กิตกรรมประกาศ

ผู้วิจัยขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) ที่ให้การสนับสนุนโครงการวิจัยนี้จนเสร็จสมบูรณ์ ผู้วิจัยขอขอบคุณ Professor Barbara A. Osborne (University of Massachusetts at Amherst, USA) และ Professor Stefan H. E. Kaufmann (Max Planck Institute of Infection Biology, Germany) ที่ให้ความร่วมมือในงานวิจัยนี้และเอื้อเฟื้อพลาสมิด เซลล์คัลเจอร์ และสัตว์ทดลอง ผู้วิจัยขอขอบคุณ ดร. สาธิต พิชญางกูร (AFRIMS) ที่กรุณาเอื้อเฟื้อ CpG DNA และ รองศาสตราจารย์ ดร. อังคณา ฉายประเสริฐ (ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ ศิริราชพยาบาล มหาวิทยาลัยมหิดล) ที่กรุณาให้คำแนะนำเรื่องการเลี้ยงเชื้อ BCG สุดท้านนี้ผู้วิจัยขอขอบคุณนักวิจัยที่ปรึกษา รองศาสตราจารย์ ดร. ศิริรัตน์ เร่งพิพัฒน์ และภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

Output ที่ได้จากโครงการ

1. เข้าร่วมเสนอผลงานใน Annual Meeting of American Association of Immunologist 2006 ณ เมืองบอสตัน สหรัฐอเมริกา ระหว่างวันที่ 12-16 พฤษภาคม 2549 โดยนำเสนอในรูปแบบโปสเตอร์ หัวข้อ Toll-like receptor (TLR) agonists upregulate Notch receptors in murine macrophages
2. เข้าร่วมเสนอผลงานใน Keystone Symposia Tuberculosis: From Lab Research to Field Trials 2007 ณ เมืองแวนคูเวอร์ แคนาดา ระหว่างวันที่ 20-25 มีนาคม 2550 โดยนำเสนอในรูปแบบโปสเตอร์ หัวข้อ Notch1 is upregulated during BCG infection in macrophages
3. Manuscript ที่ส่งไปพิจารณาเพื่อตีพิมพ์ใน European Journal of Immunology ซึ่งกำลังอยู่ระหว่างการ revision

ภาคผนวก

1. บทคัดย่อการไปเสนอผลงานใน Annual Meeting of American Association of Immunologist 2006
2. บทคัดย่อการไปเสนอผลงานใน Keystone Symposia Tuberculosis: From Lab Research to Field Trials 2007
3. Manuscript ที่กำลังส่งไปเพื่อพิจารณาตีพิมพ์และอยู่ระหว่างการ revision จาก European Journal of Immunology

Notch Signaling is Activated by TLR Stimulation and Regulates Macrophage Functions

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Notch Signaling is Activated by TLR Stimulation and Regulates Macrophage Functions

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Key words: macrophages, Notch signaling, γ-secretase inhibitor, Toll-like receptors

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Abbreviations: GSI, Gamma secretase inhibitor

Summary

Notch signaling is a well-conserved pathway involved in cell fate decisions, proliferation and apoptosis. We report on the involvement of Notch signaling in regulating gene expression in activated macrophages. TLR agonists such as bacterial lipopeptide, polyI:C, LPS and unmethylated CpG DNA all induced upregulation of Notch1 in primary and macrophage-like cell lines. Notch1 upregulation was dependent on the MyD88 pathway when stimulated through TLR2, but not TLR4. Activated Notch1 and expression of the Notch target gene, *Hes1*, were detected in activated macrophages, suggesting that Notch signaling was activated upon stimulation. Inhibiting processing of Notch receptor by γ-secretase using a γ-secretase inhibitor (GSI), the expression of Notch1 was downregulated to basal levels. This treatment significantly modulated expression of *TNFα, IL-6*, and *IL-10*. While expression of *IL-6* was suppressed, *IL-10* was induced upon GSI treatment. Production of TNFα decreased in GSI-treated cells at 6 h but reached levels comparable to control at 24 h. In addition, the amount of NO produced was significantly lower and the expression of MHC class II was upregulated in GSI-treated cells. Taken together, stimulation of macrophages through the TLR signaling cascade triggered activation of Notch signaling, which in turn regulated gene expression patterns involved in proinflammatory responses.

Introduction

The innate immune response recognizes invasion of microorganisms using pattern recognition receptors (PRRs) through recognition of pathogen-associated molecular patterns (PAMPs) [1]. Toll-like receptors (TLRs), the mammalian orthologs of *Drosophila* Toll receptor, play a crucial role in recognizing PAMPs. There are more than 10 TLRs identified in mammals, each showing differential specificity for its ligands [2]. The signaling generated by engagement of TLRs with specific ligands involves the recruitment of TIR domain-containing adaptor proteins such as MyD88 and TIRAP/MAL, phosphorylation of secondary messengers which culminates in nuclear translocation of transcription factors such as NF-κB [3]. This event leads to initiation of innate immune responses including inflammation through cytokine production and secretion of reactive mediators. The outcome of stimulation through the TLR signaling pathway can be varied depending on combination of receptor-ligand interactions. It is still largely unknown how individual TLR signals are eventually shaped for ligand-specific outcomes [2, 3].

Notch signaling is a well-conserved signaling pathway involved in cell fate decisions during development of various systems, including the hematopoietic and neuronal systems [4]. The Notch receptor is a heterodimeric transmembrane protein expressed on the cell surface of signal receiving cells. The extracellular domain is comprised of EGF-like repeats and is responsible for ligand binding. Receptor-ligand interactions lead to the cleavage of the transmembrane peptide by two proteolytic

enzymes, *i.e.* tumor necrosis factor α (TNFα)-converting enzyme (TACE) and γ-secretase. These events release the intracellular domain of Notch (NIC) and promote migration into the nucleus, where it forms a transactivation complex with the DNA-binding protein, RBP-J/CBF-1. The transactivation complex further recruits coactivators such as p300 via a scaffold protein MAML and drives the transcription of target genes, such as *Hes-1*, *CD25* as well as *Notch* itself [5]. Mammalian Notch receptors are encoded by four different genes, *i.e. Notch1-4*, and genes encoding five mammalian Notch ligands have been identified, *i.e. Jagged1, 2, Delta-like 1, 3* and *4*.

