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Dysregulated response by the body towards an infection leads to tissue and organ damage is known as sepsis. Currently, approximately 18 million people worldwide are affected by this deadly disease annually. Septic shock, a severe form of sepsis, continues to have a very high mortality rate. When the immune system fails to regulate an infection, this results in the buildup of reactive oxygen species (ROS) and cytokine storm due to an increase in pro-inflammatory cytokines. This ultimately results in organ failure and death. Currently, no viable treatment method is available to combat sepsis. Lipopolysaccharide (LPS), also known as endotoxin, is commonly used in controlled experiments to trigger the symptoms of sepsis. 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 could be ameliorated via the endogenous upregulation of antioxidant defense in macrophage cells. CDDO-IM (2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline), a novel triterpenoid compound, was used to upregulate endogenous antioxidant defenses. Phorbol-12-myristate-13- acetate (PMA) was used to differentiate ML-1 monocytes into macrophages. This differentiation was analyzed through the increase in the expression of CD206, differentiation marker present on macrophage cells. Data from this study show that gene expression levels of inflammatory cytokine genes such as interleukin-1 beta (IL-1beta), interleukin-8 (IL-8), tumor necrosis factor-α (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. Additionally, CDDO-IM has been shown to protect against LPS-induced cytotoxicity in macrophages, and NF-kB transcriptional activity was also noted to decrease upon treatment with CDDO-IM in macrophages. *In vivo* mice trials indicated that levels of serum TNF-alpha had been significantly reduced by the administration of CDDO-IM. Also, pro-inflammatory cytokine levels of TNF-alpha, IL-6, and IL-1β in hepatic tissue were significantly decreased as a result of CDDO-IM treatment in LPS-induced mice. This data demonstrated that the endogenous upregulation of a multitude of antioxidants by CDDO-IM attenuated LPS-induced inflammation and injury. This study may contribute to the advancement of our understanding of treating life-threatening inflammatory diseases such as sepsis.

UPREGULATION OF ROS DETOXIFICATION GENES BY TRITERPENOID CDDO-IM IN MACROPHAGES: PROTECTION AGAINST LIPOPOLYSACCHARIDE-INDUCED INFLAMMATORY INJURY

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Sepsis and Septic Shock

Infection and symptoms of infections including fever, tachycardia, tachypnea, and leukocytosis are defining elements in the development of sepsis, a condition, if left untreated, results in systemic organ failure, and ultimately death. Sepsis, affecting nearly 18 million people annually, is one of the leading causes of death globally in developed as well as developing nations. When patients suffering from sepsis are receiving proper treatment including adequate amount of fluids, septic shock occurs when the body is unable to respond efficiently to the treatment leading to hypotension [1]. In the United States, specifically, sepsis accounts for the tenth leading cause of death, affecting approximately 700,000 people annually who experience severe sepsis leading to septic shock. These cases result in the death of approximately 250,000 individuals in the United States annually. An extensive amount of research has been dedicated to targeting this deadly condition for the past three decades. Despite the extensive focus placed on understanding the underlying role of sepsis, it remains to be the primary cause of death in hospital intensive care units (ICU). In the ICUs of hospitals in both the United States and Europe, there is an alarming 82% mortality rate in the half-million patients that suffer from septic shock annually [2].

Developing proper treatment for sepsis has been extremely challenging as evidenced by the fact that the only drug approved by the FDA, drotrecogin alfa, experienced failures in the PROWESS shock clinical trials throughout the world. Thus, this drug was recently removed from the market [3]. These failures indicate a serious need to develop a proper therapeutic regimen to combat sepsis. Accordingly, unfolding the pathophysiology of sepsis will allow for the framing of an efficient, mechanistic-based approach to ameliorate and ultimately eliminate the symptoms of sepsis in patients [4-6].

Sepsis is Associated with the Overproduction of ROS

Sepsis is caused by a dysregulation in the host immuno-inflammatory response during an infection. Due to the complexity of the molecular events which alter gene expression caused by infection, an excessive or inappropriate release of mediators involved in the host defense mechanism cause cellular damage, which leads to tissue and organ damage and failure [7]. Oxidative stress is involved in multiorgan failure and death in critically ill patients. The human body's inability to balance the production of ROS and potent removal by antioxidants and ROS scavengers presents a major obstacle in treating pathological conditions such as sepsis.

Molecular oxygen plays a vital role as an electron acceptor in the mitochondria during synthesis of ATP. Although oxygen plays an important role in the synthesis of ATP, incomplete reduction or excitation of oxygen results in the generation of reactive oxygen species (ROS) [8]. ROS are chemical particles located inside cells and are reactive towards a multitude of molecules composed of lipids, proteins, and DNA. These

molecules include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH ·). The chemical composition and nature of each of these reactive molecules is unique. Thus, the reactivity and biological targets of every ROS vary appreciably. Oxidants are synthesized and deployed in cells and tissues to serve as signaling molecules to maintain cell and tissue homeostasis [9]. Targets of ROS include cellular macromolecules, lipid membranes and various organelles [10]. Cellular ROS can be generated endogenously from the mitochondria through oxidative phosphorylation or from exogenous sources through various xenobiotic compounds. ROS plays a pivotal role in countless cellular pathways to maintain cell survival, proliferation, and cytokine stimulation [11]. However, cells have antioxidant defense mechanisms designed to alleviate ROS levels to prevent oxidative stress. When these defense mechanisms are inundated by increased ROS levels, nucleic acids, proteins, and lipids are targeted by ROS resulting in multiple tissue and organ failure.

ROS is generated during the reduction of oxygen into water. Initially, oxygen gains electrons to generate superoxide anion. Further reduction of superoxide anion results in the generation of hydrogen peroxide which is further reduced to the hydroxyl radical. the hydroxy radical can undergo further reduction to generate water. During this phase of oxygen reduction, ROS can buildup in the cell, therefore it is vital that enzymes and molecules involved in the detoxification process work efficiently to balance ROS levels. Enzymatic and non-enzymatic methods are utilized by organisms to detoxify and balance ROS levels in the cells. Non-enzymatic antioxidant compounds include glutathione (GSH), vitamins C and E, ß-carotene, and uric acid. Enzymes include

superoxide dismutase (SOD), catalase, and glutathione peroxidase. SOD is involved in the dismutation of superoxide anion to generate hydrogen peroxide. As shown in Figure 1 during the reduction of superoxide into hydrogen peroxide, GSH is oxidized into GSSG, and the electrons from GSH are gained by superoxide to generate hydrogen peroxide. Catalase is an enzyme involved in the reduction of hydrogen peroxide into water [8]. Together, the molecular pathways involved in the detoxification process work hand-in-hand to maintain homeostatic ROS levels in the cell.

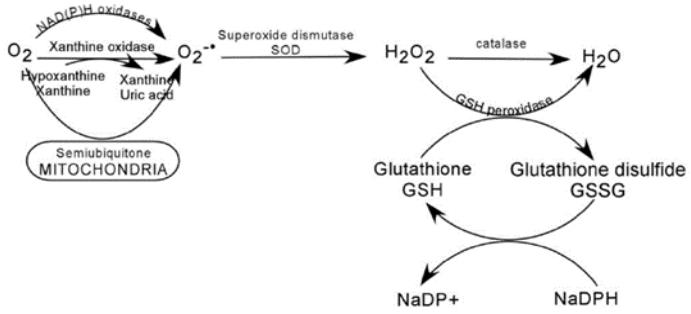


Figure 1. ROS Detoxification Mechanisms. Adapted from Marambe [12]

The immune and inflammatory response system is activated in both infectious and non-infectious stimuli. Gram-negative, as well as gram-positive bacteria, play a vital role in infectious diseases [13]. An infection develops from the stimulation of the innate, or nonspecific, immune response. The innate immune response is the first line of defense against an infection and therefore occurs in a rapid manner, which typically does not require an antigen, hence it is a nonspecific response. This response is mediated mainly by circulating leukocytes, such as monocytes, macrophages, and neutrophils. These cells are activated rapidly during infection and through phagocytosis, engulf and disintegrate

foreign organisms and molecules. During this response, various cytokines and reactive oxygen species (ROS) are synthesized by the leukocytes as mediators to fight the infection. If the infection becomes uncontrollable or the leukocytes are unable to properly manage to combat the infection, ROS builds up in the cells and tissues leading to increased inflammation and tissue damage.

