

Bacterial LPS up-regulated TLR3 expression is critical for antiviral response in human monocytes: evidence for negative regulation by CYLD

Zhixing K. Pan^{1,2}, Chris Fisher¹, Jian-Dong Li³, Yong Jiang⁴, Shuang Huang² and Ling-Yu Chen¹

¹Department of Medical Microbiology and Immunology and Medicine, University of Toledo Medical Center, 3035 Arlington Avenue, Toledo, OH 43614, USA

²Department of Immunology and Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037, USA

³Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA

⁴Department of Pathophysiology and Key Laboratory of Proteomics of Guangdong Province, Southern Medical University, Guangzhou 510515, People's Republic of China

Correspondence to: Z. K. Pan; E-mail: kevin.pan@utoledo.edu

Received 2 December 2010, accepted 17 March 2011

Abstract

In the host immune system, the leukocytes are often exposed to multiple pathogens including bacteria and viruses. The principal challenge for the host is to efficiently detect the invading pathogen and mount a rapid defensive response. Leukocytes recognize invading pathogens by directly interacting with pathogen-associated molecular patterns via Toll-like receptors (TLRs) expressed on the leukocyte surfaces. In this study, we provide direct evidence that bacterial LPS enhances the host antiviral response by up-regulating TLR3 expression in human peripheral blood monocytes and monocytic cell lines, THP1 cells. Moreover, LPS induces TLR3 expression via a TLR4-MyD88-IRAK-TRAF6-NF- κ B-dependent signaling pathway. Interestingly, CYLD, an important deubiquitinase, acts as a negative regulator of TLR3 induction by LPS. Our study thus provides new insights into a novel role for bacterial infection in enhancing host antiviral response; furthermore, it identifies CYLD for the first time as a critical negative regulator of bacterial LPS-induced response.

Keywords: gene expression, inflammation, signal transduction, toll-like receptor

Introduction

The major outer membrane component of Gram-negative bacteria, LPS, is one of the most important activators of the innate immune system, which involves the host defense against bacterial infection (1–3). The Toll-like receptor (TLR) family is comprised of various germ line-encoded receptors that are constitutively expressed by most cells and have the ability to recognize microbial conserved molecular structures known as pathogen-associated molecular patterns (PAMPs). Human blood leukocytes recognize invading pathogens by directly interacting with PAMPs on a variety of pathogens via TLRs expression on the leukocytes. Activation of TLRs, in turn, leads to induction of direct antimicrobial activity that can result in elimination of the invading pathogen before a full adaptive immune response takes effect. To date, 11 members of the human TLR family have been identified. Of these, TLR4 is critically involved in host response to a Gram-negative bacterial product like LPS (4–7).

Our recent studies demonstrated that TLR4 and downstream molecules MyD88, IRAK1 and small GTPase Rho play key roles in activating host immune and inflammatory

response induced by LPS (8, 9). In contrast, TLR3 is involved in sensing viral infections and initiating the appropriate immune responses. Many RNA viruses express dsRNA during their replication cycle, and dsRNA is involved in the activation of immune system during virus infection. Signaling initiated by TLR3 is thought to occur upon dsRNA binding, however, the details of which are still being determined (10). Although this is the case, TLR3 is thought to bind to poly(I:C), a synthetic analogue of dsRNA, and is the most widely utilized ligand in TLR3 studies (11–13). TLR3 binding of viral dsRNA activates IFN- α/β . This function of TLR3 may be involved in activation of NF- κ B (14).

Multiple cell types are known to express TLR3, but human peripheral blood monocytes do not express TLR3 (15–17). Although a great deal has been explored during the past few years about the synthesis and release of proinflammatory cytokines by monocytes, relatively little is known about the nature of activated monocytes in the detection and response of a viral infection within the blood.

In the present study, we demonstrate that bacterial LPS pre-treatment strongly enhances TLR3-mediated antiviral cytokine expression in human peripheral blood monocytes and in THP1 cells. These functions of LPS appear to be mediated through the up-regulation of TLR3 expression in human monocytes. Furthermore, the mechanisms of bacterial LPS induction of TLR3 RNA and protein expression may be positively regulated by TLR4-MyD88-IRAK-TRAF6-NF- κ B-dependent signaling pathway, while it is negatively regulated by CYLD in human blood monocytes and monocytic cells. These results suggest a potential different antiviral response in human blood monocytes and other immune cells, and that the signal mechanisms involved in mediating this effect in human blood monocytes is stimulated by bacterial LPS.

Methods

Reagents

LPS was isolated from *Salmonella minnesota* Re595 bacteria as described (18). Poly(I:C) was purchased from Calbiochem (San Diego, CA, USA). A monoclonal antibody against TLR3 was purchased from ebioscience (San Diego, CA, USA). The MyD88-dominant-negative plasmid, the luciferase reporter plasmids containing nucleotide sequences encoding the NF- κ B response element and the TNF- α and IFN- β promoters were obtained as previously described (19). The pRL-tk-LUC internal control reporter plasmid was provided in the Dual Luciferase Reporter kit (Promega). The siRNA vector mediating knockdown with or without nucleotide sequences targeting CYLD was obtained as previously described (20).

Preparation of monocytes from peripheral blood

Heparinized human peripheral blood from health donors was fractionated on Percoll (Pharmacia) density gradients. Mononuclear cells and neutrophils were initially separated by centrifugation through a 55%/74% discontinuous Percoll gradient. Monocytes were further prepared from the mononuclear cell population with gelatin/plasma coated flasks as described (21). The purity of monocytes was >85–90% as determined by staining with the anti-CD14 monoclonal antibody (Coulter Immunology, Miami, FL, USA), and cell viability was >95% as measured by trypan blue exclusion. Monocytes were resuspended in RPMI-1640 medium (Irvine Scientific, Santa Ana, CA, USA) with 10% (V/V) heat-inactivated fetal bovine serum (FBS), penicillin (100 U ml⁻¹), streptomycin (100 μ g ml⁻¹) and L-glutamine (2 mM).

