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Original Article

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Parthenolide inhibits LPS-induced inflammatory cytokines through the toll-like receptor 4 signal pathway in THP-1 cells

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Abstract

Parthenolide (PTL) shows potent anti-inflammatory and anti-cancer activities. In the present study, the molecular mechanisms of PTL's activities were explored in lipopolysaccharide (LPS)-induced human leukemia monocytic THP-1 cells and human primary monocytes. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay was used to analyze the effect of PTL on THP-1 cell viability. Enzyme-linked immunosorbent assay was used to determine the effect of PTL on LPS-induced inflammatory cytokine secretion. Flow cytometry and quantitative real-time polymerase chain reaction were used to assess the effect of PTL on LPS-induced toll-like receptor 4 (TLR4) expression. Phosphorylation levels of signaling molecules were determined by western blot analysis. Results showed that PTL <12.5 μM did not significantly affect THP-1 cells viability. LPS treatment led to a marked up-regulation of interleukin (IL)-6, IL-1β, IL-8, IL-12p40, tumor necrosis factor-α, IL-18, and NO in THP-1 cells. However, PTL inhibited the expression of these cytokines in a dose-dependent manner, with IC_{50} values of 1.091–2.620 μM. PTL blocked TLR4 expression with an IC_{50} value of 1.373 μM as determined by the flow cytometry analysis, and this blocking effect was verified at both protein and mRNA levels. Up-regulation of phosphorylation levels of extracellular signal-regulated kinase 1/2, Jun N-terminal kinase, p38, nuclear factor κB (NF-κB) p65, and IκBα and up-regulation of expressions of other molecules (inducible nitric oxide synthase, TLR4, and TNF receptor-associated factor 6) induced by LPS were abolished by PTL in a dose-dependent manner. The anti-inflammatory mechanisms of PTL operate partly through the TLR4-mediated mitogen-activated protein kinase and NF-κB signaling pathways. Therefore, TLR4 may be a new target for anti-inflammation therapies.

Key words: anti-inflammation, inflammatory cytokine, MAPK, parthenolide, TLR4

Introduction

Toll-like receptors (TLRs) play a critical role in the innate immune system, particularly in the inflammatory response by recognizing pathogen-associated molecular patterns [1]. TLRs are essential for the protective immunity against infection, but inappropriate TLR responses contribute to acute and chronic inflammation [2]. TLR4, a member of the TLR family, is expressed on cell surface and mainly recognizes microbial membrane components such as lipids, lipoproteins, and proteins. TLR4 is identified as the lipopolysaccharide

(LPS) receptor, which is critical in LPS-induced inflammatory actions [3]. LPS is a well-characterized inducer of inflammatory response and the pro-inflammatory activity of LPS-induced TLR4 expression plays an important role in pathological responses in autoimmune disorders and chronic inflammatory conditions, with the development of atherosclerosis, neurodegenerative diseases, and other chronic diseases [4]. Once activated, TLR4 initiates downstream signaling pathways [mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB)], resulting in the release of cytokines such as interleukin (IL)-6, IL-1β, IL-8, IL-12p40, tumor necrosis factor (TNF)-α, IL-18, and NO, which then mediate the innate immune response and inflammatory response [5,6].

Accumulating evidence indicates that chronic inflammation plays a critical role in several chronic degenerative pathologies, including cardiovascular diseases, renal damage, and cancer [7,8]. Several anti-LPS or anti-cytokine clinical trials have been conducted, but none has so far been successful. Parthenolide (PTL), a sesquiterpene lactone (SL) originally purified from the shoots of feverfew (*Tanacetum parthenium*), has shown potent anti-cancer and anti-inflammatory activities [9]. Some observations revealed that the anti-inflammatory property of PTL in THP-1 cells, a human leukemia monocytic cell line, is partly due to its inhibition of inducible nitric oxide synthase (iNOS) promoter activation [9]. What's more, there is a study showing that PTL can attenuate NF-κB activation after an LPS challenge in rat myocardium [10]. Other studies indicated its inhibition of inflammatory cytokines secretion such as NO, IL-6, and TNF-α release and related signaling pathways such as MAPK and NF-κB activation in central nervous system diseases [11,12]. However, the mechanism of PTL inhibition of TLR4 and related molecules involved in pro-inflammatory signaling pathways remains unclear. Findings in RAW 264.7 macrophages suggested that PTL modulated toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent downstream signaling pathways such as MAPK and NF-κB of TLRs can be used in anti-chronic inflammatory diseases [13,14].