Notch receptors and ligands are expressed at various stages during hematopoiesis and the signal initiated by receptor–ligand interactions plays a crucial role in lineage commitment of certain cell lineages [4, 5]. Disruption of Notch signaling using either genetic or pharmacological approaches in T-lineage cells results in arrest of thymic development and functional aberrations in peripheral T lymphocytes [6-8]. Similarly, Notch signaling has been shown to influence various myeloid lineage choices [9, 10]. Analyses of expression patterns of Notch receptors/ligands in monocyte/macrophage lineage cells reveal that both Notch receptors and ligands are temporally expressed during development, and this expression persists even after completion of development [11-13]. Differential effects of TLR agonists in regulating the expression of Notch ligands have been described, and the importance of Notch ligand expression by antigen-presenting cells in regulating the outcomes of immune responses is highlighted by its influence on

Th1/Th2 immunity [7]. Furthermore, Notch signaling has been shown to directly regulate immune responses in the periphery such as production of several cytokines in T lymphocytes, including IFNγ, IL-4 and IL-10 [7, 14-16].

In addition to Notch ligands, Notch receptors are also expressed in mature macrophages and, recently, Notch signaling has been reported to be involved in regulating expression of genes involved in antigen presenting capacity and cytotoxicity [12, 17]. However, it is unclear whether activation through TLR regulates Notch expression and the role of Notch signaling in regulating cytokine production in this cell type has not been addressed. In this study, we investigate the effects of different TLR agonists on the expression of Notch receptors and the role Notch signaling plays in macrophages using pharmacological approaches to inhibit Notch signaling. We report that various TLR ligands similarly trigger upregulation of Notch1 in murine bone marrow (BM)-derived macrophages and the macrophage-like cell line RAW264.7, both in an MyD88-dependent and MyD88-independent manner. Furthermore, Notch signaling is activated by stimulation through TLR signaling. Using a γ-secretase inhibitor (GSI) to block Notch signaling we demonstrate that GSI blocks the upregulation of Notch induced by LPS/IFNγ stimulation. Expression of several cytokine genes and NO production at late stages of activation (12–24 h), were significantly altered by GSI treatment. These results suggest that Notch signaling triggered by TLR stimulation regulates gene expression in activated macrophages.

Results

Notch receptors are upregulated by activation of macrophages with LPS/IFNγ

It has been shown previously that mature macrophages express Notch1 and Notch 4 [12]. In order to investigate the effects of stimulation through TLR on the expression of Notch receptors, we treated the macrophage-like cell line RAW264.7 with LPS (TLR4 ligand), rIFNγ or LPS plus rIFNγ for 0 h, 2 h, 4 h, and 6 h and analyzed the expression of Notch1 by RT-PCR. As shown in Fig. 1A, stimulation with LPS, rIFNγ and LPS plus rIFNγ significantly triggered upregulation of Notch1 transcription, with stimulation by LPS plus rIFNγ being the strongest inducer. Four different Notch receptors are differentially expressed during hematopoiesis. Functional diversity and functional redundancy among these four receptors have been reported [18, 19]. Since Notch1 is upregulated by LPS treatment, we investigated effects of LPS on the expression of other Notch receptors in RAW264.7 cell line by RT-PCR. The expression of Notch2 was not affected by stimulation, while expression of Notch4 was downregulated by treatment with IFNγ. In BM-derived macrophages, similar upregulation of Notch1 was confirmed, while Notch2 was upregulated as early as 3 h after LPS treatment, in contrast to that seen in RAW264.7 cell line (Fig. 1B, Fig. 3A). The expression of Notch3 could not be detected at any time point during this experiment (data not shown). The expression of Notch4, on the other hand, was detected at 0 h, but readily downregulated at 3 h after treatment.

In addition, we examined the expression of the Notch target gene, *Hes1* as marker for activation of Notch signaling. As shown in Fig. 1C, treatment of RAW264.7 cells by LPS or LPS plus IFNγ both upregulated *Hes1* expression. Stimulation with LPS plus IFNγ induced more rapid upregulation of *Hes1* at 2 h post treatment, while LPS alone induced *Hes1* upregulation at 4 h post treatment. Taken together, treatment with LPS plus IFNγ affects Notch receptor expression in primary BM-derived macrophages and a macrophage-like cell line, and this stimulation also triggers activation of Notch signaling.

Macrophages change their functional phenotypes based on the local cytokine microenvironment [20]. They can be activated by treatment with LPS alone, IFNγ alone or a combination of LPS plus IFNγ. Each stimulus induces a different biological outcome, and the combination of both signals often shows synergistic effects in macrophages. Therefore, we determined effects of IFNγ on LPS-induced Notch1 upregulation in macrophages. Treatment by IFNγ alone for 18 h induced slight upregulation of Notch1 protein in BM-derived macrophages (Fig. 2A). Treatment with both IFNγ plus LPS simultaneously yielded an upregulation pattern of Notch1 similar to that in cells treated with LPS alone (Fig. 2A). In addition, the order of IFNγ or LPS addition did not influence the expression of Notch1, as compared to LPS treatment alone (Fig. 2B). NO was produced by activated macrophages (data not shown) verifying the biological synergy of LPS and IFNγ treatment. Taken together, LPS alone or in combination with IFNγ induced

upregulation of Notch1 in macrophages. These data also confirm and extend the PCR analyses shown in Fig. 1 by demonstrating the increase in Notch protein levels following treatment with LPS plus IFNγ.

Different TLR agonists upregulate Notch1 expression in macrophages in an MyD88-dependent and MyD88-independent fashion

Macrophages express abundant TLRs which enable them to sense the presence of pathogens via recognition of PAMPs and alert other immune cells to the insult. Because treatment with LPS (a TLR4 agonist as shown in Fig. 3A and above), influences the expression pattern of Notch1 in macrophages, we next examined the effects of treatment of BM-derived macrophages with other TLR agonists, e.g. synthetic lipopeptide Pam_3Cys lipopeptide (TLR2), polyI:C (TLR3) and CpG DNA (TLR9), on Notch1 expression. All TLR agonists tested here induced Notch1 upregulation at 6 h post treatment, similar to that seen when stimulated with LPS (Fig. 3). In addition, treatments of BM-derived macrophages and RAW264.7 cells with TLR agonists all induced Notch1 upregulation in a dose-dependent manner (data not shown). Therefore, stimulation through TLRs similarly upregulated Notch1 expression at the protein level.

Upon recognition of microbial products, TLRs transduce signals through multiple pathways by recruiting different adaptor molecules. MyD88 is one of the adaptor molecules utilized by multiple TLRs. While TLR2 agonists signal solely

through MyD88, TLR4 agonists signal via MyD88-dependent and MyD88-independent pathways [21]. We asked whether Notch1 upregulation mediated by stimulation through TLR2 and TLR4 in macrophages depends on MyD88. BM-derived macrophages from MyD88$^{-/-}$ mice upregulated Notch1 at 6 h after stimulation with LPS, similar to WT controls. However, defects in sustaining Notch1 expression for longer periods were detected in MyD88$^{-/-}$ BM-derived macrophages (Fig. 4A). In contrast, when BM-derived macrophages from MyD88$^{-/-}$ mice were stimulated by TLR2-specific Pam_3Cys lipopeptide, upregulation of Notch1 was completely absent both at early and late time points (Fig. 4B). Therefore, Notch1 upregulation induced by signaling through TLR4 at an early stage was MyD88 independent whereas sustaining Notch1 expression over longer time periods was MyD88 dependent. On the other hand, TLR2-mediated Notch1 upregulation relied completely on the MyD88 adaptor molecule.

