Notch1 Signaling Contributed To TLR4-Triggered NF-kB Activation In Macrophages

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Abstract

Background: Macrophages substantially shape the development, progression, and complications of inflammation-driven diseases. Although numerous researches support a critical role for Notch signaling in most inflammatory diseases, there is limited data on the role of Notch signaling in TLR4-induced macrophage activation and the interaction of Notch signaling with other signaling pathways(e.g., the NF-kB pathway during inflammation) in macrophages.

Methods and Results: In this study, we confirmed that stimulation with TLR4 ligand LPS up-regulates Notch1 expression in RAW264.7 monocyte/macrophage-like cell line. LPS also induced the expression of Notch target genes Notch1 and Hes1 mRNA in macrophages, suggesting that TLR4 signaling enhances Notch pathway activation. The upregulation of Notch1, NICD, and Hes1 protein by LPS treatment was inhibited by the Notch1 inhibitor of DAPT. The increase of TNF-a, IL-6, and IL-1b induced by LPS was inhibited by DAPT while jagged1, the Notch1 ligand, rescued them. Furthermore, the suppression of Notch signaling by DAPT up-regulated CYLD expression but down-regulated TRAF6, IKKa/bphosphorylation, and subsequently phosphorylation and degradation of IκB-α, indicating the inhibition of TLR4-triggered NF-kB activation by DAPT. Interestingly, DAPT showed no inhibitory effect on the increase of MyD88 expression induced by LPS in our study.

Conclusions: Our study shows that the stimulation of macrophages via the TLR4 signaling cascade triggers the activation of Notch1 signaling, which regulates the expression patterns of genes involved in pro-inflammatory responses through by activating NF-kB. It may be dependent on the CYLD-TRAF6-IKK pathway. The Notch1 signaling may be considered as a potential therapeutic target against infectious and inflammatory driven diseases.

Background

Macrophages activation also mediates inflammation through the secretion of a variety of pro-inflammatory mediators, including tumor necrosis factor-a(TNF-α), interleukin-1β (IL-1β), IL-6, nitric oxide (NO), and prostaglandin E2 (PGE2) [1]. When these mediators are produced in excess, they cause excessive inflammatory responses [2]. Toll-like receptors (TLRs) [3] are essential pattern recognition receptors expressed on antigen-presenting cells, which can recognize conserved microbial structures and induce macrophage activation, resulting in the production of inflammatory cytokines[4,5]. Free activation of the TLR response can lead to excessive inflammation and tissue damage, contributing to the pathogenesis of inflammatory diseases. Therefore, the tight regulation of TLR signaling pathways is significant for controlling inflammatory response[6,7]. However, it is not clear whether other signaling pathways primarily signals through cell membrane receptors play a vital role in TLR signaling activation and regulation of inflammatory responses.

Notch signaling is a highly conserved pathway that determines cell fate, including cell growth, differentiation, and survival [8, 9]. There is Increasing evidence that Notch receptor signaling regulates

innate immune responses, especially the activation and polarization of macrophages [10, 11, 12]. Ligand binding to the Notch receptor triggers sequentially cleaved receptors via disintegrin and metalloproteinase domain (ADAM)-type metalloproteinases and g-secretase, leading to the release of Notch intracellular domain (NICD) nuclear translocation. The NICD translocates to the nucleus and binds to the DNA-binding protein RBP-J/CSL/CBF1. This transcription-activation complicated drives the expression of Notch target genes, partiuclarly the hairy and split enhancer (Hes) and hairy and split enhancer with the transcription factors YRPW motif (Hey) family [13]. The Notch-RBP-J pathway contributes to the expression of canonical pro-inflammatory genes, resulting in a more robust immune response in these cells.

Recent evidence suggests a cross-talk between Notch and TLR signaling pathways [14, 15, 16]. Different studies have showed that the expression of several cytokines in response to TLR activation of macrophage depends on the activition of Notch receptors. In addition, increased expression of Notch receptors and Notch signaling has been reported to cause pathological inflammation in many inflammatory diseases [17, 18]. Activation of TLR upregulates the expression of Notch ligands, receptors, and RBP-J, which is beneficial to the Notch signaling pathway. Notch and TLR signaling pathways together activate Notch target gene Hes1 and Hey, increasing TLR-triggered cytokine production [19].. So dysregulation of the interaction between Notch and TLR pathways may have critical involvement of many inflammatory disorders.

This study will describe the Notch1 signaling in inflammatory macrophages in response to LPS (TLR4 agonist) stimulation using RAW 264.7, a murine macrophage cell line. In addition, we will investigate whether Notch1 plays a role in enhancing inflammation in response to TLR4 stimulation on macrophages and identify the role of Notch1 TLR4-mediated activaty of nuclear factor kappa-B (NF-κB).

Methods

Reagents and Cell Culture

RAW 264.7 cells (ATCC No. TIB-71) DMEM (Gibco BRL, Grand Island, NY, USA) was cultured in plates. Cells were activated with 100 ng/ml LPS (Sigma-Aldrich, SL, USA). Notch signaling was inhibited in vitro with a g-secretase inhibitor DAPT (Sigma-Aldrich, SL, USA) at a final concentration of 10 μM., Notch signal was activated by adding recombinant soluble Jagged1 (Abcam, Cambridge, UK) into the medium, and the final concentration was 1 μg/ml.