Cell culture and transfections

THP-1 cells were cultured in RPMI-1640 with glutamax and 25 mM HEPES (Invitrogen), penicillin streptomycin (Invitrogen) and supplemented with 10% heat-inactivated FBS. To transfect cells for reporter assays, an appropriate number of THP-1 cells were split 24 h prior to transfection in fresh medium. Cells were transfected with 1.5 μ g of the designated reporter plasmid and 0.5 μ g of the *Renella* luciferase reporter control (Promega) utilizing the Nucleofector Kit V system (Amaxa).

Immunoprecipitations and immunoblots

To detect TLR-3 expression, human blood monocytes or THP-1 cells (1×10^6 cells per dish) were placed in 100 mm dishes and stimulated with 100 ng/ml of LPS. Cells were cultured for indicated times, harvested and utilized to prepare cytosolic extracts. Determination of total protein in extracts occurred by Bradford assays using the Coomassie Protein Assay Reagent (Pierce). Extract volumes for all samples containing total protein amounts equal to the lowest protein amount were brought up to 250 μ l and mixed with TLR3 monoclonal antibody (0.75 μ g/250 μ l) (ebioscience). Reactions were allowed to proceed for 1 h. After 1 h, washed protein G-Sepharose 4 Fast Flow (Amersham) was added to the reaction for an additional 1 h. The protein G-sepharose beads with bound complexes were then washed three times with Dulbecco's PBS (Invitrogen) and bound TLR3 was eluted with 5 \times SDS buffer. The protein elutes were resolved using a 12% SDS-PAGE gel, transferred to nitrocellulose and probed with the same TLR3 monoclonal antibody utilized during the immunoprecipitations. The blots were then incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) secondary antibody for 2 h at room temperature. Detection of nitrocellulose bound proteins occurred utilizing the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and the fluorchem 890 (Alpha Innotech) detection system.

Luciferase assays

THP-1 cells were placed in six-well plates (1×10^5 cells per well), transfected with appropriate reporters and cultured for 24 h. After 24 h, transfected cells were stimulated with 100 ng/ml LPS and cultured for 10 h. Then 100 ng of poly(I:C) was added to the medium and cells were cultured for an additional 24 h. After stimulating the cells with poly(I:C) for 24 h, cells were pelleted and lysed utilizing passive lysis buffer (Sigma). Luciferase activity present in resulting cell extracts was quantified by performing luciferase assays utilizing the Dual-Luciferase Reporter Assay System (Promega) following the protocol provided. To detect luciferase fluorescence the Monolight 3010 luminometer (Pharmingen) was utilized.

siRNA

The TLR3 small interfering RNA and control siRNA were purchased from Dharmacon (Lafayette, CO, USA). A final concentration of 33 nM TLR3 siRNA or control siRNA was transfected into THP1 cells with amaxa electroporation technique utilizing nucleofector Kit (Amaxa Biosystems, Gaithersburg, MD, USA) following the manufacturer's protocol. Transfection efficiency of the siRNA's in THP1 cells was determined by co-transfecting siGLO control with either of the siRNA and then visualizing the effect using fluorescence microscopy. To determine the efficiency of gene silencing, the whole cell lysate of the above co-transfected cells was used for western blotting as described above and probed using specific antibody for TLR3. At 24 h after transfection, cells were used for each experiment.

Quantitative real-time PCR

Human blood monocytes or THP-1 cells were placed in six-well plates (1×10^5 cells per well) and stimulated with LPS

at indicated concentrations and times. The cells were then stimulated with or without 100 ng/ml of poly(I:C) for experiments involving TNF- α , IFN- β and TLR3. After stimulation, cells were collected and total RNA was prepared using the RNeasy mini kit (Qiagen). Any residual DNA present in the resulting RNA was removed by Dnase I treatment utilizing the DNA-free kit (Ambion) following the protocol provided. The RNA was quantified and 1 μ g of total RNA was reverse transcribed using the Omniscript RT kit (Qiagen). Resulting cDNA at a concentration of 0.5 μ g/ μ l was used to PCR amplify during the QRT-PCR assay. To amplify the cDNA product, 0.5 U of Platinum *taq* DNA polymerase (Invitrogen) was used in a reaction containing 1 μ M of TLR3, TNF- α , IFN- α , IFN- β , CYLD or β -actin primers, 0.2 mM dNTPs, 0.2 mM dNTPs (Idaho Technology Inc.), PCR reaction buffer (Idaho Technology Inc.), Enzyme Diluent (Idaho Technology Inc.) and SYBR Green I (Molecular Probes). The cDNA containing reaction mixtures were placed in a 96-well Optical Reaction Plate (Applied Biosystems), amplified and fluorescence emitted from binding of the SYBR Green I dye to target PCR products was detected using the ABI prism 9500 detection instrument. Results were analyzed using the Δ Ct and the $\Delta\Delta$ Ct method.

Enzyme linked immunosorbent assay

To detect TNF- α protein concentrations in medium of cultured human blood monocytes or THP-1 cells, cells (5.0×10^4 cells per well) were plated in 24-well plates and stimulated with LPS or Poly(I:C) for 10 h, medium was then utilized to perform TNF- α ELISA assays (Beckman Coulter) following the protocol supplied. Fluorescence emitted by addition of substrate corresponding to target was detected by the VERSA max plate reader (Molecular Devices). In some experiments (Fig. 1C and D), the cells were treated with LPS for 10 h prior to stimulation with Poly(I:C) as indicated in the text and legends for figures.

Statistical analysis

Statistical analysis was performed with Student *t*-test. *P* values of <0.05 were considered statistically significant.