Effect of γ-secretase inhibitor on Notch1 expression

To investigate the role of Notch signaling in regulating biological function(s) of macrophages, we employed a pharmacological approach by GSI, IL-CHO [22]. Since all Notch receptors are proteolytically cleaved and activated by the multi-subunit enzyme, γ-secretase, the problem of functional redundancy among receptors can be overcome by using GSI [23]. Using GSI as a tool, several studies have reported phenotypes similar to Notch-loss-of-function mutations [8, 14, 24, 25].

Stimulation of macrophages with LPS plus rIFNγ generated cleaved Notch1 and upegulation of Notch1, confirming that Notch signaling is activated by this treatment (Fig. 5 A–B). Pretreatment with IL-CHO prior to LPS and IFNγ stimulation resulted in elimination of cleaved Notch1 and a marked decrease in Notch1 upregulation at concentrations between 10 and 25 μM. This result is consistent with previous findings suggesting that transcription of Notch receptors is under the control of a feed-forward loop of Notch signaling itself [14]. To exclude cytotoxicity of IL-CHO treatment, we verified morphological changes of cells stimulated with LPS plus rIFNγ in the presence or absence of IL-CHO up to 36 h (Fig. 5C). We did not detect any obvious changes of cell death and analysis of cytotoxicity did not reveal any significant differences in viability (data not shown). However, cells cultured in the presence of IL-CHO appeared more flat and attached to the surface than cells cultured with vehicle control. Taken together, IL-CHO treatment completely abrogated Notch1 expression in activated macrophages at doses that were not toxic to cells.

Effects of IL-CHO on biological macrophage functions

To investigate the effects of interfering with Notch signaling using IL-CHO on biological macrophage functions, we examined the expression of several cytokines and effector molecules typically produced by activated macrophages upon stimulation with LPS plus rIFNγ. Treatment with IL-CHO of stimulated

macrophages did not affect mRNA expression of *TNFα, IL-10* and *iNos* at 6 h after stimulation (data not shown). However, effects of IL-CHO were seen at 12 h and 24 h after treatment. While the expression level of *IL-10* mRNA increased, *IL-6* mRNA significantly decreased in the presence of IL-CHO (Fig. 6A). When the abundance of secreted TNFα was measured at 6 h, 12 h and 24 h after stimulation, IL-CHO treatment modified TNFα production at 6 h but this effect was not seen at later time points (Fig. 6B). In addition, NO production was significantly suppressed in the presence of IL-CHO. Treatment with IL-CHO also enhanced cell surface expression of MHC class II (Fig. 6C–D). Hence, inhibition of Notch signaling using GSI altered biological functions of macrophages, suggesting a pivotal role for Notch signaling in innate immune responses by activated macrophages.

Discussion

Notch receptors and ligands are expressed during development of myeloid lineage cells and their expression persists after maturation to monocytes or tissue macrophages[26]. A role for Notch signaling during cell fate decision of dendritic cells, monocytes/macrophages, and granulocytes has been reported [27-29]. In addition to a crucial role during development, it has become apparent that Notch signaling also regulates the function of peripheral immune cells, in particular T lymphocytes [5]. It has been suggested that different TLR agonists can modulate the expression of Notch receptors and Notch ligands on antigen-presenting cells and, as

a result, influence the outcomes of a given immune response. In this study, we examined effects of stimulating macrophages through TLRs on the expressions of Notch receptors. We found that all TLR agonists (TLR2, TLR3, TLR4 and TLR9) tested upregulate Notch1 protein expression in both the macrophage-like cell line RAW264.7- and BM-derived primary macrophages. TLR2-mediated Notch1 upregulation was completely abrogated in MyD88$^{-/-}$ macrophages, whilst TLR4 signaling did not rely on MyD88 for early Notch1 upregulation. Notch2 mRNA was also upregulated by the TLR4 agonist in primary macrophage cultures, but not in RAW264.7 cells. In addition, downregulation of Notch4 mRNA was seen in both cell types. Furthermore, both cleaved Notch1 protein and *Hes1* mRNA were readily detected upon macrophage stimulation, providing strong evidence that Notch signaling was activated. This result suggests that Notch signaling regulated biological activities of macrophages in response to activation by microbes through TLRs.

To verify the role of Notch signaling in regulating inflammatory responses triggered by macrophages, we tested effects of GSI on cytokine production, NO production and MHC class II expression. GSI treatment did not alter mRNA expression of *TNFα* but the amount of secreted protein was markedly decreased at the early stage of stimulation, suggesting that Notch signaling regulates TNFα production at the post-translational level. Expression of *IL-10* mRNA was increased in GSI-treated cells, suggesting that Notch signaling can inhibit production of IL-10

in macrophages. Interestingly, Notch signaling has been shown to be required for IL-10 production by T cells where the Notch receptor colocalizes with CD4 [16]. In contrast to IL-10, expression of *IL-6* mRNA was suppressed in GSI-treated cells, suggesting that Notch signaling is required for IL-6 expression. In fact, the binding partner of Notch, CBF-1, binds to and regulates the *IL-6* gene [30]. In addition to cytokines, decreased production of NO and increased expression of MHC class II were detected in GSI-treated macrophages. It appears that Notch plays a critical role in steering an immune response toward inflammation by regulating expression of various cytokines and effector molecules generated by macrophages.

Recently, Monsalve *et al.* [17] showed that increasing Notch1 expression upon stimulation with LPS plus rIFNγ in RAW264.7 cells modulates STAT1 transactivation capacity. Using an overexpression strategy, they also found decreased NO production, but increased ICAM1 and MHC class II expression by stable transfectants with a truncated active form of Notch [17]. The discrepancy between the results obtained in this study and those from Monslave *et al.* [17] may lie in experimental conditions and the different approaches used. The major differences are that we used primary BM-derived macrophages, not a cell line, for studying effects of GSI treatment. As shown here, expression of some Notch receptors was affected differentially in RAW264.7 cells and primary macrophages. In addition, we cannot rule out the possibility that GSI treatment affected other signaling pathways resulting in the phenotypes observed in our study.

