*Original Research*

# Triterpenoid CDDO-IM protects against lipopolysaccharide-induced inflammatory response and cytotoxicity in macrophages: The involvement of the NF-κB signaling pathway

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## Impact Statement

Septic shock, a severe form of sepsis, continues to have a very high mortality rate. Approximately 18 million people worldwide are affected by this deadly disease annually. Currently, no viable treatment method is available to combat sepsis. Lipopolysaccharide (LPS), also known as endotoxin, is well known to cause sepsis. This study provides convincing evidence that LPS-induced inflammation and cytotoxicity in macrophages can be ameliorated via CDDO-IM (2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline), a novel triterpenoid compound. CDDO-IM also inhibits activation of NF-kB transcriptional activity induced by LPS, indicating the involvement of the NF-κB signaling. This study may contribute to the advancement of our understanding of treating life-threatening inflammatory diseases such as sepsis.

## Abstract

Lipopolysaccharide (LPS), also known as endotoxin, can trigger septic shock, a severe form of inflammation-mediated sepsis with a very high mortality rate. However, the precise mechanisms underlying this endotoxin remain to be defined and detoxification of LPS is yet to be established. Macrophages, a type of immune cells, initiate a key response responsible for the cascade of events leading to the surge in inflammatory cytokines and immunopathology of septic shock. This study was undertaken to determine whether the LPS-induced inflammation in macrophage cells could be ameliorated via CDDO-IM (2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline), a novel triterpenoid compound. Data from this study show that gene expression levels of inflammatory cytokine genes such as interleukin-1 beta (IL-1β), interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) were considerably increased by treatment with LPS in macrophages differentiated from ML-1 monocytes. Interestingly, LPS-induced increase in expression of pro-inflammatory cytokine levels is reduced by CDDO-IM. In addition, endogenous upregulation of a series of antioxidant molecules by CDDO-IM provided protection against LPS-induced cytotoxicity in macrophages. LPS-mediated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) transcriptional activity was also noted to decrease upon treatment with CDDO-IM in macrophages suggesting the involvement of the NF-κB signaling. This study would contribute to improve our understanding of the detoxification of endotoxin LPS by the triterpenoid CDDO-IM.

**Keywords:** Lipopolysaccharide (LPS), endotoxin, macrophages, inflammation, detoxification, CDDO-IM

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## Introduction

Lipopolysaccharide (LPS), also known as lipoglycan, is composed of large lipid and polysaccharide molecules. LPS is referred to as an endotoxin. These molecules are embedded in the outer membrane of Gram-negative bacteria, which are bacteria that contain a thin peptidoglycan cell wall between the inner and outer membranes of the bacterium. These bacteria are ubiquitous and some examples include *Escherichia coli, Salmonella*, and cyanobacteria.[1] LPS is a portion of the cell membrane of bacteria and is released in response to damage of the cell wall and is mainly involved in activating cells, such as macrophages.[2] Previous studies have shown that LPS can stimulate a cascade of events leading to the synthesis and activation of multiple cytokines, chemokines, and antimicrobial polypeptides.[2–5]

Inflammation is a product of several biochemical responses caused by injury to cells and tissue. These responses include vascular migration and leukocyte activation. During inflammation, lymphocytes and macrophages are mainly involved

in suppressing and eliminating the threat. The primary focus of the initial immune response is the ability of immune cells to recognize foreign substances. Macrophages are specialized immune cells differentiated from circulating monocytes. Due to the extreme diversity of infectious microbes as well as tissue structure, it is very reasonable to say that macrophages contain one of the most evolved phenotypic characteristics.[3,6–8] These specialized cells receive various cues from the surrounding environment which allows them to assume different functions and phenotypes.[9] The specificity and complexity of macrophages allow these immune cells to respond in a precise and very specific manner to different stimuli in different tissue types. Macrophages are responsible for digestion of foreign substances through phagocytosis by recognizing LPS present on the surface of certain bacteria.[3,6–8,10]

Triterpenoids are naturally occurring compounds extracted from plants. These compounds are steroid-like and are known for their medicinal properties.[11] 2-Cyano-3,12-dixooleana-1,9-dien-28-imidazolide (CDDO-IM)[12] is a triterpenoid compound and has been used because of its use as an anti-inflammatory as well as an antioxidant agent.[13,14] CDDO-IM has been used in liver cells at nanomolar levels to study its antioxidant effects and it has been found that liver cells treated with CDDO-IM have increased levels of NAD(P)H dehydrogenase quinone 1 (NQO1), an antioxidant enzyme.[13,15] Macrophages have been suggested to play an essential role in initiating the cascade of inflammatory cytokines induced by endotoxin LPS. However, the role of CDDO-IM in LPS-induced inflammation and cytotoxicity in macrophages remains unknown. We found that through endogenously upregulating antioxidants in macrophages by CDDO, the levels of several inflammatory cytokines are significantly reduced. Also, CDDO contains cytoprotective properties that significantly increase cell viability which was decreased by LPS-mediated toxicity.

