GAJE, GABRIELLA M., M.S. *In vitro* and *in vivo* roles of 3H-1,2-Dithiole-3-Thione in Lipopolysaccharide-Induced Toxicity and Inflammatory Injury (2021).
Directed by Dr. Zhenquan Jia. 48 pp.

Lipopolysaccharide (LPS) is a bacterial endotoxin known for being critical in triggering septic shock. It is widely used for studies to understand systemic inflammation that mimics the inflammatory storm of human sepsis patients. 3H-1,2-dithiole-3-thione (D3T) is a common dithiolethione that works as an anti-inflammatory agent in cancers and other inflammatory diseases. However, it is unknown whether D3T can reduce the LPS-induced expression of inflammatory and oxidative stress responses. This study aimed to examine the effect of D3T on LPS-induced expressions of anti-oxidative and anti-inflammatory genes *in vivo* and *in vitro*. *In vivo* mice trials demonstrated that the administration of LPS showed a significant increase in expression of two key pro-inflammatory genes interleukin-6 (IL-6) and monocyte chemoattractant protein (MCP-1) in liver tissues. D3T significantly reduced the LPS-induced gene expression of MCP-1 and IL-6, in mouse kidney tissue. Similarly, D3T mitigated the LPS-induced gene expression of MCP-1 in mouse liver tissue. Mouse liver tissue treated with D3T showed significant increases in the antioxidant gene expression of GCLM and HO-1. The results indicate that D3T has an inflammatory effect *in vivo*. For the in vitro portion of the study, 100 μM D3T was able to significantly increase the gene expression of HO-1 and GCLC in THP-1 cells. However, the high concentration of D3T also induced the pro-inflammatory gene expression of IL-8 in THP-1 cells. D3T at this concentration was not effective in mediating the expression of LPS-induced cytotoxicity or expression of pro-inflammatory cytokines. This is likely due to the increase of IL-6 by D3T alone. D3T displayed anti-inflammatory properties *in vivo*, but further studies are needed to explore

the effects of D3T in low doses against pro-inflammatory gene expression in THP-1 cells and other cell lines induced by LPS. In conclusion, the results of the current study could contribute to D3T as a potential therapeutic agent to reducing the inflammatory responses caused by the LPS endotoxin *in vivo*.

IN VITRO AND *IN VIVO* ROLES OF 3H-1,2-DITHOLE-3-THIONE IN LIPOPOLYSACCHARIDE-INDUCED TOXICITY AND INFLAMMATORY INJURY

by

Gabriella M Gaje

A Thesis

Submitted to

the Faculty of The Graduate School at

The University of North Carolina at Greensboro

in Partial Fulfillment

of the Requirements for the Degree

Master of Science

Greensboro

2021

Approved by

Dr. Zhenquan Jia
Committee Chair

APPROVAL PAGE

This thesis written by Gabriella M Gaje has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair

Dr. Zhenquan Jia

Committee Members

Dr. Paul Steimle

Dr. John Lepri

June 23, 2021

Date of Acceptance by Committee

June 23, 2021

Date of Final Oral Examination

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ILLUSTRATIONS

CHAPTER I: INTRODUCTION

Sepsis and Lipopolysaccharides (LPS, also termed endotoxin)

Sepsis:

Sepsis is defined as being a storm of various abnormalities induced in an organism by infection. Sepsis has many physiological, pathological, and biochemical effects on the body. Unfortunately, sepsis affects a large amount of the population and is a mounting public health concern [1]. Sepsis was defined by a consensus conference in 1991 as "the combination of an infection with two or more features of what was called the 'systemic inflammatory response syndrome' (SIRS)" [1].

Mortality rates for sepsis are inexplicably high, most noticeably in elder populations and those with many comorbidities. A large risk factor for developing sepsis is some form of pre-existing immune system weakness. Despite a definition update in 2003, doctors and researchers have found it very difficult to pinpoint an exact infection based on clinical signs and symptoms. This led to the thought process that the problem may not lie in the infection itself, but rather the mechanistic immune response [2].