Signaling through TLRs often causes activation of the NF-κB pathway. Modulating Notch signaling also has a profound effect on the NF-κB signaling cascade in T lymphocytes and hematopoietic precursor cells [14, 25, 31]. Therefore, it is tempting to speculate that Notch regulates activities of macrophages by interfering with the NF-κB pathway. However, Monsalve *et al*. [17] observed unaltered NF-κB reporter activity after treatment with LPS plus rIFNγ, when Notch is constitutively activated. Instead, they found that constitutively activated Notch1 decreases AP-1 and increases STAT1/3 reporter activity.

Notch signaling has been reported to directly regulate cytokine production in T lymphocytes, such as IFNγ, IL-4, IL-6 and IL-10 [7, 14, 16, 32]. Since macrophages also produce IL-6, IL-10 and IFNγ, it will be interesting to explore the role Notch signaling plays in regulating cytokine production in macrophages. The effects of GSI on cytokine production are reminiscent of those seen with overexpression of SOCS3 [33]. SOCS3 plays an important role in regulating expression of *TNFα*, *iNOS* and *IL-6* in macrophages, and its expression is induced by IL-10 [33, 34]. Expression of IL-6 and iNOS are negatively regulated by SOCS3 at mRNA and protein levels, while TNFα expression is negatively regulated at the protein level [33]. Since mRNA of IL-10 increased in the presence of GSI, it will be of interest to explore the relationships between Notch signaling, IL-10 and SOCS3 in macrophages. In Th cells, Notch has been shown to colocalize with CD4 and to be required for IL-10 production [16]. However, GSI enhanced mRNA expression of

IL-10 in stimulated macrophages, suggesting that Notch signaling inhibits IL-10 production in this cell type. Interestingly, chromatin remodeling studies revealed differences in DNaseI hypersensitivity sites in the promoter of IL-10 in T lymphocytes and macrophages, suggesting distinct regulatory mechanisms in these two cell types [35].

Our study reveals that Notch signaling is activated in LPS plus IFNγ-stimulated macrophages and Notch signaling regulates critical biological functions of activated macrophages. Notch signaling is a crucial regulator of biological functions in macrophages during the effector phase by promoting inflammatory responses. Consistent with this notion, components of Notch signaling are upregulated in lesions of chronic inflammation [36, 37]. It will be of interest to investigate whether Notch is involved in initiating inflammation by interacting with the TLR signaling cascade or with other signaling pathways involving TLRs.

Materials and methods

Mice, BM-derived macrophages and RAW264.7 cell line

Female C57BL6 mice (aged between 10–12 weeks) were purchased from the National Laboratory Animal Centre (Mahidol University, Salaya, Thailand). All procedures involving laboratory animals were conducted according to guidelines issued by Chulalongkorn University. MyD88$^{-/-}$ mice back-crossed onto the C57/BL6 background were kindly provided by Dr. Shizuo Akira and the experiments involving these mice were conducted at the Max Planck Institute for Infection Biology according to German protection law [38]. Mice were sacrificed, and femoral bone marrows were isolated. BM-derived macrophages were generated as described elsewhere [39]. In brief, cells were washed from BM cavity and incubated for 7 days in RPMI 1640 media supplemented with 10% fetal bovine serum purchased from HyClone Laboratory (Logan, UT), 100 μM gentamycin and 20% L929-conditioned media. Media were changed every 2 days and cells were harvested at the end of culture using 5 mM EDTA in phosphate buffer saline (PBS). Cells were confirmed to be of macrophage/monocyte lineage by cell surface staining with anti-CD11b and anti-Mac1 mAbs and by analysis using flow cytometry. More than 95% were found to be CD11b^{+} and Mac1^{+}. RAW264.7 cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 μM gentamycin and L-glutamine.

Reagents and antibodies

LPS from *Escherichia coli* O26:B6, polyinosinic:polycytidylic acid were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic TLR2 agonist Pam_3Cys lipopeptide was purchased from EMC microcollections GmbH (Germany). TLR agonist CpG DNA has been described elsewhere [40]. Mouse r-IFNγ was purchased from R&D Systems (Minneapolis, MN) and kept at –80°C. The γ-secretase inhibitor, IL-CHO, has been described elsewhere [22]. IL-CHO was dissolved in DMSO at a concentration of 5 mM and aliquots were kept at –80°C. TriZol reagent for total RNA isolation was purchased from Life Technologies (Grand Island, NY). The mAb for cell surface staining, i.e. FITC-conjugated anti-Mac1 mAb, PE-conjugated anti-CD11b, mAb and biotinylated anti-mouse MHC class II (IA^b) mAb were purchased from Caltag (Carlsbad, CA). Rabbit polyclonal Ab against Notch1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Ab against cleaved Notch1 (Val1744) was purchased from Cell Signaling Technology (Boston, MA). The mAb against βactin was purchased from Chemicon International (Temecula, CA). Secondary Ab conjugated with HRP was purchased from Amersham Biosciences (Piscataway, NJ).

RT-PCR

Total RNA was isolated from cells treated as indicated using TriZol reagent according to the manufacturer's instruction. RNA (500 ng) was used to generate

cDNA using random hexamer primers and RevertAid M-MuLV reverse transcriptase (Fermetas; ON, Canada). Obtained cDNAs were used as templates to amplify *Notch1-4*, *Hes1*, *IL-6*, *IL-10* and *β-actin*. The primer pairs used in this study are as follows.

Primer	Sequence (5' to 3')
Murine Notch1forward	GTGAGGGTGATGTCAATG
Murine Notch1 reverse	TGAAGTTGAGGGAGCAGT
Murine Notch2 forward	TGGAGGTAAATGAATGCCAGAGC
Murine Notch2 reverse	TGTAGCGATTGATGCCGTCC
Murine Notch3 forward	ACACTGGGAGTTCTCTGT
Murine Notch3 reverse	GTCTGCTGGCATGGGATA
Murine Notch4 forward	CACCTCCTGCCATAACACCTTG
Murine Notch4 reverse	ACACAGTCATCTGGGTTCATCTCAC
Murine Hes1 forward	CCGGTCTACACCAGCAACAGT
Murine Hes1 reverse	CACATGGAGTCCGAAGTGAGC
Murine IL-6 forward	CATGTTCTCTGGGAAATCGTGG
Murine IL-6 reverse	AACGCACTAGGTTTGCCGAGTA
Murine IL-10 forward	TCAAACAAAGGACCAGCTGGACAACATACTGC
Murine IL-10 reverse	CTGTCTAGGTCCTGGAGTCCAGCAGACTCAA
β actin forward	ACCAACTGGGACGACATGGAGAA

β actin reverse	GTGGTGGTGAAGCTGTAGCC

The PCR reactions were carried out using Taq DNA polymerase (Fermentas). The PCR products were analyzed on 1.5% agarose gel by electrophoresis, except those for *Hes1* which were analyzed on 5% polyacrylamide gel, and visualized after staining with ethidium bromide using gel documentation system (BioRad; Hercules, CA).