## Materials and methods

### Cell culture and growth

ML-1 monocytes are human immune cells derived from the leukemia cell line. These leukocytes play important roles in the immune system through phagocytosis, cytokine synthesis, and antigen presentation. Macrophages, which are differentiated from monocytes, are mainly involved in phagocytosis of cellular debris and microbes, and any organism that does not contain the specific antibodies on its surface can be recognized by macrophages. ML-1 monocytes was cultured in T-150 flasks containing RPMI media with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics. Cells were split when density reached approximately $6 \times 10^5$ cells/mL media. Because monocytes float in the media, cells can be split by simply obtaining the desired number of cells in a conical tube and resuspending the cells in fresh media where cell density is approximately $1.5 \times 10^5$ cells/mL.

### ML-1 differentiation into macrophages

Approximately 20 million ML-1 monocytes were counted and treated with 0.3 ng/mL of 12-O-tetra-decanoylphorbol (TPA) in 60 mL of RPMI complete media for 72 h. After 72 h, the old media containing the TPA and non-adhering cells, including monocytes, were removed and replaced with fresh media leaving only the adhering macrophages. Macrophages were cultured for an additional 72 h so that most cells would become non-adherent. Following this 6-day differentiation process, the macrophages were ready for treatment.

### CDDO-IM treatment

1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imida-zole (CDDO-IM) is a chemical used to suppress the proliferation of cancer cells, including human leukemia and breast cancer cells. CDDO-IM can be used to endogenously enhance the glutathione (GSH) levels as well as other antioxidants in cells. Initially, macrophages were treated with various concentrations of CDDO-IM ranging from 100 to 800 nM. Approximately six million cells were treated with CDDO-IM per petri dish for 24 h. Following this, treatment cells were collected for antioxidant enzyme studies.

### Glutathione and NAD(P)H: quinone oxidoreductase 1 assays

Macrophages differentiated from ML-1 monocytes were treated with various concentrations of CDDO-IM described previously. After 24 h, cells were collected in a 15 mL conical tube using a cell scraper as some macrophages float in the media and some macrophages adhere to the petri dish. The cells were centrifuged at 250×*g* for 7 min at 4℃. The pellet of cells was suspended in 1 mL phosphate buffer saline (PBS). The cells were centrifuged at 250×*g* for 5 min, and the resulting pellet was resuspended in 300 µL sterile potassium phosphate tissue buffer containing 2 mM ethylenediaminetetraacetic acid (EDTA). Sonication carried out at 15-s intervals three times was used to break the cell membranes and release the protein lysates into the supernatant, which was collected for further tests.

Total protein concentration was measured using the lysate collected previously. For this measurement, 4 µL of lysate was mixed with 796 µL of Bio-Rad® Protein assay dye reagent concentrates. The absorbance was compared with a 1.48 mg/mL bovine serum albumin (BSA) standard to determine total protein concentration. This mixture was vortexed and transferred to cuvettes for analysis. The Beckman-Coulter® DU-800 spectrophotometer was used to measure absorbance at 595 nm.

For the glutathione assay, the lysate from macrophages treated with CDDO-IM was collected as described previously. In brief, 10 µL of the sample lysate was mixed with 12.5 µL meta-phosphoric acid and 0.1M sodium phosphate buffer at pH 8.0 (GSH buffer). The mixture was centrifuged at 13,000×*g* for 5 min at 4℃. After that, 10 µL of the supernatant was mixed with 0.1 mL o-phthalaldehyde and 1.89 mL of GSH buffer per sample and was incubated for 15 min at room temperature. GSH activity was measured using fluorescence intensity by excitation at 350 nm and emission at 420 nm. Using a standard curve devised by our lab, the GSH content was calculated.

NAD(P)H: quinone oxidoreductase 1 (NQO1) activity was detected using the Beckman-Coulter® DU-800

spectrophotometer. Briefly, 20 mL of 50 mM Tris-HCL buffer (NQO1 buffer), 60 µL of 50 mM nicotinamide adenine dinucleotide phosphate (NADPH), and 80 µL of dichlorophenolindophenol (DCPIP) were mixed to create a reaction mixture. To an assay cuvette, 6 µL of sample lysate was added to 694 µL of reaction mixture. NQO1 activity was measured by DCPIP reduction rate measured at 600 nm.

### MTT assay

Macrophages were grown in 24-well Costar® treated plates in 0.5 mL/well RPMI media at a density of $8 \times 10^4$ cells/well. Half of the plate (12 wells) were treated with 400 nM CDDO-IM dissolved in DMSO and the remaining 12 wells were treated with DMSO as a control for 24 h at 37℃ in 5% $CO_2$ incubator. Following the 24-h CDDO-IM treatment, three control wells were treated with 200 ng/mL LPS and three CDDO-IM-treated wells were treated with 200 ng/mL LPS. Three control wells were treated with 400 ng/mL LPS and three CDDO-IM-treated wells were treated with 400 ng/mL LPS. Macrophages underwent LPS treatments for a further 3 h. Following this treatment, cell viability was determined using 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) for 3.5 h. Following the MTT treatment, macrophages were collected in 2 mL microcentrifuge tubes and washed with PBS. Cells were centrifuged at 200×*g* for 6 min and the supernatant was removed and washed with 0.5 mL PBS and centrifuged again at 1200×*g* for 6 min. The supernatant was removed and 175 µL of MTT reagent containing 10% DMSO, 40% isopropanol, 50% diethylpyrocarbonate (DEPC) water was transferred to a 96-well plate. Afterward, the plate was covered and placed on a shaker for 5 min at low speed to dissolve the formazan crystals. Once the formazan crystals were dissolved, absorbance was measured at 570 nm test cell viability. This assay will use the Bio-Tek® Synergy 2™ plate reader for quantification and analysis.

