Chloroquine attenuates LPS-induced inflammatory responses through up-regulation of USP25
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Abstract

Lipopolysaccharide (LPS) is a key pathogen for sepsis, and its recognition with toll-like receptor 4 (TLR4) could activate two distinct signaling pathways, leading to activation of transcription factors including NF-κB and interferon regulatory factor 3 (IRF3). Chloroquine (CQ) has been shown to affect LPS-TLR4 colocalization and inhibit both MyD88-dependent and TRAM/TRIF-dependent pathways, while the involved mechanism is still poorly understood. Here we found that ubiquitin-proteasome system might be involved in this process. CQ increased USP25, a deubiquitinating enzyme, mRNA and protein expressions in a dose-dependent manner, which might to some degree be involved in its attenuation of LPS-induced macrophage activation. Overexpression of USP25 decreased LPS-induced inflammatory cytokines like TNF-α, IL-6 and IFN-β, while specific siRNA-mediated USP25 silencing increased TNF-α, IL-6 and IFN-β production and secretion. In addition, USP25 deletion strengthened mitogen-activated protein kinases (MAPKs) phosphorylation and IκB degradation. Moreover, USP25 interference increased NF-κB and IRF3 nuclear translocation. Taken together, our data demonstrated a new possible regulator of LPS-induced macrophage activation mediated by CQ, through up-regulation of USP25.

Key words: chloroquine (CQ); LPS; NF-κB; ubiquitin-specific protease 25 (USP25); sepsis
1. Introduction

Gram-negative bacteria and their pathogen associated molecular patterns (PAMPs) could result in systemic inflammatory response syndrome (SIRS), also known as sepsis, which could cause multiple organ dysfunction syndrome (MODS), septic shock and ultimately death (Angus and van der Poll 2013; Stearns-Kurosawa et al. 2011). Lipopolysaccharide (LPS/endotoxin), one of the most well-known and recognized triggers, ends up in the release of various inflammatory mediators and chemokines via MyD88-dependent and MyD88-independent pathways (Kawai and Akira 2010; Lu et al. 2008).

Chloroquine (CQ), an anti-malaria drug, is also widely used to treat auto-immune disorders, infectious diseases and cancer due to its autophagy inhibitor capacity (Jang et al. 2006; Zhang et al. 2015). CQ was demonstrated to activate host defense responses and block the synthesis of most pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interferon-β (IFN-β) in response to different stimuli (Nujic et al. 2012; Yang et al. 2013; Yasuda et al. 2008). However, nothing is yet known about the underlying molecular mechanisms. Over the past decade, we have studied the effect of CQ on LPS-induced macrophages activation and its detailed pharmacological mechanism. We have found that CQ could reduce the release of pro-inflammatory cytokines and affect internalization of LPS-TLR4 compounds in LPS-stimulated RAW264.7 cells (Hong et al. 2004; Wang et al. 2012). In addition, we also found that CQ dose-dependently increased SENP6 (SUMO-specific protease 6) protein expression, which has been reported to suppress LPS-induced macrophages activation, and SENP6 may be responsible...
for the decrease of TNF-α and IL-6 (Long et al. 2015).

Ubiquitin-proteasome system (UPS) is the major lysosomal-independent degradation pathway in eukaryotic cells, which is necessary for inflammation, signaling conduction, DNA damage repair and other biological activities (Coombs et al. 2011; Herrmann et al. 2005; Meiners et al. 2002; Moutzouris et al. 2010; Wang and Maldonado 2006). Ubiquitination is an ATP-dependent, post-translational modification process in which the 8kD protein ubiquitin is covalently attached to the lysine residues of substrate proteins in the sequential reaction of three different enzymes (Hershko et al. 1983; Hershko and Ciechanover 1998). Proteasome, commonly named as 26S proteasome, consists of one 20S lysis core and two 19S regulatory particles, which is responsible for degradation of ubiquitin-labeled proteins (Wang and Maldonado 2006). There has 8 different ubiquitination categories since ubiquitin has 7 lysine and 1 methionine residues to be modified (Kirisako et al. 2006; Zinngrebe et al. 2014). Among these eight ubiquitination modifications, K63 and K48 linkages have been got widely studies. K63 ubiquitination accounts for signaling transduction, whereas K48 ubiquitination has an impact on protein degradation (Chau et al. 1989; Chen and Sun 2009; Pickart 2004). So the proteasome is one, but not the only destination of these modifications. Similar to ubiquitination, the reverse process of deubiquitination through a family of deubiquitinases (DUBs) is the dissociation of ubiquitin chains from substrate proteins, which plays a critical role in many pathways converging on NF-κB and terminates excessive immune responses (Ciechanover et al. 1982). Ubiquitin-specific protease (USP), the largest and most studied family of DUB, has been drawn more and more attentions in its regulatory roles in various inflammatory responses. USP2a was identified as a regulator in
TLR/IL-1R-induced NF-κB activation through deubiquitination of TRAF6 (He et al. 2013). USP4 was shown to regulate innate immune responses (Zhou et al. 2012). USP7 was found to modify NF-κB ubiquitination level, which changed its transcriptional activity (Colleran et al. 2013). USP25 might regulate IL-17-dependent inflammation in vitro and in vivo (Zhong et al. 2012).