Western blot

Cells treated as indicated were harvested and cell lysates were prepared as described previously [14]. Amounts of proteins were measured using BCA protein assay kit (Pierce; Rockford, IL). Cell lysates (30 μg) were separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis using Protein III system (Bio-Rad). After gel separation, proteins were transferred onto PVDF membrane (Amersham Biosciences) and blocked in PBS containing 3% nonfat dry milk and 0.05% Tween 20. Blots were probed with rabbit anti-Notch1 Ab at 1:1000 dilution, rabbit anti-cleaved Notch1 Ab at 1:1000 dilution or anti-βactin mAb at 1:5000 dilution, followed by washing and probing with HRP-conjugated donkey anti-rabbit IgG Ab or sheep anti-mouse IgG Ab at 1:4000 dilution. After washing, signals were detected using ECL Western blotting analysis system (Amersham Biosciences).

Measurement of nitrites

To measure the amount of NO produced by macrophages, BM-derived macrophages were treated as indicated. At the end of culture, culture supernatants were harvested by centrifugation and kept at –80°C. Culture supernatants were subjected to assay for NO production using Griess reagents as described previously [41].

Measurement of TNFα by ELISA

Quantitative ELISA was carried out using mouse TNFα ELISA test kit purchased from eBioscience (San Diego, CA). Culture supernatants were collected after treating cells as indicated and kept at –80°C until subjecting to ELISA. ELISA was carried out according to manufacturer's instructions.

Statistical analysis

To calculate the statistical differences between control and samples, Student's paired t-test was used. Values of $p<0.05$ were considered significant.

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Figure Legends

Figure 1. Expression profiles of *Notch1-4* and *Hes1* in activated macrophages.

A–B. RAW264.7 or BM macrophages were treated with LPS (100 ng/ml), rIFNγ (10 ng/ml) or LPS plus rIFNγ and total RNA was isolated at indicated times. Expressions of *Notch 1-4* were analyzed by RT-PCR. The *β actin* was used as loading control.

C. RAW264.7 cells were treated with LPS or LPS plus rIFNγ for indicated times and total RNA was isolated. *Hes1* expression was analyzed by RT-PCR.

Figure 2. Notch1 is upregulated in macrophages stimulated with IFNγ and LPS.

A. BM macrophages were left untreated or treated with LPS (100 ng/ml), rIFNγ (10 ng/ml) or LPS plus rIFNγ for 18 h. Cell lysates were analyzed for Notch1 expression by Western blot. The β actin was used as loading control.

B. BM macrophages were left untreated or treated with rIFNγ (10 ng/ml) alone, LPS (100 ng/ml) alone for 12 h. For co-treatment with LPS plus rIFNγ, macrophages were pretreated with LPS (100 ng/ml) for 4 h and rIFNγ was added and incubated for a further 8 h, or pretreated with rIFNγ (10 ng/ml) for 4 h and LPS was added and further incubated for 8 h. Cell lysates were analyzed for Notch1 expression by Western blot. The β actin was used as loading control.

Figure 3. TLR agonists upregulate Notch1 expression in activated macrophages.

BM macrophages were treated with LPS (100 ng/ml) (A), poly I:C (100 μg/ml) (B), Pam3Cys lipopeptide (1 μg/ml) (C) or CpG DNA (500 ng/ml) (D) for indicated times and cell lysates were analyzed for Notch1 expression by Western blot. The β actin was used as loading control.

Figure 4. Upregulation of Notch1 in MyD88$^{-/-}$ BM.

BM macrophages from C57BL6 WT mice or MyD88$^{-/-}$ mice were stimulated with LPS (A) or Pam3Cys lipopeptide (B) for indicated times. Cell lysates were analyzed for Notch1 expression by Western blot.

Figure 5. Effects of GSI treatment on Notch1 expression and morphology of macrophages.

A–B. BM macrophages were pretreated with IL-CHO or DMSO mock control for 1 h, and treated with LPS (100 ng/ml) plus rIFNγ (10 ng/ml) for 18 h. Cell lysates were analyzed expression of cleaved Notch 1 (A) or Notch1 (B) by Western blot. The β actin was used as loading control

C. Morphology of BM macrophages treated as described above for 36 h.

Figure 6. Effects of GSI treatment on production of cytokines and NO and expression of MHC class II in macrophages.

A. BM macrophages were pretreated with mock control DMSO or IL-CHO (25 μM) for 1 h before stimulation with LPS and rIFNγ for indicated periods. Total RNA was isolated and expressions of *IL6*, *IL-10* were analyzed using RT-PCR. Expression of *β-actin* was used as loading control.

B. BM macrophages were treated as described in A and culture supernanats were harvested at 6 h, 12 h and 24 h. The amount of TNFα in culture supernatant was measured by ELISA. The results shown are representative of two independent experiments carried out in duplicate. (*p<0.05 between DMSO-treated and IL-CHO-treated samples).

C. BM macrophages were pretreated as described in B. Culture supernatants were harvested and subjected to the assays for nitrite production using Griess reaction. The results correspond to representatives of the means ± SD of triplicate experiments. (*p<0.05 between DMSO-treated and IL-CHO-treated samples).

D. BM macrophages were treated as described in B for 24 h and cells were harvested and stained using biotinylated anti-MHC class II (I-A^b) and avidin-FITC. Expression of MHC class II was analyzed by flow cytometry.

