Aldrich, St Louis, MO) were added to activate macrophages. In some experiments, recombinant murine TNFα (10 ng/mL) (BioLegend, San Diego, CA) were added to activated macrophages.

2.3 Gamma Secretase Inhibitor (GSI)

The GSIs, GSI (a kind gift from Dr. Todd Golde, University of Florida, FL, USA) or DAPT (Merck, NJ), have been used previously (Monsalve et al., 2009; Palaga et al., 2008). GSI was dissolved in DMSO to a final concentration of 50 mM and stored at -80ºC until use. For treatment of activated macrophages, cells were treated with GSI (25 μM) or vehicle control DMSO during the priming by IFNγ overnight and the stimulation with LPS.

2.4 Western Blotting

Cells were treated as described, and cell lysates were harvested as described previously (Palaga et al., 2008). Upon separation via SDS-PAGE, Notch1 and cleaved Notch1 were detected using rabbit antibodies against Notch1 (C20) (Santa Cruz Biotech, Santa Cruz, CA, USA) and cleaved Notch1 (Val1744) (Cell Signaling Technology, Danvers, MA, USA). RIPA buffer with the addition of phosphatase inhibitor cocktail (Sigma Aldrich) was used to prepare cell lysates to detect phosphoproteins. Antibodies for detecting molecules in the MAPK pathways were from the MAPK and phospho-MAPK family antibody sampler kit (Cell Signaling Technology). Horseradish peroxidase-conjugated secondary antibodies against rabbit and mouse IgG were obtained from GE Healthcare (Buckinghamshire, UK). Signals were detected by chemiluminescence.

For separation of cytoplasmic and nuclear extracts, cells were treated as indicated and NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL) were used to prepare the extracts according to the manufacturer's instruction. Western blot was carried out for detecting c-Rel as described above.

2.5 Quantitative Real-time RT-PCR (qPCR)

Total RNA was isolated from cells treated as indicated using TRIzol reagent (Invitrogen, Paisley, UK). cDNA was prepared using reverse transcriptase (Fermentas, Glen Burnie, MD, USA) and random hexamers (Invitrogen). qPCR amplifications were performed with 1xMaxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) according to the manufacturer's protocol. Primers specific for *Hes1* have been described previously (Narayana and Balaji, 2008) and primers specific for *Hes5* were designed in this study. Primers used for the amplification of *il12p40*, *il23p19* and *irf5* have also been previously described (Edwards et al., 2006; Krausgruber et al., 2011; Palaga et al., 2008; Tada et al., 2000). *β-actin* was used as a reference gene. qPCR was carried out using an MJ Mini personal Thermal cycler (BioRad, USA). The relative gene expression levels were calculated and analyzed using the $2^{-\Delta\ \Delta CP}$ method (Livak and Schmittgen, 2001).

2.6 Overexpression of Truncated Intracellular Notch1

Overexpression of the truncated intracellular form of Notch1 (N^{IC}) in RAW 264.7 cells (ATCC No. TIB-71) was carried out using pcDNA3 plasmid containing the intracellular Notch1 (N^{IC})-encoding sequences corresponding to amino acid residues 1759–2556 (pcDNA3N^{IC}), and empty pcDNA3 plasmid was used as a control vector (both were kind gifts from Professor Barbara Osborne, University of Massachusetts at Amherst, USA). All plasmids were prepared using the Endo-free® Plasmid Maxi kit (Qiagen, Hilden, Germany). Transient transfection was performed using FuGeneHD transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instruction. N^{ic} overexpression was confirmed by Western blot detection.

2.7 Retroviral Transduction

The retroviral vector for the expression of dominant negative MAML1 (DN-MAML; MSCV-Mam (12–74)-EGFP) was a kind gift from Dr. Warren Pear (University of Pennsylvania, PA, USA). The empty control vector, MSCV-IRES-GFP (Addgene plasmid 20672), was purchased from Addgene (Cambridge, MA, USA). The retroviral vectors and packaging construct pCL-Eco (Imagenex) were co-transfected into 293T cells using FuGene® HD transfection reagent (Roche), according to the manufacturer's instructions. Culture supernatants containing retroviruses were harvested twice, at 48 and 72 hr after transfection, and used to transduce BMMs twice. The transduction efficiency was confirmed by fluorescent microscopy and flow cytometry.