### Quantitative real-time polymerase chain reaction

Macrophages were treated with the appropriate concentrations of CDDO-IM and LPS and RNA was extracted from the cells using TRIzol™ reagent. Purified RNA was diluted in 15 µL RNase-free diethylpyrocarbonate (DEPC)-treated water. The concentration and purity of the RNA were measured using the Nanodrop UV–Vis spectrophotometer. RNA concentrations from all treatments were normalized to 500 ng/µL. cDNA from the RNA was synthesized using reverse transcriptase. The reagents for cDNA synthesis are as follows: 14.875 µL DEPC-treated water, 5 µL 5× First Strand Buffer, 1.25 µL deoxynucleotide triphosphate (dNTP) solution, 1.25 µL Random Primers, 0.625 µL Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), and 2 µL RNA.

Following cDNA synthesis, genes of interest were targeted using the appropriate forward and reverse primers. Target genes included interleukin (IL)-8, IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The Applied Biosystems™ StepOnePlus™ Real-Time PCR System ran for 40 cycles at the following settings: 95℃ for 15 s, 58℃ for 1 min, and 60℃ for 15 s. Comparative threshold cycle (CT) values were used to quantify the gene expression.

The primer sequences are listed as followed: GAPDH (Forward: 5′-AGA ACG GGA AGC TTG TCA TC -3′, Reverse:5′- GGA GGC ATT GCT GAT GAT CT-3′); IL-8 (Forward: 5′-CTC TGT GTG AAG GTG CAG TT-3′, Reverse:5′-AAA CTT CTC CAC AAC CCT CTG-3′); IL-1β (Forward: 5′-CAG CCA ATC TTC ATT GCT CA-3′, Reverse: 5′-GGA GGC ATT GCT GAT GAT CT -3′); TNF-α (Forward: 5′-CTA TCT GGG AGG GGT CTT CC-3′, Reverse:5′-GGT TGA GGG TGT CTG AAG GA-3′); HO-1 (Forward: 5′-CAC GCA TAT ACC CGC TAC CT-3′, Reverse: 5′-CCA GAG TGT TCA TTC GAG CA-3′); MCP-1 (Forward: 5′-TTC CTC CAC CAC CAT GCA G-3′, Reverse: 5′-CCA GCC GGC AAC TGT GA-3′); GCLC (Forward 5′-ACCATCATCAATGGGAAGGA-3′, Reverse: 5′-GCGATAAACTCCCTCATCCA-3′); NQO1 (Forward:5′-TTACTATGGGATGGGGTCCA-3′, Reverse: 5′-TCTCCCATTTTTCAGGCAAC-3′); CD206 (Forward: 5′-CCT ACT GGA CAC CAG GCAAT-3′, Reverse: 5′-CAACCCAGTCCGTTTTTGAT-3′); glutathione reductase (GR; Forward: 5′-CAG TGG GAC TCA CGG AAGAT-3′, Reverse: 5′-AAA CCC TGC AGC ATT TCATC-3′); and GCLM (Forward:5′-CTCCCTCTCGGGTCTCTCTC-3′, Reverse: 5′-GCGATAAACTCCCTCATCCA-3′). The mean quantities of genes of interest listed above were normalized based on the mean of housekeeping gene GAPDH.

### NF-kB RAW 264 Renilla luciferase assay

Approximately $1 \times 10^5$ macrophages were grown in 6-well plates for 24 h. This was followed by a 24-h 400 nM CDDO-IM treatment. Following the CDDO-IM treatment, cells were added 200 ng/mL LPS for 6 h. Following this treatment, media was removed from the cells and cells were washed twice with 0.5 mL PBS. Following this, 0.5 mL of PBS was added into each well and 0.5 µL of coelenterazine (1 mM) was dissolved in the PBS in each well. The luciferase reaction was measured using the Biotech Synergy 2 plate reader.

## Results

### Human ML-1 monocytes differentiation

Macrophages were differentiated from human ML-1 monocytes using 0.3 ng/mL TPA for 72 h. Following this treatment, gene expression levels of CD206 were measured. CD206 is a receptor protein that is abundantly expressed on the membranes of macrophages but this protein is less expressed in monocytes. A significant increase in CD206 expression levels was analyzed in macrophages where there was an approximately eightfold increase in the expression of the CD206 gene (Figure 1).

### Induction of GSH and NQO1 by CDDO-IM

The activities of glutathione (GSH) (Figure 2(a)) and NQO1 (Figure 2(b)) were measured in macrophages treated with varying concentration of CDDO-IM ranging from 100 to 800 nM. There was a significant increase in GSH and NQO1 activity in macrophages treated with 100, 200, 400, and 800 nM CDDO-IM for 24 h.