Most infections occur in the tissue, therefore a proper mechanism to extravasate leukocytes to the site of infection is essential. This mechanism involves upregulation of chemokines such as inflammatory cell adhesion molecule (ICAM) in the cells located at the site of infection, creating a chemotactic gradient allowing the leukocytes to migrate to the site of infection [14].

Lipopolysaccharide (LPS) Mechanism of Action

Lipopolysaccharide (LPS), also known as lipoglycan, are composed of large lipid and polysaccharide molecules. 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 [15]. LPS is referred to as an endotoxin, which denotes the method by which LPS induces its toxic effects. Endotoxins are a portion of the cell membrane of bacteria and are released in response to damage of the cell wall and are mainly involved in activating cells, such as macrophages, containing the respective antigen, whereas the counterpart, exotoxins, are released by the bacterium into the surrounding environment [16].

The structure of LPS includes a polysaccharide component consisting of an O-Antigen, outer core, and inner core followed by a lipid portion of the molecule referred to as Lipid A. The O-Antigen is a hydrophilic component and is very specific to different species of bacteria. The hydrophilic inner and outer core consists of a polysaccharide chain linking the O-Antigen with the lipid A. Lipid A is a hydrophobic component of LPS and creates an interface with the outer membrane of the bacteria. Lipid A is the component of LPS that physically makes up the outer membrane of bacterial cells [13]. This is the portion of LPS that exerts toxic effects. LPS also plays a vital role in the integrity of the bacterial membrane by increasing the overall negative charge of the membrane. Mutation of LPS results in the death of bacterial cells [15].

Sepsis and Inflammation-cytokine, NF-kB, the Role of Macrophages in Inflammation

Inflammation is a product of several biochemical responses caused by injury to cells and tissue. These responses include vascular, migration, and leukocyte activation. Inflammation can be relatively short-lived which is referred to as acute inflammation. If the inflammatory response lasts for a longer period of time chronic inflammation occurs, which can lead to tissue and organ pathology. During acute inflammation, mainly neutrophils relocate to the site of injury to alleviate and eliminate the cause of injury. During chronic 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 and 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. These specialized cells receive various cues from the surrounding environment which allows them to assume different functions and phenotypes [17]. 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 surface molecules present on the surface of certain bacteria, such as LPS. Each bacterial surface molecule will trigger a unique inflammatory response in the macrophage, which allows the macrophage to recognize the foreign substance to trigger an appropriate response. The receptors on the macrophage that recognize bacterial surface molecules are referred to as toll-like receptors (TLR). TLR recognition is the key to maintaining the homeostatic regulation of the host immune system. Binding of TLR to antigens results in a cascade of events leading to the synthesis and activation of multiple cytokines, chemokines, and antimicrobial polypeptides. Chemokines will allow more macrophages to be recruited to the site of inflammation. Activation of various cytokines will result in the activation of the NF- κB pathway [18].

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a well-defined redox-sensitive transcription factor which is activated during oxidative stress by regulating the gene expression of pro-inflammatory cytokines as well as antioxidants [19]. NF-κB is a heteromeric dimer composed of the p50 and p65 subunits bound to the inhibitor, κB. This transcription factor is normally present in the cytoplasm of the cell in its inactive form. During oxidative stress, the IκB subunit undergoes phosphorylation and

ubiquitination resulting in the degradation of this subunit by the 28S proteasome. Following this degradation, the heterodimer composed of p50 and p65 subunits translocate to the nucleus where, acting as transcription factors, bind to promoters of multiple genes involved in regulating oxidative stress [14].

Antioxidant-based Therapeutic Intervention in the Management of Sepsis

Previous studies have demonstrated that oxidative stress caused by the overproduction of ROS has shown to exhibit symptoms of sepsis in humans [20, 21]. Elevated levels of biomarkers used to measure oxidative stress such as lipid peroxidation have been measured in patients suffering from sepsis. Additionally, increased activity of the transcription factor, NF-kB, as wells as a decrease in the levels of antioxidants have been analyzed in patients experiencing sepsis [22, 23]. Thus, researchers have been provoked to use exogenous compounds carrying antioxidant properties such as vitamins A, C, E, N-acetylcysteine, and mimetics of antioxidant enzymes to combat sepsis *in vitro, in vivo,* as well as in clinical trials [24, 25]. Despite exhibiting promising results from the administration of exogenous antioxidants in *vitro* studies as well as a few clinical trials [26, 27], most randomized clinical trials have been unsuccessful in treating sepsis. In fact, some clinical trials have shown to exert harmful effects upon administering exogenous antioxidant therapy [28, 29]. For example, studies from these trials have shown that patients who are administered N-acetylcysteine (NAC), which is a precursor to glutathione (GSH), have increased levels of GSH in the blood [30]. This shows that although the body is able to convert NAC to GSH, increased levels of GSH in the blood does not necessarily correlate to increased well-being of the body. Physiological effects

of increased GSH in the blood have not shown to be positive, and no improvement in mortality rates have been observed. Another approach to combat oxidative stress involves the administration of supplements such as vitamins A, C, and E or selenium in various combinations to restore antioxidant levels. However, the increase in selenium and glutamine levels in the plasma, failed to reduce in mortality rate. In fact, patients who received glutamine, an amino acid involved in the synthesis of glutathione, had an increase in mortality rate when compared to patients receiving antioxidants. Inconsistent clinical trial results have been observed in patients undergoing antioxidant therapy. As a matter of fact, some patients receiving vitamins A and C and phenolic compound treatment have increased levels of oxidative stress. As presented in Figure 1, ROS detoxification involves the coordinated effort of all cellular ROS detoxification molecules and enzymes, rather than a single molecule or enzyme. Systematic detoxification of ROS is necessary to prevent tissues and organs to undergo further oxidative stress and failure. Knowing this, it is imperative that protection against endotoxin-induced septic shock should involve a pharmacological instigation of the expression and coordination of a string of enzymes and molecules**.**

Triterpenoids are naturally occurring compounds extracted from plants. These compounds are steroid-like and are known for their medicinal properties [32]. 2-Cyano-3,12-dixooleana-1,9-dien-28-imidazolide (CDDO-Im) (Figure 2) [33] is a synthetic compound and has been used extensively because of its use as an anti-inflammatory as well as an antioxidant agent. Pharmacological control of both inflammation and oxidation is vital to detoxify ROS in cells. [34, 35]. 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 NQO1, an antioxidant enzyme [34, 36]. Additionally, treatment with CDDO-Im has targeted the Nrf2-ARE pathway in mononuclear cells and neutrophils by upregulating gene expression of proteins involved in this pathway [37, 38].

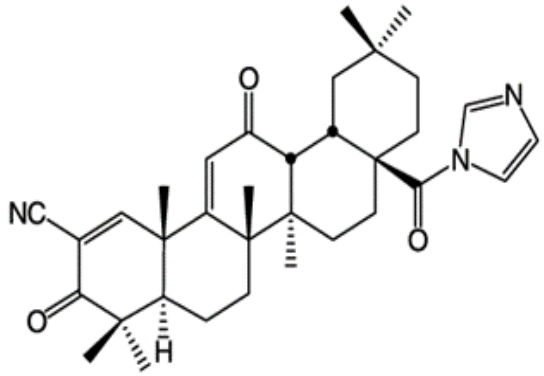


Figure 2. CDDO-Im (CDDO-Imidazolide) Chemical Structure. Adapted from Yates [31]

A multitude of events in the body are responsible for eliminating harmful organisms and substances. During these events, if the body is unable to eliminate this threat, sepsis can develop, which leads to tissue and organ damage. During this response, inflammatory cytokines are activated in macrophages, which play a critical role in the pathology of sepsis [39]. The oxidative and inflammatory response in the body is a major factor in the pathophysiology of sepsis. I hypothesize that the antioxidative and anti-inflammatory properties of CDDO-IM can reduce LPS-activated expression of a series of pro-inflammatory cytokines in macrophages and the effect is via inhibition of NF-κB pathway. 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. *In vivo* results show that mice treated with CDDO contain significantly reduced amounts of inflammatory cytokines such as TNF-a. This study can provide a better understanding of the role of endogenous antioxidants and the various ROS detoxification mechanisms by CDDO-Im to prevent sepsis.