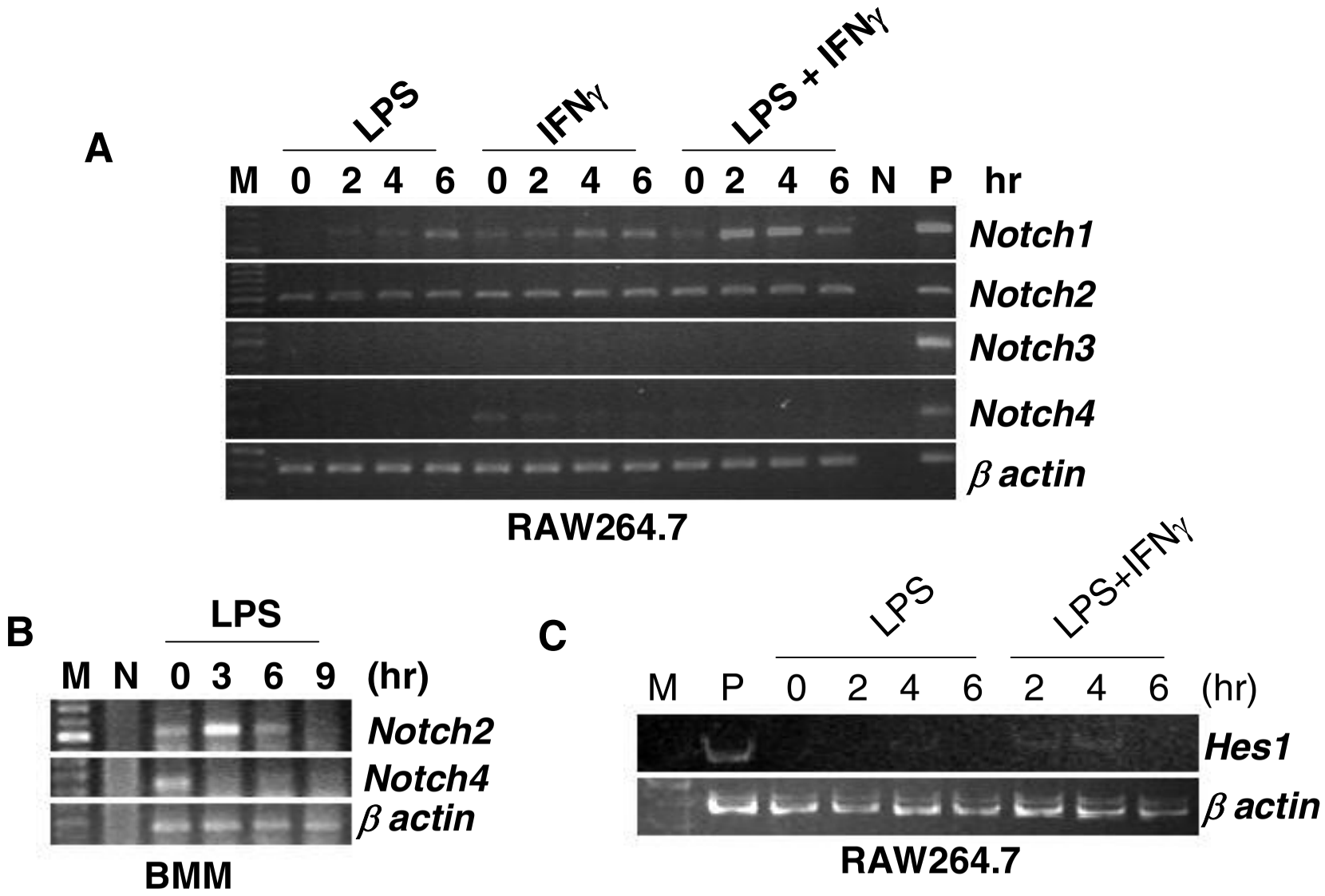
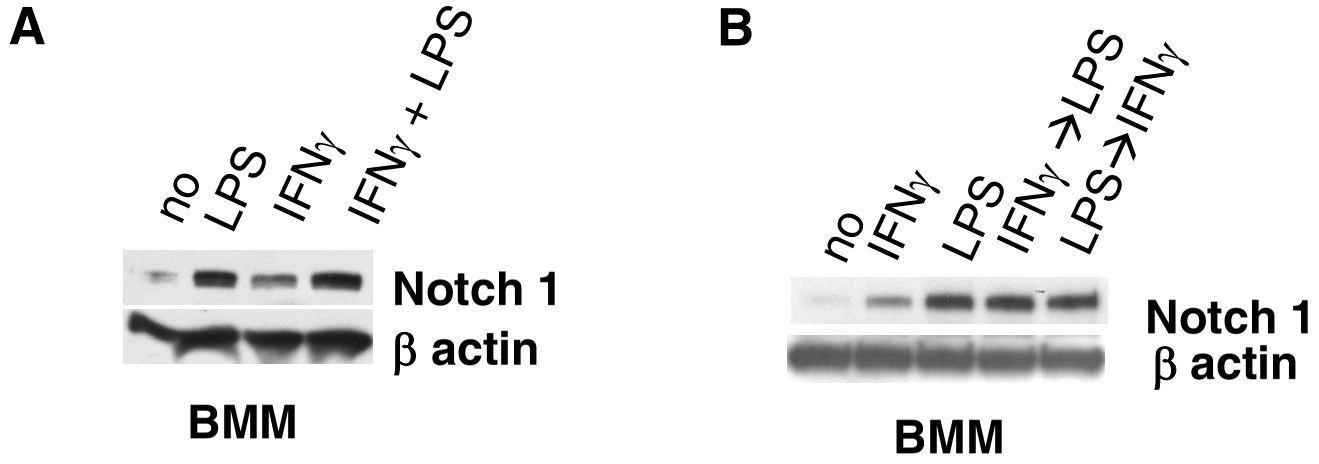


Figure 1
Palaga et al., 2006



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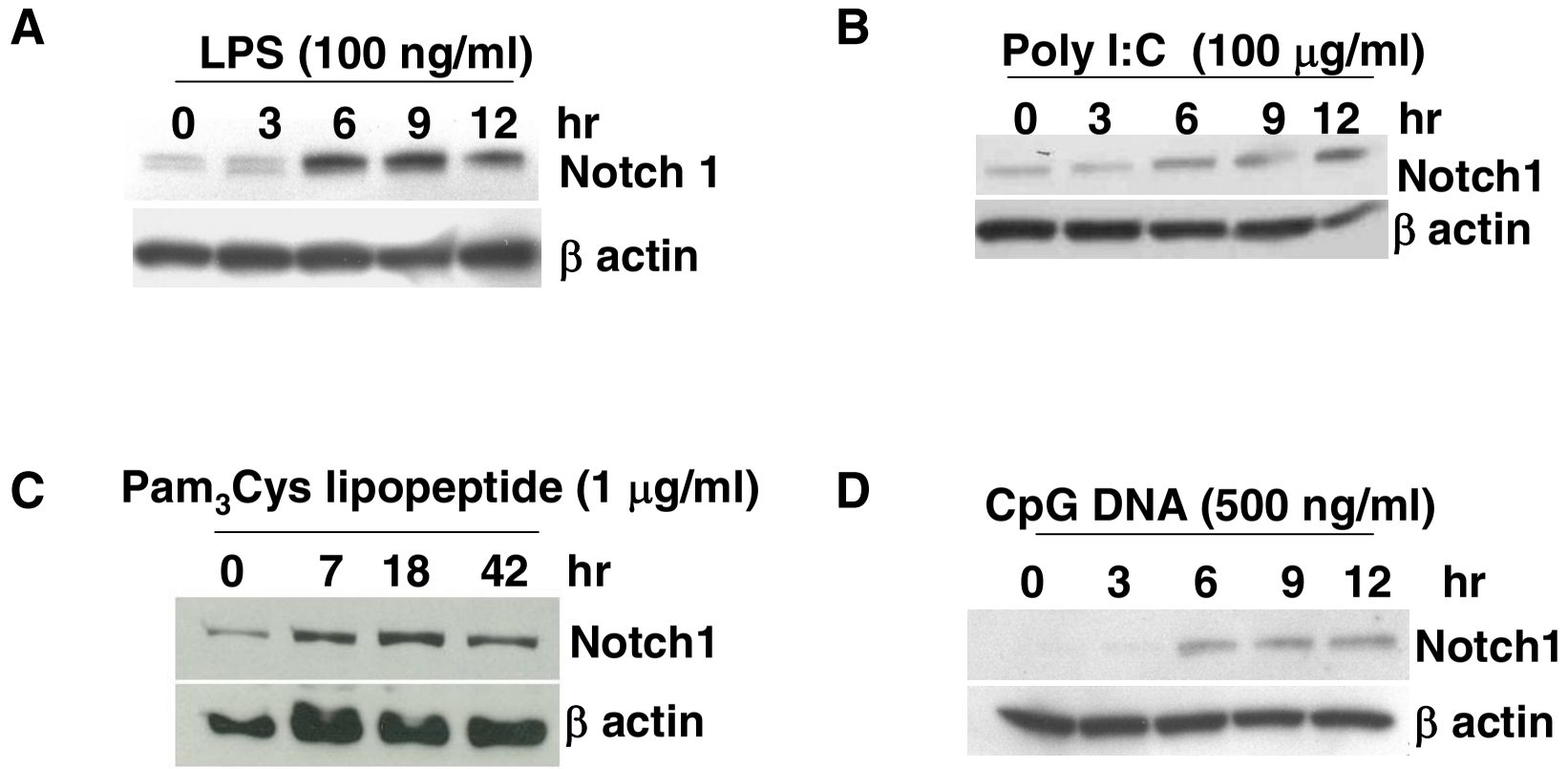