Results

Poly(I:C) induces the production of TNF- α by LPS-primed human monocytes and THP-1 cells

The TNF- α cytokine is a pleiotropic factor that is released from cells during antiviral responses in an autocrine and paracrine fashion. To assess the relationship between poly(I:C) stimulation and synthesis of cytokines, we examined the effects of poly(I:C) on TNF- α release in human blood monocytes and THP1 cells by ELISA assays. As can be seen in Fig. 1, poly(I:C) was not able to activate TNF- α expression in both human peripheral blood monocytes (Fig. 1A, lane 3) and THP1 cells (Fig. 1B, lane 3). However, the induction of TNF- α protein expression by LPS in both human peripheral blood monocytes (Fig. 1A, lane 2) and THP1 cells (Fig. 1B, lane 2) indicates that these cells can produce TNF- α in response to TLR4 stimulation. Interestingly, when monocytes (Fig. 1C) and THP1 cells (Fig. 1D) were pre-treated

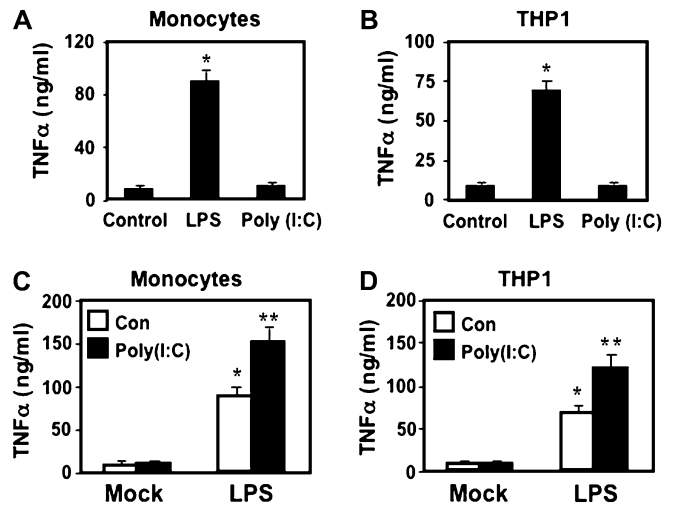


Fig. 1. Poly(I:C) induces the production of TNF- α by LPS-primed human monocytes and THP-1 cells. Human peripheral blood monocytes (Fig. 1A and C) or THP1 cells (Fig. 1B and D) were stimulated with 100 ng/ml LPS (Fig. 1A and B, lane 2) and 100 ng Poly(I:C) (Fig. 1A and B, lane 3) for 16 h. Figure 1(C and D), monocytes or THP1 cells were pre-treated with (Fig. 1C and D, lane 4) or without (Fig. 1C and D, lanes 1–3) LPS for 10 h and then treated with Poly(I:C)(100 ng) for 6 h. The supernatants were collected, and the secreted TNF- α was measured by ELISA as described in Methods. These results are representative of three separate experiments. **P* < 0.05 compared with control; ***P* < 0.05 compared with LPS-treated groups.

with 10 ng/ml LPS for 10 h, significant TNF- α was seen by the stimulation of 100 ng of poly(I:C) (Fig. 1C and D, lane 4). This experiment suggests that poly(I:C) induces the production of TNF- α in LPS-primed human monocytes and THP-1 cells.

Poly(I:C) increases the NF- κ B, TNF- α and IFN- β promoters in LPS-primed THP-1 cells

Since signals initiated from activated TLRs are mediated by NF- κ B and are hypothesized to result in the production of TNF- α and IFN- β , we quantified the activities of the NF- κ B, TNF- α and IFN- β promoters. Therefore, to determine if LPS in poly(I:C) stimulates these promoters, we performed luciferase assays using luciferase reporters that had the luciferase gene under the control of the stated promoters. Specifically, quantification of activities was done using luciferase reporters that contained either repeats of a consensus NF- κ B-binding site or the promoters of TNF- α and IFN- β .

In response to LPS, a significant increase in NF- κ B activity quantified by luciferase activity occurs but this does not occur in the absence of LPS. Importantly, no luciferase activity is detected in extracts produced from cells stimulated with 100 ng/ml of poly(I:C) only. Upon stimulation with both LPS and poly(I:C), a further increase in luciferase activity occurs when compared with luciferase activity induced by LPS only (Fig. 2A). The determination of poly(I:C) with or without LPS to induce TNF- α and IFN- β promoter activity was assessed. In both cases, an increase in luciferase activity occurred in response to LPS. Similar to NF- κ B, poly(I:C) did not activate TNF- α or IFN- β promoter activity in the absence

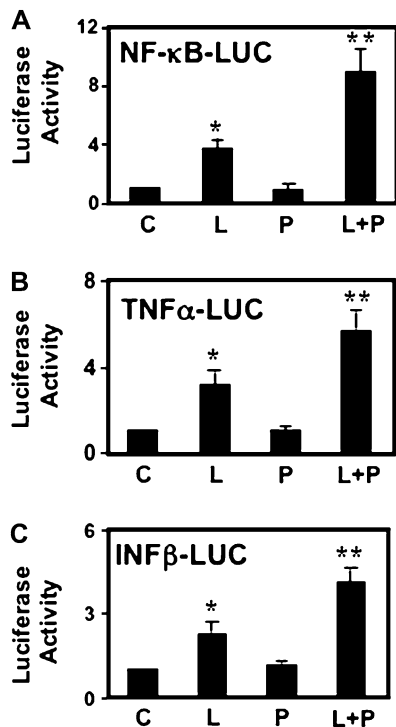


Fig. 2. Poly(I:C) increases TNF- α , IFN β and NF- κ B promoter activity by LPS-primed THP-1 cells. THP-1 cells were transfected with NF- κ B-LUC, IFN- β -LUC or TNF- α -LUC and with the control reporter pRL-tk-LUC. Cells were cultured for 24 h after transfection. Cells were then treated with 100 ng of LPS for 10 h and then added 100 ng of poly(I:C) for an additional 24 h. At the end of the 24 h poly(I:C) stimulation, luciferase assays were performed using the THP-1 cellular extracts. These results are representative of six separate experiments. * $P < 0.05$ compared with control; ** $P < 0.05$ compared with LPS-treated groups. C: Control; L: LPS; P: poly(I:C).

of LPS. However, when THP1 cells were pre-treated with 10 ng ml⁻¹ LPS for 10 h, significant NF- κ B, TNF α and IFN- β luciferase induction was seen by stimulation of 100 ng of poly(I:C) compared with that induced by LPS only (Fig. 2A–C, lane 4).

LPS induces TLR3 expression

The above experiments indicated that poly(I:C) stimulates TNF- α and IFN- β gene expression only in LPS-primed monocytes and THP1 cells. The function of this LPS is not fully understood. The induction of TNF- α protein expression by LPS in both human peripheral blood monocytes and THP1 cells indicates that these cells can produce TNF- α in response to LPS stimulation. Published data suggest that TLR3 is expressed in extremely low level in human monocytes (17), we, therefore, examined whether LPS up-regulates TLR3 in human peripheral blood monocytes and THP1 cells.

