Notch Signal Suppresses Toll-like Receptor-triggered Inflammatory Responses in Macrophages by Inhibiting Extracellular Signal-regulated Kinase 1/2-mediated Nuclear Factor κB Activation

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Multiple signaling pathways are involved in the tight regulation of Toll-like receptor (TLR) signaling, which is important for the tailoring of inflammatory response to pathogens in macrophages. It is widely accepted that TLR signaling can activate Notch pathway; however, whether full activation of Notch signaling can feedback modulate TLR signaling pathway so as to control inflammation response remains unclear. Here, we demonstrated that stimulation with TLR ligands up-regulated Notch1 and Notch2 expression in macrophages. The expression of Notch target genes including Hes1 and Hes5 was also induced in macrophages by LPS, suggesting that TLR signaling enhances the activation of Notch pathway. Importantly, overexpression of constitutive active form of Notch1 (NICD1) and Notch2 (NICD2) suppressed production of TLR4-triggered proinflammatory cytokines such as TNF-α and IL-6 but promoted production of anti-inflammatory cytokine IL-10, which is dependent on the PEST domain of NICD. In addition, NICD1 and NICD2 suppressed TLR-triggered ERK phosphorylation, which is indispensable for Notch-mediated inhibition of TLR4-triggered proinflammatory cytokine production. Furthermore, activation of Notch signaling inhibited NF-κB transcription activity by MyD88/TRAF6 and TRIF pathways, which was dependent on ERK activity. Therefore, our results showed that Notch signaling negatively regulates TLR-triggered inflammatory responses, revealing a new mechanism for negative regulation of TLR signaling via Notch pathway.

Toll-like receptors (TLRs), the key pattern-recognition receptors expressed on antigen-presenting cells, play critical roles in the host defense against invading microbial pathogens. After the recognition of pathogen-associated molecule patterns, Toll-like receptors initiate shared and distinct signaling pathways depending on adapter MyD88 and/or TRIF, leading to the activation of transcription factors NF-κB, AP-1, IRF3, and/or IRF7, which consequentially induce the production of proinflammatory cytokines and type I interferon. Less efficient activation of Toll-like receptors may not evoke potent antinfection immunity; however, excessive activation of the TLR-triggered response may also induce inflammatory disorders such as endotoxin shock or inflammatory autoimmune diseases. Therefore, the tight regulation of TLR signaling pathways is important for the control of the inflammatory response. Up until now, various intracellular signaling molecules have been shown to be involved in the regulation of the TLR pathway to maintain the immunological balance. In addition, some other signaling pathways, such as integrin CD11b (5) and immunoreceptor tyrosine-based activation-associated receptors (6), have cross-talk with TLR signaling pathways, resulting in fine tuning of TLR-triggered innate inflammatory responses. This raises one important question, that other new signaling pathways, especially signals via cell membrane receptors, should be further clarified for their involve-

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4 The abbreviations used are: TLR, Toll-like receptor; DLL, Delta-like; GSI, γ-secretase inhibitor; NICD, Notch intracellular domain; ODN, oligodeoxy-nucleotide; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adapter-inducing interferon-β.
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Notch signaling is a highly conserved pathway determining cell fate including cell proliferation, differentiation, and survival (7–9). Up to date, four Notch receptor family members (Notch1–4) and five Notch ligands Delta-like-1 (DLL-1), DLL-3, DLL-4, Jagged-1 and Jagged-2 have been identified in mammals cells (10, 11). The Notch receptors are large type I transmembrane receptors that undergo proteolytic processing by a furin-like convertase during transit to the cell surface. Binding of a ligand triggers sequential receptor cleavage by a disintegrin and metalloproteinase domain (ADAM)-type metalloproteases and γ-secretase, resulting in the release of a nuclear translocation of Notch intracellular domain (NICD). The NICD translocates to the nucleus, where it interacts with the transcription factor RBP-J at the jk site (also named CSL), leading to the formation of a transcriptional activator complex and induction of the transcription of Notch downstream target genes such as basic helix-loop-helix family (Hes1 and Hes5) and hairy and enhancer of split-related (HESR) family (Hey1 and Hey2). However, in the absence of NICD, CSL protein complexes with co-repressor proteins to repress gene transcription (12).