RNA isolation and RT-PCR

RNA of RAW264.7 cells was homogenized in Trizol reagent following the manufactuer's instructions. Quantitative RNA was measured. With the Total RNA extraction, cDNA was synthesized in the buffer following the manufacture's introductions; 0.5 mg random primer of hexanucleotide, 2.5 mM nucleotide, 40 units RNase inhibitor, and 50 units/ml SuperScript II RT (expression vector, Invitrogen, Carlsbad, CA), in the last roll of 20 ml. This mixture was hatched for 40 minutes at 42°C and finally digested with 2 units/ml E. coli RNase H for 30 min at 37°C. PCR was performed with 3 ml of the RT reaction mixture and

47 ml buffer containing 2.5 U of Taq DNA polymerase (Promega, Madison, WI) and 30 pM mouse Notch1 and Hes1 cDNA specific primers. The primers used are as follows: Notch1 (predicted length 452 bp) forward: 5’- CTTGCCAGGTTTTGCTGGAC -3’, reverse: 5’- CTTTGCCGTTGACAGGGTTG -3’, Hes1 (predicted length 325 bp) forward: 5’- GTCTACCTCTCTCCTTGGTCCT -3’, reverse: 5’- AGGCTTTGATGACTTTCTGTGCT -3’ and GAPDH (predicted length 635 bp) forward: 5’- CATCTTCCAGGAGCGAGACC -3’, reverse : 5’- TGAAGTCGCAGGAGACAACC -3’. PCR was performed in a thermocycler (GeneAmp® PCR System 2400, Foster City, CA). RT-PCR products was loaded onto a 2% agarose gel with ethidium bromide. Bands of DNA are visible under UV light.

Measurement of TNF-α , IL-1β ,IL-6

Following the manufacturer’s intruction, TNF-a, IL-6, and IL-1b in conditioned medium induced by LPS was measured using an ELISA kit (Santa Cruz Biotechnology, Dallas, TX, USA) and read at 450 nm.

Confocal microscopy

RAW 264.7 cells were inoculated on the film and activated with 100ng/ml LPS for 8 h. After rinsing twice with cold PBS, fixation with 4% PAF for 15 min. Wash with PBS twice, infiltrate with 0.2% Triton X-100, seal with 5% BSA for 1 h, then incubate with anti-Notch1 Ab (Cell Signaling Technology, Beverly, MA) at 37℃ for 4 hours. Cells were washed and incubated with Alexa 488 combined with secondary Abs (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour. Images were observed under an SP5 II confocal microscope (Leica).

Nuclear protein extractions

Nuclear protein was extracted using the method described previously. Cells were harvested, lysed in buffer and centrifuged for 15 min. The precipitate was harvested and then suspended in potassium phosphate buffer (pH 7.2). It was centrifuged for 1 h with 2.2 M sucrose and 1.0 mM MgCl2. The nuclear pellets was washed with buffer and centrifuged for 10 min. The pellets were reconstituted with the buffer. The mixture was kept on ice for 1 h with agitation and centrifuged for 30 min. The supernatant was used for western blot.

Western blot analysis

RAW264.7 cell lysates were prepared, and the protein concentrations was quantitatively determined with Bradford reagent (Bio-Rad, Hercules, CA). The exact amount (30 mg) of protein was resolved in 10% SDS-PAGE gels and after electrophoresis, was transferred to the PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ), then incubate anti-Hes1, MyD88, TRAF6, IKKa/b , IkBa Abs (Santa Cruz Biotechnology, Dallas, TX, USA), anti-Notch1, NF-κB, CYLD Abs (Cell Signalling Technology, Beverly, MA) overnight at 4℃, followed by horseradish peroxidase-couple goat anti-rabbit IgG. The membranes were stripped and reimprinted with anti-actin Ab (Santa Cruz Biotechnology, Dallas, TX, USA) to verify that each channel was equally loaded with proteins. The bands were displayed by enhanced

chemiluminescence system substrate (Amersham Pharmacia Biotech) and density scanning (Gel Doc XR; Bio-Rad).

Statistics

All data are presented as mean ± SEM. One-way ANOVA and Fisher's test performed a Statistical comparison among multiple groups (≥3). Data were analyzed by unpaired samples T-test . The difference was statistically significant ($P < 0.05$).

Results

Notch1 signaling was activated by TLR4 ligation in RAW264.7 cells

The activation and differentiation of macrophages, an essential component of chronic inflammation, are involved in diverse inflammation-driven human diseases. M1 macrophages are typically activated by lipopolysaccharide (LPS), which is an established TLR4 ligant, and produce proinflammatory cytokines. To investigate if Notch1 pathway is also activated after the activation of macrophage induced by LPS, we measured the associated-gene expression of Notch1 by RT-PCR on RAW264.7 cells. RAW264.7 cells were treated with 100 ng/ml LPS for 0, 2, 4, 8 and 24 h. As shown in Figure 1, the mRNA level of Notch1 was significantly increased from 4 h to 24 h after stimulation with LPS and peaking at 4 h. In addition, as the Notch target genes, Hes1 mRNA was concurrently increased from 4 h and there is more than 8.32 folds increase when it reached peak at 8 h compared with the medium control in RAW264.7 cells.

DAPT, as Notch1 inhibitor, suppressed the activation of Notch1 pathway and mitigated the inflammation response on LPS induced RAW264.7 cells

To confirm the deactivation of Notch1/Hes1 signaling pathway by DAPT, we tested the key proteins on RAW264.7 cells. 100 ng/ml LPS stimulated the macrophages for 8 hours and DAPT was added to the culture medium prior to LPS. No cytotoxic effect of DAPT (10 μM) was observed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (data not shown). Consistent with above mRNA results, the Notch1/Hes1 pathways were activated by LPS stimulation. In contrast, DAPT significantly inhibited the Notch1 upregulation on RAW264.7 cells compared with LPS shown by immunoflurescence in Figure 2A. Furthermore, DAPT attenuated the upregulation of NICD and Hes1 after incubation with LPS shown by Western Blot analysis in Figure 2B. There is no change of translocation of intracellular Notch receptor domain (NICD) and Hes1 in RAW267.7 without LPS stimulation (Fig. 2B).