In sepsis patients, it appears that the failure of one organ will often lead to the failure of other subsequent organs. This can occur through a multitude of processes, including inter-organ crosstalk, where one organ's dysfunction influences the function of another. For example, sepsis-induced acute kidney injury can eventually lead to the dysfunction of the heart and lungs, associated with fluid overload (hypervolemia). Endothelial dysfunction is another major player, as the endothelium provides the inner layer of the cells of blood vessels. Endothelial permeability is increased in sepsis, leading

to hypervolemia and subsequently decreased tissue perfusion. The combination often causes multiple organ failure seen in sepsis patients [3].

Lipopolysaccharides (LPS, also termed endotoxin)

LPS is classified as endotoxin and found in the walls of gram-negative bacteria. Endotoxins are a form of pathogen-associated molecular patterns (PAMPs). LPS is known as the most well-understood endotoxin because it has an established role in pro-inflammatory pathways. With LPS being recognized as a major gram-negative PAMP, many researchers use LPS as the main toxin in their research. Different strains of LPS have different levels of immune responses in mammals. In a review by Kayle Dickson and Christian Lehmann, it is mentioned that LPS from *Helicobacter pylori* is much less toxic than LPS from Enterobacteriaceae. These variations can be seen in the chemical structure of the toxin. There are three portions that makeup LPS: Lipid A, an O side chain, and core oligosaccharide, with Lipid A being the main PAMP of LPS [4]. LPS is also indicated in causing neutrophil degranulation, which is an essential function of neutrophils [5]. When there is an inflammatory response, such as sepsis, in an organism, neutrophils are shown to secrete MPO or myeloperoxidase. MPO can be used as a marker for tissues being infiltrated by inflammatory cells [6].

The hydrophobic Lipid A keeps LPS attached to the outer membrane, the oligosaccharide is known as "the core," and the O antigen attaches to this core. Lipid A has two factors that affect its potential to cause a greater immune response. These factors include the length and quantity of the acyl chains and the number of phosphate groups. When there are longer acyl chains with two phosphate groups, there is an increase in the

intensity of the immune response. For optimal TLR-4 activation, six acyl chains are needed, while four acyl chains would cause Lipid A to become an antagonist to TLR-4 [7]. Due to the unique nature of LPS's structure, it makes it very difficult for hydrophobic solutes to permeate across the outer membrane. The biosynthesis of LPS occurs over three locations: the cytoplasm, IM, and periplasm. Multiple independent pathways ultimately end up working in an assembly line fashion to biosynthesize LPS [8].

Lipid A is the main component for LPS toxicity in sepsis. For bacteria to survive in LPS, both Lipid A and the core oligosaccharide must be present. When the O antigen is attached, LPS is referred to as smooth. When the O antigen is missing, then LPS is referred to as rough. The status of the LPS can greatly impact its susceptibility to toxins and mutagens. CD36 is a scavenging receptor that can activate macrophages in the presence of both rough and smooth LPS. Research suggests that smooth LPS may be more efficient at inducing cytokine gene expression [9].

Why is LPS so important for the simulation of sepsis? For starters, LPS is relatively non-toxic to rodent models in comparison to humans. Larger doses are necessary to induce septic shock and pro-inflammatory cytokines in the rodent model. LPS can induce hypothermia and cause decreased cardiac output with increased peripheral vascular resistance. Additionally, the effects of increased cytokine levels can happen relatively quickly, with high doses of LPS administration. In general, LPS is a reliable model for its convenience and ease of reproducibility. It is able to be measured reliably and can be easily standardized. Overall, the LPS induced rodent model has more long-standing physiological responses, which makes it ideal to study [10].

Multiple studies have shown that the synthetic creation of monophosphoryl Lipid A (MPLA) may be able to reduce the inflammatory effects of sepsis caused by LPS. MPLA is created by cleaving the C1 phosphate group on Lipid A [4]. While MPLA also has six acyl chains and similar properties to LPS, it is much less toxic due to the cleaved phosphate group. Many factors that LPS can heavily induce the expression of, such as IL-1β, have much less effect with MPLA. MPLA has been tested in vaccine trials and what found was that the administration of MPLA intravenously induced endotoxin tolerance, leading to lower levels of cytokine gene expression and less symptoms exhibited by human/primate test subjects [9].