To determine the effect of LPS to increase TLR3 expression, real-time PCR (RT-PCR) and western blot experiments were performed. Total RNA was isolated for RT-PCR experiments from monocytes that did not express TLR3 mRNA (Fig. 3). Upon stimulation of monocytes with increasing concentrations of LPS, induction of TLR3 mRNA

occurs. Expression of TLR3 is at a significant level when stimulated with 10 ng/ml of LPS (Fig. 3A). To determine the time at which maximal induction of TLR3 occurs, monocytes were stimulated for the indicated times with 100 ng/ml of LPS. As can be seen, the induction of TLR3 is minimal at 4 h of LPS stimulation, but the expression levels of TLR3 RNA increase with time to a maximal level at 6 h. From 6 to 12 h, TLR3 expression decreases at which time it is still higher than TLR3 expression at 4 h of LPS stimulation (Fig. 3B).

Further demonstration that LPS induces TLR3 expression was obtained by western blot to detect TLR3 protein expression in both human peripheral blood monocytes. Due to previous reports supporting low expression levels of TLRs, immunoprecipitations utilizing a TLR3-specific antibody subsequent to western blot analysis. Specifically, stimulation of human blood monocytes cells for 2, 4, 6 and 12 h with LPS was performed. The ability of LPS to induce TLR3 expression is minimal at 4–6 h where little TLR3 expression is detected. After 12 h of LPS stimulation, TLR3 expression increases to a maximal level in human blood monocytes (Fig. 3C).

A TLR4-dependent MyD88-IRAK-TRAF6-NF- κ B signaling pathway is required for LPS-induced TLR3 expression

We next sought to determine which surface receptor and downstream adaptors are involved in TLR3 induction by LPS. Because TLR4 is important for mediating LPS-induced gene transcription, we first investigated the role of TLR4 in LPS-induced TLR3 up-regulation using a TLR4 functional antibody (TLR4fAb) that blocks binding of LPS to TLR4. Pre-treatment of human monocytes or THP1 cells with the TLR4fAb abrogates the LPS-induced TLR3 expression (Fig. 4A), indicating that the LPS-induced TLR3 up-regulation in monocytes requires the binding of LPS to TLR4. To further confirm the requirement of TLR4 in mediating LPS-induced TLR3 up-regulation, we examined TLR3 induction by LPS in HEK293-pcDNA, HEK293-TLR2 or HEK293-TLR4 cells, stably transfected with pcDNA, TLR2 or TLR4, respectively. As expected, LPS induced TLR3 mRNA expression in HEK293-TLR4 cells but not in HEK293-pcDNA or HEK293-TLR2 cells (Fig. 4B). We next investigated the involvement of MyD88 in LPS-induced TLR3 up-regulation. As shown in Fig. 4(C), over-expression of a dominant-negative mutant form of MyD88 attenuated LPS-induced TLR3 up-regulation in THP1 cells. Because activated MyD88 recruits IRAK-1 and subsequently interacts with TRAF6, we investigated if IRAK-1 and TRAF6 are also involved in TLR3 induction. As shown in Fig. 4(C), co-expressing dominant-negative IRAK-1 or TRAF6 inhibited LPS-induced TLR3 expression.

We previously demonstrated that LPS stimulates the activity of NF- κ B and that this activity in monocytes is required for the induction of inflammatory cytokine induced by LPS (8). We therefore tested whether the activation of NF- κ B was important for this LPS-induced TLR3 up-regulation by assessing the effect of the proteasome inhibitor, MG-132, on LPS-induced TLR3 up-regulation. Figure 4(D) shows that MG-132 inhibited LPS-induced up-regulation of TLR3. We further confirmed the requirement for NF- κ B by knockdown of p65 with p65

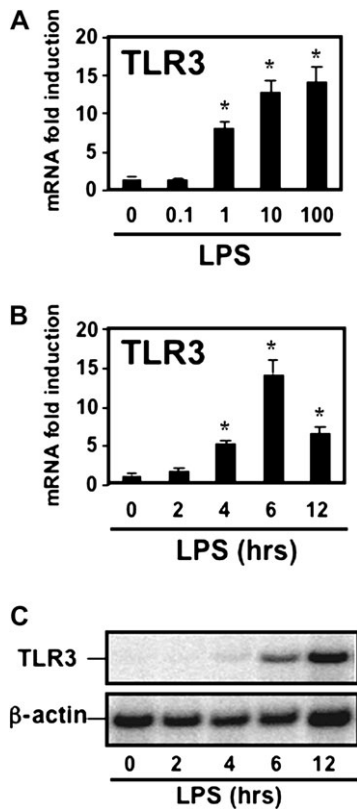


Fig. 3. LPS increases TLR3 RNA and protein expression. (A and B) LPS-induced TLR3 expression at the mRNA level in human blood monocytes in a dose-dependent (0, 0.1, 1, 10 and 100 ng/ml of LPS) and time-dependent (0, 2, 4, 6 and 12 h of LPS) manner, as assessed by real-time quantitative-PCR analysis. (C) LPS-induced TLR3 expression at the protein level in human blood monocytes in a time-dependent manner, as assessed by western blot analysis. * $P < 0.05$, compared with untreated control. P value was determined by Student's t -test. Values are the mean \pm SD ($n = 3$ for A and B). Data shown in (C) are representative of three or more independent experiments.

siRNA. As shown in Fig. 4(E), p65 siRNA markedly inhibited TLR3 induction by LPS. Taken together, these results provide evidence that TLR4/MyD88/IRAK/TRAF6/NF- κ B signaling pathway is required for LPS-induced TLR3 up-regulation in human monocytes.