Although the role of Notch signaling in maturation, differentiation, and activation of lymphocytes has been comprehensively reported, limited data are available about the role of Notch signaling in macrophages during inflammation. To investigate if Notch1 signaling contributed to production of proinflammatory cytokines from activated macrophage induced by LPS, the established TLR4 agonist. The data shown in Figure 1 indicated us LPS can activate the macrophages through TLR4 and Notch1 pathways on macrophages is also upregulated. Activated macrophages will release various inflammatory

cytokines. In our study, the expression and release of TNF-α, IL-6 and IL-1βin macrophages were increased after 100 ng/ml LPS 8 hour-incubation by Western Blot (Fig. 3A) or ElASA analysis (Fig. 3B). However, these cytokines were significantly enhanced by Jagged 1, the Notch1 ligand, but partially inhibited by DAPT, the Notch1 inhibitor.

The inflammatory cytokines production may depend on NF-κB pathway and Notch1 pathways regulated the nuclear translocation of NF-κB

NF-κB has emerged as a key regulator of inflammatory cytokines genes. In order to investigate if Notch1 pathways regulated the activation of NF-κB, we first confirmed the activation of NF-κB induced by LPS and further tested if Notch1 was involved in the activation of NF-κB. RAW264.7 cells were incubated by DAPT, prior to LPS, for 8 hour, the cytosolic and nuclear protein extracts were prepared. DAPT inhibited the NF-κB p65 nuclear translocation enhanced by LPS shown by Western Bloting analysis (Fig. 4) and DAPT without LPS had no effect on NF-κB activation.

To address the potential crosstalk of Notch1 in NF-κB pathways, we investigated if DAPT would have regulate the key signaling molecules in activation of NF-κB. LPS acts as the classical agonist of TLR4 and may depend on MyD88-TRAF6-IKKα/βapathway to activate NF-κB. DAPT could inhibit the upregulation of TRAF6, but not MyD88 by Western Blot analysis in Figure 5A. Furthermore, DAPT could stabilize the IKKα/βby inhibiting IKKα/βaphosphorylation and reduce the phosphorylation and degradation of IκB-α (Fig. 5B and C).

The blockade of Notch1 signaling inhibited CYLD expression

CYLD physically interacts with and induces the deubiquitination of many different proteins, including IKK, to inhibit the NF-kB signaling. So we next investigated the CYLD expression after Notch1 blockade. Our data showed LPS lead to CYLD decrease and Jagged1 could deepen this reduction but DAPT significantly enhanced the CYLD expression (Fig. 6).

Discussion

Activating macrophages by Toll receptor is vital in developing resistance to pathogens and inflammatory reponses [20]. Notch signaling plays a role in the controlling macrophage activation and inflammation [21], To verify the role of Notch1 signaling in regulating macrophage-triggered inflammatory responses, we perform the study to characterize Notch1 signaling in macrophages activated by TLR4 triggering and determined that the role of Notch1 in regulation of NF-kB and sequencing inflammation cytokines production. We found DAPT, the inhibitor of Notch1, not only suppressed the Notch1 and associated protein expression but deactivated the TRAF6 and reduced the IKKa/bphosphorylation, lead to the attenuation of nuclear translocation of NF-kB. In addition, DAPT could regulate NF-kB activation by stablizing CYLD, which has recently been identified as a negative regulator of NF-kB [22]. We confirmed that Notch1 signaling contributed to TLR4-triggered NF-kB activation in macrophages and it may dependent on the CYLD-TRAF6-IKK pathway.

The Notch signaling pathway is a conserved signaling pathway that is involved in determining cell fate during development. Dysregulation of the Notch signaling pathway is associated with various pathological processes, including inflammatory diseases [23]. The Notch pathway is derived from the interaction of Notch1–4 with different types of ligands. Notch receptors and ligands are continuously expressed after monocytes or tissue macrophages [24] . Studies have shown that different TLR agonists can regulate the expression of Notch receptors and Notch ligands on antigen-presenting cells, thus influence the outcome of a given immune response [25]. We investigated the role of LPS as a TLR4 agonist in the regulation of Notch1 signaling in the activation of macrophages. LPS also induced the expression of Notch1 target genes including Hes1 and NICD induced in macrophages [26], suggesting that TLR4 signaling enhances the activation of the Notch1 pathway in our study.

In our study, we have confirmed that TLR signaling could activate the Notch pathway; however, it is not clear whether full activation of Notch signaling can feedback regulation of TLR signaling pathway to control the inflammatory response. It is well known that the Notch pathway can be formally activated by ligands Jagged1, Jagged2, DLL1, DLL3 or DLL4, as well as informally activated by various molecules common to other pathways [27, 28, 29] . We use Jagged1 as a ligant of Notch1, DAPT, as an inhibitor, to further investigated the role of Notch1 to regulate TLR4 signaling and sequencing inflammatory response. We found Jagged1 could enhance the production of LPS-induced inflammatory cytokines, including TNF-α, IL-6 and IL-1β. However, DAPT negatively regulated Notch1 signaling and reduced the production of TNF-α, IL-6 and IL-1β.It is well known that depending on environmental signals, macrophages can be differentially activated to classically activated (M1 phenotype) or alternatively activated (M2 phenotype) according to environmental signals [30, 31]. In comparison, M1 macrophages are characterized by the production of inflammatory mediators such as TNF-α, IL-6 and IL-1β in response to microbial product-mediated Toll-like receptors activation [32, 33, 34]. Our study shows that the activation of TLR4 by LPS could prompt macrophage to M1 switch and produce M1 cytokines, which could be mediated by Notch1 pathway. In addition, There are reports that TLR2-mediated Notch1 upregulation is ultimately canceled in MyD88-/-macrophages, while the TLR4 signaling pathway dose not depend on MyD88 for early Notch1 up-regulation. Interestingly, we found DAPT could inhibit the upregulation of TRAF6, but not MyD88. It also implied that Notch1 signaling can feedback regulate TLR4 signaling, but not dependent on MyD88.