Innate immune recognition of microbial components and its critical role in host defense against infection have been intensely studied in recent years. Monocytes are key cells of the innate immune system and are responsible for the initiation, progression and resolution of inflammation, pathogen clearance, wound healing, and tissue homeostasis [6,15]. THP-1 is widely used as a model to study inflammatory response. Base on the findings in RAW264.7 macrophages [13,14], whether PTL plays a role in THP-1 cell line and primary monocytes after an LPS challenge, and whether its anti-inflammatory effects function through TLR4 signaling pathway are studied. LPS-induced THP-1 cells are used as a model to investigate the anti-inflammatory effects of PTL and to explore the underlying mechanism. Our results showed that PTL inhibits LPS-induced inflammation mainly by affecting TLR4 signaling, suggesting that targeting TLR4 expression may be a novel approach for anti-inflammatory treatment and cancer prevention and therapy.

Materials and Methods

Cell culture and treatment

THP-1 cells were purchased from American Type Culture Collection (ATCC; Rockville, USA). Fresh bone marrow samples were obtained from two newly diagnosed acute myelocytic leukemia (AML) patients and one healthy volunteer. Bone marrow mononuclear cells were harvested using Ficoll-Hypaque density centrifugation (Lymphoprep; Cedarlane Laboratories, Burlington, USA) and then collected and subject to subsequent isolation of human primary monocytes by the MACS magnetic separation system with a Human Monocyte IsolationKit II (Negative isolation; Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$ cells were >95% pure as determined by flow cytometry [15]. THP-1 cells or primary monocytes (5×10^5 cells/ml in 24-well plates) were maintained in a humidified incubator at 37°C under 5% CO_2, in RPMI-1640 medium containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen, Carlsbad, USA), and 10% fetal bovine serum (FBS; PAA, Morningside, Australia). Cells were pretreated with PTL for 2 h and divided into five different treatment groups: cells without LPS (Sigma, St Louis, USA) or PTL (Sigma) as the control group; cells treated with LPS (1 μg/ml) alone; and cells treated with PTL at different concentrations (0.75, 3, and 12 μM) in the presence of LPS. Cells treated with PTL for another 4–18 h were analyzed by western blot analysis, enzyme-linked immunosorbent assay (ELISA), quantitative real-time polymerase chain reaction (qRT-PCR), and flow cytometry.

Cell viability assay

The cytotoxic effects of PTL were determined before further studies. Briefly, THP-1 cells (5×10^4 cells/well) in 96-well plates (Promega, Madison, USA) were treated with PTL (0, 12.5, 25, and 50 μM) for 24 h and cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay. An electron coupling reagent, phenazine methosulfate (PMS), was also used in the assay. After incubation for the indicated time in the appropriate medium, 20 μl of MTS/PMS mixture was added to each well and incubated for 2 h. Viable cells reduced MTS into formazan. Absorbance was determined at 490 nm for each well using a spectrophotometer. The background absorbance from the control wells was subtracted from the measured absorbance values. Three duplicate studies were performed for each experimental condition.

Evaluation of cytokine secretion by ELISA

The inhibitory effect of PTL on IL-6, IL-1β, IL-8, IL-12p40, TNF-α, IL-18, and NO production in THP-1 was determined by ELISA kit (R&D Systems, Minneapolis, USA) with the mean minimum detectable doses of 0.7, 1, 3.5, 15, 1.6, 1, and 0.25 pg/ml, respectively. THP-1 cells were treated as described earlier. The supernatants were then collected from cell cultures and stored at −20°C until further use for determining the levels of the various cytokines. All tests were carried out in strict accordance with manufacturer's instructions, and each sample was analyzed in triplicate.

Evaluation of TLR4 by flow cytometry

The expression levels of TLR4 were monitored after different treatments with PTL and LPS. THP-1 cells were treated as indicated above then collected, washed twice with phosphate-buffered saline (PBS), re-suspended in 100 μl of PBS containing 2% FBS, and finally stained with rabbit monoclonal antibody against TLR4 (Cell Signaling, Beverly, USA) for 2 h. After being washed twice with PBS, cells were incubated with goat anti-rabbit IgG-FITC (Cell Signaling) for 1 h and washed twice with PBS, finally re-suspended in 100 μl of PBS containing 2% FBS. Unstimulated cells and LPS stimulated cells without PTL were used as controls. Flow cytometry was performed with a 488 nm laser coupled to a cell sorter (FacsCalibur; BD Biosciences, San Jose, USA).

Western blot analysis

THP-1 cells were treated as indicated and then collected and lyzed with a lysis buffer (20 mM Tris–HCl, pH 7.5, containing 150 mM

NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. The extraction of nuclear proteins (tubublin, NF-κB, and IκBα) was carried out using nuclear and cytoplasmic extraction reagents (Pierce, Rockford, USA) according to the manufacturer's instructions. The concentration of protein in each cell lysate was determined using a bicinchoninic acid protein assay kit (Pierce). Twenty micrograms of cell protein lysates per sample were mixed with 2× sodium dodecyl sufate (SDS) loading buffer containing dithiothreitol and heated at 100°C for 10 min before being resolved by SDS–polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes and blocked for 2 h at room temperature with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and 5% non-fat milk, and then incubated overnight with primary antibodies (1:1000; Cell Signaling) at 4°C. After thorough washes, membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:5000; Cell Signaling), and then detected using Electro-Chemi-Luminescence western detection reagents and a VersaDocMP5000 imaging system (Bio-Rad, Hercules, USA).