Inflammation and Cytokines/Chemokines

It is known that inflammatory cytokines and chemokines such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) can contribute to the underlying pathologies of sepsis [11]. Inflammation is an immune response that the body uses to fight foreign pathogens. The immune system is divided into two parts when responding to the pathogens: innate and adaptive. The innate immune system initiates the inflammatory response, which leads to vasodilation, leukocyte emigration, and vascular leakage. The innate immune system acknowledges the pathogens via pattern recognition receptors, including toll-like receptors. The pattern recognition receptors are expressed through a variety of immune cells such as macrophages, neutrophils, and monocytes. Neutrophils adhere to endothelial cells and migrate past the vascular wall to adhere to the site of infection. If the innate immune system cannot control the site of infection, the adaptive immune system is activated. The adaptive immune system activates specific T

or B cells to clear foreign pathogens. However, if the adaptive immune system also fails to control the infection, the cells eventually end up in a cyclical state of inflammation, which produces reactive oxygen species [12].

Cytokines are proteins that are released by cells and have specific effects on the interactions between cells. Cytokine is a general term that can be modified based on the type of cells that produce the cytokines. Different cell types are capable of secreting the same cytokine and there are certain types of cytokines that can act on several cell types. Cytokines are rarely produced singularly and often happen in a cascade. Pro-inflammatory cytokines are produced mainly by activated macrophages and involved with the upregulation of inflammatory reactions. They are often indicated in the process of pathologic pain. IL-1β is released by monocytes during cell injury or infection. TNF-α, also known as cachectin, can act on several signaling pathways to regulate apoptotic pathways. Chemokines are a class of cytokines known to induce chemotaxis. They primarily function to activate and migrate leukocytes. There are reserved cysteine residues in chemokines that allow them to be classified into four groups: CC, CXC, C, or CXXXC. There are a variety of chemokines that include MCP-1 [13].

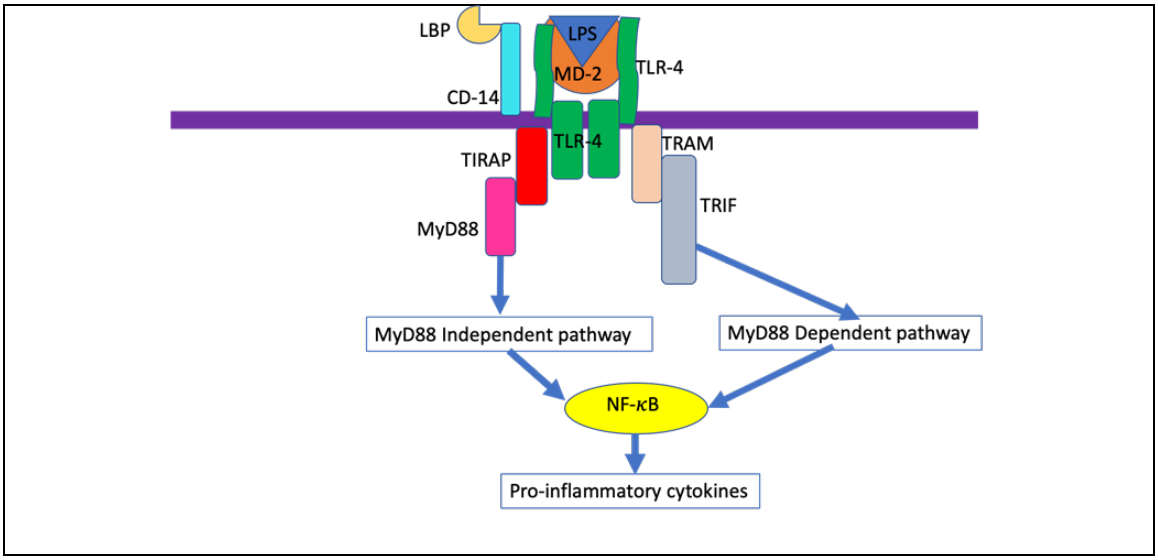
Toll-like Receptor 4 Signaling and NF-κB pathway

Toll-like receptors: Toll-like receptors, or TLRs, are indicated in causing immune responses in mammals. They are a group of pattern recognition receptors that are expressed by leukocytes, endothelial cells and parenchymal cells. LPS is known as a TLR-4 agonist, which means that the application of LPS in the cells will initiate pathways involving TLR-4. Although initially thought to just be expressed in the immune

system, TLR-4 can also be expressed in mesangial cells and tubular cells of the kidney [14]. Working in concurrence with myeloid-differentiation factor 2 (MD-2), LPS activates TLR-4. This is caused when the lipopolysaccharide-binding protein (LBP) and CD14 allow LPS to bind to its immune cell receptor. Once LPS has bound to its receptor, adaptor proteins come into the intracellular domain and activate the MyD88 pathways [9]. This pathway can be seen below.