CHAPTER II

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 that can be recognized by macrophages ML-1 monocytes were cultured in T-150 flasks containing RPMI media with 10% FBS and 1% penicillin/streptomycin antibodies. Cells were split when density reached approximately $6x10^5$ 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 $1.5x10^5$ 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 hours. After 72 hours, 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 hours so that most cells

would become non-adherent. Following this six-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 nM to 800 nM. Approximately 6 million cells were treated with CDDO-Im per petri dish for 24 hours. Following this treatment cells were collected for antioxidant enzyme studies.

Antioxidant Enzyme Assay

Macrophages differentiated from ML-1 monocytes were treated with various concentrations of CDDO-Im described previously. After 24 hours 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 minutes at 4ºC. The pellet of cells was suspended in 1 mL Phosphate Buffer Saline (PBS). The cells were centrifuged at 5,000 RPM for 5 minutes, and the resulting pellet was resuspended in 300 µL sterile potassium phosphate tissue buffer containing 2 mM EDTA. Sonication carried out at 15-second intervals 3 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. 4 µL of lysate was mixed with 796 µL of Bio-Rad® Protein assay dye reagent concentrates. The absorbance was compared to 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 collected from macrophages treated with CDDO-Im was collected as described previously. 10 µL of the sample lysate was mixed with 12.5 µL meta-phosphoric acid and 0.1% sodium phosphate buffer at pH 8.0 (GSH buffer). The mixture was centrifuged at 13,000 RPM for 5 minutes at 4ºC. 10 µL of the supernatant was mixed with 0.1 mL o-phthalaldehyde (OPT) and 1.89 mL of GSH buffer per sample and was incubated for 15 minutes at room temperature. GSH activity was measured used 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.

NQO1 activity was detected using the Beckman-Coulter® DU-800 spectrophotometer. 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) was mixed to create a reaction mixture. 6 µL of sample lysate was added to 694 µL of reaction mixture. NQO1 activity was measured by the rate of 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 x 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 hours at 37 ºC in 5% CO_2 incubator. Following the 24-hour CDDO-IM treatment, 3 control wells were treated with 200 ng/mL LPS, and 3 CDDO-IM treated wells were treated with 200 ng/mL LPS. 3 control wells were treated with 400 ng/mL LPS, and 3 CDDO-IM treated wells were treated with 400 ng/mL LPS. Macrophages underwent LPS treatments for a further 3 hours. Following this treatment, cell viability was determined using 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) for 3.5 hours. Following the MTT treatment, macrophages were collected in 2 mL microcentrifuge tubes and washed with PBS. Cells were centrifuged at 1200 g for 6 minutes, and the supernatant was removed and washed with 0.5 mL PBS and centrifuged again at 1200 g for 6 minutes. The supernatant was removed and 175 µL of MTT reagent containing 10% DMSO, 40% Isopropanol, 50% DEPC water and transferred to a 96 well plate. Afterward, the plate was covered and placed on a shaker for 5 minutes at low speed to dissolve the formazan crystals. Once the formazan crystals were dissolved, absorbance was measured at 570 nm to test to cell viability. This assay will use the Bio-Tek® Synergy 2™ plate reader for quantification and analysis.

ROS Assay

Macrophages were grown in Petri dishes treated with 400 nM of CDDO-IM as well as 0 nM CDDO-IM for 24 hours. Approximately 0.5 x 10^6 cells were collected and

suspended in 1 ml air-saturated complete PBS (PBS containing 0.5 mM MgCl2, 0.7 mM CaCl2, and 0.1% glucose) in each tube. It is very vital to keep the cell number stable throughout this experiment. The reaction mixture for each sample contained 10 µM of lucigenin, 10 µM of rotenone and 10 µM of myxothiazol. Lucigenin-derived chemiluminescence (LDCL) was initiated by adding LPS at various concentrations ranging from 1 ng/mL to 1 µg/ and LDCL was recorded for 30 minutes.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

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 5x 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 IL-8, IL1-beta, IL-6, TNF-alpha, and MCP-1. GAPDH was used as the housekeeping gene. The Applied Biosystems™ StepOnePlus™ Real-Time PCR System ran for 40 cycles at the following settings: 95 °C

for 15 seconds, 58 °C for 1 minute, and 60 °C for 15 seconds. Comparative threshold cycle (CT) values were used to quantify the gene expression.

Table 1. Human Primer Sequence. DNA sequence of human primers used to conduct PCR reactions of various genes

Target gene	Forward primer	Reverse primer
GAPDH	5'- AGA ACG GGA AGC TTG TCA TC – 3'	5'- GGA GGC ATT GCT GAT GAT CT – 3'
IL-8	5'- CTC TGT GTG AAG GTG CAG TT – 3'	5'- AAA CTT CTC CAC AAC CCT CTG – 3'
IL-6	5' - TAC CCC CAG GAG AAG ATT CC – 3'	5' – GCC ATC TTT GGA AGG TTC AG – 3'
IL1-beta	5' – CAG CCA ATC TTC ATT GCT CA – 3'	5' – TCG GAG ATT CGT AGC TGG AT – 3'
ICAM	5'- ACA GTG ACC ATC TAC AGC TTT C – 3'	5'- CGG GTC TGG TTC TTG TGT ATA A – 3'
TNF-α	5'- CTA TCT GGG AGG GGT CTT CC – 3'	5'- GGT TGA GGG TGT CTG AAG GA-3'
HO-1	5' - CAC GCA TAT ACC CGC TAC CT - 3'	5' - CCA GAG TGT TCA TTC GAG CA -3'
GST	5' - CGG TGA CAG CGT TTA ACA AA – 3'	5' - GCA CTT GCT GGA ACA TCA AA- 3'
MCP-1	5'- TTC CTC CAC CAC CAT GCA G– 3'	5'- CCA GCC GGC AAC TGT GA– 3'

NF-kB RAW 264 Renilla Luciferase Assay

Approximately 1 x10^5 macrophages were grown in 6 well plates for 24 hours. This was followed by a 24-hour 40 nM CDDO-IM treatment. Following the CDDO-IM treatment, cells were added 10 ng/mL and 100 ng/mL LPS for 6 hours. 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.

In Vivo Analysis of Antioxidative Defense by CDDO-Im in LPS Mediated Multiorgan Endotoxemia

As previously stated, sepsis is a disease characterized by the pathophysiological overproduction of ROS leading to oxidative and inflammatory stress. It is hypothesized that *in vivo* administration of CDDO-Im can increase the expression of a multitude of antioxidant defense mechanisms in multiple organs of mice dosed with LPS to induce sepsis. LPS is commonly used to induce sepsis in animal models [50]. To test his hypothesis, i*n vivo* studies were carried out in murine models at Campbell University School of Osteopathic Medicine. Tissue samples were analyzed in the Jia lab to understand LPS-induced endotoxemia. For this experiment, LPS was injected into mice via an intraperitoneal injection based on body weight (0.25 mg/30 g b.w.). Five minutes following LPS injection CDDO-Im was given orally to mice at varying concentrations based on body weight (0, 0.1, 0.2, 0.3 mg/kg b.w.) as shown in Table 1. Organs and tissues were harvested from the mice following euthanasia 6 hours and 7 days post LPS injection. 6-hour treatment will allow us to understand the acute inflammatory response, whereas the 7-day treatment will allow us to understand the chronic response involved in LPS mediated sepsis. The organs and tissues as well as blood samples were collected to measure levels of antioxidant enzymes and evaluate the level of LPS mediated endotoxemia. Our collaborator's lab at Campbell University have devised these doses and times based on preliminary experiments.

Following the *in vivo* treatment, mice were euthanized via cervical dislocation using isoflurane as an anesthetic. Cardiac puncture was used to remove blood, and major organs (kidney, heart, liver, lung, colon, small intestine) were collected to measure antioxidant enzyme levels. For histological analysis, a portion of these organs were preserved in 10% buffered formaldehyde.