We have previously demonstrated that CQ attenuates LPS-induced macrophages activation, but the involved mechanism remains ambiguous. Moreover, we have found that overexpression of SENP6 mediated by miR669 after treatment with CQ decreased the release of pro-inflammatory cytokines like TNF-α and IFN-γ in mouse RAW264.7 cells (Long et al. 2015), which indicated us that SUMOylation, a ubiquitination-like process that share similar steps and biological events with ubiquitination, may be involved in the CQ-mediated termination of LPS-TLR4 pathways. In other words, whether ubiquitination is also involved in the above process? As described earlier, USPs might regulate IL-17- or TLR- dependent inflammation in vitro and in vivo (Zhong et al. 2012; Zhong et al. 2013). This prompted us to further examine whether USPs may also be involved in CQ-induced attenuation of macrophage activation. Based on these, in the present study, we further explored the effect of CQ on USPs expression and investigated its role in its anti-infection process.

2. Materials and methods

2.1. Cells

Murine macrophage-like cell line RAW264.7 cells (purchased from ATCC, Manassas, VA, USA) were maintained in DMEM medium (gibco, Grand Island, NY, USA) supplemented with 10% endotoxin-free fetal bovine serum (gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100
µg/ml streptomycin sulfate (Sigma, MO, USA) at 37°C humidified incubator with 5% CO₂.

2.2. Antibodies and reagents

The lipopolysaccharide/endotoxin (LPS) from *Escherichia coli* O55:B5, chloroquine (CQ) and 3-(4,5)-dimethylthiazole(-2-yl)-2,5-diphenyltetrazoliumromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). MG132 were purchased from MedChem Express (NJ, USA). Bortezomib (BTZ) were obtained from LC laboratories (MA, USA). The small interfering RNA(siRNA) for USP25 (sc-76830) was purchased from Santa Cruz biotechnology (CA, USA). PCR primers were synthesized by Sangog Biotech Co., Ltd (Shanghai, China). Mouse TNF-α (887324) and IL-6 (887064) ELISA kits were obtained from eBioscience Inc. (CA, USA). Mouse IFN-β (SEA222Eq) ELISA kits were purchased from Cloud-Clone Corp. (TX, USA). Primary antibodies against MAPK (including p38, ERK and JNK) (#9926S), phospho-MAPK (#9910S), NF-κB p65 (#4764S), IRF3 (#3036S) were from cell signaling technology (Beverly, MA, USA). Antibodies against USP25 (ab187156), IκB (ab32518), phospho-IκB (ab133462) were purchased from Abcam (Cambridge, UK).

2.3. Methods

2.3.1. Cytotoxicity analysis

RAW264.7 cells (1×10⁶/ml) were plated in 96-well plates and incubated overnight. Then, gradient concentrations of CQ were added for 24 hours to determine the best working dosage. Gradient concentrations of 26S proteasome inhibitor MG132 or 20S proteasome inhibitor bortezomib (BTZ) were added for 1 hour. Alternatively, for time-dependent analysis, MG132 or
BTZ were added for different hours. The culture supernatant was removed and 20µl of the MTT working solution was added to a 200µl serum-free total medium for 4 hours. The culture supernatant was removed again and 150µl of DMSO was added for the dissolution of the formazan crystals, the optical densities were then measured at 490nm using the Varioskan Flash microplate reader (thermo scientific, RA, USA).

2.3.2. Cytokine quantification using ELISA

RAW264.7 cells (5×10⁵/ml) were pretreated with MG132 or BTZ for 1 hour and stimulated by 100ng/ml LPS in the presence or absence of 20µg/ml CQ. The supernatants were harvested and the TNF-α and IL-6 levels were measured using ELISA kits according to the manufacturer's instructions. For the effect of siRNA transfection on cytokines release, cells were transfected with specific siRNA and challenged by LPS with or without CQ. Then the supernatants were harvested and stored at -20°C for analysis. TNF-α IL-6 and IFN-β levels were measured using ELISA kits according to the manufacturer's instructions.

2.3.3. RNA extraction, reverse transcription and quantitative PCR

Total RNA was isolated with RNA-simple Total RNA kit (DP419) (Tiangen biotech Co., Beijing, China) and retro-transcribed with ReverTra Ace-α-RNA easy kit (FSQ-101) (TOYOBO, Osaka, Japan). The real-time PCR assays were performed with SYBR Green PCR Master- Mix- (QPK-212) (TOYOBO, Osaka, Japan) on the MyIQ real time PCR detection system (Bio-Rad, CA, USA) according to the manufacturer's instructions. Sequences of gene-specific primers used in real-time PCR assay are listed in table 1 as follows. The housekeeping gene β-actin was used as a
control. The mRNA expressions were normalized to β-actin and the \(2^{-\Delta\Delta Ct}\) method was used to analyze as described previously (Livak and Schmittgen 2001).