Next comes the interaction with (TRAM)/TIR-domain-containing adaptor-inducing interferon B (TRIF) proteins which activates another pathway. Coupled with the MyD88 pathways, TRIF can produce the previously mentioned pro-inflammatory cytokines [7]. MyD88 is essential for TLR-4 to activate a downstream signaling pathway, which induces an inflammatory response.

Illustration 1: An overview of the LPS/TLR-4 signaling pathway



LPS is recognized by the facilitation of LBP and CD14, then mediated by the TLR-4/MD-2 receptor complex. The MyD88 pathway mediates the activation of pro-inflammatory cytokines via the NF-κB pathway.

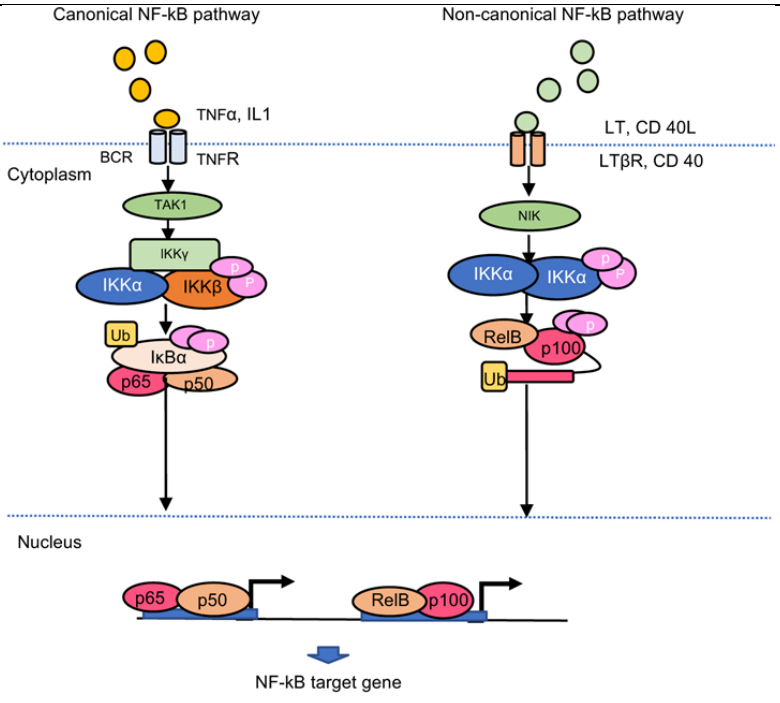
NF-κB pathway

When TLRs are activated by the LPS stimulus, it in turn, activates NF-κB, which is a nuclear factor. It is composed of many dimeric transcription factors that can organize the inflammatory responses seen in ailments such as sepsis or different types of cancer. A misregulation in the system causes the responses and has been shown in therapeutic studies. Once NFκB is activated, it can cause the expression of various chemokines, cytokines, and cell surface molecules such as IL-1, IL-6, TNF-α, and IL-8 [14]. A study by Jian Wan et al. tested the effects of inhibiting NF-κB in relation to LPS-induced TLR 4 activation in monocytes. The study utilized helenalin as a NFκB inhibitor. What the study found was that LPS is a strong activator of NF-κB transcription activity. By inhibiting NF-κB, the induction of TLR-4 by LPS was also inhibited [15].