C57/BL6 mice were dosed with LPS and CDDO as stated in Table 1. Following these treatments. ELISA kits were used to measure the levels of pro-inflammatory cytokines in serum samples of mice. RNA was extracted from liver samples similar to the *in vitro* study of mice to study the gene expression levels of antioxidant genes GCLC, GCLM, NQO1, and GST.

Table 2. C57/BL6 Mice CDDO-IM and LPS Treatment Dosage and Time.
12 mice were used per treatment. 6 mice were sacrificed 6 hours post-treatment.
6 mice were sacrificed 7 days post-treatment.

Cytoprotective Properties of CDDO-Im via LPS Induced Sepsis in C57Bl/6 Mice			
LPS Dosage	CDDO-Im Dosage	6 hour	7 days
0 mg/30 g b.w.	0 mmol/kg b.w.	6 mice	6 mice
0.25 mg/30 g b.w.	0.1 mmol/kg b.w.	6 mice	6 mice
0.25 mg/30 g b.w.	0.2 mmol/kg b.w.	6 mice	6 mice
0.25 mg/30 g b.w.	0.3 mmol/kg b.w.	6 mice	6 mice

CHAPTER III

RESULTS

ML-1 Differentiation

Macrophages were differentiated from human ML-1 monocytes using 0.3 ng/mL TPA for 72 hours. Following this treatment, gene expression levels of CD206 were measured. CD206 is a receptor protein and profoundly expresses on the membranes of macrophages but is less expression in monocytes. A significant increase in CD206 expression levels was analyzed in macrophages where there was an approximately 8-fold increase in the expression of the CD206 gene (Figure 3).

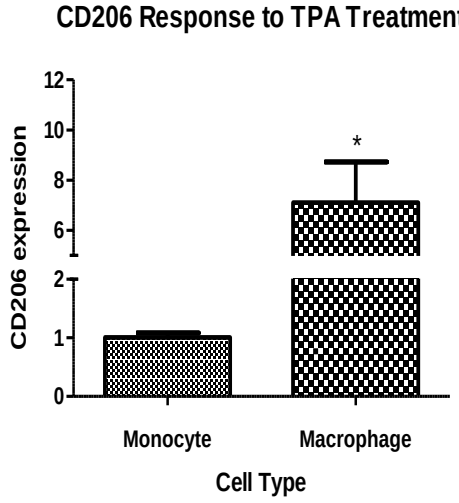


Figure 3. CD206 Response to TPA Treatment in ML-1 Monocytes. TPA increases the expression levels of CD206, a biomarker for the differentiation of ML-1 monocytes to macrophages. ML-1 Monocytes were treated with 0.3 ng/mL LPS for 72 hours which was followed by replacement of old media with fresh media followed by re-culturing of cells for 72 hours. The relative CD206 mRNA was evaluated by real-time PCR and data were normalized using GAPDH as the house keeping gene (Data represent means ± standard errors of the mean, n = 3. *, P<0.05 vs. monocyte)

Induction of GSH and NQO1 by CDDO-IM

The activities of glutathione (GSH) (Figure 4), as well as NQO1 (Figure 5), were measured in macrophages treated with varying concentration of CDDO-IM ranging from 100 nM to 800 nM. There was a significant increase in GSH and NQO1 activity in macrophages treated with 100 nM, 200 nM, 400 nM, and 800 nM CDDO-IM for 24 hours.

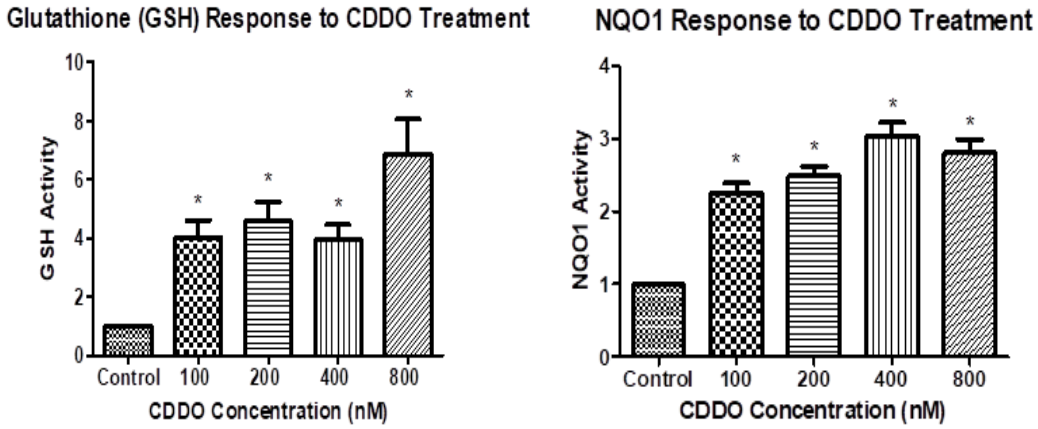


Figure 4. Antioxidant Enzyme Activity Response to CDDO Treatment. Glutathione (GSH) levels (A) and NQO1 activity (B) in macrophage cells differentiated from ML-1 Monocytes in response to 24-hour CDDO-IM treatment. CDDO-IM concentrations ranged from 100 - 800 nM. (Data represents mean ± SEM, *, P<0.05 vs. control)

CDDO-IM Treatment Induces Antioxidant Molecule Gene Expression

Gene expression levels of various antioxidant molecules involved in the ROS scavenging pathways was studied. In this study gene expression levels of GCLM, GCLC, HO-1, GR, and NQO1 was analyzed in response to a 12-hour 400 nM CDDO-IM treatment. There was an approximately 17-fold increase in GCLM levels (Figure 5A). For GCLC, there was an approximate 2.5-fold significant increase in gene expression levels

with respect to the control (Figure 5B). Expression levels of HO-1 were significantly increased as well upon treatment with CDDO-IM. There was an approximately 850-fold increase in the gene expression level of HO-1 (Figure 5C). 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 5D). 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 (Figure 5E).

CDDO-IM Inhibits LPS- Induced Expression of Pro-Inflammatory Cytokines

As shown in Figure 6, LPS significantly increased the expressions of proinflammatory cytokines such as IL-8, IL1-beta, TNF-a, 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 to with treatment of LPS at 100 ng/mL only (Figure 6A). IL1-beta, TNF-a, and MCP-1 levels were also significantly reduced when treated with CDDO-IM and LPS (Figure 6) compared to with treatment of LPS only. These results suggest that CDDO-IM possess the anti-inflammatory activities necessary to regulate LPS-induced inflammation.