2.8 Immunofluorescent Staining

Cells were grown in cell culture chambers and activated as indicated. After washing with PBS, cells were fixed with 4% paraformaldehyde and incubated with rabbit anti-c-Rel polyclonal antibody (sc-71, Santa Cruz Biotech, USA), followed by anti-rabbit IgG (H+L, $(Fab')_2$ fragment) Alexa Fluor® 555 (Cell Signaling Technology). Cells were observed under an inverted fluorescent microscope or a confocal microscope.

2.9 IL-12p70 ELISA

Culture supernatant from activated BMM treated as indicated was harvested 24 hr after treatment. Secreted IL-12p70 levels were detected according to the manufacturer's

instruction using LEGEND MAX^{TM} mouse IL-12p70 ELISA kit (BioLegend, San Diego, CA, USA). The ELISA kit has a detection limit of 15 pg/mL.

2.10 Statistical Analysis

All data were analyzed using an independent *t*-test by SPSS software. A p value of < 0.05 was considered statistically significant.

3. Results

3.1 Expression of Notch receptors and the target gene Hes1 in activated macrophages

To investigate the involvement of Notch signaling in the activation of BMMs, we first confirmed the expression of Notch1 and cleaved Notch1 (Val1744), which is an indicator that Notch signaling is activated, in LPS/IFNγ stimulated BMMs. The activated BMMs showed the highest levels of Notch1 at 1 and 6 hr after stimulation while the appearance of cleaved Notch1 was obvious at 1 hr after stimulation (Figure 1A). The expression of *Notch2* mRNA, but not *Notch3* and *Notch4*, was also detected in activated BMMs (data not shown). To investigate the status of Notch signaling in activated BMMs, we investigated the expression levels of some well-studied Notch signaling target genes, *Hes1*, *Hey1* and *Hes5*. As shown in Figure 1B and C, activated BMMs slightly, but not significantly, increased expression level of *Hes5* and *Hes1* at 1 and 6 hr, respectively, (Figure 1B-C). The expression levels of *Hes1* and *Hey1* at 1–3 hr after stimulation were decreased, compared to the 0 hr time point (data not shown). Taken together, these data indicate that LPS/IFNγ-stimulated macrophages activate Notch signaling during an early phase of activation.

3.2 Inhibition of Notch signaling affects il12p40 expression in activated macrophages

To further investigate the involvement of Notch signaling in BMM activation, we used the GSI to inhibit the processing of all Notch receptors by gamma secretase and thus suppress all Notch signaling (Palaga et al., 2008). First, we examined the effect of GSI treatment on the expression of Notch1 and cleaved Notch1 using Western blotting. As shown in Figure 1A (the right panel), treatment with GSI (25 μM) resulted in decreased total Notch1 levels and a complete disappearance of cleaved Notch1 at all time points tested, which is consistent with previous studies (Palaga et al., 2008; Palaga et al., 2003). The disappearance of cleaved Notch1 strongly indicates that GSI inhibited Notch1 processing. Treatment with GSI at the indicated concentration did not affect the cell viability of the activated BMMs (data not shown).

The effect of GSI treatment on the expression of *il12p40* mRNA was examined. Activated BMMs expressed high level of *il12p40* but a significant decrease in *il12p40* mRNA levels was detected when cells were treated with GSI (Figure 1D). Furthermore, *il23p19* mRNA, which encodes a unique subunit of the pro-inflammatory cytokine IL-23, was also significantly decreased in GSI-treated activated macrophages (Figure 1E). Taken together, these data suggest that the activation of Notch signaling is involved in the regulation of *il12p40* and *il23p19* in activated macrophages. To confirm the effect of GSI treatment on cytokine secretion, IL-12p70 level was detected using ELISA. As shown in Figure 1F, GSI treatment significantly decreased the level of IL-12p70 in activated BMMs.