Recent evidence suggests that there is cross-talk between Notch and TLR signaling pathways (13, 14). TLR activation up-regulates the expression of Notch ligands, receptors, and RBP-J, favoring Notch signaling pathway. For example, LPS activates Notch signaling through a JNK-dependent pathway that subsequently regulates the inflammatory response (15). However, LPS was also reported to suppresses Notch signaling via nitric oxide in macrophages (16). A recent study demonstrated that Notch and TLR signaling pathways cooperate to activate the transcription of Notch target gene Hes1 and Hey and to increase the production of TLR-triggered cytokines such as TNF-α, IL-6, and IL-12 (17). Several studies also indicated that Notch signaling plays an important role in inflammatory disorders (18, 19). So it seems that there is reciprocal regulation between Notch and TLR pathway. However, the detailed relationship and underlying mechanisms between Notch and TLR signaling remain far from being understood.

In this study, we showed that the TLR4 signal up-regulated the expression of Notch and its target gene in macrophages. Overexpression of NICD1 and NICD2 decreased TLR4-triggered proinflammatory cytokines but increased antiinflammatory cytokine production. Furthermore, we found that NICD1 and NICD2 suppressed TLR-triggered ERK phosphorylation, which is indispensable for Notch-mediated inhibition of TLR4-triggered inflammatory cytokine production. Besides, we found that Notch inhibited NF-κB transcription activity by MyD88/TRAF6 and TRIF pathways, which was dependent on ERK inactivation by Notch signaling. Therefore, we show for the first time that Notch signaling negatively regulates the TLR-triggered inflammatory response.

MATERIALS AND METHODS

Reagents and Antibodies—RAW264.7 and HEK293T cells were obtained from ATCC (Manassas, VA). LPS (0111:B4) was from Sigma-Aldrich. Phosphorothioate-modified CpG oligodeoxynucleotide (ODN) was synthesized by Shenggong, and its sequence is 5’-TCC ATG AC TG TTC CTG ATG CT-3’. PD98059 and γ-secretase inhibitor X (GSI) were purchased from Calbiochem. Primary antibodies against Hes1 and Hes5 were from Santa Cruz Biotechnology, anticleaved Notch1, cleaved Notch2, phosphor-ERK1/2, JNK1/2, and p38 antibodies were from Cell Signaling Technology, and anti-β-actin antibody was from Sigma.

Plasmids and Vectors—NICD1, NICD2, and pCMV plasmids were a kind gift from Dr. Xiaolin Tu (Indiana University School of Medicine, Indianapolis, IN). N1-6MT plasmid was a kind gift from Prof. Raphael Kopan (Washington University School of Medicine, St. Louis, MO). pCS2 plasmid was a kind gift from Dr. Junyu Zhang (Fudan University, Shanghai, China). MyD88, TRAF6, and TRIF constructs and NF-κB luciferase reporter plasmids were described previously by us (5, 20, 21). All plasmids were confirmed by DNA sequencing.

Cell Culture and Transfection—Mouse macrophage cell line RAW264.7 and human HEK293T cell line were cultured and transfected with JetPEI (Polyplus Transfection, Illkirch, France) as described (20). Thioglycolate-elicited mouse peritoneal macrophages were isolated from C57BL/6 mice (6–8 weeks) obtained from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China) and cultured as described previously (5, 20, 21) and nucleoinfected with the Mouse Macrophage Nucleofekt kit using Nucleofector II Biosystems (Amaxa, Gaithersburg, MD) (20, 22).