NF- kB has emerged become a key regulator of immune responses and inflammation. Members of the NF- kB family form homo- and heterodimers that are associated with IkB proteins and held in the cytoplasm of resting cells [35] . Upon activation, the IkB kinase (IKK) complex phosphorylates IkB proteins and targets them for degradation, releasing the NF- kB subunits for nuclear translocation [36] . In our study, DAPT could inhibit the nuclear translocation of NF- kB by hindering IKK phosphorylation. CYLD has recently been identified as a negative regulator of NF- kB, characterized by the C terminal catalytic domain of deubiquitinating enzyme, which is required for CYLD to remove ubiquitin from specific proteins that positively mediate signaling through the NF-kB [37] . In addition, upon NF-kB pathway stimulation the inhibitory function of CYLD is inactivated by IKKb mediated phosphorylation [38] . We found that

DAPT could enhance the expression of CYLD but Jagged 1 is opposite. This finding provide a potential mechanism that Notch1 could inference with the negative regulation of NF- kB activation.

Conclusions

In summary, our findings suggest that Notch1 enhances macrophage inflammation in response to TLR4 stimulation by modulating NF-κB activation. Excessive of Notch1 and TLR4 crosstalk may aggravate inflammatory responses. Notch signaling could be as a potential therapeutic target against infection- and inflammation-driven diseases.

Declarations

Declaration of competing interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Credit authorship contribution

Li Li and Jin-hua Jin: performed the experiments. **Yilan Song , Chongyang Wang, Jingzhi Jiang and Guanghai Yan:** collected the material in compliance with ethical guidelines and performed part of the experiments. **Hanye Liu, Xiaofei Ma and Dandan Wang**: quantified the data. **Xiangzheng Qin and Liangchang Li**: designed the study, analysed the data and wrote the manuscript.

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DATA AVAIABILITY STATEMENT

Data supporting the findings of this study will be available from the corresponding author upon reasonable request.