Quantitative real-time PCR

For qRT-PCR, THP-1 cells and primary monocytes were treated as indicated and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). qRT-PCR was carried out using SYBR Green I (Peq-Lab, Erlangen, Germany). Primer sequences for human *TLR4* were (product size 147 bp): 5′-TGGAAGTTGAACGAATGGAATGTG-3′ and 5′-ACCAGAACTGCTACAACAGATACT-3′. Primer sequences for *GAPDH* were (product size 280 bp): 5′-GAGATCCCTCCA AAATCAAGT-3′ and 5′-TGAGTCCTTCCACGATACCAA-3′. PCR was performed with 40 cycles on a Linegene real-time PCR detection system (Bioer Technology, Hangzhou, China) as follows: initial denaturation at 94°C for 3 min; denaturation at 94°C for 30 s; annealing at 60°C for 30 s; and extension at 72°C for 60 s. Finally, products were stained with ethidium bromide and electrophoresed through a 2% agarose gel. Data were analyzed using the $2^{-\Delta\Delta C_T}$ method [16,17]. The level of *TLR4* mRNA was normalized to that of *GADPH* mRNA.

Statistical analysis

All experiments were performed three times. Results were expressed as mean ± standard deviation (SD). The method of one-way analysis of variance was used for analysis of statistical significance and $P < 0.05$ was considered to be statistically significant.

Results

The cytotoxic effect of PTL on THP-1 cells

The cytotoxic effects of PTL were first evaluated. The viability of THP-1 cells after incubation with different concentrations of PTL for 24 h was determined by MTS assay (**Fig. 1**). Exposure to 0–12.5 μM PTL for 24 h did not affect the viability of THP-1 cells, while PTL concentrations >12.5 μM ($P < 0.05$) markedly decreased the viability. Therefore, PTL concentrations <12.5 μM were suitable to evaluate the selective pharmacological action of PTL without interfering with normal cell function. An IC_{10} = 12.5 μM (10% inhibiting concentration) was calculated using Prism. Hence, we chose concentrations under 12.5 μM (0.75, 3, and 12 μM) in the subsequent experiments.

Inhibitory effect of PTL on the LPS-induced up-regulation of inflammatory factor production in THP-1 cells

To evaluate the inhibitory effect of PTL on LPS-mediated production of inflammatory cytokines, the levels of IL-6, IL-1β, IL-8, IL-12p40, TNF-α, IL-18, and NO in the supernatant of THP-1 cells were determined by ELISA. These inflammatory cytokines play important roles in human immunity and inflammatory response. Cells were pretreated with various concentrations of PTL (0.75, 3, and 12 μM) in the presence or absence of LPS (1 μg/ml) for 18 h and then inflammatory cytokines in the supernatants were detected by ELISA (**Table 1**). LPS treatment led to a marked up-regulation of the levels of IL-6, IL-1β, IL-8, IL-12p40, TNF-α, IL-18, and NO in THP-1 cells. However, PTL inhibited the up-regulation in a dose-dependent manner, with IC_{50} (50% inhibiting concentration) values of 2.620, 2.594, 1.858, 2.157, 1.091, 2.518, and 2.175 μM, respectively. These results indicated that PTL can affect LPS-mediated pro-inflammatory responses in THP-1 cells.

Pretreatment with PTL attenuates LPS-induced TLR4 expression

Figure 2A shows that LPS significantly stimulated TLR4 expression in THP-1 cells. PTL inhibited the LPS-induced up-regulation of TLR4 in a dose-dependent manner. The IC_{50} value of PTL on LPS-induced TLR4 expression was 1.373 μM. Results expressed as mean fluorescence intensity in **Fig. 2B** showed that PTL at 3 and 12 μM significantly diminished LPS-induced TLR4 expression compared with LPS stimulated group ($P < 0.01$). The inhibition rates of PTL on TLR4 expression at 3 and 12 μM were 61.54 and 65.53%, respectively. Western blot analysis (**Fig. 2C**) and relative density (**Fig. 2D**) showed that the expression of TLR4, LPS-induced up-regulation of adaptor protein TNF receptor-associated factor 6 (TRAF6), and effector iNOS were all inhibited by PTL in a dose-dependent manner ($P < 0.01$). Although these results show that PTL down-regulates TLR4 expression, it is still unclear how this drug affects TLR4 expression. To clarify this problem, mRNA levels of TLR4 were evaluated by qRT-PCR in THP-1 cells and human primary monocytes. As shown in **Fig. 2C,E**, LPS-induced TLR4 expression at both protein and mRNA levels, and pretreatment with PTL could block TLR4 expression in a dose-dependent manner.