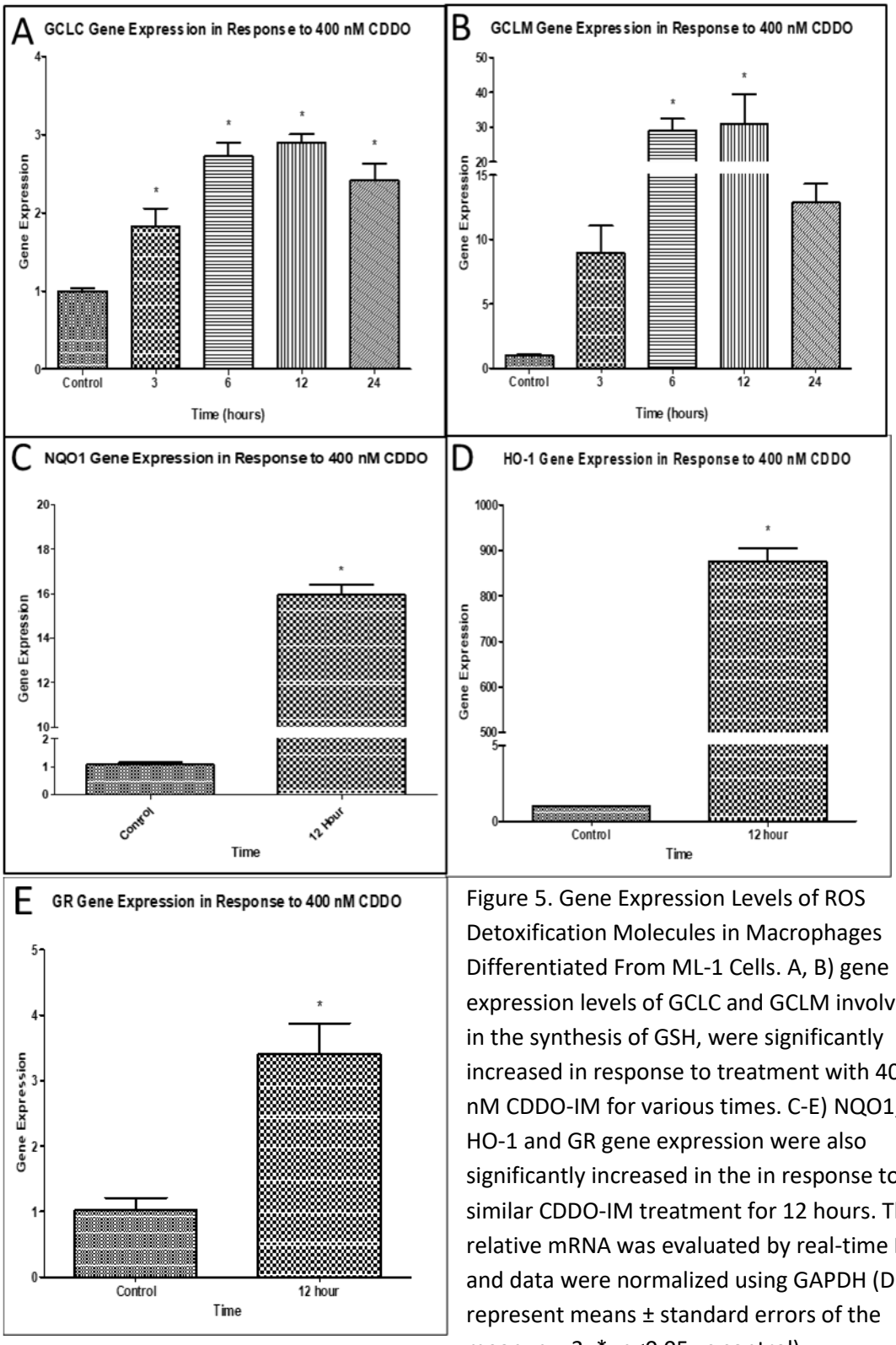


Figure 5. Gene Expression Levels of ROS Detoxification Molecules in Macrophages Differentiated From ML-1 Cells. A, B) gene expression levels of GCLC and GCLM involved in the synthesis of GSH, were significantly increased in response to treatment with 400 nM CDDO-IM for various times. C-E) NQO1, HO-1 and GR gene expression were also significantly increased in the in response to similar CDDO-IM treatment for 12 hours. The relative mRNA was evaluated by real-time PCR and data were normalized using GAPDH (Data represent means ± standard errors of the mean, n = 3. *, $p<0.05$ vs control)

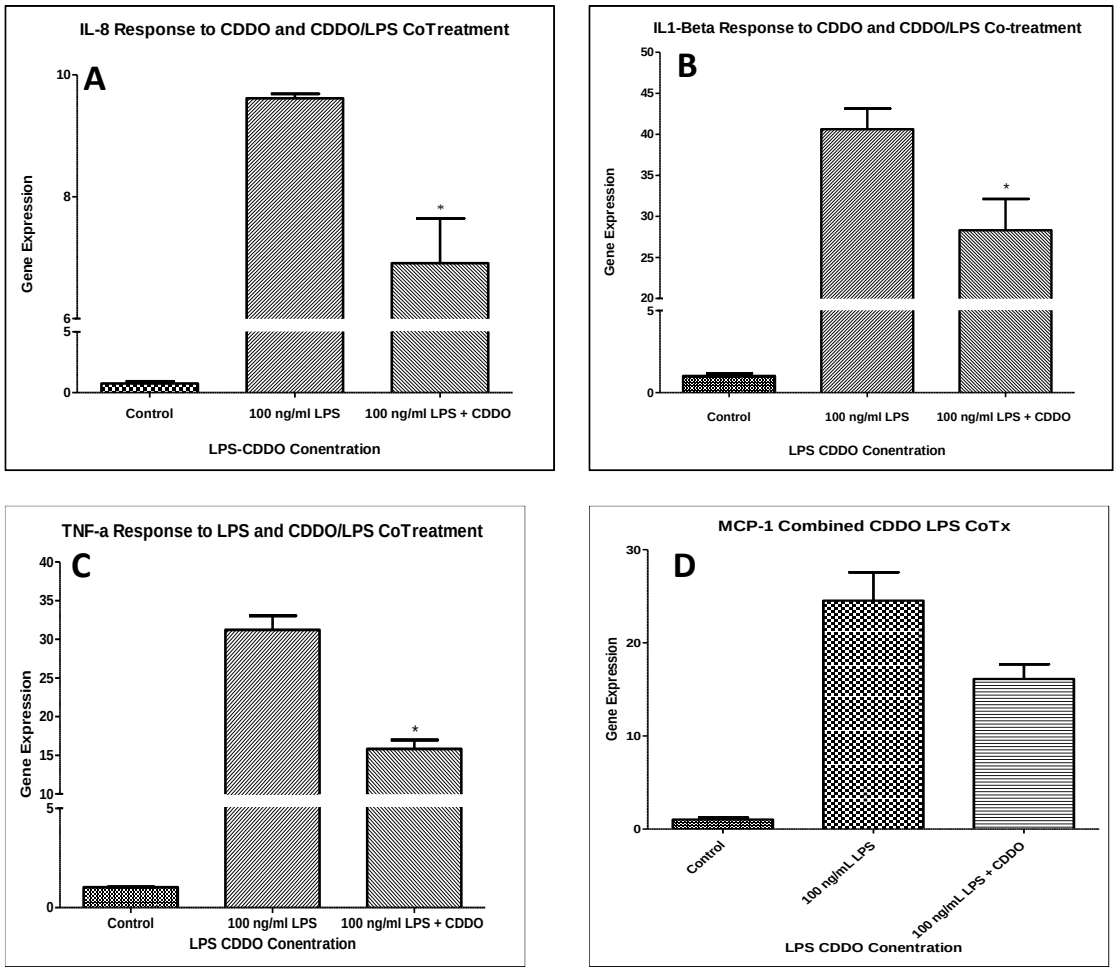


Figure 6. Pro-Inflammatory Cytokine Expression Response to LPS/CDDO Treatment. Co-treatment with CDDO-IM in macrophages resulted in a significant reduction in the upregulation of pro-inflammatory cytokines by LPS in the macrophages differentiated from ML-1 cells. Cells were treated with 400 nM CDDO-IM for 24 hours followed by a 3-hour 100ng/mL LPS treatment. Gene expression levels of IL-8 (A), IL-1-beta (B), TNF-a (C), and MCP-1 (D) were measured by real-time PCR and data were normalized using GAPDH. Data represents mean ± SEM (, n = 3, *, P<0.05 vs. 100 ng/mL LPS).

CDDO-IM Inhibits LPS-Induced ROS Generation

To examine the role of ROS and its involvement in the antioxidative and anti-inflammatory effects of CDDO-IM, ROS generation via LPS was measured by a reaction with lucigenin in macrophages treated with and without CDDO-IM. Lucigenin is a

bioluminescent compound used to measure levels of superoxide anion. A luminometer was used to measure this luminescent response for 30 minutes real-time at 37 ºC. Indicated in figure 7A, 100 ng/mL LPS treatment enhanced levels of superoxide generation whereas 1 µg/mL LPS treatment did not enhance ROS levels. A higher concentration of LPS can result in a decrease in cell viability which results in a decrease in the measurable levels of ROS. Co-treatment of macrophages with CDDO-IM and LPS was carried out to examine whether CDDO-IM influences the ROS overproduction by LPS. As indicated in figure 7B, intracellular superoxide overproduction by LPS was reduced by CDDO-IM.