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Figures

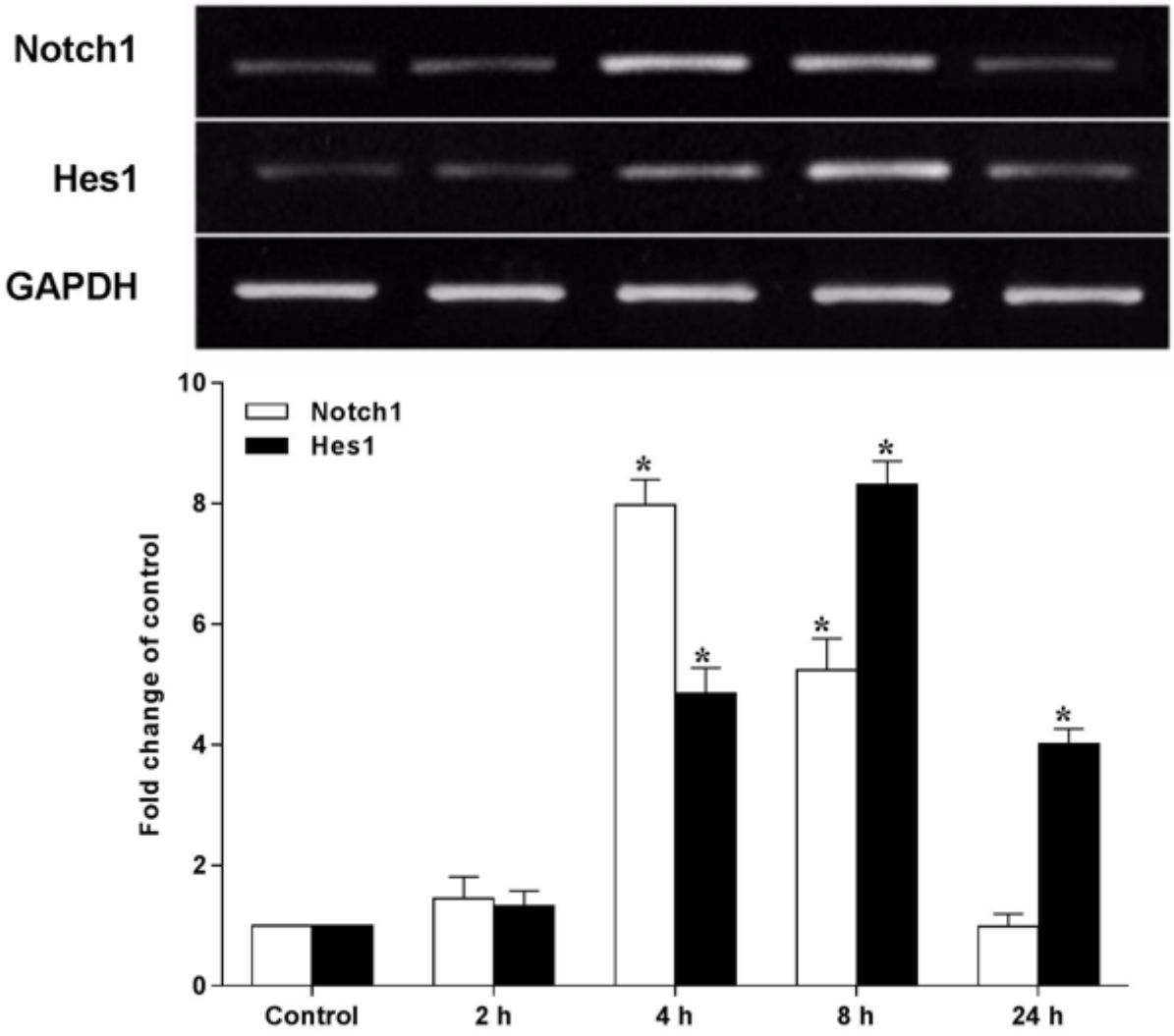

Figure 1

Notch1 and Hes1 mRNA expression were up-regulated after stimulating with LPS in RAW264.7 cells. RAW264.7 cells were seeded on 6 well plate and incubated with 100 ng/ml LPS for 2, 4, 8 and 24 h and medium control without LPS. Notch-1 and Hes1 mRNA level were quantified by reverse transcription (RT)-PCR analysis. Value represents mean ± S.E.M. of five independent experiments. * $p < 0.05$ significant compared with control group.

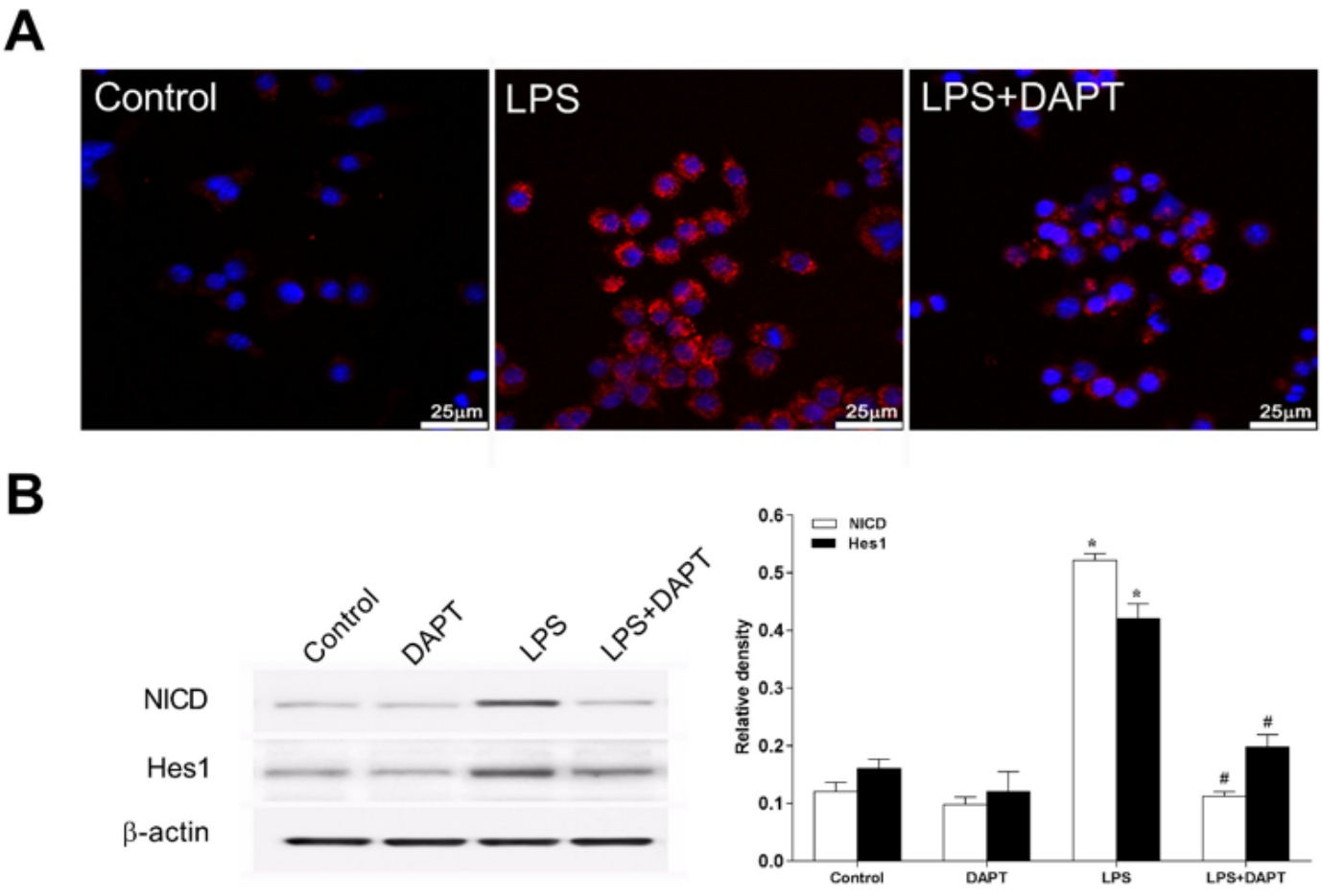

Figure 2

Notch1 inhibitor of DAPT suppressed the upregulation of Notch1, NICD and Hes1 by LPS on RAW264.7 cells. (A) Notch1 expression was enhanced after 100 ng/ml LPS stimulation at 8 hours on RAW264.7 cells. Notch1 is red on the immunofluorescence image and nuclei are stained with DAPI (blue). In contrast, the increased Notch-1 was significant downregulated after treatment with DAPT (10 μM) prior to stimulation with LPS. (B) NICD and Hes-1 were enhanced by stimulation with LPS on RAW264.7 cells and DAPT, added prior to LPS, reversed this increase quantified by Western blotting. Value represents mean ± S.E.M. of five independent experiments. * $p < 0.05$ significant compared with control group. # $p < 0.05$ significant compared with LPS group. Scale bar=200um