To explore a direct role of Notch signaling in controlling the expression of *il12p40* in activated macrophages, a truncated intracellular Notch1 (N^{ic}), mimicking an active form of Notch receptor, was overexpressed in the macrophage cell line, RAW 264.7, and the expression of *il12p40* was determined by qPCR. Overexpression of Nic alone was not sufficient in inducing the transcription of *il12p40* (data not shown). Upon stimulation using LPS/IFNγ, however, N^{ic} overexpession significantly increased the level of *il12p40* mRNA

at 6 hr but not at earlier time point after stimulation (Figure 2A). Taken together, these results strongly suggest that Notch signaling is involved in regulating transcription of *il12p40* in macrophages upon activation by LPS/IFNγ.

3.3 Mechanisms of regulating il12p40 in activated macrophages by the Notch signaling pathway

IRF5 promotes pro-inflammatory responses by acting as a master regulator of M1-specific gene expression by directly activating the transcription of *il12p40*, *il12p35* and *il23p19* while repressing *il10* expression (Krausgruber et al., 2011). Because we observed a decrease in *il12p40* and *il23p19* levels upon GSI treatment, *irf5* mRNA levels were investigated in BMMs activated in the presence or absence of GSI. As shown in Figure 2B, *irf5* mRNA levels is increased significantly upon GSI treatment, suggesting that *irf5* mRNA expression is not the cause of decreased *il12p40* expression in BMMs when Notch signaling is suppressed. Furthermore, increased *irf5* expression upon inhibition of Notch signaling may suggest that *irf5* may be negatively regulated by Notch signaling.

To elucidate the molecular mechanisms by which the inhibition of Notch signaling affects *il12p40* expression in activated BMMs, we examined MAPK (p38 MAPK, ERK1/2) and NF-κB (p65 and c-Rel) activation. Treatment with GSI selectively interfered with Erk1/2 activation, but no defect was found in p38 MAPK activation (Figure 2C). Furthermore, as shown in Figure 3A-I, c-Rel was readily detected in the nuclei of activated BMMs, whereas the cytoplasmic retention of c-Rel was found upon GSI treatment. Decreased c-Rel in nuclear extract as detected by Western blot also confirmed this observation (Figure 4B). We also investigated activation of the NF-κB p65 subunit (phosphorylated p65) and IκB levels using Western blotting but did not detect any differences between DMSO-treated and GSI-treated macrophages (data not shown). Taken together, these results strongly implicate Notch signaling as a regulator of *il12p40* in activated macrophages in an IRF5-independent manner, possibly via regulating c-Rel and Erk1/2.

Since TNFα is one of the activators of NF-κB including c-Rel and its expression was compromised upon GSI treatment in LPS/IFNγ-stimulated macrophages (Martin et al., 2001; Palaga et al., 2008), we asked whether addition of exogenous TNFα would rescue the defective c-Rel activation in activated BMM treated with GSI. As shown in Figure 4A, exogenous TNFα only partially rescued the level of *il12p40* mRNA in the presence of GSI. Consistent with this result, nuclear localization of c-Rel was also slightly recovered with addition of TNFα (Figure 4B-J). Taken together, these results suggest that Notch signaling regulates IL-12p40 expression partly by regulating TNFα production. Furthermore, they also indicate that Notch signaling may directly regulate nuclear localization of c-Rel in activated macrophages.

3.4 Activation of c-Rel and Transcriptional Activities of Notch signaling

To investigate whether the transcriptional activities of Notch signaling are required for c-Rel activation, we transduced BMMs with retroviral vectors with GFP alone or dominant-negative Mastermind-like (DN-MAML) fused with GFP. MAML is a scaffold protein required for stable Notch/CSL/MAML formation, and DN-MAML was shown to suppress the transcriptional activity of the Notch/CSL transcriptional activation complex (Maillard et al., 2004; Nam et al., 2003). Using GFP as marker for transduction, c-Rel localization was investigated in CA macrophages. As shown in Figure 5A-K, c-Rel was detected throughout the cytoplasm in unstimulated cells, while it was detected exclusively in the nuclei of activated BMMs whether they were transduced with control vector or DN-MAML vector. This unexpected result implies that the transcriptional activation of Notch signaling is

dispensable for regulating c-Rel activation in activated macrophages. Similar levels of *il12p40* mRNA in transduced activated BMMs confirmed this result (Figure 5L).