2.3.4. Western blot

Briefly, cells \((1\times10^6/ml)\) pretreated with siRNA, followed by challenged with or without CQ and LPS, were washed with ice-cold PBS and lysed with lysis buffer (thermo pierce, IL, USA) plus protease inhibitors (Roche, Germany) for 30 min at 4°C. After centrifugation at 12000rpm for 10 min, the protein concentration was measured, and equal amounts of total proteins were then separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Western blot analysis was performed with rabbit anti-USP25 monoclonal antibody (1:1000, Abcam, catalog No. ab187156), rabbit anti-IκB monoclonal antibody (1:1000, Abcam, catalog No. ab32518), rabbit anti-p-IκB monoclonal antibody (1:5000, Abcam, catalog No. ab133462), rabbit anti-p38 monoclonal antibody (1:1000, CST, catalog No. 9926S), rabbit anti-ERK monoclonal antibody (1:1000, CST, catalog No. 9926S), rabbit anti-JNK monoclonal antibody (1:1000, CST, catalog No. 9926S), rabbit anti-p-p38 monoclonal antibody (1:1000, CST, catalog No. 9910S), rabbit anti-p-ERK monoclonal antibody (1:1000, CST, catalog No. 9910S), rabbit anti-p-JNK monoclonal antibody (1:1000, CST, catalog No. 9910S) or mouse anti-tubulin monoclonal antibody (1:1000, Beyotime, catalog No. AT819), followed by either anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:2000, Beyotime). Visualization was achieved with enhanced SuperSignal West Femto Maximum Sensitivity Substrate kit (thermo pierce, IL, USA) for chemiluminescence assay under a ChemiDoc XRS gel imaging system (Bio-Rad, CA, USA).
2.3.5. Cell transfection

RAW264.7 cells (4×10⁵/ml) were cultured in 6-well plates. After growth of cell fusion, 40 pmol of siRNA was mixed with transfection reagent (04476093001) (Roche, Germany) and transfected into the cells for 18-24 hours according to the manufacturer's instructions. Thereafter the cells were challenged by 100ng/ml LPS for 24 hours with or without 20µg/ml CQ pretreatment for 1 hour, and supernatants were collected for cytokine analysis. For the quantitative PCR and western blot assays, RAW264.7 cells were transfected with the USP25 siRNA and stimulated with LPS for 4 hours and then harvested. For all transfections, an empty vector (sc-36869) (Santa Cruz, CA, USA) was used as a negative control.

2.3.6. Detection of NF-κB activity using ELISA

RAW264.7 cells (1×10⁶/ml) pretreated with specific siRNA in 6-well plates were treated with 100ng/ml LPS in the presence or absence of 20µg/ml CQ for 4 or 12 hours. Then the nucleic proteins were extracted with the nuclear and cytoplasmic protein extraction kit (P0028) (Beyotime, Jiangsu, China). The DNA binding activity of NF-κB was quantified by ELISA using Trans-AM p65 (40096) Transcription Factor Assay Kit (Active Motif, Tokyo, Japan) according to the manufacturer's instructions. Briefly, each sample was added into a 96-well pre-coated with immobilized oligonucleotide containing a 5’-GGGACCTTCC-3’ binding site plates. After incubation with specific primary antibody and horseradish peroxidase - conjugated secondary antibody, visualized and detected at 450nm to quantify the capacity of NF-κB binding to target oligonucleotide using the Varioskan Flash microplate reader (thermo scientific, RA, USA).
2.3.7 Immunofluorescence assays

RAW264.7 cells were grown on confocal observation dishes, transfected with specific siRNA followed by treated with LPS in the presence or absence of CQ for 1 h. Then the cells were fixed with 4% (w/v) paraformaldehyde at room temperature for 10 min, and 0.1% Triton X-100 for 5 min was used for permeabilization. After blocked in 3% BSA for 1 h, primary antibody of NF-κB p65 (1:200, CST, catalog No. 4764S) or IRF3 (1:200, CST, catalog No. 3036S) were incubated at 4ºC overnight, followed by treatment with anti-rabbit secondary antibody labeled with fluorescence (1:250, Beyotime, catalog No. A0516) for 1 h and nuclear staining with DAPI (Beyotime, catalog No. C1005). Each dish was observed under a 780 laser confocal microscopy (Zeiss, Germany) and nuclear translocation was quantified as overlapping images using the LSM examiner software.

2.4. Statistical analysis

All experiments were performed at least three times and data were presented as mean ± standard deviation (S.D.). Statistical significance between groups was determined by an analysis of variance (ANOVA) test followed by Bonferroni test, with a value of $p<0.05$ considered to be statistically significant.