RNA Interference—The Notch1-specific siRNA (siNotch1-#1084, 5’-CGCGUGAAACCUACAATT-3'; siNotch1-#3635, 5’-GAGGGAGAUAAACAUUACTT-3'), and scrambled control siRNA (siNon, 5’-UUCUCGUGU-ACGU TT-3') were synthesized by GenePharma (Shanghai, China) (23). ERK1- and ERK2-specific siRNA (siERK1, sc-29308, and siERK2, sc-35336) were purchased from Santa Cruz Biotechnology. siRNA duplexes were delivered into RAW264.7 cells or mouse peritoneal macrophages at a final concentration of 10 nM using INTERFERin (Illkirch), according to the standard protocol.

Western Blot Analysis—Extraction protein from cells was measured by the BCA protein assay reagent kit (Pierce). 50 μg of protein was resolved by 10% SDS-PAGE and immunoblotted with the indicated antibodies with appropriate HRP-conjugated antibodies as secondary antibodies (Cell Signaling Technology) (5, 23).

ELISA—IL-6, TNF-α, and IL-10 levels in the culture supernatants were quantified with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer’s instructions (5, 20, 21).

Real-time Quantitative PCR—Total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Real-time quantitative RT-PCR analysis was performed as described previously (20, 23) using Light Cycler (Roche Diagnostics) and the SYBR RT-PCR kit (Takara, Kyoto, Japan). The primers used are shown in supplemental Table 1.

Luciferase Reporter Assay—RAW264.7 cells or HEK293T cells were co-transfected with the mixture of indicated luciferase reporter plasmid and NF-κB reporter plasmid and the indicated amounts of NICD1, NICD2, N1-6MT, MyD88, TRAF6,
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or TRIF construct for 24 h, with PRL-TK-Renilla as the luciferase internal control reporter gene. Total amounts of plasmid DNA were equalized with empty control vector. The cells were left treated or untreated with LPS. Luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer’s instructions. Data are normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase as we described previously (22).

Statistical Analysis—Statistical significance was determined by Student’s t test, with a p value <0.05 considered to be statistically significant.

RESULTS

TLR Ligation Up-regulates Notch Expression and Activates Notch Signaling in Macrophages—As shown in Fig. 1A, Notch1 and Notch2 were expressed in RAW264.7 cells and peritoneal macrophages, whereas the expression of Notch3 and Notch4 was weak. Therefore we focused on the roles of Notch1 and Notch2 in the TLR response. Notch ligands including DLL-1, DLL-3, DLL-4, Jagged-1, and Jagged-2 were also expressed in RAW264.7 cells and peritoneal macrophages (Fig. 1B). In addition, we further examined the activation of Notch signaling pathway by detecting Notch target gene, Hes1 and Hes5 expression. As shown in Fig. 1C, Hes1 and Hes5 were expressed in RAW264.7 cells and peritoneal macrophages, indicating that Notch signaling was active in macrophages without any stimulation.

Previous studies demonstrated that TLR signaling could modulate Notch signaling in macrophages (13–15). So, we first detected Notch expression under stimulation with LPS (100 ng/ml) and CpG ODN (0.33 μM). As shown in Fig. 1D, stimulation of macrophages with LPS and CpG ODN up-regulated the expression of Notch1 and Notch2. By detecting the expression of Hes1 and Hes5, we found that LPS could induce Notch activation in peritoneal macrophages (Fig. 1E), which was in agreement with a previous report (13). Taken together, these data indicate that Notch activation could be further enhanced by TLR ligands in macrophages, raising a possibility that Notch signaling may be involved in the regulation of TLR-triggered inflammatory responses.