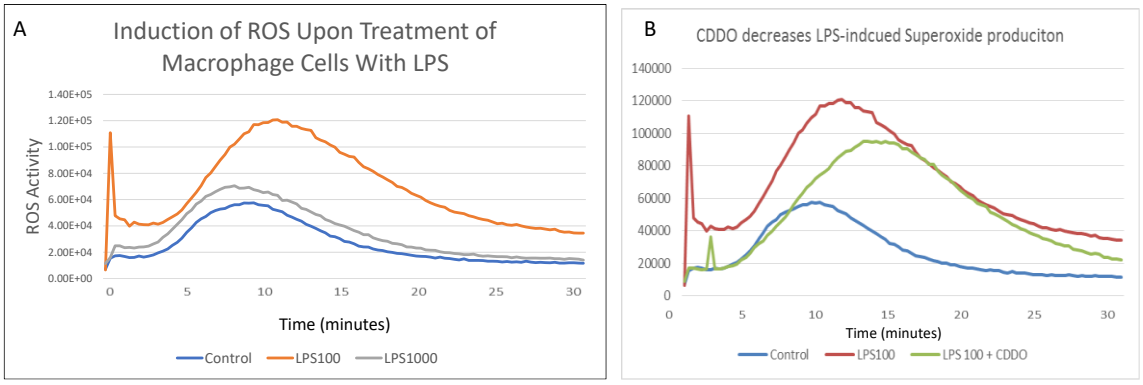
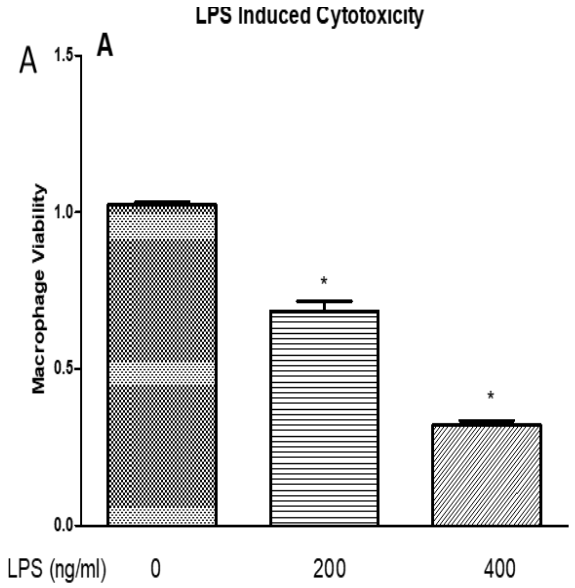


Figure 7. Induction of ROS in Macrophage Cells. A representative profile of superoxide production in macrophages incubated with LPS at various concentrations for 30 minutes (A). Intracellular superoxide production in macrophages treated with or without CDDO-IM for 24 hours followed by LPS stimulation for another 30 minutes (B).

CDDO-IM Protects Against LPS Toxicity

As shown in figure 7 macrophages treated with 100 ng/mL LPS resulted in an excessive amount of ROS production. Overproduction of ROS is known to increase oxidative stress and cell death, either through necrosis or apoptosis. The cytoprotective role of CDDO-IM was measured using the MTT assay. As shown in Figure 8A, macrophages treated with 200 ng/mL 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 40 nM CDDO-IM with respect to cells treated with only 200 ng/mL LPS (Figure 8B).



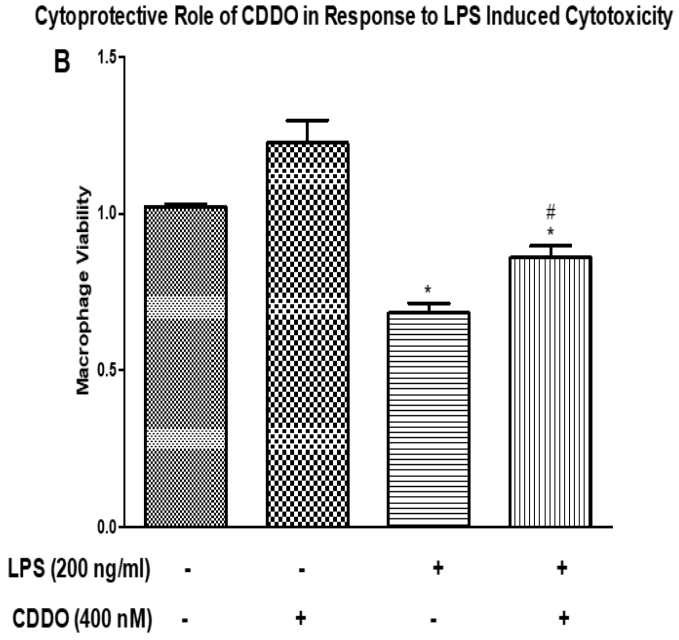


Figure 8. Cytoprotective Role of CDDO in Response to LPS-Induced Cytotoxicity. LPS-induced cytotoxicity (A) and cytoprotective effects of CDDO-IM against LPS Toxicity. The cells were treated with LPS as indicated concentrations for 24 hours and the cell viability was measured by MTT assay (A). The cells were pretreated with CDDO-IM at 400 nM for 24 hours followed by incubating the cells with LPS for another 24 hours (B). 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).*

CDDO-IM Attenuated LPS-Mediated NF-κB Activation

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 NFk-B 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 9). This result suggests that CDDO-IM can inhibit LPS-induced NF-κB activation.

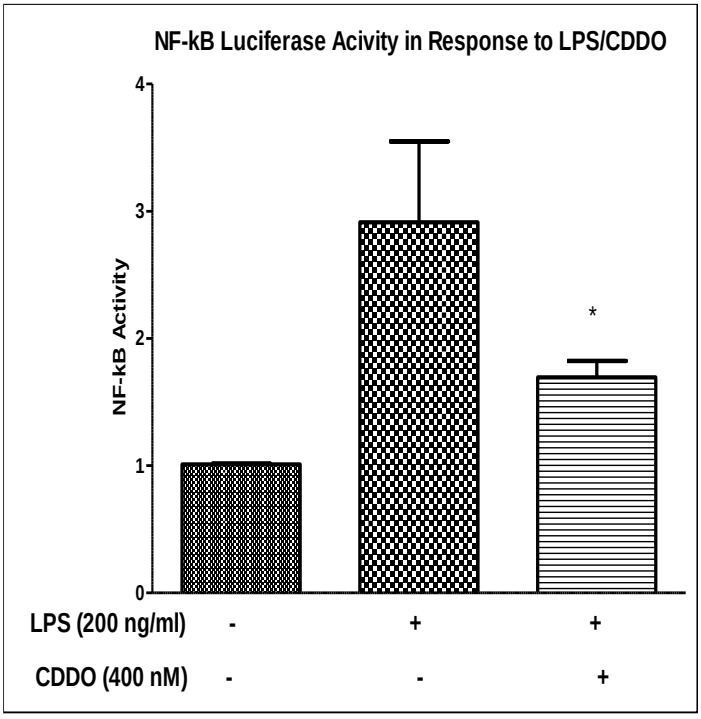


Figure 9. NF-kB Luciferase Activity in Response to LPS/CDDO. CDDO-IM reduced LPS-mediated NF-kB Activity in NFκB RAW264.7 macrophages. NFκB RAW264.7 cells (1 x10^5 cells/well) were plated in a 6-well format in assay medium and were pretreated with or without CDDO-IM at 400 nM for 24 hours followed by incubating the cells with 200 ng/ml LPS for another 3 hours. 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 represents mean ± SEM (*n = 3.*, P < 0.05 vs. LPS only).*

In Vivo Studies of CDDO-IM and LPS

The protective role of CDDO-IM was examined in C57/BL6 mice treated with LPS as well as co-treated with LPS and CDDO-IM. Body weight of mice was measured prior to and after treatments, and there was no statistical change in the body weight of mice in all treatment groups with respect to control (data not shown). Serum levels of the

cytokine TNF-alpha were measured in mice. A significant decrease in the levels of serum TNF-a was found in mice treated with LPS + CDDO-IM with respect to mice treated with LPS alone suggesting the anti-inflammatory property of CDDO-IM (Figure 10). Gene expression levels of inflammatory cytokine levels were also measured in liver samples. Significant reduction in the expression of TNF-alpha, IL1-beta, and IL-6 was found in the mice treated with LPS + CDDO-IM when compared to mice treated with only LPS (Figure 11).