TLR3 is critical for induction of type I IFNs and pro-inflammatory cytokines

The above experiments indicated that LPS up-regulates TLR3 in a TLR4-dependent manner. We next sought to determine the physiological relevance of TLR3 up-regulation. We assessed the effect of over-expressing wild-type TLR3 or knockdown TLR3 on expression of cytokines induced by Poly(I:C), a synthetic ligand for TLR3. As shown in Fig. 5, over-expression of wild-type TLR3 enhanced Poly(I:C)-induced expression of all these genes (Fig. 5A–D) and knockdown of TLR3 reduced Poly(I:C)-induced expression of cytokine (Fig. 5F), indicating that enhanced TLR3 expression indeed enhances host antiviral responses. Taken together with the data of Figs 1 and 2, these results suggest

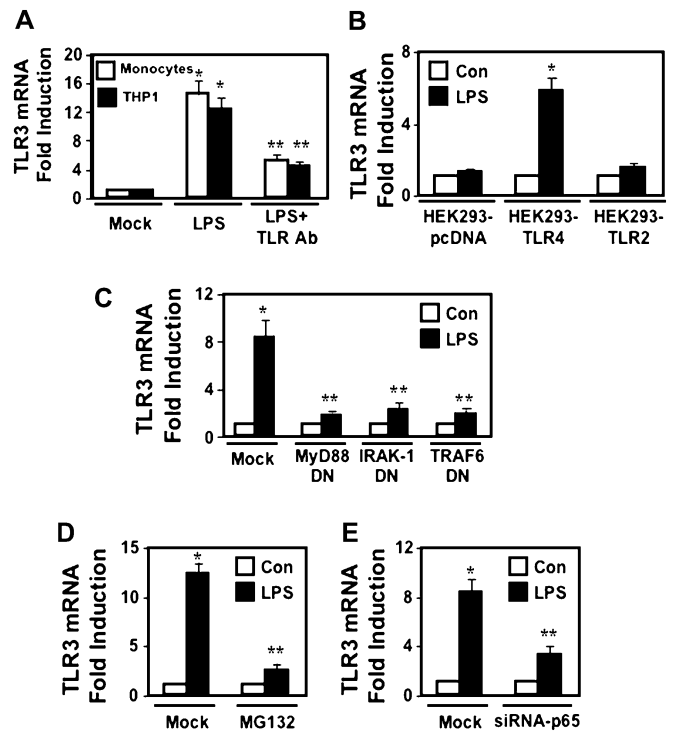


Fig. 4. A TLR4-dependent MyD88-IRAK-TRAF6-NF- κ B signaling pathway is required for LPS-induced TLR3 expression. (A) Human blood monocytes or THP1 cells were pre-treated with functional antibody of TLR4 (TLR4fAb) for 1 h and then stimulated with LPS for 6 h as assessed by performing real-time Q-PCR analysis. (B) LPS markedly induced TLR3 expression at the mRNA level in HEK293-TLR4 cells, but only weakly in HEK293-pcDNA cells and HEK293-TLR2 cells. (C) Over-expression of dominant-negative MyD88, IRAK-1 or TRAF6 reduced LPS-induced TLR3 expression at the mRNA level in THP1 cells. (D) MG-132, a proteasome inhibitor that can inhibit NF- κ B translocation, reduced LPS-induced TLR3 expression at the mRNA level in THP1 cells. The cells were pre-treated with MG-132 (1 μ M) for 2 h and then treated with LPS for 6 h as assessed by performing real-time Q-PCR analysis. (E) p65 knockdown using p65 siRNA inhibited LPS-induced TLR3 expression at the mRNA level. These results are representative of three separate experiments. * $P < 0.05$ compared with control; ** $P < 0.05$ compared with only LPS-treated groups.

that LPS potentiates TLR3-dependent expression of host antiviral genes by inducing TLR3 expression in human blood monocytes.

CYLD acts as a negative regulator of LPS-induced TLR3 up-regulation

Our results presented above suggest that activation of NF- κ B may be involved in LPS-stimulated TLR3 gene expression. Published data have shown that the cylindromatosis tumor suppressor (CYLD), a recently identified novel deubiquitinase, decreases the activation of NF- κ B in the context of the tumor necrosis factor receptor signaling (22, 23). We next sought to determine whether CYLD is involved in LPS-induced TLR3 expression. We first evaluated the efficiency of CYLD siRNA in reducing CYLD expression and inhibiting the LPS-induced phosphorylation and degradation of I κ B α . As shown in Figure 6(A), CYLD siRNA efficiently reduced CYLD expression in THP1 cells transfected with

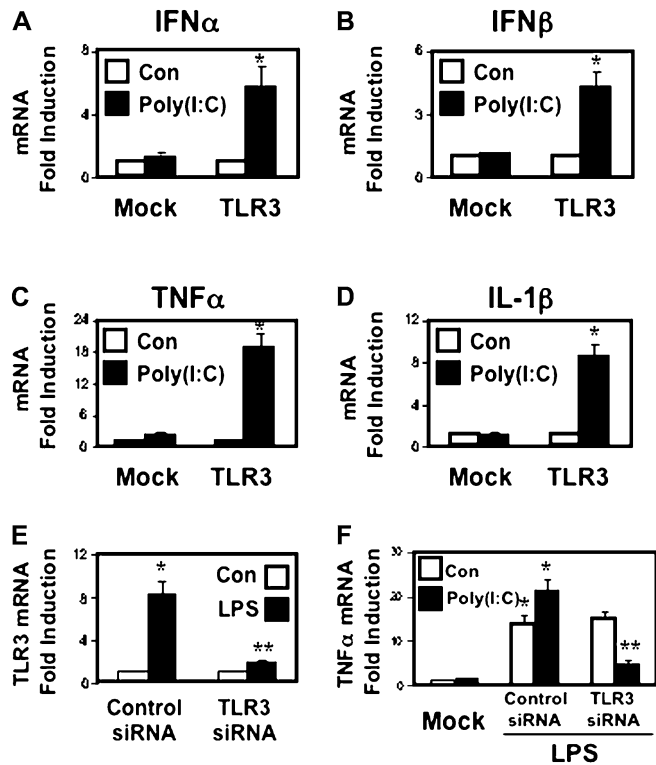


Fig. 5. TLR3 is critical for induction of type I IFNs and pro-inflammatory cytokines. Over-expression of wild-type TLR3 enhanced Poly(I:C)-induced expression of IFN- α , IFN- β , TNF- α and IL-1 β in THP1 cells. THP1 cells were transfected with wild-type TLR3. Cells were treated with 100 ng Poly(I:C) and relative quantity of IFN- α (A), IFN- β (B), TNF- α (C) and IL-1 β (D) mRNA expression were measured by Q-PCR analysis 3 h after treatment. (E and F) THP1 cells were transfected with TLR3 siRNA or control siRNA and then stimulated with LPS and Poly(I:C) as assessed by performing real-time Q-PCR analysis for TLR3 (E) or TNF α mRNA (F). These results are representative of three separate experiments. * $P < 0.05$ compared with control; ** $P < 0.05$ compared with control siRNA groups.