Notch Suppresses Proinflammatory Cytokine Production but Promotes Antinflammatory Cytokine Production in TLR-triggered Macrophages—TLR4 can initiate a complex signaling pathway leading to inflammatory cytokine production in macrophages, resulting in inflammation response (24). So we evaluated whether Notch signaling is involved in TLR-triggered inflammatory response in macrophages. Constitutive expression of NICD in target cells could produce an “activated” Notch phenotype. We transfected the intracellular domain of Notch1 or Notch2 (referred to as NICD1 and NICD2, respectively) into peritoneal macrophages. The activation of Notch was confirmed by detecting cleaved Notch using Western blotting in peritoneal macrophages when transfected with NICD1 and NICD2 (supplemental Fig. S1A). As shown in Fig. 2A, overexpression of NICD1 decreased LPS- and CpG ODN-induced secretion of proinflammatory cytokines such as IL-6 and TNF-α while increased the production of antiinflammatory cytokine IL-10. A similar result was also observed in NICD2-overexpressing macrophages in response to the stimuli with LPS and CpG ODN. We further found that the replacement of the 3′ PEST domain of NICD1 with a 6Myc tag (N1–6MT) reversed the NICD1-mediated down-regulation of TNF-α and IL-6 production and the up-regulation of IL-10 production (Fig. 2B). Therefore, the PEST domain, whose phosphorylation triggers Notch degradation (25, 26), plays key roles in mediating regulatory effects of Notch on TLR-mediated inflammatory response.

We next silenced Notch1 expression in mouse peritoneal macrophages with specific siRNA (si-N1–1084 and #3635). The silencing efficacy was confirmed by Western blotting (supplemental Fig. S1B). We found that silencing of Notch1 expression increased LPS-induced TNF-α and IL-6 production but decreased IL-10 production (Fig. 3A). Furthermore, blockade of Notch signaling by a GSI, γ-secretase inhibitor X, significantly increased TNF-α and IL-6 production but decreased IL-10 production (Fig. 3B). These results further confirmed the role of Notch signaling in the negative regulation of TLR-triggered inflammatory response.

Notch Suppresses TLR-triggered Inflammatory Response through ERK1/2 Inactivation—We then investigated whether Notch signaling participates in regulation of TLR-mediated signaling pathway in macrophages. Multiple signaling pathways were involved in the TLR-mediated inflammatory response, including MAPKs and NF-κB (27–29). We first detected the effect of Notch on the MAPK pathway in peritoneal macrophages. As shown in Fig. 4A, whereas LPS stimulation alone enhanced the phosphorylation of ERK1/2, JNK1/2, and p38, overexpression of NICD1 significantly inhibited LPS-induced ERK1/2 phosphorylation compared with mock. A similar result was observed in NICD2-transfected macrophages in response to LPS stimulation (Fig. 4B). On the other hand, overexpression of N1-6MT reversed Notch-mediated inhibition of ERK1/2 activation (Fig. 4C). Blockade of Notch signaling by GSI also promoted LPS-induced ERK activation (supplemental Fig. S2).

We pretreated peritoneal macrophages with a specific MEK inhibitor, PD98059, for 30 min before LPS stimulation and observed that the effect of Notch on TLR-mediated inflammatory cytokine production. Blockade of ERK activation by PD98059 completely reversed the increase in GSI or N1-6MT overexpression-mediated IL-6 and TNF-α production, and the decrease in GSI or N1–6MT overexpression-mediated IL-10 production (Fig. 4, D and E). These results suggest that ERK1/2 inactivation is required for Notch-mediated inhibition of TLR-triggered inflammatory cytokine production.