4. Discussion

The Notch signaling pathway is involved in various processes of cellular differentiation, as is clearly shown in $CD4^+$ helper T cell differentiation (Amsen et al., 2009). Different stimuli activate macrophages to become distinct effector cells, and the identification of specific markers and their biological significance of each type have been described in various diseases, including chronic inflammation and cancer (Biswas and Mantovani, 2010; Mosser and Edwards, 2008; Stout and Suttles, 2004). In this study, we investigated the involvement of the Notch signaling pathway in regulating IL-12 expression in LPS/IFNγ activated macrophages and uncovered a novel link between Notch signaling and c-Rel.

Notch1 and Notch2 were found to be expressed and upregulated in activated macrophages. This result strongly indicates that Notch1 and 2 may play important roles in regulating gene expression in activated macrophages. The appearance of cleaved Notch1 confirmed our observation that Notch signaling is activated in macrophages. The involvement of Notch2 needs further investigation and Notch1 and 2 may exhibit redundant functions in this context.

Previously, it was reported that IFNγ treatment led to the suppression of several Notch downstream target genes such as *Hes1* and *Hey1* which are induced by TLR stimulation (Hu et al., 2008). In the same study, they also reported the effect of IFNγ treatment specifically on Notch2 by decreasing the level of an intracellular Notch2. Interestingly, this report stated that level of intracellular Notch1 was not affected by IFNγtreatment (Hu et al., 2008). On the other hand, we clearly detected cleaved Notch1, indicating that Notch1 is cleaved upon stimulation. We also observed the decreased *Hey1* expression at 1–6 hr after stimulation but the levels of *Hes1* and *Hes5* were slightly increased. The differences in expression level of *Hes1* and *Hes5* between our study and Hu *et al*. may be due to the difference between human and mouse. Hu *et al*. reported this observation using mainly human macrophages while ours used exclusively murine macrophages. In addition, we have focused more on the presence of cleaved Notch1 but not Notch2, and proposed that it acts independently on its transcriptional activities (see below). Therefore, the contradictory findings between the two studies highlight the possibility that Notch signaling may function in two different ways in macrophages, *i.e.* CSL/RBP-Jκ-dependent (Hes/Hey induction) and -independent manners (activation of NF-κB pathway).

As previously reported, we found that Notch signaling supports pro-inflammatory macrophages because the inhibition of Notch signaling resulted in decreased expression of *il12p40* and *il23p19* (Fung et al., 2007; Hu et al., 2008; Palaga et al., 2008). IL-12p40 expression from APCs is considered to be a critical factor in biasing helper T cells towards Th1 differentiation, whereas IL-23p19 expression is important for the maintenance and expansion of the Th17 lineage (Langrish et al., 2005). Therefore, using GSI on macrophages may decrease their capacity to induce helper T cells to become Th1/Th17 effectors. Interestingly, when GSI was administered *in vivo* in a model of experimental autoimmune encephalomyelitis (EAE), the severity of the disease was alleviated (Minter et al., 2005). Given that EAE is mediated mainly by pathogenic Th1/Th17 cells, the results of GSI treatment in this animal model may derive partly from the direct effect on macrophages.

Recently, IRF5 was identified as a master regulator of M1 macrophages by directly activating *il12p40*, *il12p35* and *il23p19* and suppressing anti-inflammatory *il10* (Krausgruber et al., 2011). To our surprise, *irf5* mRNA levels were higher when macrophages were activated in the presence of GSI. Because NF-κB (c-Rel/p50) and MAPK

pathways are involved in IL-12p40 expression (Cao et al., 2006; Sanjabi et al., 2000), we investigated these pathways and found defects in c-Rel nuclear translocation upon GSI treatment. In addition, Erk1/2 activation was decreased. In T cells, Notch signaling is shown to regulate nuclear localization of c-Rel and p50 upon activation (Shin et al., 2006). This study reported that overexpression of the active form of Notch1 interacts with c-Rel/p50 in the nucleus and maintains NF-κB activity, suggesting that the transcriptional activity of Notch signaling by Notch/CSL complex may not be required for this activity. To this end, we overexpressed DN-MAML in BMMs to investigate the effect of inhibition of Notch/CSL transcriptional activity. We found that DN-MAML did not decrease *il12p40* expression or retention of c-Rel in the cytosolic compartment. This result does not contradict to the study by Shin et al. which focused mainly on the direct role of activated Notch receptor in activation of NF-κB pathways in T cells. Our study, together with the results reported by Shin et al., provides a possible transcriptional-independent Notch functions for activation of NF-κB pathways. Taken together, we conclude that Notch signaling regulates c-Rel activation independent of its transcriptional activity. Furthermore, IRF5 alone is not sufficient to induce optimum *il12p40* expression in CA macrophages.