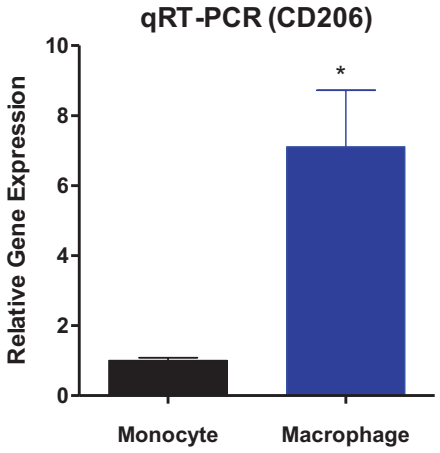


**Figure 1.** CD206 response to TPA treatment in ML-1 monocytes. TPA increases the expression levels of CD206, a biomarker for the differentiation of ML-1 monocytes to macrophages. ML-1 monocytes were treated with 0.3 ng/mL TPA for 72 h which was followed by replacement of old media with fresh media, followed by re-culturing of cells for 72 h. The relative CD206 mRNA was evaluated by real-time PCR and data were normalized using GAPDH as the housekeeping gene (data represent mean ± standard errors of the mean, $n = 3$. *$p < 0.05$ vs monocyte). Student's $t$ tests were performed to obtain statistical significance ($p$ value). (A color version of this figure is available in the online journal.)

## CDDO-IM treatment induces antioxidant molecule gene expression

Gene expression levels of various antioxidant molecules involved in the reactive oxygen species (ROS) scavenging pathways were studied. Gamma-glutamylcysteine ligase (GCL) is a key enzyme for the synthesis of GSH. The enzyme has two subunits, GCLC and GCLM. Macrophage cells differentiated from ML-1 monocytes were treated with 400 nM CDDO-IM at different time points from 3 to 24 h and the relative gene expression of GCLC and GCLM was measured. The results of quantitative real-time polymerase chain reaction (qRT-PCR) showed that GCLC was significantly induced after 3–24 h of treatment ($p < 0.05$) (Figure 3(a)), and the gene expression of GCLM increased significantly after 6–24 h of treatment (Figure 3(b)). Both genes showed peaks after 12 h of CDDO-IM treatment. For GCLC, there was an approximate 2.5-fold significant increase in gene expression levels with respect to the control after 12 h of CDDO-IM treatment (Figure 3(a)). There was an approximately 30-fold increase in GCLM levels after 12 h of CDDO-IM treatment (Figure 3(b)). Therefore, the 12-h time point was chosen to study the response of cells to other antioxidant genes, including NQO1, HO-1, and GR, after 400 nM CDDO-IM treatment. NQO1 gene expression levels were significantly increased in response to CDDO-IM treatment. An approximately 16-fold increase in NQO1 gene expression was analyzed in macrophages treated with 400 nM CDDO-IM for 12 h (Figure 3(c)). Expression levels of HO-1 were significantly increased as well upon treatment with CDDO-IM (Figure 3(d)). There was an approximately 850-fold increase in the gene expression level of HO-1 (Figure 3(d)). GR expression levels were also significantly increased in response to CDDO-IM treatment where an approximately 3.5-fold increase was measured in macrophages (Figure 3(e)).

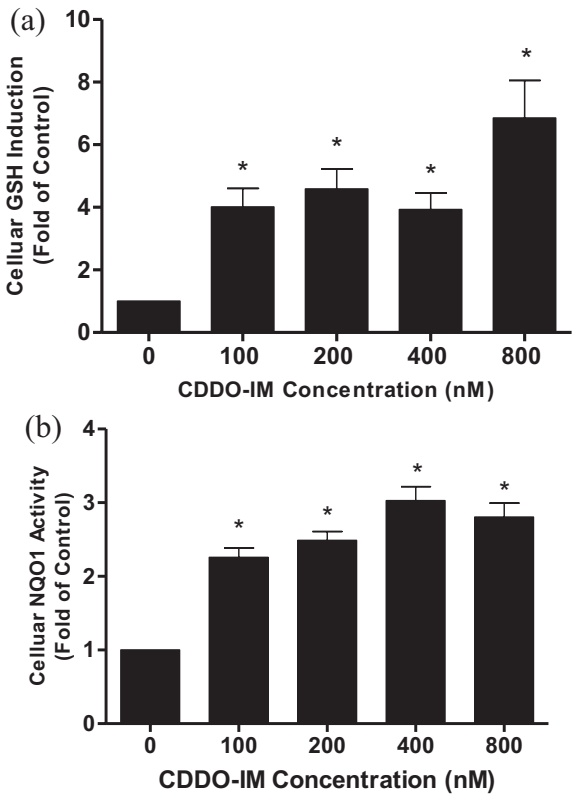


**Figure 2.** Antioxidant enzyme activity response to CDDO treatment. (a) Glutathione (GSH) levels and (b) NQO1 activity in macrophage cells differentiated from ML-1 monocytes in response to 24-h CDDO-IM treatment. CDDO-IM concentrations ranged from 100 to 800 nM (data represent mean ± SEM, *$p < 0.05$ vs control). The differences between treatments were measured by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test.