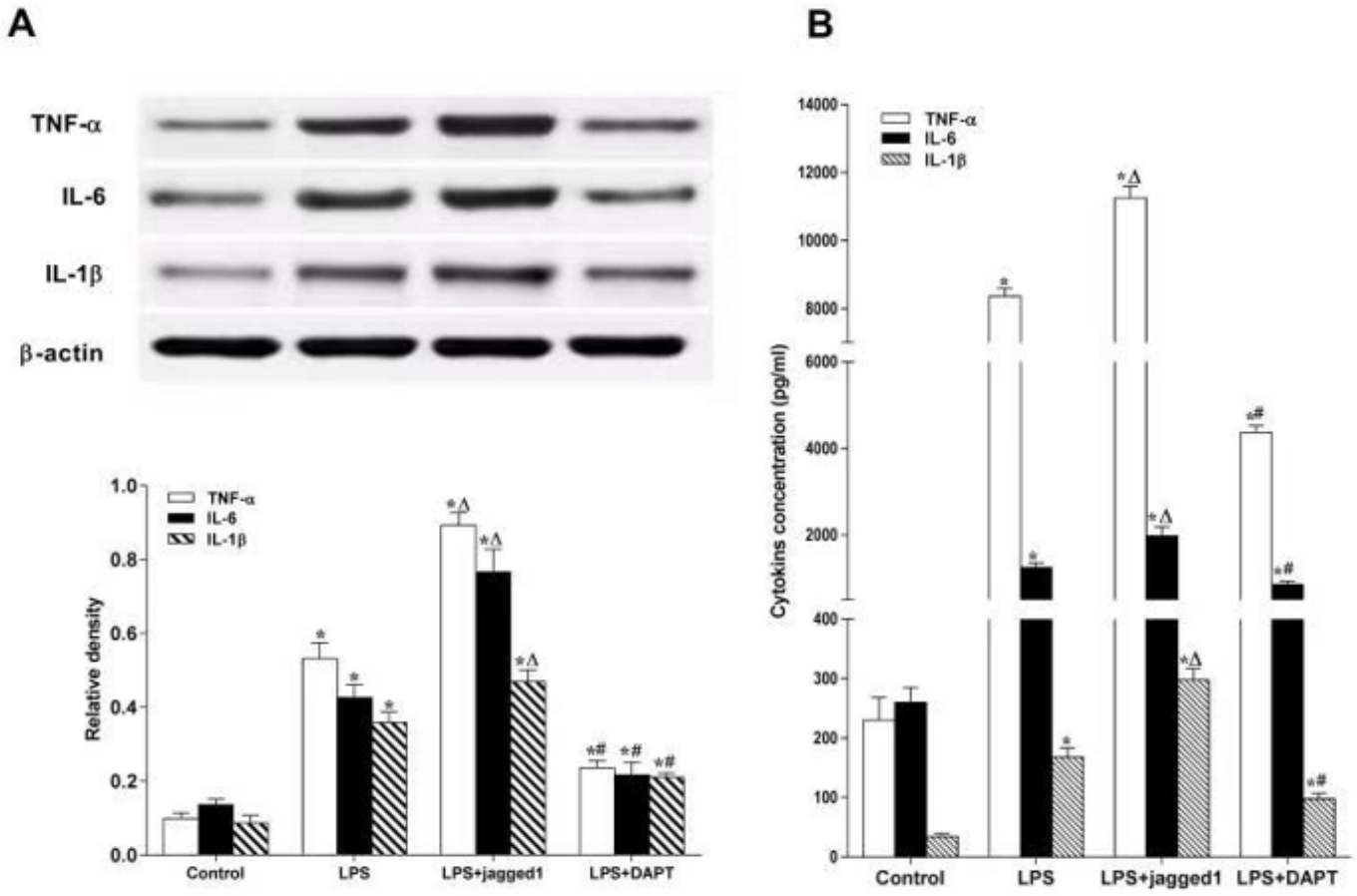

Figure 3

Inhibition of Notch1 suppresses but activation of Notch1 enhances LPS-induced inflammatory response on RAW264.7 cells. RAW264.7 cells were stimulated with LPS (100 ng/ml) for 8 h. Either DAPT (10 μM), as the inhibitor of Notch1, or Jagged1 (1 μg/ml), as the ligand of Notch1, was added to cells prior to LPS. (A) The cell lysate were prepared and TNF-α, IL-6 and IL-1β were detected by Western blotting. (B) The culture medium were collected and TNF-α, IL-6 and IL-1β were analyzed by ELISA. Value represents mean ± S.E.M. of five independent experiments. * $p < 0.05$ significant compared with control group. # $p < 0.05$ significant compared with LPS group.