We have previously reported that the production of TNFα was compromised at early time point upon activation of macrophages in the presence of GSI (Palaga et al., 2008). Since TNFα can induce nuclear translocation of c-Rel, it is possible that the defect seen in c-Rel activation when Notch signaling is suppressed is due to decrease in the level of TNFα produced. This indeed is the case because exogenous TNFα partially rescued the level of *il12p40* mRNA and nuclear localization of c-Rel when Notch signaling is suppressed. More importantly, however, this rescue is not complete. This result strongly indicates that Notch signaling may directly regulate the nuclear localization of c-Rel via other mechanisms. How Notch signaling regulates c-Rel nuclear localization requires further investigation.

Notch signaling is involved in regulating transcription of cytokines in T cells, NKT cells and macrophages (Benson et al., 2005; Tanaka et al., 2006; Wongchana and Palaga, 2011). The mechanisms of these regulatory functions of Notch signaling can be direct as in IL-4 and IL-6 in CD4+T cell and macrophages, respectively. The results from our study added another piece of evidences suggesting a critical role of Notch signaling in regulating the expression of IL-12/IL-23, two major cytokines in Th1/Th17 responses. When constitutively active form of Notch1 was transiently overexpressed in RAW264.7 cell line, increase in *il12p40* mRNA was not detected without stimuli, suggesting that activation of Notch signaling pathway alone is not sufficient to turn on transcription of *il12p40*. Thus, it is likely that Notch signaling is cooperating with other signaling pathways such as NF-κB in regulating the transcription of this gene.

The results obtained from this study led us to propose a model of involvement of Notch signaling in macrophage activation (Figure 5M). In activated macrophages (LPS+IFNγ), Notch signaling is activated to regulate NF-κB (c-Rel) activation and TNFα production among other activities. This Notch/c-Rel crosstalk does not require the transcriptional activities of Notch/CSL. Together with IRF5 and other signaling pathways, macrophages become fully activated and produce pro-inflammatory cytokines including IL-12p40. Notch signaling has been demonstrated in various systems to function in a cellular context-dependent manner. Our results add another piece of evidences implying Notch signaling as a regulator of the pro-inflammatory and Th1/Th17 type immune responses in macrophages activated by different stimuli.

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List of Abbreviation

APC	Antigen presenting cell
BMM	Bone marrow derived macrophages
DMSO	Dimethyl sulfoxide
DN	Dominant negative
GSI	Gamma secretase inhibitor
IFN	Interferon
LPS	Lipopolysaccharide
MAML	Mastermind-like
N^{IC}	intracellular Notch1
PAMP	pathogen associated molecular pattern
qPCR	Quantitative real-time RT-PCR
TNF	Tumor necrosis factor

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Highlights

- Notch signaling is activated in LPS/IFNγ-stimulated macrophages
- Inhibiting Notch signaling decreased the level of *il12p40* mRNA and secreted IL-12p70
- Inhibiting Notch signaling delayed c-Rel nuclear translocation upon activation of macrophages
- Exogenous TNFα partially rescued defects in c-Rel activation when Notch signaling is suppressed