## CDDO-IM inhibits LPS-induced expression of pro-inflammatory cytokines

As shown in Figure 4, LPS significantly increased the expressions of pro-inflammatory cytokines such as IL-8, IL-1β, TNF-α, and MCP-1 in macrophages differentiated from ML-1 monocytes. IL-8 levels were significantly reduced in cells treated with CDDO-IM and LPS (400 nM CDDO-IM and 100 ng/mL LPS) compared with treatment with LPS at 100 ng/mL only (Figure 4(a)). IL-1β and TNF-α levels were also significantly reduced when treated with CDDO-IM and LPS (Figure 4) compared with treatment with LPS only. As shown in Figure 4(d), for the pro-inflammatory gene expression of MCP-1, the LPS treatment group showed about 24-fold change, while the LPS + CDDO-IM treatment group showed only a 14-fold change compared with the control group. However, compared with the LPS treatment only, CDDO-IM treatment did not significantly affect the LPS-induced MCP-1 gene expression (Figure 4(d), $p = 0.07$). These results suggest that CDDO-IM possess the anti-inflammatory activities necessary to regulate LPS-induced inflammation.

## CDDO-IM protects against LPS toxicity

The cytoprotective role of CDDO-IM was measured using the MTT assay. As shown in Figure 5(a), macrophages

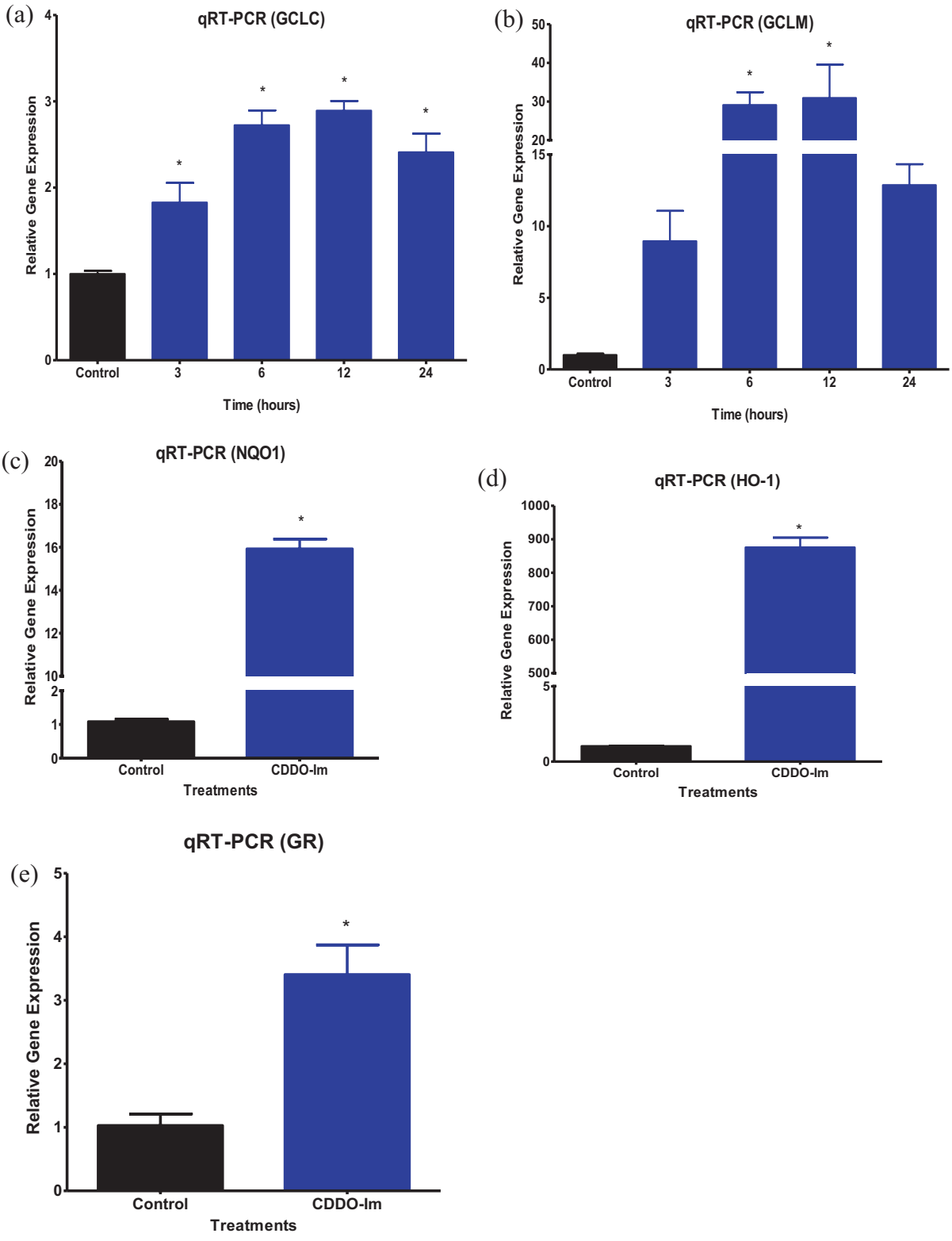


**Figure 3.** Gene expression levels of ROS detoxification molecules in macrophages differentiated from ML-1 cells. (a) and (b) Gene expression levels of GCLC and GCLM involved in the synthesis of GSH, were significantly increased in response to treatment with 400 nM CDDO-IM for various times. (c) to (e) NQO1, HO-1, and GR gene expression were also significantly increased in the in response to similar CDDO-IM treatment for 12 h. The relative mRNA was evaluated by real-time PCR and data were normalized using GAPDH (data represent mean ± standard errors of the mean, $n = 3$. *$p < 0.05$ vs control). The differences between treatments were measured by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. (A color version of this figure is available in the online journal.)

treated with 200 and 400 ng/mL LPS resulted in a significant decrease in cell viability. However, there was a significant increase in cell viability in macrophages when co-treated with 200 ng/mL LPS and 400 nM CDDO-IM with respect to cells treated with only 200 ng/mL LPS (Figure 5(b)).