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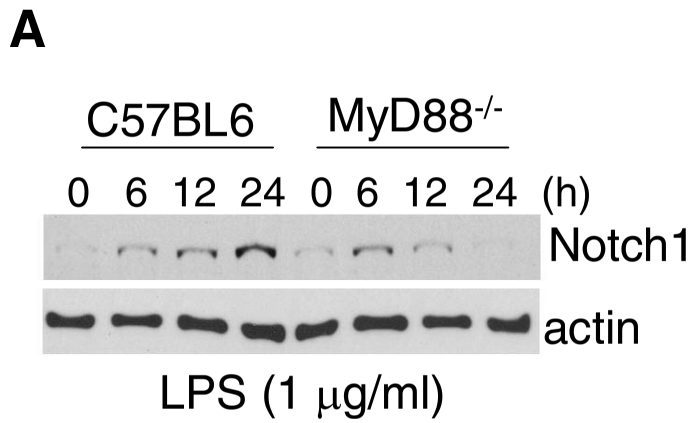

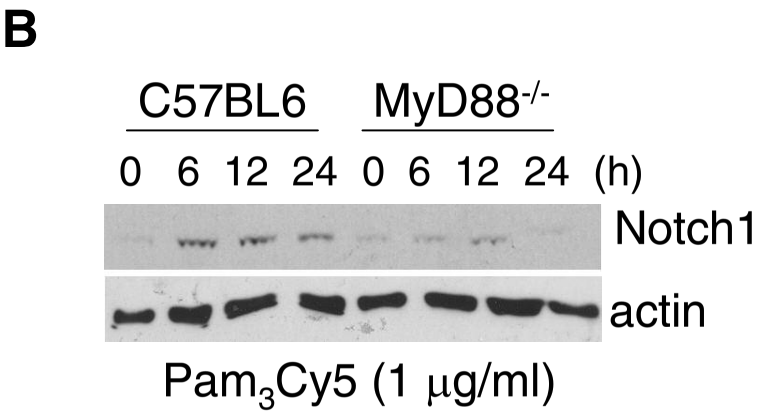

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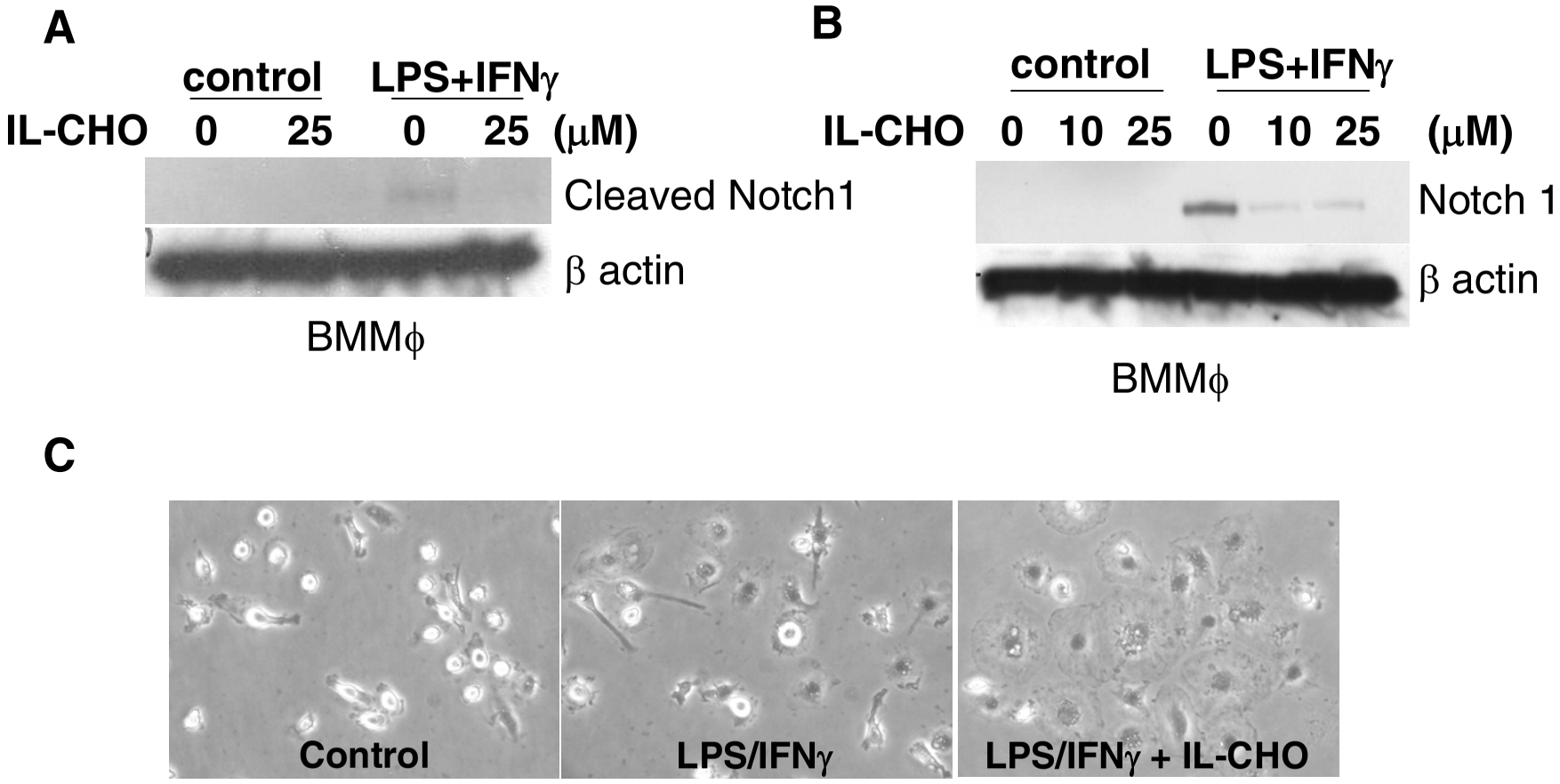