The NF-κB pathway is critical in the involvement of various inflammatory reactions. For mammals, there are five protein monomers that make up the NFκB network: p65/RelA, RelB, cRel, p50 and p52. These monomers are regulated by two separate pathways: NFκB essential modulator (NEMO)-dependent (Canonical) and (NEMO) independent pathway (non-canonical). These activation pathways regulate NF-κB activity through many mechanisms. Signals for TNFR, TLR and IL-1R receptors become integrated by the IκB/ NF-κB signaling network [1]. The canonical pathway is

induced by TNF-α and other various stimuli. It is an IKKβ-dependent cascade. Activation of the cascade leads to the phosphorylation of IkBα, which results in degradation by the proteasome. The degradation releases the NF-κB complex and allows it to translocate to the nucleus. The non-canonical pathway is caused by much more specific stimuli such as lymphotoxin α and is an IKKα-dependent cascade. Cascade activation results in phosphorylation of NIK, then phosphorylation of IKKα and subsequent phosphorylation of the p100 NF-KB subunit. This subunit is processed by p52, which leads to the activation of the p52-RelB heterodimer. The heterodimer is then translocated to the nucleus where it has control of the transcriptional processing of target genes [16]. These processes can be seen in the LPS/TLR4 pathway schematic.

Illustration 2: The NF-κB pathway



The NF-κB pathway demonstrating both the canonical and non-canonical pathways.

Following activation, IkBα is phosphorylated by IKK at two N-terminal serines, which triggers the ubiquitin-dependent IkBα degradation in the proteasome. This results in a quick, transient nuclear translocation of the canonical NF-κB members. The dominant members are the p50/RelA and p50/c-Rel dimers. The nuclear translocation of NF-κB will soon cause the transcription of pro-inflammatory cytokines such as IL-6 and TNF-α. Th canonical pathway is known as being rapid while the non-canonical pathway is indicated as being a much slower process [17]

Nrf2 Pathway

The Keap-1/Nrf2/ARE signaling pathway regulates this inflammation and ultimately releases anti-inflammatory cytokines [18]. NF-E2 related factor 2 (Nrf2) is controlled by Kelch-like erythroid-associated protein 1 (Keap-1) which allows for the proteasomal degradation of Nrf2. Nrf2 belongs to the Cap 'n' Collar (CNC) subfamily which is comprised in seven functional domains that are essential for the interactions that occur between Keap-1 and Nrf2 [19]. Once this degradation occurs, Keap-1 and Nrf2 are no longer combined and Nrf2 can move into the nucleus. Once it is in the nucleus, it will bind to the antioxidant response element that will eventually encode for cytoprotective, anti-inflammatory phase II enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferase (GST).

There are two types of Nrf2 regulation that can occur: Keap1-dependent and Keap1-independent. Keap1- dependent is the mechanism by which Nrf2 translocates. New evidence is emerging that show that Nrf2 may be able to regulate independently of Keap1. This mechanism involves the serine rich Neh6 domain of Nrf2. It binds with two

motifs (DSGIS and DSAPGS) to B-transducin repeat-containing protein (B-TrCP). B-TrCP is a substrate receptor that pinpoints Nrf2 in the process of ubiquination and proteasomal degradation. Another important player is glycogen synthase kinase-3, which phosphorylates Nrf2 in the Neh6 domain. This will facilitate B-TrCP to recognize Nrf2 and begin protein degradation [19].

The activation of the Nrf2 pathway can inhibit the activation of the NF-κB pathway [19]. Ultimately this pathway serves to relieve the deleterious effects in reaction to dangerous xenobiotic compound toxification and oxidative stress that can be caused by a multitude of stressors [20].

Dithiolethione compound 3H-1,2-dithiole-3-thione (D3T)

3H-1,2-dithiole-3-thione, also known as D3T, is a common dithiolethione. It is a sulfur containing compound that is bioavailable via the ingestion of cruciferous vegetables such as broccoli and kale. These compounds are known for their chemoprotective properties that also code for phase II and antioxidant enzymes. The cytoprotective effects of D3T include the upregulation of phase II enzymes during the Keap-1/Nrf2/ARE pathway as seen in a study with zebrafish embryos [20]. D3T can specifically target the Keap-1/Nrf2 interaction, which translocates Nrf2. When Nrf2 is translocated, it binds to ARE, which then causes the release of phase II antioxidant genes [21].