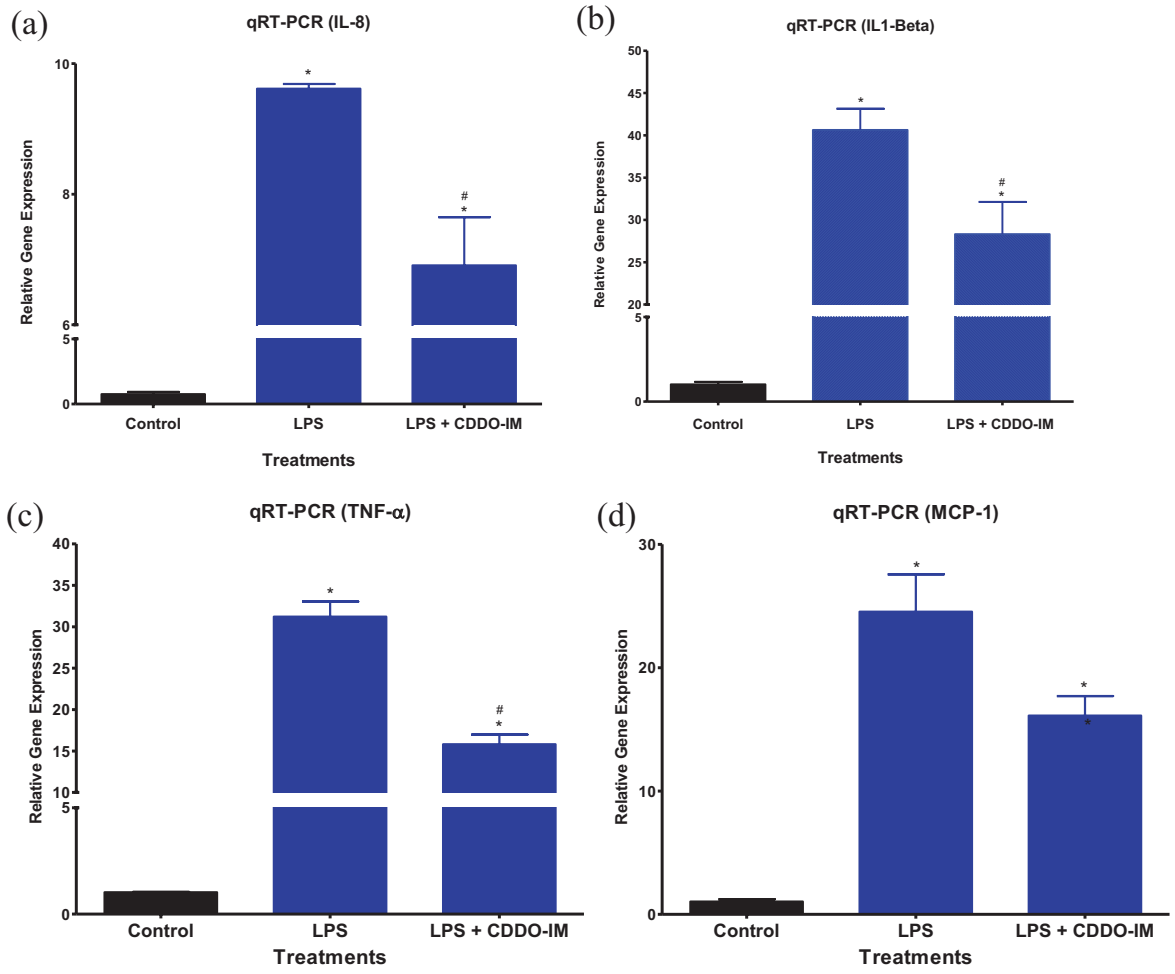


**Figure 4.** Pro-inflammatory cytokine expression response to LPS/CDDO-IM treatment. Co-treatment with CDDO-IM in macrophages resulted in a significant reduction in the upregulation of pro-inflammatory cytokines by LPS in the macrophages differentiated from ML-1 cells. Cells were treated with 400 nM CDDO-IM for 24 h, followed by a 3-h 100 ng/mL LPS treatment. Gene expression levels of (a) IL-8, (b) IL-1β, (c) TNF-α, and (d) MCP-1 were measured by real-time PCR and data were normalized using GAPDH. Data represent mean ± SEM ($n = 3$, $^{*}p < 0.05$ vs control; $^{\#}p < 0.05$ vs LPS only). The differences between treatments were measured by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. (A color version of this figure is available in the online journal.)