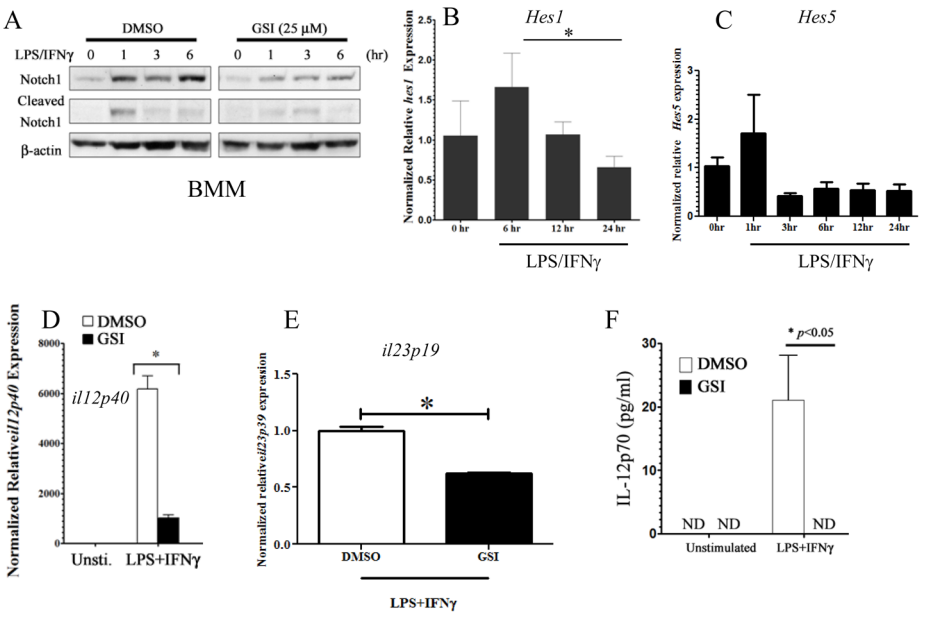

Figure 1. Activation of Notch Signaling in Activated Macrophages and Effect of GSI Treatment on IL-12p40 Expression

(A) BMMs were stimulated by LPS/IFNγ for indicated times in the presence of DMSO vehicle control or GSI (25 μM) as described in the materials and methods. Total cell lysates were harvested and analyzed for Notch1 and cleaved Notch1 (Val1744) using Western blotting. The results represent three independent experiments. (B-C) BMMs were activated as described in (A) without GSI for indicated times, and the expression levels of *Hes1* and *Hes5* were measured using qPCR. The results are the mean±SD and represent two independent experiments. (D-E) BMMs were activated as described in (A) for 4 hrs, and the expression levels of *il12p40* and *il23p19* were measured using qPCR. The results are the mean±SD and represent two independent experiments. (*) indicates where $p < 0.05$, which was considered to be statistically significant. (F) BMMs were pretreated with GSI (25 μM) or vehicle control DMSO and activated for 24 hr to become CA and regulatory macrophages as described above. The amount of IL-12p70 was measured in the culture supernatants using ELISA. The results are the mean±SD and represent two independent experiments. ND = not detectable.

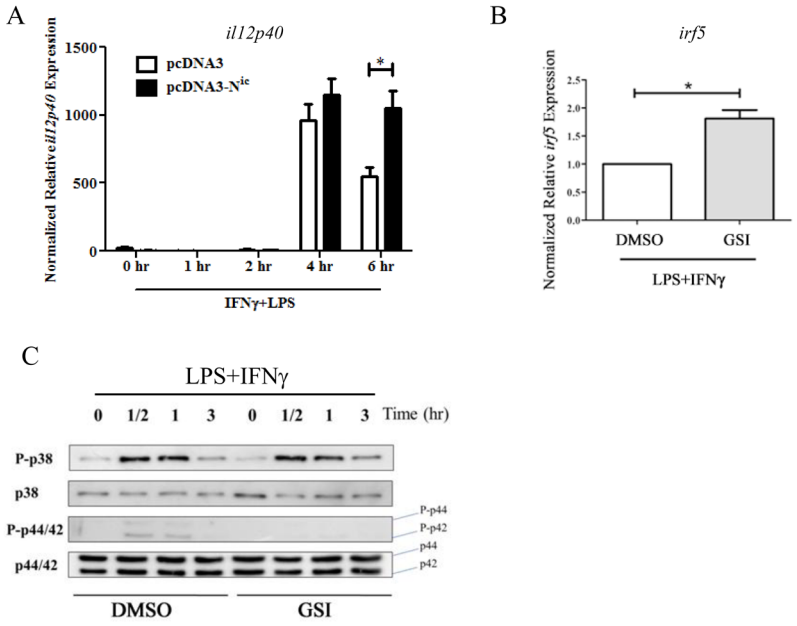

Figure 2. Overexpression of N^{ic} on the Level of *il12p40* mRNA in RAW264.7 Cell Line and Effects of GSI Treatment on the Expression of *irf5* and MAPK Pathways in activated BMMs