Notch Signaling Inhibits TLR-triggered NF-κB Transcription Activity by MyD88/TRAf6 and TRIF Pathways—NF-κB is one of the most important regulators of proinflammatory gene expression including TNF-α and IL-6. We found that TLR-triggered NF-κB transcription was significantly suppressed by overexpression of NICD1 and NICD2 (Fig. 5A). It is well known that TLR signaling pathway is mediated by both the MyD88-dependent pathway and the TRIF (MyD88-independent) pathway. As shown in Fig. 5A, co-transfection of NICD1 or NICD2 with MyD88 inhibited NF-κB transcription activity in a dose-dependent manner in HEK293T cells; and transfection of
FIGURE 1. TLR ligands up-regulate Notch expression and activate Notch signaling on macrophages. A–C, RAW264.7 cells and mouse peritoneal macrophages were subjected to real-time quantitative PCR analysis for mRNA expression of Notch receptors (A) and Notch ligands (B), and Notch target genes (C). Mouse β-actin was amplified as a control. D, mouse peritoneal macrophages were stimulated with LPS (100 ng/ml) or CpG-ODN (0.33 μM) for the indicated time, and Notch1 and Notch2 mRNA expression were detected by real-time quantitative PCR. Data are shown as mean ± S.D. (error bars; n = 4) of one representative experiment. Similar results were obtained in three independent experiments. E, mouse peritoneal macrophages were stimulated with LPS (100 ng/ml) for the indicated time, and then Hes1 and Hes5 protein expression was detected by immunoblotting. Data are representative of three separate experiments.
NICD1 or NICD2 together with TRAF6 has a similar effect (Fig. 5B). However, co-transfection of N1–6MT with MyD88, TRAF6, or TRIF reversed Notch-mediated inhibition of NF-\(\kappa\)B transcription activity (supplemental Fig. S3). In addition, co-transfection of NICD1 or NICD2 with TRIF also dose-dependently inhibited NF-\(\kappa\)B transcription activity (Fig. 5C). These results suggest that Notch signaling inhibits TLR-induced NF-\(\kappa\)B transcription activity, which is dependent on both MyD88/TRAF6 and TRIF pathways.

Notch-mediated ERK Inactivation Is Responsible for Inhibition of NF-\(\kappa\)B Transcription Activity—Because both the MAPK cascade and the NF-\(\kappa\)B signaling pathway participate in the TLR-triggered inflammatory response (30, 31), we investigated the relationship between ERK1/2 activation and NF-\(\kappa\)B activity in the Notch-mediated inhibitory effect on TLR inflammatory response. We used PD98059, the specific MEK inhibitor, to treat RAW264.7 cells and then observed the effect of Notch inhibition by GSI on LPS-triggered NF-\(\kappa\)B transcription activity. As shown in Fig. 6A, inhibition of Notch signaling by GSI increased LPS-triggered NF-\(\kappa\)B transcription activity, whereas PD98059 reversed the GSI-mediated effect on NF-\(\kappa\)B transcription activity. To confirm further the role of ERK1/2 in the Notch-mediated inhibitory effect of NF-\(\kappa\)B, we silenced ERK1/2 expression in RAW246.7 cells with spe-
cific siRNA for ERK1 and ERK2 (siERK1 and siERK2), and then observed the effect of the inhibition of Notch by GSI on LPS-triggered NF-κB transcription activity. As shown in Fig. 6B, GSI-mediated promotion of NF-κB activation was also reversed by down-regulation of ERK1/2 expression. These results suggest that Notch-mediated ERK inactivation is
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DISCUSSION

The present study affirms the hypothesis that the Notch pathway plays an important role in TLR response in macrophages. Evidence supporting this idea includes the expression of multiple Notch receptors and ligands in macrophages; markedly increased Notch1 and Notch2 expression in macrophages stimulated with LPS or CpG ODN, an event that likely involves TLR4/9 and NF-κB; the LPS-induced expression of Notch target genes; the suppression of TLR4-triggered secretion of proinflammatory cytokines by Notch activation; and the regulation of TLR-induced MAPK and NF-κB pathways in macrophages overexpressing NICD1/2.