Antioxidant genes GST and NQO1 were measured in the mice liver samples, and a 30-fold increase in the gene expression levels of GST was found with respect to control samples (Figure 12A). However, there was not a significant change in the gene expression levels of GST in these mice ($p>0.05$) due to limited sample size as well as large sampling variance. Gene expression levels of NQO1 remained unchanged in the liver tissue samples of all treatment groups (Figure 12B).

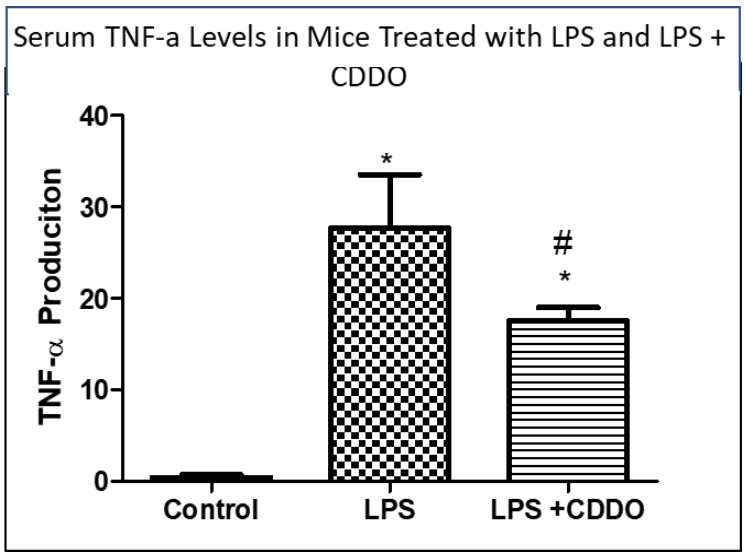


Figure 10. Serum TNF-a Levels in C57/BL6 Mice. CDDO-IM reduced the secretion of serum TNF-α in LPS treated mice. Serum TNF-α levels were measured by ELISA. Values are mean ± SEM, n=4. *, $p<0.05$ vs. control; #, $p<0.05$ vs. LPS alone-treated mice.

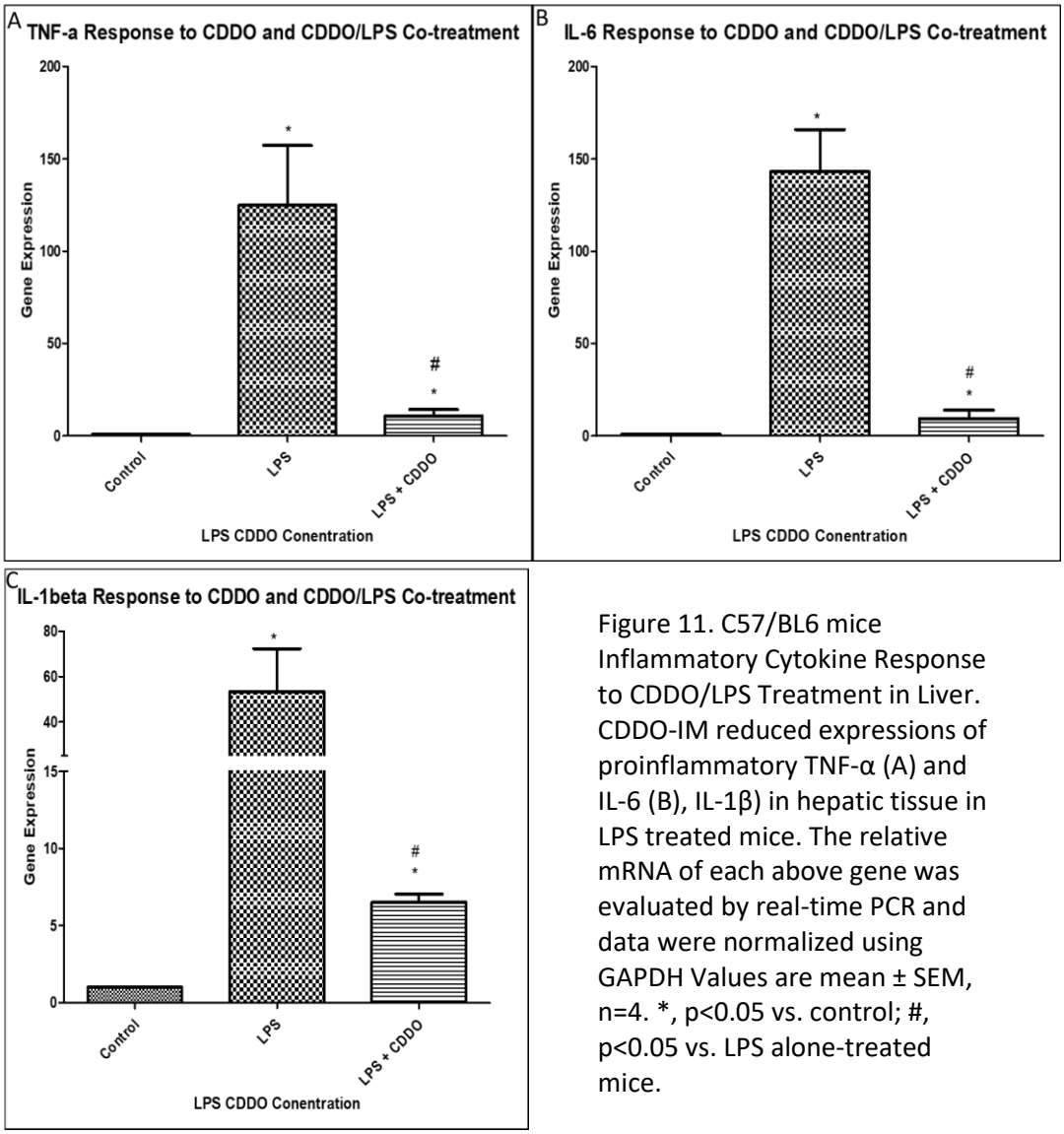


Figure 11. C57/BL6 mice Inflammatory Cytokine Response to CDDO/LPS Treatment in Liver. CDDO-IM reduced expressions of proinflammatory TNF-α (A) and IL-6 (B), IL-1β) in hepatic tissue in LPS treated mice. The relative mRNA of each above gene was evaluated by real-time PCR and data were normalized using GAPDH Values are mean ± SEM, n=4. *, $p<0.05$ vs. control; #, $p<0.05$ vs. LPS alone-treated mice.