3. Results

3.1. Proteasome plays a role in LPS-induced inflammatory responses and CQ-mediated attenuation.
We have previously found that SENP6, a deSUMOylation enzyme, was overexpressed by CQ, which decreased the release of pro-inflammatory cytokines (Long et al. 2015). SUMOylation, a ubiquitination-like process, share similar steps and biological functions with ubiquitination. Taking that into consideration, we hypothesized that ubiquitination might be involved in CQ attenuation in LPS-induced macrophage activation. So we selected 26S and 20S proteasome inhibitor in order to conform this assumption. Hence, mouse macrophage RAW264.7 cells were treated with gradient concentrations of CQ to confirm its best working dose again. And cells were treated with MG132 or BTZ to determine their optimal dose. Then cells were treated for different time in order to get the longest working hour. Thereafter, TNF-α and IL-6 levels were detected by ELISA. As shown in Fig. 1D, TNF-α and IL-6 levels were reduced after proteasome inhibitor MG132 treatment compared with normal stimulation \((p<0.01)\). Interestingly, there seems to be an inconsistent effect on TNF-α and IL-6 release with BTZ treatment, which displayed substantially reduced TNF-α \((p<0.01)\) and invariable IL-6 \((p>0.05)\) release. Hence, we know that proteasome might be to some degree participated in LPS-induced inflammatory pathways and inhibition of this pathway mediated by CQ.

3.2. CQ increased some USPs mRNA expression and upregulated USP25 mRNA and protein expressions in a dose-dependent manner

Given that proteasome, one destination of the UPS, joined the LPS-TLR4 pathways and attenuation induced by CQ, accompanied with DUBs is one of the components of the UPS. We firstly examined some UPSs mRNA expressions in CQ-pretreated and LPS-stimulated Raw264.7 cells. We found that CQ slightly increased USP2, USP7, USP25 and USP28 mRNA expressions in
LPS-activated cells (Fig 2A). Considering that recent published data have considered USP25 as a possible negative regulator of LPS-TLR4 pathways (Zhong et al. 2013), we further observed the effect of CQ on USP25 mRNA and protein expressions. RAW264.7 cells were treated with gradient concentrations of CQ and then USP25 mRNA expression was assayed by quantitative real-time PCR. As the results Fig. 2B indicated, pretreatment with different dose of CQ (5µg/ml, 10µg/ml, 20µg/ml) increased USP25 mRNA expression in RAW264.7 cells (0.81 fold, 1.21 fold and 2.25 fold, \( p < 0.01 \), respectively). Simultaneously protein expressions were examined, as shown in Fig. 2B, USP25 protein expressions were enhanced gradually as increasing dose of CQ (\( p < 0.01 \)).

Next, we detected the USP25 expressions in CQ-pretreated and LPS-activated cells. We found that USP25 mRNA and protein expressions were increased as well (Fig. 2C). Thus we know that there was an increase in mRNA and protein expression levels in 20µg/ml dose. These results suggested that CQ increased USP25 expression in a dose-dependent manner, probably leading to CQ-induced inhibition of LPS-TLR4 pathways and disruption of macrophages activation.

### 3.3. Verification of USP25 gene silencing model

Since CQ increased USP25 expressions, we hypothesized that upexpression of USP25 mediated by CQ may be involved in its suppression of macrophages activation induced by LPS. In order to observe the biological role of USP25 in this process, we used specific siRNA to transfect RAW264.7 cells to knockdown its gene expression. PCR and western blot analysis were used to confirm modification of USP25 mRNA and protein expression. As shown in Fig. 3A, after siRNA transfection, USP25 mRNA relative fold expression decreased significantly (0.15-fold, \( p < 0.01 \)). Similarly, western blot analysis showed siRNA-treated cells exhibited low-expression (Fig. 3A).
Therefore, the specific interference model was established. Afterwards, this modified method was used to investigate the possibility that over-expression of USP25 mediated by CQ suppressed LPS-induced NF-κB and IRF3 transcriptional activity.

3.4. USP25 interference reversed suppression of pro-inflammatory cytokine production triggered by LPS

Pro-inflammatory cytokines as TNF-α, IL-6, and IFN-β are traditionally regarded as the marker of macrophages activation mediated by LPS (Chaudhry et al. 2013; Yao et al. 1998). We have found that CQ mediated overexpression of USP25. However, how CQ altered LPS-induced signaling pathways through overexpression of USP25 has not been elucidated. Consequently, we first examined the expression of TNF-α, IL-6, and IFN-β in siRNA-treated and LPS-activated RAW264.7 cells. We found that mRNA expressions of TNF-α, IL-6, and IFN-β were increased in USP25-knockdown cells compared to that in normal cells (35.83-fold vs. 20.92-fold, 385.35-fold vs. 125.08-fold and 41.79-fold vs. 3.63-fold, \( p < 0.01 \), respectively). In addition, release of these cytokines was detected using appropriate ELISA kits. Similar to mRNA level, as shown in Fig. 3C, depletion of USP25 exhibited increased TNF-α, IL-6, and IFN-β release levels (+25.85%, +32.54% and +50.11%, \( p < 0.01 \), respectively), suggesting that CQ-mediated overexpression of USP25 suppressed LPS-induced inflammatory responses.