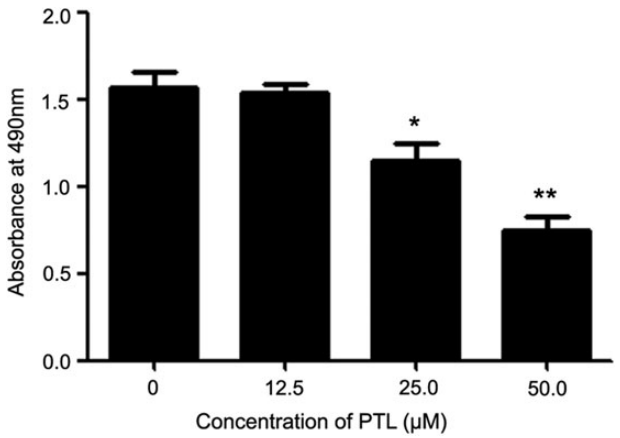

Figure 1. The effect of PTL on the viability of THP-1 cells THP-1 cells (5×10^4 cells/well) were treated with PTL (0, 12.5, 25, and 50 μM) for 24 h. Cell viability was determined by MTS assay. Data are from three independent experiments (the mean ± SD). *$P < 0.05$ vs. the control group (cells without PTL treatment), **$P < 0.01$ vs. the control group (cells without PTL treatment).

Table 1. Inhibitory effect of PTL on the LPS-induced up-regulation of inflammatory factor production

Groups						
LPS (μg/ml)	0	1	1	1	1	IC_{50}
PTL (μM)	0	0	0.75	3	12	(μM)
Cytokines						
IL-6 (pg/ml)	109.697 ± 21.035	667.273 ± 107.345	499.307 ± 38.048*	297.576 ± 33.524**	153.420 ± 18.924**	2.620
IL-1β (pg/ml)	99.308 ± 50.287	1171.006 ± 198.664	1011.258 ± 62.045	448.994 ± 79.275**	162.830 ± 52.118**	2.594
IL-8 (pg/ml)	2844.444 ± 549.972	35455.556 ± 6321.952	25381.482 ± 12573.208*	12233.331 ± 1571.348**	5011.114 ± 693.889**	1.858
IL-12p40 (pg/ml)	2.667 ± 3.214	986.909 ± 115.626	744.788 ± 103.198*	408.727 ± 67.193**	93.879 ± 20.436**	2.157
TNF-α (pg/ml)	85.455 ± 12.893	4194.545 ± 725.267	2382.424 ± 198.462**	1330.909 ± 180.048**	291.515 ± 153.898**	1.091
IL-18 (pg/ml)	10.957 ± 2.460	453.710 ± 71.199	355.304 ± 24.912*	174.000 ± 21.856**	103.275 ± 34.675**	2.518
NO (μM)	12.609 ± 13.298	730.488 ± 95.744	573.961 ± 77.048*	263.333 ± 69.338**	90.870 ± 46.648**	2.175

THP-1 cells were pretreated with PTL (0.75, 3, and 12 μM) for 2 h and further incubated in the presence or absence of LPS (1 μg/ml) for 18 h. Levels of IL-6, IL-1β, IL-8, IL-12p40, NO, TNF-α, and IL-18 were determined by ELISA. Data are from three independent experiments (the mean ± SD).

*$P < 0.05$ vs. LPS alone.

**$P < 0.01$ vs. LPS alone.

As shown in **Fig. 2F**, PTL at 3 and 12 μM could obviously inhibit TLR4 mRNA expression ($P < 0.01$). As shown in **Fig. 2G,F**, in human primary monocytes, TLR4 mRNA levels were simultaneously up-regulated after an LPS challenge. The TLR4 mRNA level from AML patients (cases 1 and 2) was slightly higher than that from the healthy volunteer (case 3), which was partly due to the activation of monocytes in AML patients. However, pretreatment with 3 μM PTL could inhibit LPS-induced TLR4 mRNA expression both in AML primary monocytes and normal primary monocytes. The result indicated that PTL also exhibited anti-inflammatory properties in human primary monocytes both in AML patients and healthy people, which was in line with the results from THP-1 cell line. These results indicated that PTL inhibits TLR4 expression

Effect of PTL on the MAPK signaling pathway in THP-1 cells

To understand the molecular mechanism by which PTL blocks various inflammatory processes, the effects of PTL on LPS-induced signaling pathways (MAPK and NF-κB) were investigated. **Figure 3A** shows that LPS stimulation triggered the activation of MAPK signaling molecules, extracellular signal-regulated kinase 1/2 (ERK1/2), Jun N-terminal kinase (JNK), and p38, as assessed by their phosphorylation levels determined by phosphor-specific antibodies. **Figure 3B** shows the relative density of those molecules. PTL blocked the phosphorylation of ERK1/2, JNK, and p38 in a dose-dependent manner (**Fig. 3B**; $P < 0.05$), without altering the total levels of these signaling molecules.