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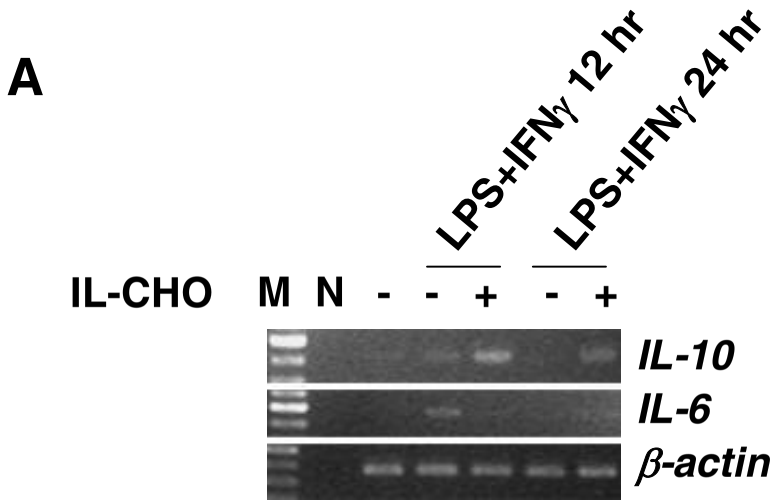

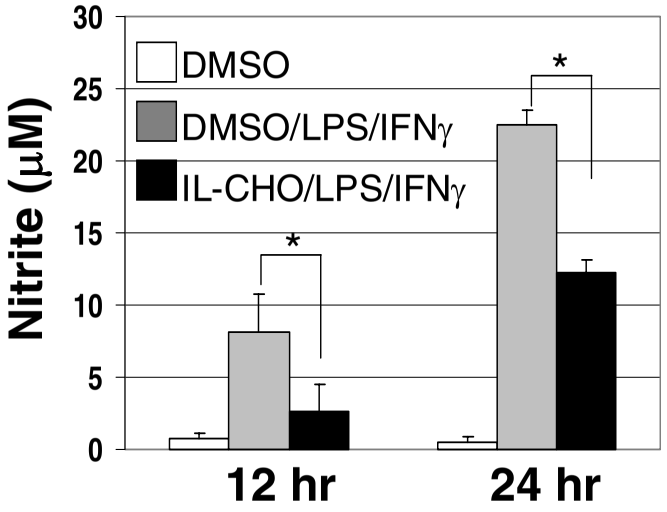

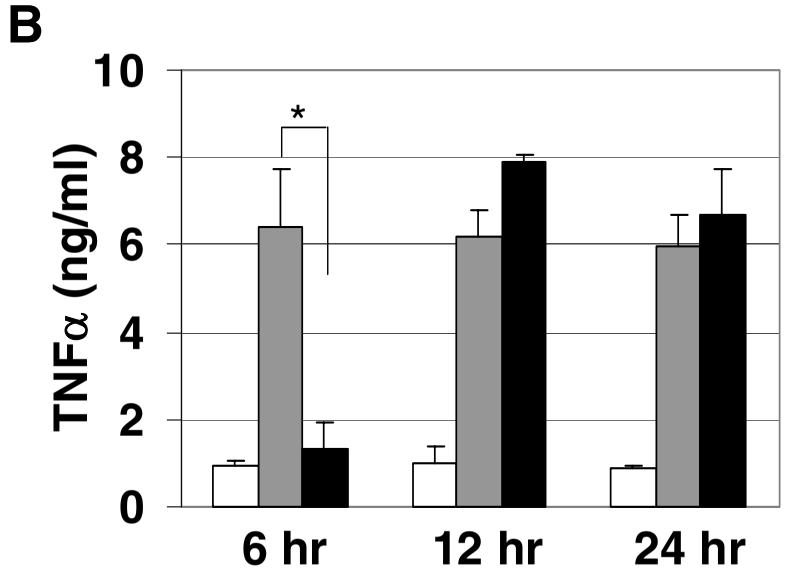

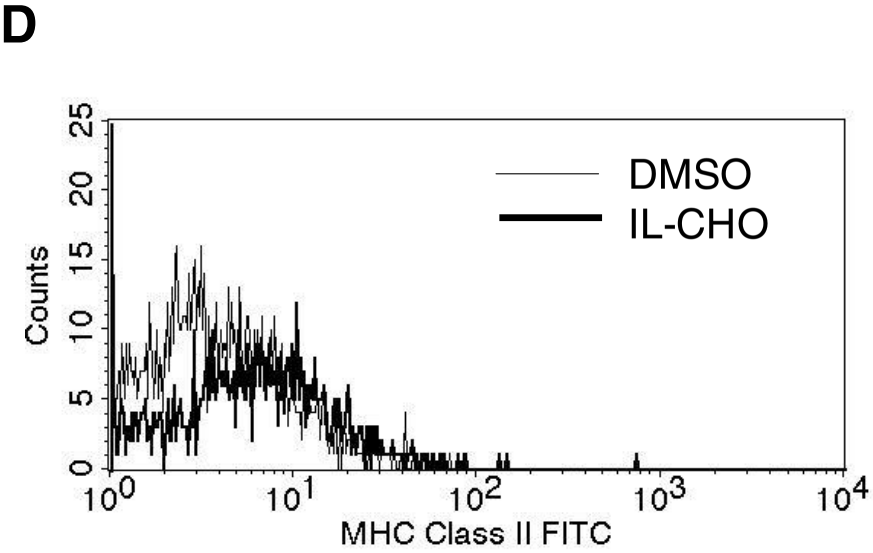

Figure 6
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