3.5. siRNA-mediated USP25 silencing augmented MAPKs phosphorylation and IκB degradation

Upon stimulation with LPS, its receptor TLR4 recruits two adaptor proteins MyD88 and
TRAM to activate two different signaling pathways, leading to the activation of NF-κB and IRF3 (Akira et al. 2006; Dunne and O'Neill 2003; Kawai and Akira 2010). MyD88-dependent pathway is a cascade of signaling events involving phosphorylation of MAPKs and degradation of IκB, which is responsible for the early activation of NF-κB and AP-1 (Kawai and Akira 2010). In order to determine the effects of CQ-mediated overexpression of USP25 on LPS-TLR4 signaling pathways, we observed phosphorylation of MAPK family members including p38, ERK, JNK as well as degradation of IκB. As shown by immunoblotting, siRNA transfection increased MAPKs phosphorylation levels challenged by LPS (Fig. 3C). CQ reduced LPS-induced IκB degradation while pretreatment with a specific siRNA augmented degradation level of IκB (Fig. 4A). Consequently, knockdown of USP25 exhibited accumulated IκB phosphorylation thus increased degradation. These data further proved that USP25 silencing reversed inhibition of CQ on LPS-TLR4 pathways, indicating that overexpression of USP25 may be required for the negative control of LPS inflammatory responses mediated by CQ.

3.6. siRNA-induced USP25 knockdown increased early and late activation of NF-κB and increased nuclear translocation of NF-κB p65 and IRF3 in murine macrophages

MyD88-dependent pathway is necessary for LPS-TLR4 signaling transduction and NF-κB early activation (Akira et al. 2006; Kawai and Akira 2010). TRAM/TRIF-dependent pathway is vital for NF-κB late activation and activation of IRF3 (Akira et al. 2006; Takeuchi and Akira 2010). Early or late NF-κB activation contributes to different cytokine reactions (Kizaki et al. 2009). Based on the fact that siRNA-induced USP25 silencing increases inflammatory cytokines reactions, we further investigated whether low expression of USP25 affected LPS-induced early
and late activation of NF-κB. Our results showed that CQ effectively inhibited both early and late activation of p65 (one subunit of NF-κB), while disruption of USP25 increased early and late NF-κB p65 activation (Fig. 4B). Having determined that USP25 knockdown enhanced NF-κB p65 activation, we next verified these effects using the nuclear translocation assay. Thus we infected siRNA-transfected cells and later analysed nuclear translocation of NF-κB p65 and IRF3. We saw nuclear accumulation of NF-κB p65 and IRF3 upon LPS infection. In CQ-treated RAW264.7 cells, however, we observed reduced accumulation of NF-κB p65 and IRF3. In accordance with our cytokines and NF-κB p65 activation ELISA observations, in USP25 silencing cells, there was a visually increase of red fluorescence within nuclear compared with control siRNA-treated cells(Fig. 4C and 4D). These data indicated that CQ-mediated overexpression of USP25 might partially act as a regulator in LPS-TLR4 inflammatory responses due to its inhibition of NF-κB and IRF3 activation. In conclusion, CQ attenuates LPS-induced macrophage activation through up-regulated USP25 expression (Fig. 4E).

4. Discussion

Over the past few decades, studies have given insights into how LPS induces NF-κB activation through MyD88-dependent or TRAM/TRIF-dependent cascades, which may play a role in immune responses in inflammatory cells(Chen 2005; Stearns-Kurosawa et al. 2011). In this NF-κB pathway, a variety of protein post-translational modifications as phosphorylation, ubiquitination or SUMOylation, were found to be involved in signal transduction. UPS is believed to be responsible for the clearance of pathogens and misfolded proteins. Nowadays, its role in infection and inflammation has been receiving more attention. Multiple forms of ubiquitination
have been demonstrated to mediate recruitment of TAK and IKK complexes, resulting in NF-κB activation (Liu and Chen 2011; Xia et al. 2009). Recent studies have shown that USPs, the largest family of DUB, might serve as signaling switches to regulate TRAF protein activation in response to inflammation, which in turn suppresses NF-κB activation (He et al. 2013; Zhou et al. 2012).

In our previous studies, we have found that acidification inhibitor CQ reduced signaling molecules activation in both MyD88-dependent and TRAM/TRIF-dependent pathways, increased internalization of LPS, thus increased the amount of LPS within macrophages (Wang et al. 2012). Given that lysosomal maturation, which is necessary for clearance of LPS, was blocked by CQ, it was reasonable that CQ might inhibit LPS-induced macrophage activation in a lysosomal-independent such as ubiquitination degradation manner. Based on these, in this study, we further investigate whether the ubiquitination degradation system specifies LPS-TLR4 signaling pathways after CQ treatment.