wild-type CYLD. Over-expression of wild-type CYLD inhibited I κ B α phosphorylation and degradation, whereas CYLD siRNA enhanced it (Fig. 6B). Next, we examined the effect of over-expressing wild-type CYLD or CYLD siRNA on LPS-induced NF- κ B activation. As expected, over-expression of wild-type CYLD attenuated NF- κ B activation by LPS, whereas CYLD knockdown enhanced it (Fig. 6C and D). These data show that CYLD indeed acts as a negative regulator of LPS-induced NF- κ B activation. We next sought to determine whether CYLD is a negative regulator of LPS-induced TLR3 up-regulation. As shown in Fig. 6(E and F), over-expression of wild-type CYLD attenuated LPS-induced TLR3 up-regulation, whereas knockdown of CYLD enhanced TLR3 up-regulation. Taking these results together, it is evident that CYLD acts as a negative regulator of LPS-induced TLR3 expression.

CYLD is induced by LPS via a TLR4-dependent pathway in human monocytes

Because a variety of genes involved in the host defense response were induced during the course of infections, we

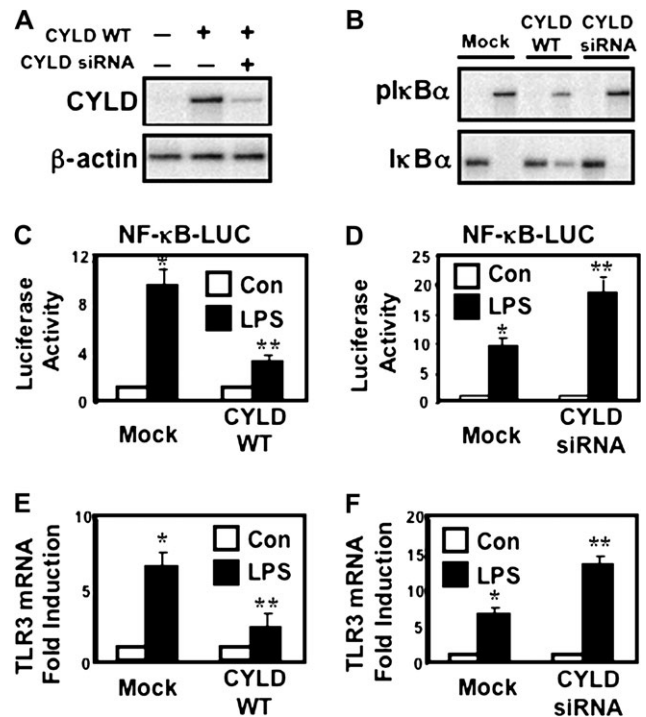


Fig. 6. CYLD is a negative regulator for LPS-induced up-regulation. (A) siRNA of CYLD efficiently reduced CYLD expression at the protein level in THP1 transfected with wild-type CYLD, as assessed by western blot analysis. (B) Over-expression of wild-type CYLD attenuated LPS-induced I κ B α phosphorylation (B) and NF- κ B luciferase assay in THP1 cells (C). (D) siRNA of CYLD enhanced NF- κ B activation as assessed by luciferase assay in THP1 cells. (E) Over-expression of wild-type CYLD reduced induction of TLR3 mRNA by LPS in THP1 cells as assessed by real-time Q-PCR analysis. (F) CYLD knockdown using CYLD siRNA enhanced LPS-induced TLR3 mRNA expression in THP1 cells. * $P < 0.05$ compared with control; ** $P < 0.05$ compared with LPS-treated control groups. These results are representative of three separate experiments.

thus examined whether LPS induces CYLD in human blood monocytes and THP1 cells. LPS stimulated a time-dependent up-regulation of CYLD message and protein in human blood monocytes and THP1 cells (Fig. 7A–C). The LPS induced increase of CYLD message was seen within 2 h of stimulation and peaked at 4 h. The kinetics of LPS-induced CYLD preceded that of LPS-induced TLR3, consistent with a role of CYLD in the expression of TLR3. We next investigated the requirement for TLR4 in LPS-induced CYLD up-regulation in human monocytes by using a TLR4 functional antibody (TLR4fAb) that blocks binding of LPS to TLR4. Pre-treatment of human monocytes or THP1 cells with the TLR4fAb completely abrogated the LPS-induced CYLD expression (Fig. 7D), indicating that LPS-induced CYLD up-regulation in monocytes requires LPS binding to TLR4. To further confirm the involvement of TLR4 in LPS-induced CYLD expression, we examined CYLD mRNA induction by LPS in HEK293-pcDNA and HEK293-TLR4 cells. As shown in Fig. 7(E), CYLD mRNA expression was greatly up-regulated in HEK293-TLR4 cells compared with HEK293-pcDNA cells. These data suggest that CYLD is induced by LPS via a TLR4-dependent pathway in human blood monocytes.