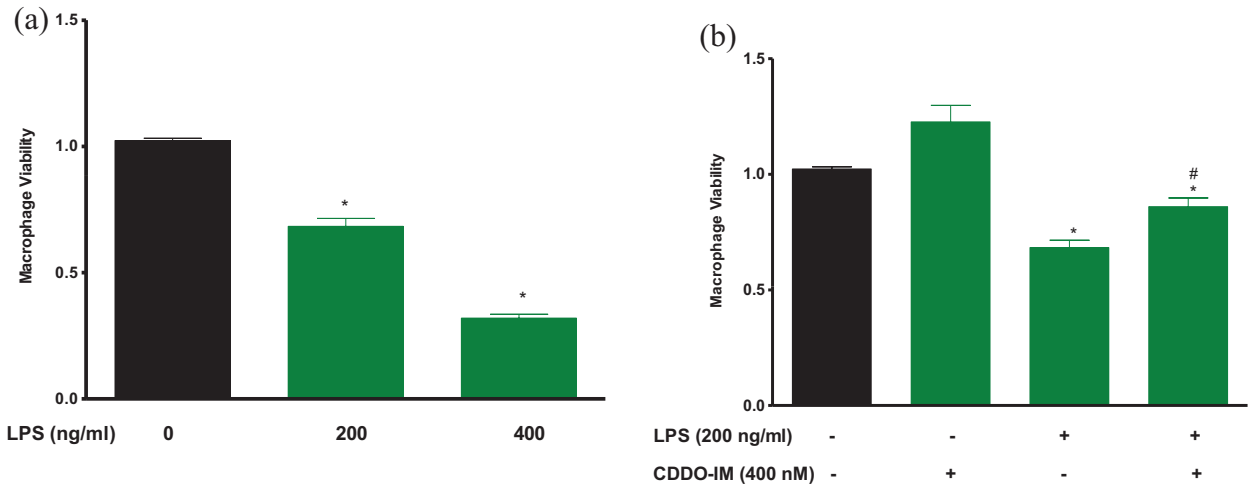


**Figure 5.** Cytoprotective role of CDDO-IM in response to LPS-induced cytotoxicity. LPS-induced cytotoxicity and cytoprotective effects of CDDO-IM against LPS toxicity. (a) The cells were treated with LPS as indicated concentrations for 24 h and the cell viability was measured by MTT assay. (b) The cells were pretreated with CDDO-IM at 400 nM for 24 h, followed by incubating the cells with LPS for another 24 h. Treatment of macrophages with LPS resulted in a significant decrease in cell viability. However, co-treatment with 400 nM CDDO-IM resulted in a significant increase in cell viability in macrophages treated with 200 ng/mL LPS. *All data represent mean ± SEM ($n = 3$.$^{*}p < 0.05$ vs control, $^{\#}p < 0.05$ vs LPS only).* The differences between treatments were measured by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. (A color version of this figure is available in the online journal.)

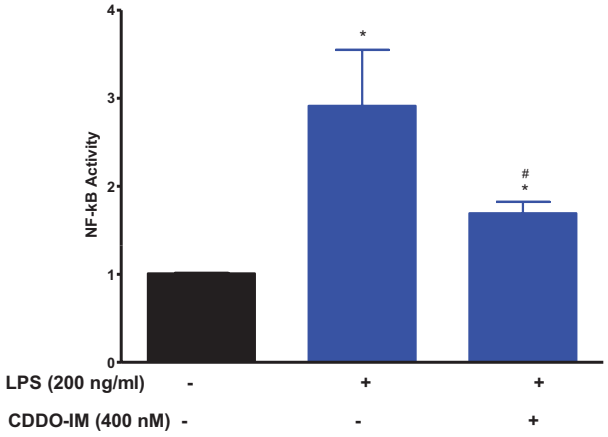


**Figure 6.** NF-kB luciferase activity in Response to LPS/CDDO-IM. CDDO-IM reduced LPS-mediated NF-kB Activity in NF-κB RAW264.7 macrophages. NF-κB RAW264.7 cells ($1 \times 10^5$ cells/well) were plated in a 6-well format in assay medium and were pretreated with or without CDDO-IM at 400 nM for 24 h, followed by incubating the cells with 200 ng/mL LPS for another 3 h. Coelenterazine was then added as a substrate at a concentration of 1 μM and luminescence was read using the Biotech Synergy 2 plate reader. All data represent mean ± SEM ($n = 3$. *$p < 0.05$ vs control; #$p < 0.05$ vs LPS only). The differences between treatments were measured by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. (A color version of this figure is available in the online journal.)

### CDDO-IM attenuated LPS-mediated NF-κB activation

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is a transcription factor which regulates the gene expression of pro-inflammatory cytokines. RAW 264 Renilla luciferase macrophage cell line was used to understand the activity level of the NF-kB gene in response to LPS stimulation in the absence and presence of CDDO-IM treatment. Results indicate that LPS stimulation dramatically increased NF-κB activation and the luciferase activity was significantly reduced in response to CDDO-IM treatment (Figure 6). This result suggests that CDDO-IM can inhibit LPS-induced NF-κB activation.