UPS is made up of ubiquitin, various enzymes and 26S proteasome. The 26S proteasome, commonly referred to as proteasome, a sandwich-like structure formed by 2 regulatory particles and 20S proteasome inside (Wang and Maldonado 2006). Regulatory particles are responsible for the capture and transportation of ubiquitin-labeled proteins, while 20S proteasome is crucial for proteolysis (Skaug et al. 2009; Wang and Maldonado 2006). Since we have imagined UPS and ubiquitination might be involved in suppression of LPS-induced macrophage activation mediated by CQ, we examined the pro-inflammatory cytokines levels in CQ-treated and LPS-stimulated RAW264.7 cells to investigate whether proteasome inhibitor affects CQ inhibition of LPS-TLR4 signaling.
pathways or not. We found a reduced pro-inflammatory cytokines release after 26S proteasome inhibitor treatment (Fig. 1D). Surprisingly, the effect of CQ on TNF-α and IL-6 release under the treatment of BTZ seems to be unparallel. We observed a decreased TNF-α release but rather stable IL-6 release after 20S proteasome inhibitor treatment (Fig. 1D). Perhaps the various roles of 20S and 26S proteasomes in the attenuation of CQ-mediated LPS-TLR4 inflammatory responses might be a possible explanation of these inconsistency. These outcome further certified UPS do play a role in the LPS-induced inflammatory and indicated proteasome might to some degree be involved in CQ attenuation of pro-inflammatory responses. In addition, a notable decrease in the level of TNF-α and IL-6 in the supernatants after treatment with bortezomib has been seen in several papers (Amiri et al. 2004).

USP is the largest and best studied family of DUBs. We screened 4 USPs molecules in order to determine the USPs expressions under the LPS and CQ co-treatment circumstances. And we found that CQ increased USP2, USP7, USP25 and USP28 mRNA expressions slightly. USP25 is a deubiquitinating enzyme belonging to USP superfamily. USP25 was once reported to negatively regulated NF-κB activation. This is supported by the fact that USP25 was associated with TRAF3 and specifically reversed ubiquitination of TRAF3 mediated by cIAP2 (cellular inhibitor of apoptosis), which is necessary for pro-inflammatory cytokines and IFN (interferon) reactions upon LPS stimulation (Zhong et al. 2013). Based on these, since USP25 might be regarded as a negative regulator of TLR signaling, we wonder whether and to which degree USP25 might be necessary for CQ-mediated inhibition of LPS-induced macrophages activation as well.
MyD88-dependent pathway includes activation of serine/threonine kinases and NF-κB early activation while TRAM/TRIF-dependent pathway is responsible for IRF3 and late NF-κB activation, as well as late-phase activation of MAPK(Kim et al. 2006; O'Neill et al. 2003). Our previous studies have demonstrated that CQ could significantly reduce early and late NF-κB activation and inhibit degradation of IRF3(Wang et al. 2012). Here we found that CQ dose-dependently mediated up-regulation of USP25 (Fig. 2), which may in turn play a role in the negative feedback to attenuate NF-κB activation. Our data indicated that USP25 interference increased both MyD88-dependent cytokines as TNF-α and IL-6, and TRAM/TRIF-dependent cytokines as IFN-β expression as well as release in LPS-stimulated RAW264.7 cells even though cells were pretreated with CQ as usual(Fig. 3). In addition, USP25 silencing induced by siRNA transfection strengthened MAPKs phosphorylation (Fig. 3) and augmented IκB degradation (Fig. 4). Furthermore, siRNA-mediated USP25 knockdown increased NF-κB p65 subunit early and late activation, promoted NF-κB and IRF3 nuclear accumulation in macrophages upon LPS infection as well (Fig. 4). In summary, USP25 low-expression seems not only increased cytokine production, but also enhanced NF-κB and IRF3 activation, suggesting its regulatory role in CQ-mediated inhibition of both LPS-TLR4 pathways. Taken together, these results show evidence that altering USP25 expression might affect both MyD88-dependent and TRAM/TRIF-dependent pathways. What's noteworthy is that mRNA and release levels of pro-inflammatory cytokines were increased slightly after USP25 silencing. Furthermore, the strengthen degree of MAPK phosphorylation and IκB degradation was relatively unapparent. Therefore, USP25 might play a relatively small but far from significant role in this process, which represents one possible molecular mechanism where CQ inhibits LPS-induced inflammatory responses. Because USP25 regulates LPS-induced signal
transduction, and overexpression of USP25 blocked SEV-induced not only the NF-κB but also IRF3 activation (Zhong et al. 2013). On the contrary, Lin et al. published that virus infection dramatically increased production of pro-inflammatory cytokines and type I IFNs, but knockout of USP25 resulted in less of an increase in TNF-α, IL-6 and IFN-β (Lin et al. 2015). A previous study revealed that USP25 caused a decrease in several of the LPS- and virus-induced pro-inflammatory cytokines and chemokines in vitro and inhibited LPS-induced septic shock in vivo (Zhong et al. 2013). In these studies, LPS or virus was treated with variable dose and time. That is to say, the effect of USP25 in LPS or virus-induced inflammatory responses seems to be controversial. And another fact we do not neglect is USPs expressions did not increased sharply after CQ treatment. Unlike any other one of cytokines or chemokines, not until cells were challenged or CQ was added did these USPs synthesize. Thus LPS-TLR4 or other signaling pathways might be regulated not by increasing USPs expressions but via altering signaling proteins post-translational modification and their functions. The original aim of the present study was to test whether modification of USP25 expression might regulate the activation of NF-κB and IRF3 induced by LPS stimulation and pretreatment with CQ. Our data displays that USP25 might play a partial part in the CQ-mediated attenuation of LPS-TLR4 signaling, thus it will be of great interest to further explore the detailed molecular mechanism.