Effect of PTL on the NF-κB signaling pathway in THP-1 cells

NF-κB also plays a critical role in the initiation of inflammation. Suppression of NF-κB could be an effective method to treat inflammatory diseases. Since the p65 subunit is responsible for the transcriptional activity of NF-κB, phosphorylation of p65 was determined using western blot analysis in this study. **Figure 4A** shows that LPS stimulation triggered the activation of NF-κB p65 and IκBα, as assessed by their phosphorylation levels determined by phosphor-specific antibodies. PTL blocked the phosphorylation of NF-κB p65 and IκBα in a dose-dependent manner (**Fig. 4B**; $P < 0.05$), without altering the total levels of these signaling molecules. Thus, the NF-κB and MAPK signaling pathways play an important role in LPS-induced inflammatory response in THP-1 cells.

Discussion

TLR signaling is principally divided into two pathways: MyD88 (myeloid differentiation factor 88)-dependent and TRIF-dependent, according to the primary adaptor usage [4,5,13]. TLR4 participates in both signaling pathways. In the canonical pathway, TLR4 recruits MyD88 to the receptor complex, initiating recruitment of IRAK-1 and IRAK-4, followed by its adaptor protein TRAF6 [13]. This results in the phosphorylation of TGF-beta-activated kinase 1 (TAK1) and finally the activation of NF-κB and MAPKs to induce pro-inflammatory cytokines release. We assumed that PTL inhibits inflammatory genes and transcription factors by blocking the signaling receptor TLR4. Therefore, we chose the activation of these two signaling pathways and expression level of their target genes, for example, IL-6, IL-1β, IL-8, IL-12p40, TNF-α, IL-18, and NO as the indicator of the activation of TLR4 induced by LPS. TRAF6 activates and further phosphorylates IκB kinase (IKK)-β and MAPKs, and then plays a critical role in TLR4-mediated inflammatory response induced by LPS. Interference with the TLR-mediated MAPK and NF-κB pathways has implications for many of the important steps in anti-inflammation and anti-tumor treatments because both signaling pathways participate in the progress of inflammation and tumors [13,14]. Early data in fetal mouse lungs implicated a critical signaling mechanism (TLR4/NF-κB pathway) in chorioamnionitis [18]. The highly specific LPS-sensing function of TLR4 is remarkable for its prevalence in Mammalia, which is the only class of the phylum Chordata known to have a gene encoding TLR4 and to display sensitivity to LPS [18].

Previous studies have revealed that in cells that TLR4 is activated during the inflammatory responses, and binding of LPS to TLR4 leads to MAPKs activation and nuclear translocation of NF-κB [12,18,19]. Therefore, THP-1 cells induced by LPS could serve as an excellent model for screening the important cellular mechanisms underlying the anti-inflammation effect of PTL [20]. The mechanism by which PTL blocks LPS-induced inflammation is unclear. A few studies indicated that PTL had inhibited angiogenesis in rheumatoid arthritis through TLR2 and TLR3 signaling pathways [21,22]. However, in this study, we mainly explored the inhibitory effect of PTL on LPS/TLR4 signaling pathway. PTL can inhibit the IKK complex, which phosphorylates the NF-κB inhibitors IκBα and IκBβ, leading to their proteasomal degradation [18,19,23]. In this study, we observed the expression levels of TLR4 protein and mRNA, using flow cytometry, western blot analysis, and qRT-PCR (**Fig. 2**). LPS stimulation led to a significant up-regulation of TLR4 and TRAF6