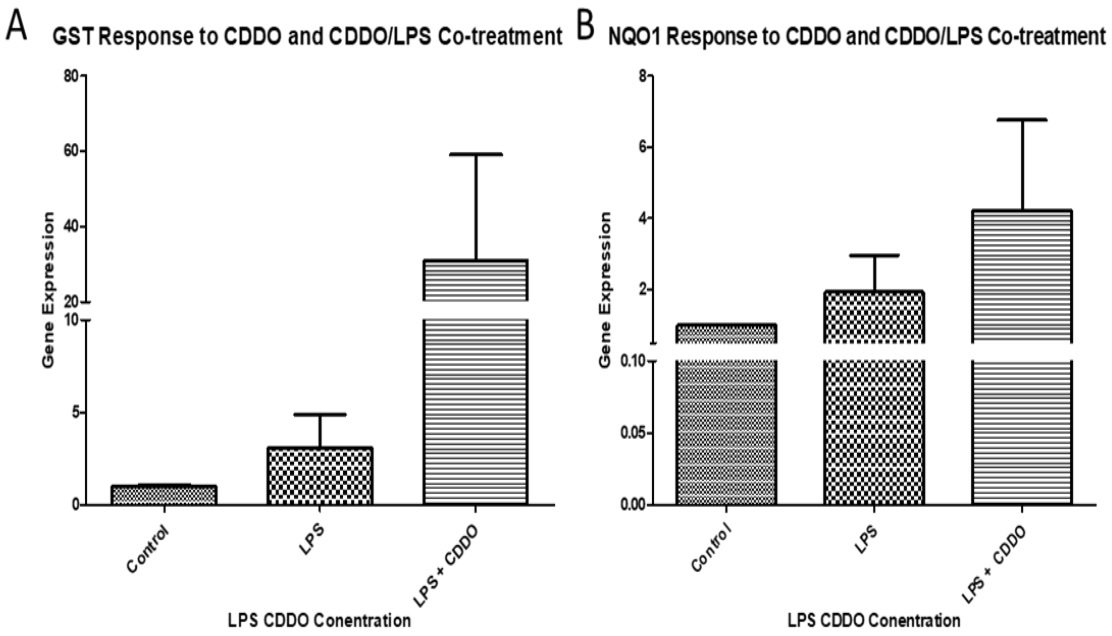


Figure 12. Antioxidant Enzyme Gene Expression in Liver of C57/BL6 Mice. Effects of CDDO-IM on the expression of GST (A) and NQO1 (B) in hepatic tissue in LPS treated mice. The relative mRNA of each above gene was evaluated by real-time PCR and data were normalized using GAPDH Values are mean ± SEM, n=4.

CHAPTER IV

DISCUSSION

Disruption in the host immune response during an infection triggers sepsis, a deadly disease. Inflammation and oxidative stress are prominent symptoms of sepsis which can be triggered by LPS, a prominent component present on the cell wall of gram-negative bacteria, in macrophages [1]. 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 [7]. 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, IL1-beta, TNF-a, and MCP-1 in macrophages were attenuated by CDDO-IM. 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-alpha, IL-8, and IL1-beta are elevated, whereas anti-inflammatory such as IL-10 and TGF-beta are reduced [13]. This elevation in pro-inflammatory cytokines and reduction in anti-inflammatory cytokines is also notable in sepsis patients[13]. 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 [13]. IL1-beta, 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 [15, 16]. TNF-a is a cytokine involved in the inflammatory response pathway and is able to induce apoptosis in cells undergoing stress via the caspase 3 pathway [15, 16]. Also, TNF-a is able to activate the NF-kB transcription factor to induce the transcription of pro-inflammatory cytokines [15, 16]. In this study, LPS-induced elevation in the expression of IL-8, IL1-beta, TNF-alpha, and MCP-1 was suppressed by CDDO-IM in the macrophages, indicative of the anti-inflammatory property of CDDO-IM. In vivo analysis showed that TNF-alpha secretion in LPS treated mice was significantly increased with respect to control mice. Consistent to our in vitro results, administration of CDDO-IM suppressed the circulating levels of TNF-alpha in the serum and reduced expressions of pro-inflammatory TNF-alpha and IL-6, IL-1β in hepatic tissue of mice treated with LPS.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is a well-known transcription factor in the regulation of the inflammatory response induced by LPS [39]. Immune cells contain Toll-like receptors (TLRs) to which LPS can bind to

triggering 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. [18]. NF-kB is activated during oxidative stress and is involved in regulating the transcription of pro-inflammatory cytokines and antioxidants [39]. 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 9), suggesting the mechanism of action of CDDO-IM may be modulated by this signaling pathway.

NF-κB is a heteromeric dimer composed of the p50 and p65 subunits bound to the inhibitor, IκB. This transcription factor is normally present in the cytoplasm of the cell in its inactive form. During oxidative stress, the IκB subunit undergoes phosphorylation and ubiquitination resulting in the degradation of this subunit by the 28S proteasome. Following this degradation, the heterodimer composed of p50 and p65 subunits translocate to the nucleus where, acting as transcription factors, bind to promoters of multiple genes involved in regulating inflammatory response [14]. To further understand the mechanism of the anti-inflammatory properties of CDDO-IM against LPS-induced surge in inflammatory cytokines, future studies can analyze NF-kB activation by examination of the phosphorylation and degradation of the inhibitory subunit IkBa. Also, nuclear translocation of the p50 and p65 subunits in macrophages can

be measured as well. The Ikb-alpha subunit when present in the cytoplasm indicates that NF-kB is inactive, whereas the absence of the Ikb-alpha subunit in the cytoplasm indicates the activation and nuclear translocation of p50 and p65 subunits of NF-kB.

Previous studies have shown that the overproduction of ROS which leads to oxidative stress and inflammatory surge plays a critical role in the development of sepsis [13]. ROS are present in cells to regulate a variety of cellular activities such as cell proliferation, gene expression, transcription factor activation, as well as DNA synthesis [13]. Despite this important role of ROS, overproduction leads to a dysregulation in the levels of inflammatory cytokines, which leads to inflammation and tissue injury and ultimately organ failure. H_2O_2 has been indicated to be involved in the activation of NF-kB, which is responsible for the regulation of many immune-inflammatory genes [17]. 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. Firstly, based on the data macrophages treated with CDDO-IM caused a concentration-dependent increase in the induction of antioxidants GSH and NQO1 (Figure 3). Also, the mRNA expression of GCLC, GCLM, HO-1, and GR were significantly increased in macrophages treated with CDDO-IM. 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 the reduction of cellular ROS levels, which in turn results in a decrease in NF-kB activity. Secondly, LPS treatment in cells has shown to increase the levels of ROS indicating that ROS plays

a vital role in LPS induced inflammation [15]. Upregulations of cellular antioxidant molecules by CDDO-IM, in turn, inhibits LPS-induced cellular ROS production (Figure 7). Lastly, LPS-induced cytotoxicity in macrophages can be reduced upon pretreatment with CDDO-IM (Figure 8).

Previous studies suggested that Nrf2 transcription plays an important role in the regulation of antioxidant molecules [36, 37]. Nuclear factor-erythroid 2–related factor 2 (Nrf2), a bZIP redox-sensitive transcription factor, is well-known to regulate the transcription of genes associated with ROS detoxification including GSH, NQO1, GR, HO-1, and heat-shock proteins [40]. For example, GST and NQO1 levels were reduced in the liver and intestine of mice that contained the disrupted Nrf2 gene [40]. The transcription factor Nrf2 can bind to the antioxidant response elements (AREs) that are regulated by direct binding of a heterodimer consisting of Nrf2 and a small Maf protein. This suggests that Nrf2/Maf directly activates the transcription of antioxidant enzymes [41]. Interesting, Nrf2 has been shown to bind upstream to the genes of pro-inflammatory molecules such as IL-6 and IL1-beta. CHIP-sequencing demonstrated that out of a total of 561 genes downregulated by Nrf2, 203 genes were downregulated directly by binding to the proximal region of these genes. Many of these genes are associated with sepsis [41]. The binding of Nrf2 halts the recruitment of RNA polymerase to the site of transcription of these genes, particularly IL-6 and IL1-beta [42]. In this context, due to the regulation of antioxidant genes by Nrf2, the reduction in LPS-induced pro-inflammatory cytokines by CDDO-IM (figure 5) can be associated with this transcription factor. Thus, future work could employ siRNA to block the expression of Nrf2 or use

Nrf2-knockout animals to understand whether the mechanism of CDDO-IM's upregulation of antioxidant genes and its anti-inflammatory action is through the Nrf2 pathway.

Dysregulation of the immune system during infection results in the development of sepsis and failure of the body to combat the infection leads to septic shock and ultimately death. During an infection, immune cells such as macrophage cells, combat bacteria, and its contents through phagocytosis. During this process, inflammatory cytokines are released to recruit more macrophages to the site of infection and induce phagocytosis, and the buildup of ROS in macrophages is imminent. The buildup of ROS and inflammatory cytokines leads to tissue and organ damage. Thus, combatting sepsis involves targeting pathways involved in the production of ROS levels and pro-inflammatory cytokine surge. In this study, LPS-induced pro-inflammatory cytokine surge, such as IL-8, IL1-beta, TNF-alpha, and MCP-1, is significantly reduced by CDDO-IM both in vitro and in vivo. Additionally, 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 and resulting in the protection against LPS-induced injury. This study can contribute towards the advancement of our understanding of treating life-threatening inflammatory diseases such as sepsis.

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