Conflicts of interest

The authors declare no conflicts of interest.

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Wang, Y., Yang, Y., Liu, X., Wang, N., Cao, H., and Lu, Y. et al. 2012. Inhibition of


### Table 1

**Gene-specific PCR primers design and synthesis**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Primer Sequences (5’-3’)</th>
<th>Product Length</th>
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<tr>
<td>mouse IL-6</td>
<td>Forward: 5’-CTGCAAGAGAAGCTTCCATCCAG-3’ 131bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-AGTGGTATAGACAGGCTGTTGG-3’</td>
<td></td>
</tr>
<tr>
<td>mouse IFN-β</td>
<td>Forward: 5’-TGGGTGGGATGAGACTATTGTTG-3’ 109bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CTCCACGTTCAATCTTCCCTC-3’</td>
<td></td>
</tr>
<tr>
<td>mouse TNF-α</td>
<td>Forward: 5’-CTGAACTTTCCGTTGGATCGG-3’ 122bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GGGGTGTCATGCAATTGAGA-3’</td>
<td></td>
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<td>mouse USP2</td>
<td>Forward: 5’-CAGGCTTCATGGGCTATAATCA-3’ 87bp</td>
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<td></td>
<td>Reverse: 5’-CCACCCGGTTCATCCTCATG-3’</td>
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<tr>
<td>mouse USP7</td>
<td>Forward: 5’-CCACAGGAAAGCGACTGGG-3’ 196bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GTAACAGTTGCTCCCTGATT-3’</td>
<td></td>
</tr>
<tr>
<td>mouse USP25</td>
<td>Forward: 5’-TCCGGCAGCAAGGACTAC-3’ 186bp</td>
<td></td>
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<tr>
<td></td>
<td>Reverse: 5’-ACGCGATGAGCCAGTGAAC-3’</td>
<td></td>
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<tr>
<td>mouse USP28</td>
<td>Forward: 5’-CTGCGAGAAATCACAGCCAT-3’ 171bp</td>
<td></td>
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<tr>
<td></td>
<td>Reverse: 5’-GGCTCTTACTTCTCTTACTT-3’</td>
<td></td>
</tr>
<tr>
<td>mouse β-actin</td>
<td>Forward: 5’-TGGAATCTCTGTGCCATCCATGAAAC-3’ 349bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TAAAACGCAGCTCAGTAACAGTCCG-3’</td>
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Figure Captions

Fig. 1:
Proteasome inhibitor MG132 or bortezomib (BTZ) involved in LPS-induced pro-inflammatory cytokines release and CQ attenuation of LPS-TLR4 signaling. (A) RAW264.7 cells (1×10^6/ml) were plated in 96-well plates and treated with gradient concentrations of CQ for 24 hours. After removed supernatants, 20µl of the MTT working solution (5mg/ml in PBS) was added to a 200µl serum-free total medium for 4 hours. Followed by removing supernatants again, 150µl of DMSO was added for the dissolution of the formazan crystals, the optical densities were then measured at 490nm using the Varioskan Flash microplate reader. **: \( p < 0.01 \) as compared to medium. (B and C) RAW264.7 cells (1×10^6/ml) were plated in 96-well plates and treated with gradient concentrations of MG132 or BTZ for 1 hour (B) or the best working dose of MG132 or BTZ for various time (C). MTT and DMSO were added in sequence, and the optical densities were then measured at 490nm using the Varioskan Flash microplate reader. **: \( p < 0.01 \) as compared to medium. (D) Cells were pretreated with MG132 or BTZ for 1 hour and stimulated with 100ng/ml LPS for 24 hours in the presence or absence of 20µg/ml CQ. The supernatants were harvested. The TNF-\( \alpha \) and IL-6 levels were measured and data were expressed as mean ± SD. ns: no-significant, **: \( p < 0.01 \) vs the same treatment without inhibitor.
Fig. 2:

USP25 expression was increased by treated with CQ in a dose-dependent manner and upregulated in LPS-stimulated RAW264.7 cells. (A) Raw264.7 cells were treated with 20µg/ml CQ for 1 hour, and then treated with 100ng/ml LPS for 2 hours. Total RNA was extracted, and real-time PCR was performed according to the manufacturer's instructions. The comparative Ct method with arithmetic formulae($2^{-\Delta\Delta CT}$) was used to determine relative quantification of gene expression. The histogram represented the data normalized by β-actin from three independent experiments. *: p<0.05, **: p<0.01. (B) Cells were treated with gradient concentrations of CQ, total RNA was extracted, and real-time PCR was performed to investigate USP25 mRNA expression according to manufacturer's instructions. Comparative Ct method with arithmetic formulae($2^{-\Delta\Delta CT}$) was used to determine relative quantification normalized by β-actin. At the same time, whole cell extracts were prepared, and the protein concentration was measured. USP25 protein expression was examined using immunoblotting analysis. Tubulin was used as a control. Data were presented from at least three independent experiments. **: p<0.01 as compared to 0ug group. (C) Cells were treated with 100ng/ml LPS in the presence or absence of 20µg/ml CQ, total RNA was extracted, and real-time PCR was performed to test its mRNA expression. Similarly, whole cell extracts were prepared, and examined for protein expression. *: p<0.05, **: p<0.01 as indicated.
Fig. 3:

siRNA-mediated USP25 interference increased TNF-α, IL-6, and IFN-β mRNA expressions and release as well as MAPKs phosphorylation in CQ-treated and LPS-stimulated RAW264.7 cells. (A) RAW264.7 cells were incubated overnight, supernatants were discarded, followed by replacement with serum-free Opti-MEM transfection medium and transfection with 80pmol of siRNA simultaneously. Real-time PCR was performed to verify gene knockdown of USP25 at the mRNA level. 2^ΔΔCt method was used to determine relative expression. Western blot analysis was conducted to certify reduced expression of USP25 at the protein level. PCR and WB results also showed the reduced mRNA and protein expressions in siRNA-transfected group. Data were presented from at least three independent experiments. ns: *p* >0.05 compared to mock, **: *p* <0.01 compared to negative control. siRNA-mediated USP25 deletion increased TNF-α, IL-6, and IFN-β expression and release in CQ-treated and LPS-stimulated RAW264.7 cells. (B and C) The cells were transfected with USP25 siRNA and later challenged with LPS in the presence or absence of CQ. The mRNA expressions of TNF-α, IL-6, and IFN-β (B) were assayed by real-time PCR and normalized by β-actin. The TNF-α IL-6 and IFN-β levels in the supernatants were measured using appropriate ELISA kits (C) and data were expressed as mean ± SD from at least three independent experiments. **: *p* <0.01 vs L group, ns: no-significant, #: *p* <0.05, #:*: *p* <0.01 vs L+C group. (D) Cells were transfected with siRNA followed by stimulation with LPS with or without CQ. Whole cell extracts were prepared, and the protein concentration was measured. The expressions of MAPK family members p38, ERK, JNK as well as phosphorylation level of these proteins were examined. Abbreviation : M - medium, L - LPS only, L+C - LPS+CQ
Fig. 4:

USP25 silencing augmented IκB degradation, enhanced NF-κB p65 early and late activation and induced nuclear accumulation of NF-κB and IRF3 in macrophages upon LPS infection. (A) RAW264.7 cells were transfected with siRNA followed by stimulation with LPS in the presence or absence of CQ. Whole cell extracts were prepared, and the protein concentration was measured. The IκB and p-IκB expressions were detected. Tubulin was used as a control. (B) RAW264.7 cells were transfected with specific siRNA for 48 hours. Cells were then treated with CQ for 1 hour, followed by infection with LPS for 4 or 12 hours. Nuclear protein was extracted, and the DNA binding activity of NF-κB p65 was detected by appropriate transcription factor ELISA kit. Values were presented as mean ± SD. ##: p<0.01 vs LPS only at the same time point; ns: no-significant, **: p<0.01 vs L+C group at the same time point. (C and D) RAW264.7 cells were transfected with siRNA. Then cells were infected with LPS in the presence or absence of CQ for 1 hour. Followed by fixed and stained for NF-κB and IRF3, and CY3 (red) labeled secondary antibody was used for imaging. DAPI (blue) was used for nuclear imagination. Independent experiments were performed at least three times and representative results were presented. (E) CQ might attenuate LPS-induced macrophage activation partially through up-regulated USP25 expression.

Abbreviation : M - medium, L - LPS only, L+C - LPS+CQ
Proteasome inhibitor MG132 or bortezomib (BTZ) involved in LPS-induced pro-inflammatory cytokines release and CQ attenuation of LPS-TLR4 signaling.

(A) RAW264.7 cells (1×10⁶/ml)
252x178mm (300 x 300 DPI)
USP25 expression was increased by treated with CQ in a dose-dependent manner and upregulated in LPS-stimulated RAW264.7 cells.

(A) Raw264.7 cells were treated

197x135mm (300 x 300 DPI)
siRNA-mediated USP25 interference increased TNF-α, IL-6, and IFN-β mRNA expressions and release as well as MAPKs phosphorylation in CQ-treated and LPS-stimulated RAW264.7 cells.

(A) RAW264.7 cells were incubated with siRNA7 and treated with CQ and LPS.
USP25 silencing augmented IkB degradation, enhanced NF-κB p65 early and late activation and induced nuclear accumulation of NF-κB and IRF3 in macrophages upon LPS infection.
(A) RAW264.7 cells were transf