A study explored maternal ethanol exposure in mouse embryos found that D3T increased the embryo Nrf2 protein expression. D3T was used to further induce the expression of Nrf2 in the embryos. Not only did it increase Nrf2 expression, D3T

significantly reduced the ROS gene expression and apoptosis caused by ethanol in the embryos. However, the study indicated that the entire mechanism underlying D3T mediated Nrf2 activation is not completely understood, and further research needs to be conducted to understand the mechanism more completely [22].

D3T has been heavily indicated in protecting cortical neurons *in vitro* from the formation of hyperoxidized perioredoxins after oxidative trauma occurs. D3T has also proven to be highly lipophilic and capable of crossing the blood brain barrier. A study of Alzheimer's disease in a mouse model demonstrated that the administration of D3T led to the amelioration of oxidative stress and improved hippocampal neurogenesis. This is indicated by the increased levels of GSH in the mice following the administration of D3T. This study also looked at the protein levels of Nrf2 and HO-1, which are both players in antioxidation. Ultimately, the study was able to show the neuroprotective effects that D3T could provide by ameliorating the oxidative stress caused by Alzheimer's disease. This could possibly indicate D3T as a treatment in other inflammatory disease processes [23]

LPS, a bacterial endotoxin, is known for triggering septic shock, which is why it has been widely used for *in vitro* and *in vivo* studies. These studies are to further understand the systemic inflammation that mimics the inflammatory storm in sepsis patients [24]. As previously mentioned, D3T can serve as an anti-inflammatory agent in inflammatory diseases such as cancer. However, it is unknown if D3T can reduce LPS-induced inflammatory and oxidative responses. This study aimed to further investigate

the findings of previous studies to determine whether D3T can be used to mitigate the effects of LPS-induced toxicity both *in vivo* and *in vitro*.

CHAPTER II: MATERIALS AND METHODS

Materials

RPMI 1640 media and Trizol RNA extraction agent were purchased from MP Biomedicals (Solon, OH). Chloroform used for RNA isolation, ethanol and lipopolysaccharide from *Escherichia coli* were purchased from Sigma-Aldrich (Sheboygan Falls, WI). isopropyl used in RNA isolation, was bought from Alfa Aesar (Ward Hill, MA). THP-1 monocyte cells were purchased from ATCC (Manassas, VA). cDNA synthesis materials nuclease-free water, MMLV-RT, 5X buffer and random primer were purchased from Invitrogen (Waltham, MA). Deoxynucleoside triphosphate used for cDNA synthesis was purchased from New England Biolabs (Ipswich, MA). Both human and mouse forward and reverse primers used for qRT-PCR were bought from Eurofins Genomics (Louisville, KY). SYBR Green fluorescent dye and MicroAmp® Fast 96-well reaction plates were purchased from Applied Biosystems by Thermo Fisher Scientific (Waltham, MA). Trypan Blue solution used for the cell viability assay was purchased from Gibco (Grand Island, NY). Other chemicals, including TRIzol Reagent, Isopropanol, horseradish peroxidase, luminol and 9 10-phenanthrenequinone (9,10-PNQ) were procured from Thermo Fisher Scientific (Waltham, MA).

Animal Study

The *in vivo* study was used to analyze LPS-induced inflammatory and oxidative stress in mice and whether D3T treatment could ameliorate this oxidative and inflammatory response. Animal protocol No. 010517-ZHU-1 was used for this experiment. 8 to 10-week male C57BL/6 mice from Jackson Laboratories located in Bar

Harbor, ME were utilized in this experiment. This strain is known to be the most widely used strain in biomedical and toxicology research [25]. Mice were maintained in a humane environment at the Campbell University School of Osteopathic Medicine compliant with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. This *in vivo* study was approved by the Campbell University Animal Welfare Committee. Mice were fed an AIN-93G diet (BioSey, NJ) as a control for cofounding variables. 4 groups of mice were generated (control, LPS, D3T, and LPS + D3T) with 4 mice randomly placed into each group. The diet was given to every mouse for one week before the treatments. D3T was prepared and administered by gastric gavage at 30 μmol/kg body weight for 24 hours followed by the administration of LPS via intraperitoneal injection at 5 mg/30 g of body weight for 15 hours. Oral gavage has been proven to be an accurate method of dosing mice. The dosages were determined by preliminary data performed at Campbell University. The following equation demonstrates how to calculate the equivalent dose in a human [26].