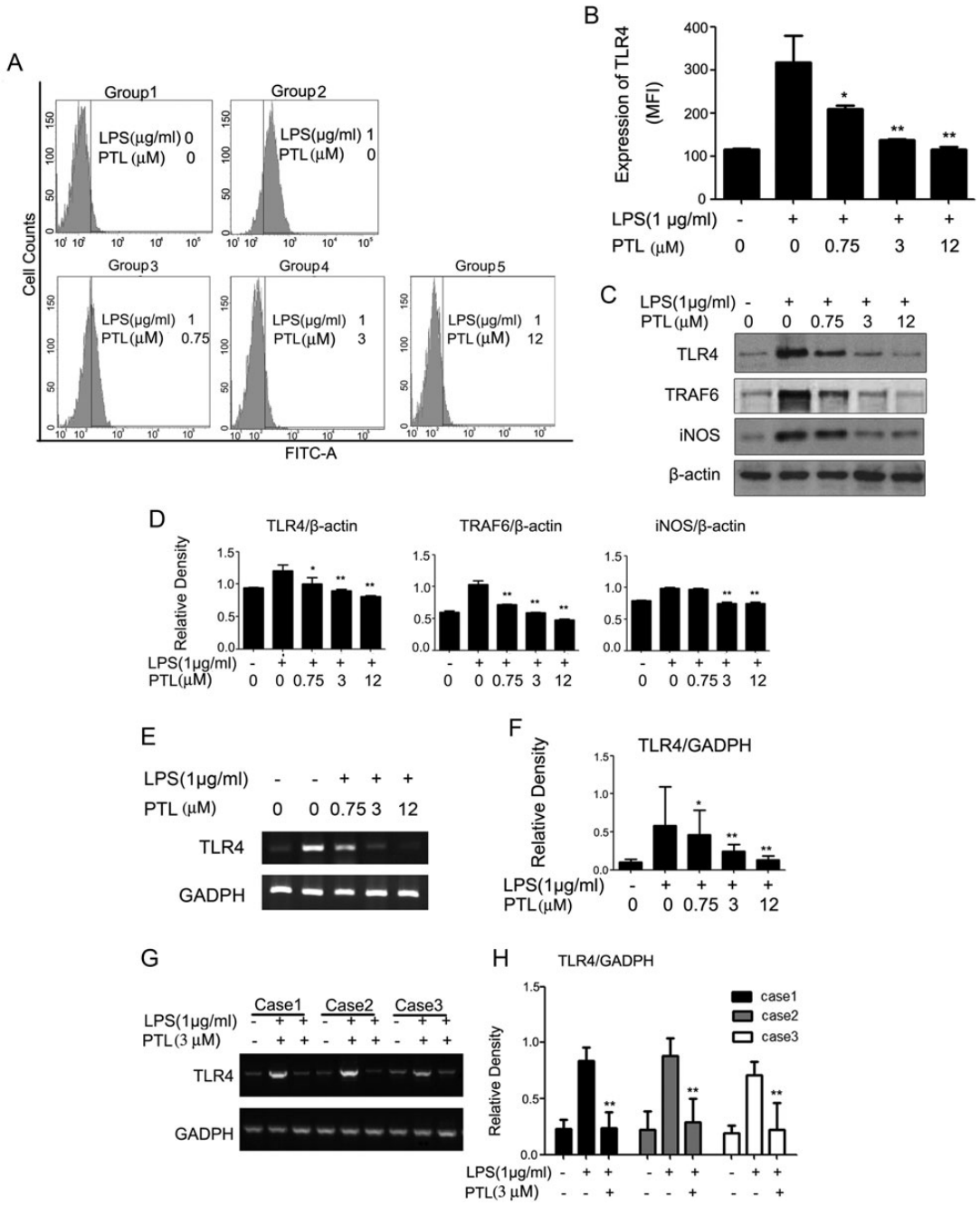

Figure 2. The effect of PTL on LPS-induced TLR4 expression THP-1 cells (5×10^5 cells/ml in 24-well plates) were pretreated with PTL for 2 h and further incubated in the presence or absence of LPS (1 μg/ml) for the indicated time, and analyzed by FACS (18 h) or western blot analysis (4 h) or qRT-PCR (4 h). Human primary monocytes (5×10^5 cells/ml in 24-well plates) isolated from two newly diagnosed AML patients (cases 1 and 2) and one healthy volunteer (case 3) were pretreated with PTL (3 μM) for 2 h and further incubated in the presence or absence of LPS (1 μg/ml) for 4 h and then analyzed by qRT-PCR. *GAPDH* served as internal control. (A,B) The effect of PTL on LPS-induced TLR4 expression determined by FACS. (C,D) The effect of PTL on LPS-induced TLR4, TRAF6, and iNOS protein expression levels detected by western blot analysis. (E,F) The effect of PTL on LPS-induced *TLR4* mRNA expression levels in THP-1 cells detected by qRT-PCR. (G,H) The effect of PTL on LPS-induced TLR4 mRNA expression levels in human primary monocytes detected by qRT-PCR. Data are representative of three independent experiments with similar results (A, C, E, and G) or are from three independent experiments (B, D, F, and H; the mean ± SD).* $P < 0.05$ vs. LPS alone, ** $P < 0.01$ vs. LPS alone.

expression levels in THP-1 cells when compared with the control group. PTL inhibited this up-regulation in a concentration-dependent manner, which was in line with previous studies [10,13,14]. Therefore, we concluded that PTL may inhibit LPS-induced inflammation through inhibition of the TLR4 signaling pathway.