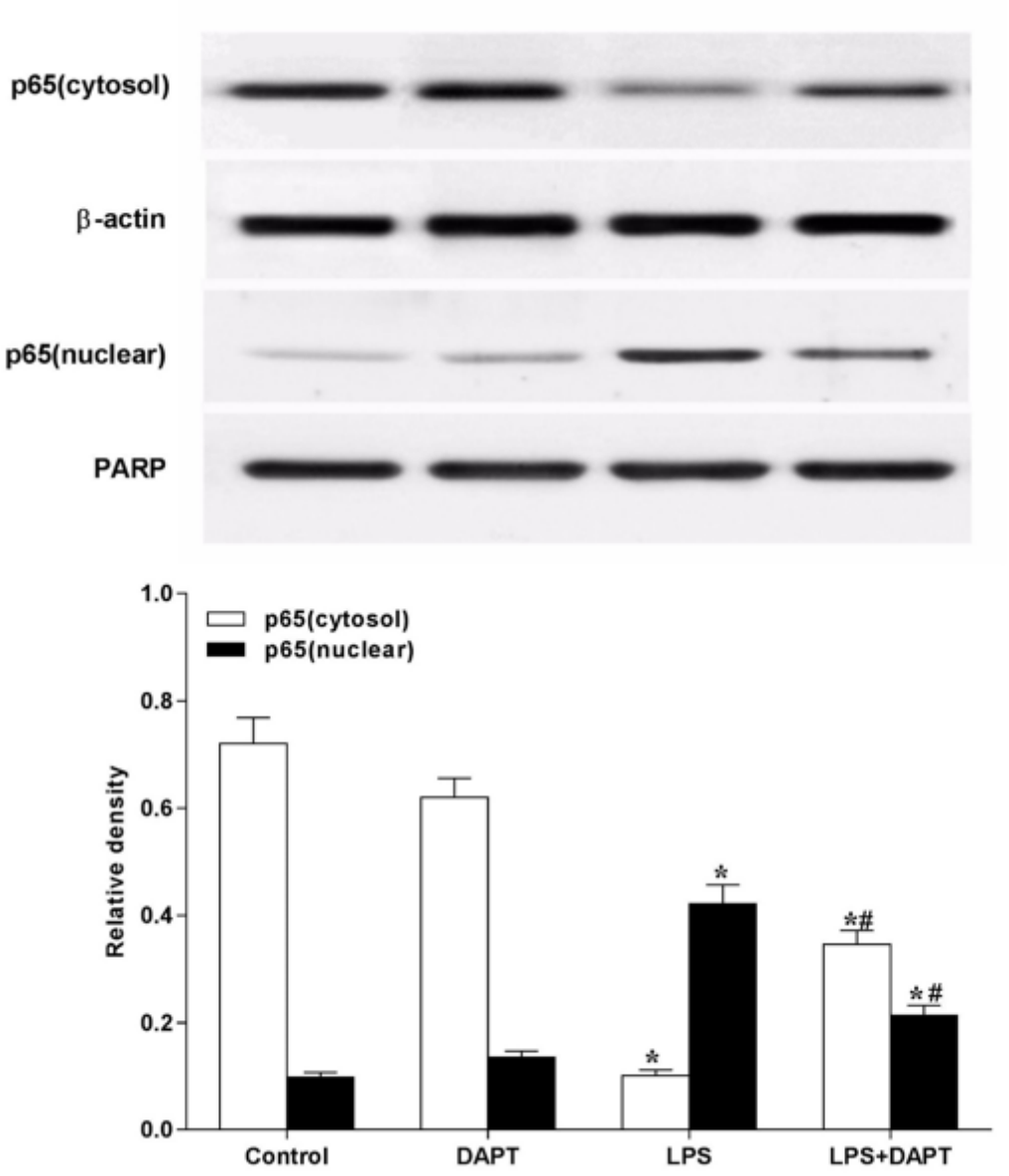

Figure 4

Inhibition of Notch1 suppressed the nuclear translocation of NF-κB stimulated by LPS on RAW264.7 cells. RAW264.7 cells were stimulated with LPS (100 ng/ml) for 8 h and prior to LPS, DAPT (10 μM) was added to the culture medium. Cytosol and nuclear protein of were isolated and Western blotting anyalysis showed reduced NF-κB p65 accumulates in the nucleus after inhibition of Notch1 with DAPT. Value represents the mean ± S.E.M. of five independent experiments. * $p < 0.05$ significant compared with control group. # $p < 0.05$ significant compared with LPS group.