Illustration 3: Human Dose Equivalent

$$\mathrm{HED}\,(\mathrm{mg/kg}) = \mathrm{Animal\ NOAEL}\,(\mathrm{mg/kg}) \times \left(\mathrm{Weight}_{\mathrm{animal}}\,[\mathrm{kg}] / \mathrm{Weigth}_{\mathrm{human}}\,[\mathrm{kg}]\right)^{(1-0.67)}$$

The equation to calculate an equivalent dosage from animal model to human.

Following treatments, Mice euthanasia was performed to obtain organ tissue and plasma samples. Organs were stored at -80 °C and transferred to UNCG. Mouse liver and kidney organ tissue samples were obtained to analyze the anti-oxidative/ anti-inflammatory gene expression as well as pro-inflammatory cytokine expression levels.

The pro-inflammatory cytokine gene expression of IL-6 and MCP-1 was measured via qRT-PCR following RNA extraction of liver and kidney samples. RNA was extracted from liver and kidney samples to measure gene expression of GCLC, GCLM, NRF2, and HO-1.

Cell Culture

THP-1 monocytes were purchased from ATCC (Manassas, VA). These cells are derived from the peripheral blood of a child with acute monocytic leukemia and held in a liquid nitrogen tank until ready to use. The cell line was grown in RPMI 1640 (Gibco, cat. No: 11875093, Gaithersburg, MD) complete media, treated with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies Corporation, Grand Island, NY).

Cell Count (Trypan Blue)

The Trypan Blue dye exclusion assay determines the number of viable cells compared to dead cells in a cell treatment. The assay abides by the principle that viable cells had intact cell membranes compared to dead cells, which would not allow cells to be stained blue in the cytoplasm [27]. $9x10^6$ monocyte cells were counted and added to two 50 mL tubes. One tube was treated with 100 uM D3T and the other was treated with DMSO to serve as a control. 1 mL of the 100 uM D3T treated cells were added to 12 wells of a 24 well plate. 1 mL of the DMSO treated cells were added to the remaining 12 wells. Treatments of LPS 100 ng/mL and LPS 1000 ng/mL were added to 18 wells, leaving 6 untreated and serving as a control. Trypan blue was used to count unstained, viable cells using a hemocytometer and the concentration was calculated.

D3T Treatment for qPCR assay

3H-1,2-dithiole-3-thione (D3T) is a common dithiolethione compound. The cells were cotreated with various concentrations of LPS and 100 μM of D3T for 3 hours, with approximately $9x10^6$ cells present in each petri dish. The cells were then used for RNA isolation and qPCR assay.

Luminescent assay for detection of cellular H_2O_2 gene expression

Levels of cellular H_2O_2 were measured using the luminol-dependent chemiluminescence assay. THP-1 cells were harvested and suspended in saturated complete phosphate buffer saline (cPBS) solution which contained 0.5 mM MgCl2, 0.7 mM $CaCl_2$, and 0.1 % glucose. Microcentrifuge tubes (1.5 mL) were created for each sample and 5 μL of luminol and 5 μL of horseradish peroxidase (HRP) were added to measure cellular H_2O_2 production. Next, 100 ng/mL and 1000 ng/ mL of LPS and 100 μM of D3T were added to aliquots of 9×10^6 cells resuspended in 1 mL cPBS at 37 ºC. Each sample was pipetted into a white opaque 96 well plate with 250 μL added to each well. Each treatment was represented on the plate in 3 wells each. The ROS luminescence assay gene expression was measured in the Bio-Tek microplate reader at the following time points: 1 hour, 2, hours, 3 hours, and 20 hours. 10-phenanthrenequinone (9,10-PNQ) was added to the plate to serve as a positive control for ROS production starting from the 1-hour time mark. The luminescence intensity amplified by ROS was measured in the same plate reader for 30 minutes for 16 intervals.