Because NF-κB belongs to a downstream signaling component of TLR4, the expressions of NF-κB p65 and IκBα can reflect TLR4 activation. In line with these observations, western blot results showed that at concentrations of 3 ($P < 0.05$) and 12 μM ($P < 0.01$), PTL obviously altered the protein levels of p-NF-κB p65 and p-IκBα.

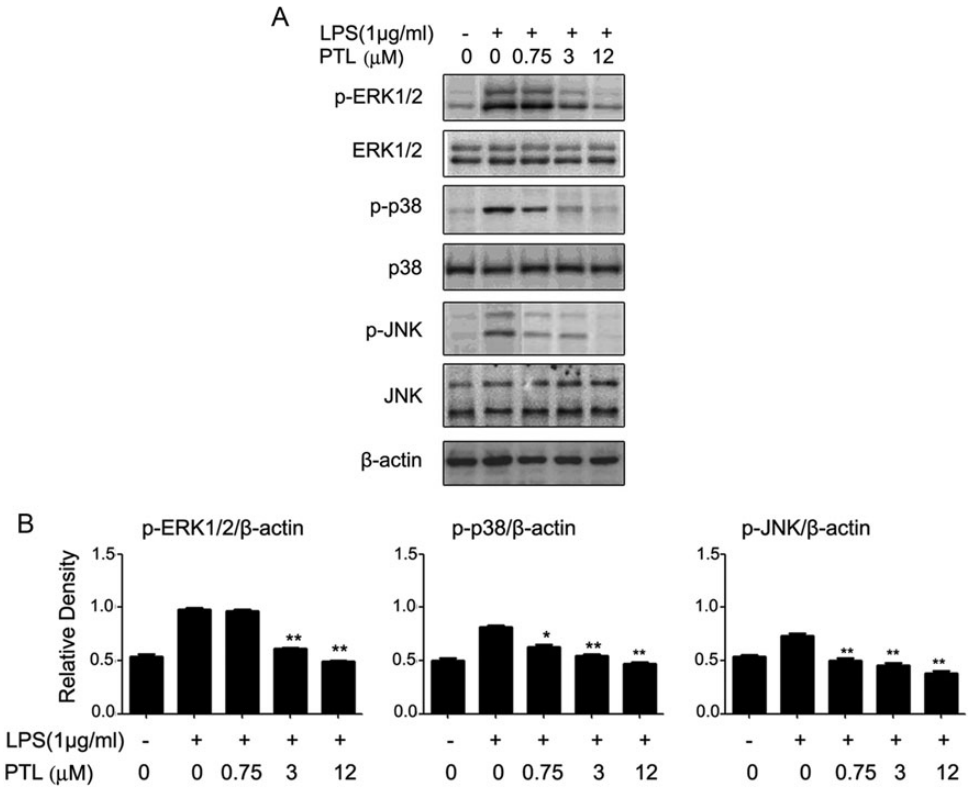

Figure 3. Effect of PTL on LPS-induced MAPKs signaling events Cells were pretreated with various concentrations of PTL in the presence or absence of LPS (1 μg/ml) for 4 h. (A) After immunoblotting, the phosphorylation of ERK1/2, JNK, p38, or their total levels was identified using phosphor-specific or non-phospho-specific antibodies. (B) The bands from western blot analysis were quantified and expressed as the ratio of p-ERK/actin, p-JNK/actin, and p-p38/actin intensities. Data are representative of three independent experiments with similar results (A) or are from three independent experiments (B; the mean ± SD). $*P < 0.05$ vs. LPS alone, $**P < 0.01$ vs. LPS alone.

Interestingly, it was found that LPS markedly increased the levels of cytokines including IL-6, IL-1β, IL-8, IL-12p40, TNFα, IL-18, and NO (Table 1), these cytokines induced by LPS can also reflect TLR4 activation after an LPS challenge. However, PTL mediated a dose-dependent inhibition of LPS-induced inflammatory cytokines in THP-1 cells. According to previous studies, NF-κB plays a critical role in inflammatory responses by regulation of iNOS protein expression level and secretion of pro-inflammatory cytokines [9,10,12]. The expressions of these pro-inflammatory cytokines are primarily regulated at the transcriptional level by the NF-κB pathway [24–26]. NO, generated by iNOS during inflammation, has various pro-inflammatory effects and is a final effector in inflammatory response [9,12]. Collectively, PTL inhibits cytokines stimulated by LPS through TLR4-mediated signaling pathway, which is indicated by that fact that LPS-induced TLR4 protein and mRNA expression are significantly blocked by PTL pretreatment.