(A) RAW264.7 cell line was transiently transfected with pcDNA3 empty vector or pcDNA3-N^{ic} for 48 hr and stimulated with LPS/IFNγ for indicated times. The expression levels of *il12p40* were measured using qPCR. The results are the mean±SD and represent two independent experiments. (B) BMMs were activated as described in (Figure 1A) for 4 hr, and the expression levels of *irf5* were measured using qPCR. The expression level in unstimulated macrophages incubated with DMSO was set as a baseline for comparison. The results are the mean±SD and represent two independent experiments. (*) indicates statistical significance where $p<0.05$. (C) Activation of p38 and Erk1/2 were detected in BMMs activated with LPS/IFNγ in the presence or absence of GSI using Western blotting. The results represent two independent experiments.

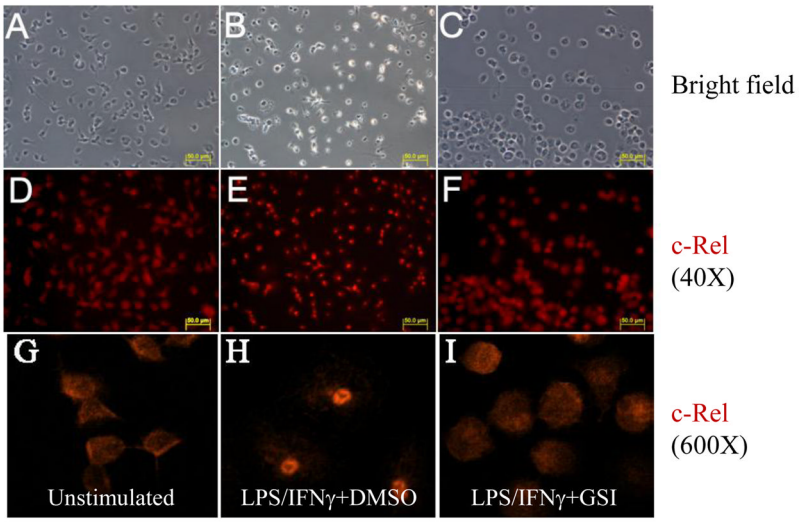

Figure 3. Effects of GSI Treatment on c-Rel in Activated BMMs

(A-I) BMMs were pretreated with GSI (25 μM) or vehicle control DMSO for 1 hr and activated as described above. Localization of c-Rel was detected at 4 hr after stimulation using immunofluorescent staining.