## Discussion

Inflammation and oxidative stress are prominent symptoms of sepsis which can be triggered by LPS, a prominent endotoxin present on the cell wall of Gram-negative bacteria.[3,16] Failure of the organism to combat the inflammatory response leads to cytokine storm surge as well as the buildup of ROS. This ultimately leads to organ failure and death.[17] Presently, a cure for this disease is yet to be established. In this study, LPS-mediated expressions of pro-inflammatory cytokines such as IL-8, IL-1β, TNF-α, and MCP-1 in macrophages were attenuated by CDDO-IM (Figure 4). Also, the LPS-induced transcriptional activity of NF-kB was also significantly reduced by CDDO-IM. This suggests that the action of inhibition of inflammation by CDDO-IM is involvement with the suppression of NF-kb transcription factor. Treatment with CDDO-IM resulted in a significant increase in the gene expression levels of various antioxidant molecules, including GCLC, GCLM, HO-1, GR, and NQO1. Also, LPS-mediated ROS generation was also reduced in macrophages treated with CDDO-IM.

During an inflammatory response, the levels of pro-inflammatory cytokines such as IL-6, TNF-α, IL-8, and IL-1β are elevated, whereas the levels of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β are reduced.[2,18–22] This elevation in pro-inflammatory cytokines and reduction in anti-inflammatory cytokines is also notable in sepsis patients.[18] Therefore, an effective strategy to combat sepsis involves the inhibition or reduction of bacterial LPS or inflammatory cytokines.

A variety of pro-inflammatory biomarkers contributing to the inflammatory response were examined in this study. IL-8, a chemokine, is synthesized and released by macrophage cells to recruit neutrophils and promote phagocytosis of bacterial content at the site of infection.[18] IL-1β, a pro-inflammatory cytokine, is involved in a series of cellular activities, including cell proliferation, differentiation, and apoptosis. During an infection, this cytokine is a key mediator in the inflammatory response to promote phagocytosis.[1,2] TNF-α is a cytokine involved in the inflammatory response pathway and is able to induce apoptosis in cells undergoing stress via the caspase 3 pathway.[1,2] Also, TNF-α is able to activate the NF-kB transcription factor to induce the transcription of pro-inflammatory cytokines.[1,2] In this study, LPS-induced elevation in the expression of IL-8, IL-1β, TNF-α, and MCP-1 was suppressed by CDDO-IM in the macrophages (Figure 4), indicative of the anti-inflammatory property of CDDO-IM.

NF-kB is a well-known transcription factor in the regulation of the inflammatory response induced by LPS.[23–29] Immune cells contain Toll-like receptors (TLRs) to which LPS can bind to trigger an immune response leading to the activation of the NF-kB transcription factor. The activation and synthesis of this transcription factor lead to the transcription and synthesis of pro-inflammatory cytokines. Increase in the levels of inflammatory cytokines results in the progression of sepsis.[10] NF-kB is activated during oxidative stress and is involved in regulating the transcription of pro-inflammatory cytokines and antioxidants.[29] In this study, the activity of NF-kB was measured using the RAW 264.7 mouse macrophage Renilla luciferase cell line. We showed that LPS significantly increased activity levels of NF-kB. This indicates that the activation of this transcription factor is essential for the inflammatory response triggered by LPS. NF-kB activation mediated by LPS was inhibited by CDDO-IM (Figure 6), suggesting the mechanism of action of CDDO-IM may be modulated by this signaling pathway.

Previous studies have shown that the overproduction of ROS which leads to oxidative stress and inflammatory surge plays a critical role in the LPS-mediated inflammation.[9,18] Our results suggested that CDDO-IM possesses antioxidative properties which are coupled with the anti-inflammatory effects against LPS-induced increase in the expression of pro-inflammatory cytokines and the activation of NF-kB. First, based on the data, macrophages treated with CDDO-IM caused a concentration-dependent increase in the induction of antioxidants GSH and NQO1 (Figure 2). Also, the mRNA expression of GCLC, GCLM, HO-1, and GR was significantly increased in macrophages treated with CDDO-IM (Figure 3). Gamma-glutamyl cysteine ligase (GCL) is an enzyme in the synthesis pathway of GSH, and GCLC and GCLM are the two subunits of this enzyme. Increase in activity and transcription of above antioxidants by CDDO-IM will result in a decrease in NF-kB activity. Second, LPS-induced cytotoxicity in macrophages can be reduced upon pretreatment with CDDO-IM (Figure 5).

In summary, in this study, LPS-induced pro-inflammatory cytokine surge, such as IL-8, IL-1β, TNF-α, and MCP-1, is significantly reduced by triterpenoid CDDO-IM in macrophages. In addition, endogenous upregulation of a series of antioxidant molecules by CDDO-IM, could, at least partially, account for the attenuated activity of the NF-kB transcription factor, resulting in the protection against LPS-induced injury. This study may help to deepen our understanding of the role of the triterpenoid CDDO-IM in the detoxification of endotoxin LPS in macrophage.

### AUTHORS' CONTRIBUTIONS

ZJ, HZ, and YL designed the experiments. HA, UA, and DRP completed the experiments and processed the experimental data. HA and ZJ drafted the manuscript. XS, YL, and HZ contributed to analysis and interpretation of results.

### DECLARATION OF CONFLICTING INTERESTS

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