In addition to the NF-κB signaling pathway, MAPK pathways, including ERK, p38, and c-JNK, also participate in LPS-induced cytokine release and inflammatory responses in THP-1 cells. As MAPKs are also downstream signaling components of TLR4, the expression of ERK, p38, and c-JNK can reflect the activation of TLR4 by LPS. In this study, western blot analysis (**Fig. 4**) shows that LPS increased the phosphorylation levels of ERK, p38, and JNK. However, PTL blocked the expression of these molecules in a dose-dependent manner. These results indicated that the TLR4-mediated MAPK pathway was also involved in the effect of PTL on LPS-induced inflammation. PTL could inhibit the activation of ERK, p38, and JNK stimulated by LPS/TLR4 signaling. Some studies have suggested that NF-κB and MAPKs are closely regulated by LPS-induced inflammatory cytokines [26,27]. Down-regulation of these cytokines and inhibition of related signal pathways would be of therapeutic benefit in treating inflammatory diseases [28]. TAK1, a member of the MAPK kinase kinase family, was an upstream signaling molecule of NF-κB. Studies have suggested that suppression of LPS-induced TAK1 phosphorylation results in the inhibition of NF-κB activation [29,30]. In addition, MyD88, an accessory protein of LPS-induced inflammation signaling, has been reported that SLs such as alantolactone can block its expression and play a role in LPS-induced anti-inflammation [31]. However, the effect of PTL on these molecules (TAK1 and MyD88) is still unclear. Further work is needed to clarify this point. Based on our recent data, we suggest that TLR4 may play a critical role in LPS-induced inflammation, and alterations in TLR4 expression may serve a novel window in anti-inflammatory response.

An important relationship has long been recognized between the immune system and cancer, and chronic inflammation has been implicated in many cancers, including adult carcinomas, pancreatic cancer, and colon cancer [5,32,33]. However, the molecular and cellular mechanisms mediating this relationship remain unresolved. Tumor-associated inflammation can drive tumor development by the recruitment of leukocytes, the production of cytokines and chemokines, and the consequent increase of angiogenesis, tumor cell survival and metastasis [33]. In this study, we investigated the TLR4-mediated NF-κB and MAPK signaling pathways that are involved not only in inflammatory responses but also in the progress and development of

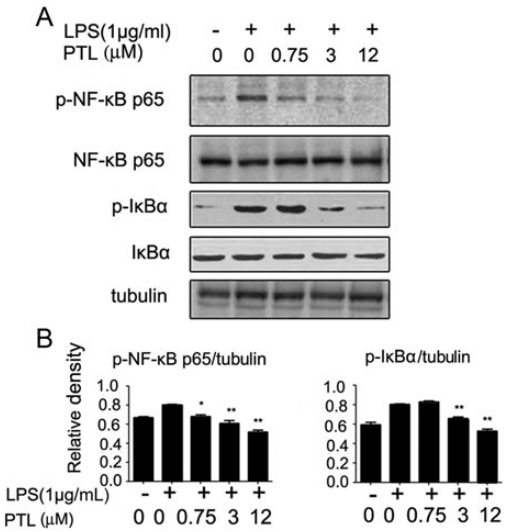

Figure 4. Effect of PTL on LPS-induced NF-κB signaling events Cells were pretreated with various concentrations of PTL in the presence or absence of LPS (1 μg/ml) for 4 h. (A) After immunoblotting, the phosphorylation of p65 and IκBα or the total protein levels was determined using phosphor-specific or non-phospho-specific antibodies. (B) The bands from western blot analysis were quantified and expressed as the ratio of p-NF-κB p65/tublin and p-IκBα/tublin intensities. Data are representative of three independent experiments with similar results (A) or are from three independent experiments (B; the mean ± SD). $*P < 0.05$ vs. LPS alone, $**P < 0.01$ vs. LPS alone.

cancer [32–34]. As shown in this study, PTL can inhibit both NF-κB and MAPK signaling activation through the LPS/TLR4 pathway, indicating that PTL could be a potential TLR4 target agent for clinical application in inflammation and cancer therapy [35]. Moreover, studies have revealed that NF-κB is constitutively active in AML cells but not in normal hematopoietic cells, and its inhibition correlates with leukemia-specific cell death, which makes PTL as a single agent in leukemia therapy due to its strong inhibitory effect on NF-κB [36–38]. Our results in human primary monocytes from AML patients were consistent with these findings. In addition, previous study has also revealed that PTL shows a high level of cytotoxicity towards B-chronic lymphocytic leukemia cells [38]. Given the fact that bacterial inflammation is a common complication in leukemia patients, PTL pretreatment may be a promising therapy for leukemia [35–38]. Taken together, PTL may play an important role in anti-leukemia process. Further studies regarding the relationships among PTL, inflammation and leukemia are important. The search for the direct targets of PTL will greatly help to better understand its pharmacological anti-inflammation and anti-cancer properties.

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