In the immune system, the role of Notch signaling in the function of professional antigen-presenting cells, especially macrophages, has recently been a hot topic. Many in vitro studies have implicated Notch signaling in macrophage TLR responses. Here, we showed that Notch signaling suppresses TLR-triggered production of proinflammatory cytokines (IL-6 and TNF-α) by inhibiting ERK1/2-mediated NF-κB activity in macrophages. Recently, several studies have reported inconsistent outcomes on the cross-talk of Notch and TLR pathways to regulate TLR responses in macrophages. Tsao et al. (15) showed that disruption of Notch signaling by DAPT (a GSI) attenuated the LPS-induced increase in the levels of released IL-1β and IL-6 but did not alter the level of TNF-α. Using another GSI (IL-CHO) to block Notch signaling, decreased LPS and IFN-γ-induced IL-6 and iNOS mRNA and increased IL-10 mRNA at late stages of activation (12–24 h) were observed in murine bone marrow-derived macrophages (13). Discrepant results were also reported concerning the activation of NF-κB pathway by DLL4-induced Notch signaling (32) or unaltered NF-κB activity by Notch activation (33). These data, together with ours, highlight the profound functional outcome of TLR...
and Notch interaction. Several issues may contribute to this complexity of interpreting these experiments. First, these studies employed different approaches to manipulate Notch activation or inhibition. For GSIs (DAPT, IL-CHO, or JLK6) (13, 15), GSI activities also regulate other signaling pathways (34); so using different GSIs may alter outcomes in a Notch-independent fashion. In addition, different ligands (DLL4 (32) or Jagged1 (15)) were used for Notch ligation. The amount of ligand used likely does not correspond to physiologically attainable levels and may be quite different among the studies. Finally, few studies have used defined models of macrophages. Macrophages from different species (human or murine) and of various levels and may be quite different among the studies. Finally, few studies have used defined models of macrophages. Macrophages from different species (human or murine) and of various

Accumulating evidence supports the existence of important but incompletely understood cross-talk between Notch and other signaling pathways like MAPK, Akt, and NF-κB that regulate cell growth and inflammation (36, 37). The present study demonstrates that Notch activation in macrophages inhibits the activation of the MAPK and NF-κB pathways (Figs. 4 and 6). Notch signaling also suppressed the TLR4-triggered transcription activity of reporter genes such as TNF-α (data not shown) that may contribute to a proinflammatory macrophage phenotype. Furthermore, whereas stimulation through the TLR4/9 pathway (e.g. by LPS or CpG ODN) induces Notch1/2 expression in macrophages (Fig. 1), Notch signaling suppresses the TLR-triggered inflammatory response (Fig. 2), which is reminiscent of a Notch target genes Hes1/Hey1-mediated feedback inhibition of cytokine production that fine tunes the TLR response (17, 35). This adds to the number of signaling pathways including the nucleotide binding domain and leucine-rich repeat-containing gene family, C-type lectins, and glucocorticoids that either positively or negatively affect the TLR responses (4). It will be important to determine whether these reciprocal regulation pathways identified here are restricted to particular cell types or are broadly operational.

Notch signaling has been reported to regulate T cells directly to produce cytokines including IL-2, IFN-γ, IL-4, IL-6, IL-10, and IL-12 (39–43). Because macrophages also produce IFN-γ, IL-6, and IL-10, it will be interesting to explore the role of Notch signaling in regulating cytokine production in macrophages. The effects of NICD and GSI on LPS-induced cytokine production are reminiscent of those of SOCS3 (44). SOCS3 plays an important role in regulating expression of TNF-α, IL-6, and iNOS in macrophages, and its expression is induced by IL-10 (44, 45). SOCS3 negatively regulates expression of IL-6 and iNOS at mRNA and protein levels and TNF-α expression at the protein level (44). It has recently been shown that Notch signaling mediates Bacillus Calmette-Guerin-induced SOCS3 up-regulation (46). Given that LPS-induced IL-10 production was increased in NICD1/2-overexpressing macrophages and decreased in the presence of GSI, and that SOCS3 is a downstream molecule of Notch signaling (46), it will be speculative that SOCS3 be involved in LPS-induced IL-10 production by Notch in macrophages. Interestingly, Notch has been shown to induce IL-10 production in T helper cells in vitro and in vivo (38, 40, 43). Therefore, the relationships among Notch signaling, IL-10, and SOCS3 need to be further investigated.

Taken together, our findings in this study demonstrate that Notch signaling negatively regulates TLR-triggered inflammatory cytokine and signaling pathway in macrophages. Thus, further understanding of the Notch signaling in the context of TLR-triggered macrophage biology will likely provide insights into the mechanisms of inflammation and new approach for rational therapeutic intervention.
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