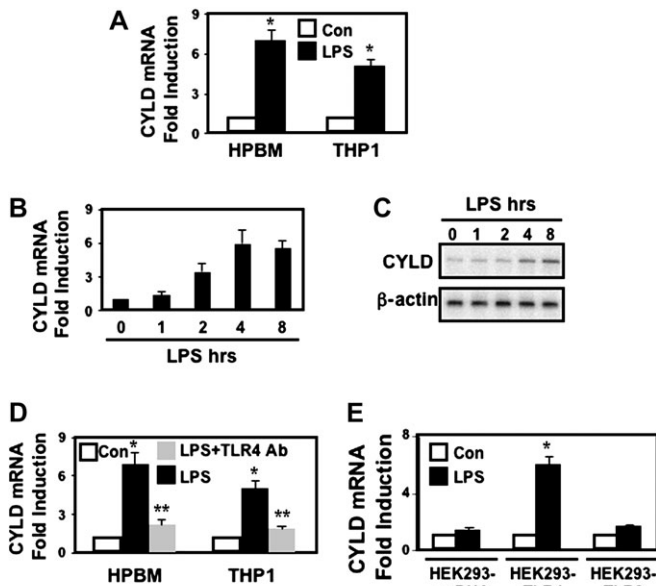


Fig. 7. CYLD is induced by LPS via a TLR4-dependent signaling pathway in human monocytes and THP1 cells. (A) CYLD was markedly induced by LPS in human blood monocytes and THP1 cells as assessed by real-time Q-PCR analysis. (B and C) LPS-induced CYLD expression in a time-dependent manner at the mRNA level as assessed by real-time Q-PCR analysis (B) and at the protein level as assessed by western blot analysis (C). (D) Functional blocking antibody of TLR4 (TLR4fAb) attenuated CYLD induction by LPS at the mRNA level. (E) LPS-induced CYLD expression was enhanced at the mRNA level in HEK293-TLR4 cells compared with HEK293-pcDNA or HEK293-TLR2 cells. These results are representative of three separate experiments. * $P < 0.05$ compared with control; ** $P < 0.05$ compared with only LPS-treated groups.

Discussion

In the host immune system, the leukocytes are often exposed to multiple pathogens including bacteria and viruses. Most studies to date have focused on investigating antibacterial response or antiviral response. There is a growing body of evidence that a significant proportion of patients have mixed infections of bacteria and virus (24–27). Moreover, inappropriate antibiotic treatment has contributed to the worldwide emergence of antibiotic-resistant strains and has led to increased incidence of polymicrobial infections. Despite the relatively well-known role of viral infections in promoting bacterial infections, it is still not clear whether bacterial infection also promotes viral infection.

In this report, we show that human peripheral blood monocytes and THP1 cells are unresponsive to the stimulation of poly(I:C), a synthetic dsRNA analog that originates as an intermediate of viral replication. We provide evidence that the bacterial LPS enhances the expression of the key genes involved in host antiviral response by up-regulating TLR3 expression in blood monocytes and THP1 cells. Moreover, LPS induces TLR3 expression via a TLR4-MyD88-IRAK-TRAF6-NF- κ B-dependent signaling pathway, and CYLD, a novel deubiquitinase, act as negative regulators of TLR3 induction by LPS. Our study thus provides new insights into a novel role for bacterial infection in enhancing host antiviral response and also identifies CYLD as a critical negative

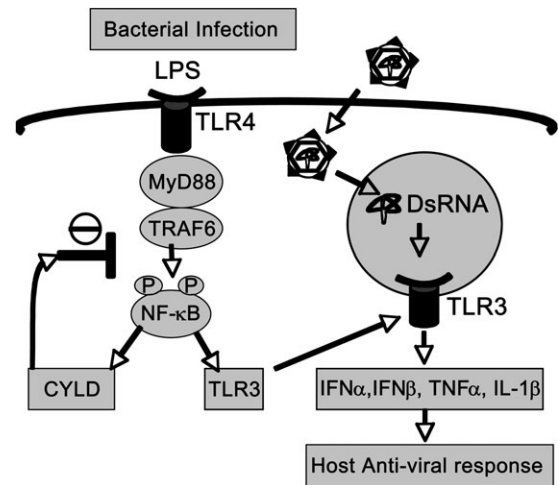


Fig. 8. Schematic representation of the signaling pathways involved in the positive and negative regulation of LPS-induced TLR3 expression.

regulator of host antiviral response (Fig. 8). Our findings may have important implications for host defense and immune response to mixed infections. Firstly, the extremely low expression of TLR3 observed in blood monocytes is an important aspect of TLR3 function because under limiting conditions, cellular responses to PAMPs can be more stringently regulated by controlling the amount of TLR protein produced. Secondly, the increased TLR3 expression contributes to the accelerated immune response of leukocytes as well as to the resensitization of monocytes to invading pathogens. Hence, regulation of TLR3 expression may be one of the immune regulatory mechanisms commonly involved in host defense against viruses. Finally, the observation that TLR3 is up-regulated by LPS suggests that bacterial products can not only initiate the host immune response, but they can also modulate the eventual responsiveness of monocytes to the invading virus by regulating the TLR3 expression level. Thus, these observations bring new insights to our understanding of the interaction between bacteria and viruses of mixed infections in our blood system. Interestingly, it has been previously reported that IFN α enhanced TLR3-mediated antiviral cytokine expression in human endothelial and epithelial cells is associated with enhanced TLR3 expression (28). Many studies also indicate that TLR3 and TLR4 use TIR domain-containing adaptor inducing IFN β (TRIF) to activate IRF3, resulting in the production of type I IFN (IFN α and IFN β) (29, 30). Therefore, it would be important to understand how human peripheral blood monocytes in the detection and clearance of a systemic viral infection.

Another major finding of this study is that LPS-induced TLR4-dependent up-regulation of TLR3 is negatively regulated by CYLD in an autoregulatory feedback manner. In contrast with the relatively well-known role of CYLD in tumorigenesis and T-cell development, the role of CYLD in host antiviral response remains largely unknown (31, 32). Our results show that LPS induces CYLD expression in human peripheral blood monocytes, which in turn results in attenuation of TLR3 expression, leading to inhibition of host antiviral responses. Thus, the involvement of CYLD may be essential to ensure the tight control of LPS-induced TLR3 up-regulation

and the host antiviral response. We can further speculate that the CYLD-dependent autoregulatory feedback loop may represent an important mechanism by which the host can self-limit serious tissue damage caused by detrimental inflammatory responses during polymicrobial infection.

In summary, our results support the idea that TLR3 signaling efficiency depends on the amount of the receptor on the cell surface and that up-regulation of TLR3 expression by bacterial LPS is necessary for dsRNA-induced antiviral cytokine production in human blood monocytes. Furthermore, we provide evidence that the mechanisms of bacterial LPS to induce TLR3 RNA and protein expression may be positively regulated by TLR4-MyD88-IRAK-TRAF6-NF- κ B-dependent signaling pathway, while is negatively regulated by CYLD in human monocytes and monocytic cells. These results suggest new insights into a novel role for bacterial infection in enhancing host antiviral response and identifies CYLD for the first time as a critical negative regulator of bacterial LPS-induced response.