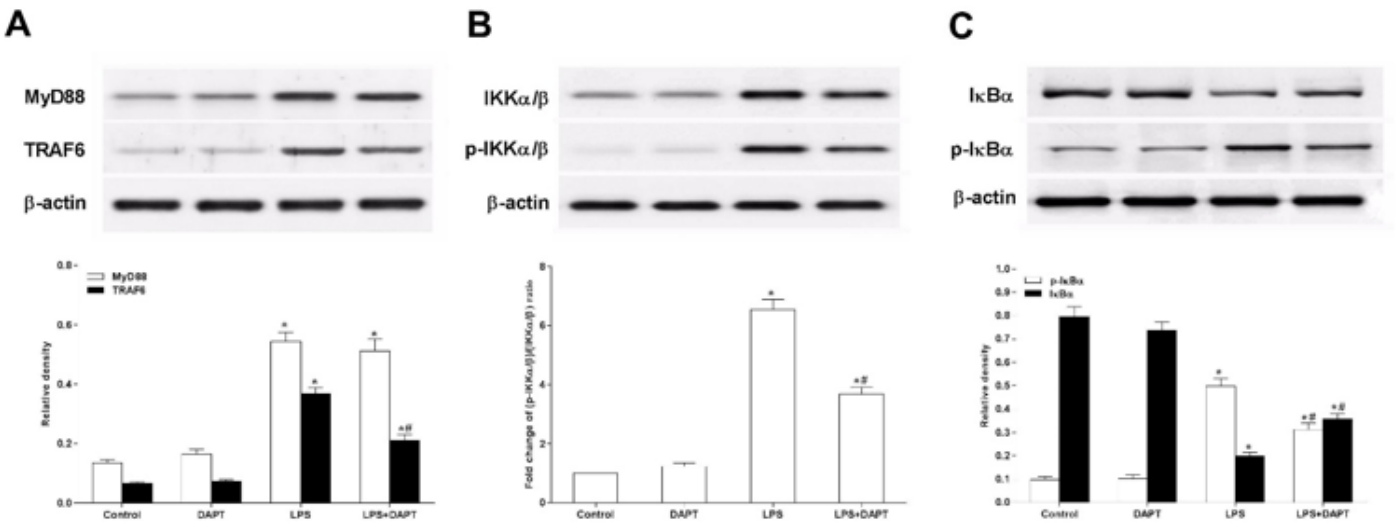

Figure 5

The suppression of nuclear translocation of NF-κB by DAPT depends on the inhibition of TRAF6 and IKKα/β phosphorylation. RAW264.7 cells were stimulated with LPS (100 ng/ml) for 8 h and were treated with DAPT (10 μM) prior to LPS. Western blotting of MyD88 and TRAF6(A), IKK α/β phosphorylation (B) and IκBα (C) in RAW264.7 cells. Value represents mean ± S.E.M. of five independent experiments. * $p < 0.05$ significant compared with control group. # $p < 0.05$ significant compared with LPS group.

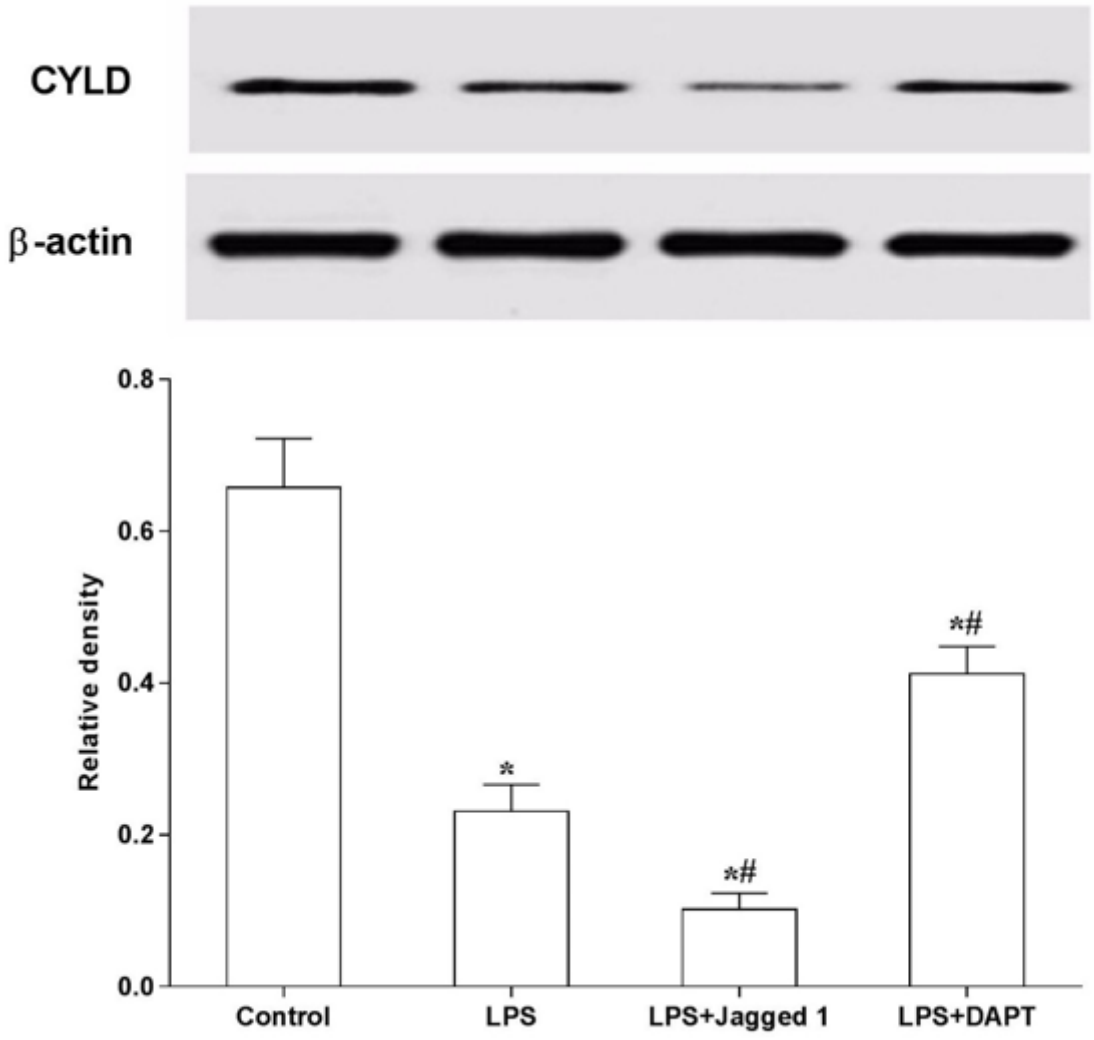

Figure 6

Blockage of Notch1 enhaced the CYLD expression in LPS-induced RAW264.7 cells. RAW264.7 cells were stimulated with LPS (100 ng/ml) for 8 h and were treated with either DAPT (10 μM) prior to LPS or with Jagged1 (1 μg/ml) prior to LPS. Western blotting of CYLD protein expression in RAW264.7 cells. Value represents the mean ± S.E.M. of five independent experiments. * $p < 0.05$ significant compared with control group. # $p < 0.05$ significant compared with LPS group.