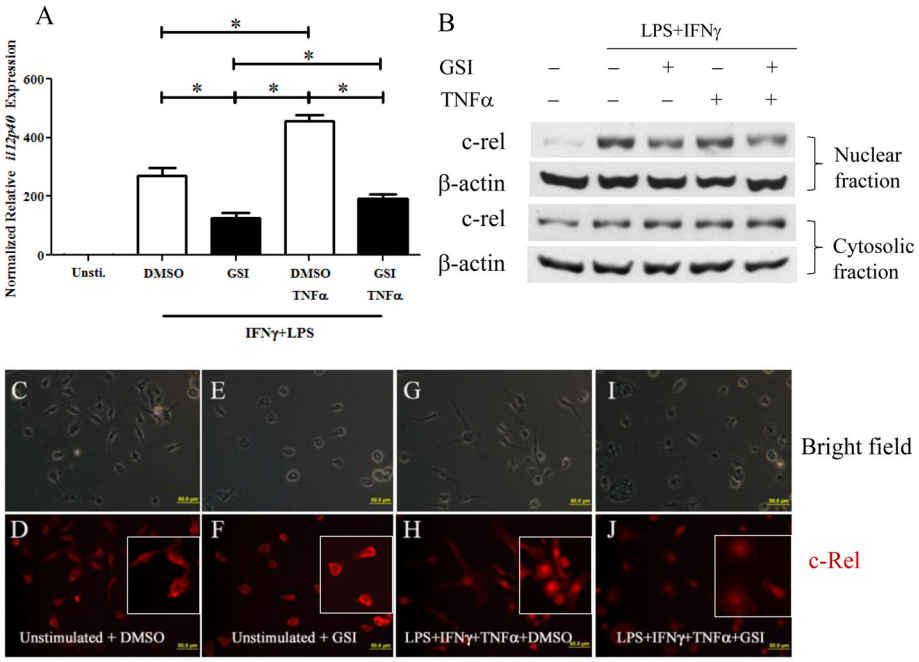

Figure 4. Effect of Exogenous TNFα on *il12p40* Expression and on c-Rel Localization in Activated BMMs

(A) BMMs were activated in the presence or absence of GSI for 4 hr as described above. Recombinant TNFα was added at the same time as LPS. The levels of *il12p40* were compared using qPCR. The results are the mean±SD and represent two independent experiments. (*) indicates statistical significance where $p<0.05$. (B) BMMs were activated in the presence or absence of GSI with or without TNFa for 4 hr. Nuclear fraction and cytosolic fraction were subjected to Western blot to detect c-Rel. (C-J) BMMs were treated as described in (A) and localization of c-Rel was detected at 4 hr after stimulation using immunofluorescent staining. The inset in each panel showed the magnified macrophages.

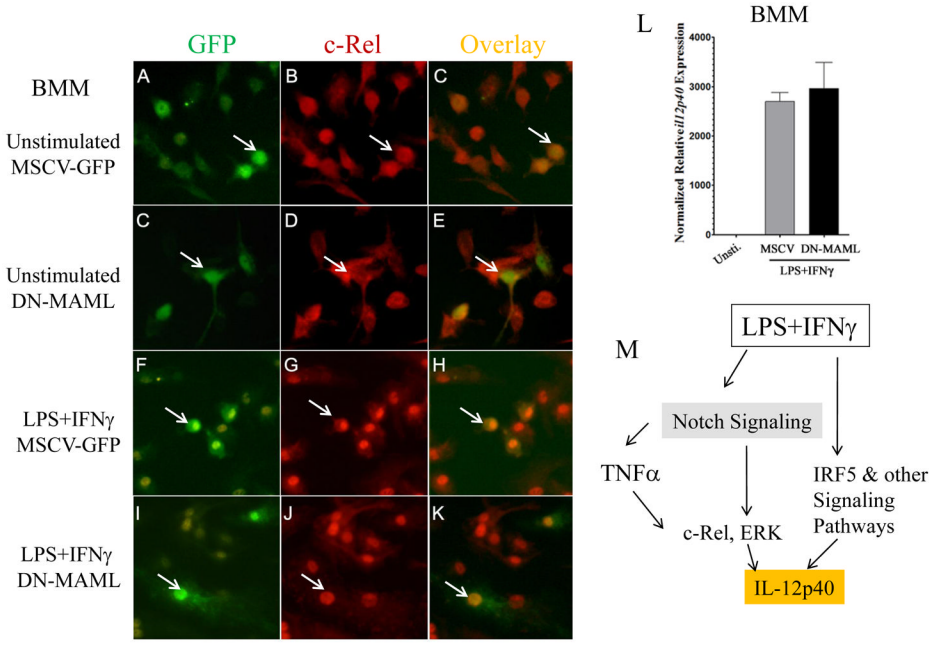

Figure 5. Effects of Overexpression of DN-MAML on c-Rel Localization and *il12p40* Expression

(A-K) BMMs were transduced with control retroviral vector MSCV-GFP or DN-MAML-EGFP before activation by LPS/IFNγ. Localization of c-Rel was detected using immunofluorescent staining at 4 hr after stimulation. White arrows indicate cells that cells are GFP+. The results represent two independent experiments. (L) BMMs were transduced by retroviral vectors and activated as described above, and total RNA was isolated at 4 hr after stimulation. Relative *il12p40* levels were analyzed using qPCR. The results are the mean±SD and represent two independent experiments. (M) Model depicting how Notch signaling is involved in regulating activation of IL-12p40 in macrophages (see text for details).