Funding

National Institutes of Health Grant AI43524 (Z.K.P.); National Institutes of Health Training Grant T32 AI 07469 (L.-Y.C).

References

- Ulevitch, R. J. 1999. Endotoxin opens the Tollgates to innate immunity. *Nat. Med.* 5:144.
- Pan, Z. K. 2004. Toll-like receptors (TLRs) and TLR-mediated signaling: more questions than answers. *Am. J. Physiol.* 286:L918.
- Ulevitch, R. J. and Tobias, P. S. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* 13:437.
- Aderem, A. and Ulevitch, R. J. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782.
- Kopp, E. and Medzhitov, R. 2003. Recognition of microbial infection by Toll-like receptors. *Curr. Opin. Immunol.* 15:396.
- Medzhitov, R. and Janeway, C. Jr. 2000. The Toll receptor family and microbial recognition. *Trends Microbiol.* 8:452.
- Akira, S. and Takeda, K. 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.* 4:499.
- Chen, L.-Y., Zuraw, B. L., Liu, F.-T., Huang, S. and Pan, Z. K. 2000. IL-1 Receptor-associated kinase and GTPase RhoA signal molecules are required for bacterial LPS-induced cytokine gene transcription. *J. Immunol.* 169:3934.
- Chen, L.-Y., Zuraw, B. L., Zhao, M., Liu, F.-T., Huang, S. and Pan, Z. K. 2003. Involvement of protein tyrosine kinase in Toll-like receptor 4 (TLR4)-mediated NF- κ B activation in human peripheral blood monocytes. *Am. J. Physiol.* 284:L607.
- Edelmann, K. H., Richardson-Burns, S., Alexopoulou, L., Tyler, K. L., Flavell, R. A. and Oldstone, M. 2004. Does Toll-like receptor 3 play a biological role in virus infections? *Virology* 322:231.
- Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R. A. 2001. Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor. *Nature* 413:732.
- Yamamoto, M., Sato, S., Mori, K. *et al.* 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN- β promoter in the Toll-like receptor signaling. *J. Immunol.* 169:6668.
- Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. and Seya, T. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat. Immunol.* 4:161.
- Meylan, E., Burns, K., Hofmann, K. *et al.* 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nat Immunol* 5:503.
- Zarembek, K. A. and Godowski, P. J. 2002. Tissue expression of human toll-like receptors and Differential regulation of toll-like receptor mRNAs in leukocytes in response to Microbes, their products, and cytokines. *J Immunol* 168:554.
- Heinz, S., Haehnel, V., Karaghiosoff, M. *et al.* 2003. Species-specific regulation of Toll-like receptor 3 genes in men and mice. *J. Biol. Chem.* 278:21502.
- Visintin, A., Mazzoni, A., Spitzer, J. H., Wyllie, D. H., Dower, S. K. and Segal, D. M. 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J. Immunol.* 166:249.
- Mathison, J. C., Wolfson, E. and Ulevitch, R. J. 1988. Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest.* 81:1925.
- Zhu, W., Downey, J. S., Gu, J., Di Padova, F., Gram, H. and Han, J. 2000. Regulation of TNF expression by multiple mitogen-activated protein kinase pathways. *J. Immunol.* 164:6349.
- Jono, H., Lim, J. H., Chen, L. F. *et al.* 2004. NF-kappaB is essential for induction of CYLD, the negative regulator of NF-kappaB: evidence for a novel inducible autoregulatory feedback pathway. *J. Biol. Chem.* 279:36171.
- Pan, Z. K., Chen, L. Y., Cochrane, C. G. and Zuraw, B. L. 2000. fMet-Leu-Phe stimulates proinflammatory cytokine gene expression in human peripheral blood monocytes: the role of phosphatidylinositol 3-kinase. *J. Immunol.* 164:404.
- Trompouki, E., Hatzivassiliou, E., Tschritzis, T., Farmer, H., Ashworth, A. and Mosialos, G. 2003. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. *Nature* 424:793.
- Yoshida, H., Jono, H., Kai, H. and Li, J. D. 2005. The tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for toll-like receptor 2 signaling via negative cross-talk with TRAF6 and TRAF7. *J. Biol. Chem.* 280:41111.
- Cameron, R. J., de Wit, D., Welsh, T. N., Ferguson, J., Grissell, T. V. and Rye, P. J. 2006. Virus infection in exacerbations of chronic obstructive pulmonary disease requiring ventilation. *Intensive Care Med.* 32:1022.
- Bandi, V., Jakubowycz, M., Kinyon, C. *et al.* 2003. Infectious exacerbations of chronic obstructive pulmonary disease associated with respiratory viruses and non-typeable *Haemophilus influenzae*. *FEMS Immunol. Med. Microbiol.* 37:69.
- Koskenvuo, M., Möttönen, M., Rahiala, J. *et al.* 2007. Mixed bacterial-viral infections in septic children with leukemia. *Pediatr. Infect. Dis. J.* 26:1133.
- Dagan, R., Hall, C. B., Powell, K. R. and Menegus, M. A. 1989. Epidemiology and laboratory diagnosis of infection with viral and bacterial pathogens in infants hospitalized for suspected sepsis. *J. Pediatr.* 115:351.
- Tissari, J., Sirén, J., Meri, S., Julkunen, I. and Matikainen, S. 2005. IFN- α enhances TLR3-mediated antiviral cytokine expression in human endothelial and epithelial cells by up-regulating TLR3 expression. *J. Immunol.* 174:4289.
- Kawai, T. and Akira, S. 2006. Innate immune recognition of viral infection. *Nat. Immunol.* 7:131.
- Kenny, E. F. and O'Neill, L. A. 2008. Signalling adaptors used by Toll-like receptors: an update. *Cytokine* 43:342.
- Reiley, W. W., Zhang, M., Jin, W. *et al.* 2006. Regulation of T cell development by the deubiquitinating enzyme CYLD. *Nat. Immunol.* 7:411.
- Massoumi, R., Chmielarska, K., Hennecke, K., Pfeifer, A. and Fassler, R. 2006. Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. *Cell* 125:665.