RNA Isolation

To prepare for RNA isolation of the tissues, frozen liver and kidney samples were processed and weighed on ice to avoid thawing. Tissue samples weighing 50-75 mg were homogenized in 1 mL of Trizol reagent using a PRO Scientific PRO250 homogenizer (Oxford, CT). The volume of the sample could not exceed 10% of the Trizol reagent volume that was used for homogenization. For the cell RNA isolation process, $9x10^6$ THP-1 cells were cultured in complete RPMI media in Corning® cell culture treated plates (Corning Life Sciences, Durham, NC, USA). Upon treatment with 100 ng/mL of LPS and 100 μM D3T and incubation, cells and media were added to 15 mL centrifuge tubes. The cells were then centrifuged at 300 g for 10 min at 4 ◦C. The supernatant media was decanted, and the remaining cell pellets were treated with 1 mL of ambion TRIzol® (Thermo Fisher Scientific, Waltham, MA, USA). The solution was pipetted into 1 mL Eppendorf tubes. 200 μL of chloroform was added, followed by agitation, and then centrifuged at 12,000 rcf for 15 min. The top aqueous phase was transferred to another set of 1 mL Eppendorf tubes and combined with 500 μL isopropanol before centrifuging again at 12,000 rcf for 10 min. The resulting pellet (RNA) was washed with 1 mL 75% ethanol and centrifuged at 7400 rcf for two intervals of 5 minutes each. The pellet was air-dried for 8-10 minutes then resuspended in 10–20 μL of DEPC H_2O.

cDNA Synthesis

RNA concentrations were measured by taking 1 μL of the sample and measuring it in the Nanodrop UV-Vis spectrophotometer. The RNA concentrations were diluted to 500 ng/μL. A reverse transcriptase was used to synthesize cDNA from the 500

ng/mLRNA. cDNA reagents include DEPC-treated water, 5x First Strand buffer, deoxynucleotide triphosphate (dNTP) solution, Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and Random primers in addition to 2 μL of RNA. Using the Applied Biosystems™ Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA), the 25 μL solution was converted to cDNA.

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

Once the cDNA was synthesized, target genes such as IL-8, GCLM, GCLC, HO-1, NRF2 and TNF-α were used compared to the constitutive gene of GAPDH for cell samples and the housekeeping gene of Beta-Actin for tissue samples. These target genes were created using forward and reverse primers. cDNA and target gene mixtures were placed in a clear 96-well plate prior to being synthesized. The StepOnePlus Real-Time PCR system ran for 40 cycles at 95 degrees C for 15 seconds, 58 degrees C for 1 minute and 60 degrees C for 15 seconds. Gene expression was quantified by using comparative threshold values.

Statistical Analysis

GraphPad Prism® software was used for statistical analyses and the results are expressed as mean± S.E.M. Student t tests were performed to obtain statistical significance (P value). Changes were considered significant when $P<0.05$.

Table 1: Human Primer Sequence

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'
GCLM	5'- CTCCCTCTCGGGTCTCTCTC- 3'	5'- ATCATGAAGCTCCTCGCTGT- 3'
TNF-α	5'- CTA TCT GGG AGG GGT CTT CC - 3'	5'- GGT TGA GGG TGT CTG AAG GA-3'
GCLC	5' – ACC ATC ATC AAT GGG AAG GA – 3'	5' – GCG ATA AAC TCC CTC ATC CA – 3'
HO-1	5'- TCCGATGGGTCCTTACACTC- 3'	5'- TAAGGAAGCCAGCCAAGAGA- 3'
NRF2	5'- GCGACGGAAAGAGTATGAGC- 3'	5'- GTTGGCAGATCCACTGGTTT- 3'

Table 2: Mouse Primer Sequence

Target Gene	Forward Primer	Reverse Primer
B-Actin	5'- CTT CCT TCC TGG GTA TGG AAT C-3'	5'- CCA GGA TAG AGC CAC CAA TC-3'
IL-6	5'- CTGCAAGAGACTTCCATCCAGTT- 3'	5'- AGGGAAGGCCGTGGTTGT- 3'
MCP-1	5'- TTCCTCCACCACCATGCAG- 3'	5'- CCAGCCGGCAACTGTGA- 3'
GCLC	5'- TGGAGCAGCTGTATCAGTGG- 3'	5'- TGGCACATTGATGACAACCT- 3'
GCLM	5'- AACACAGACCCAACCCAGAG- 3'	5'- ATCCTGGGCTTCAATGTCAG- 3'

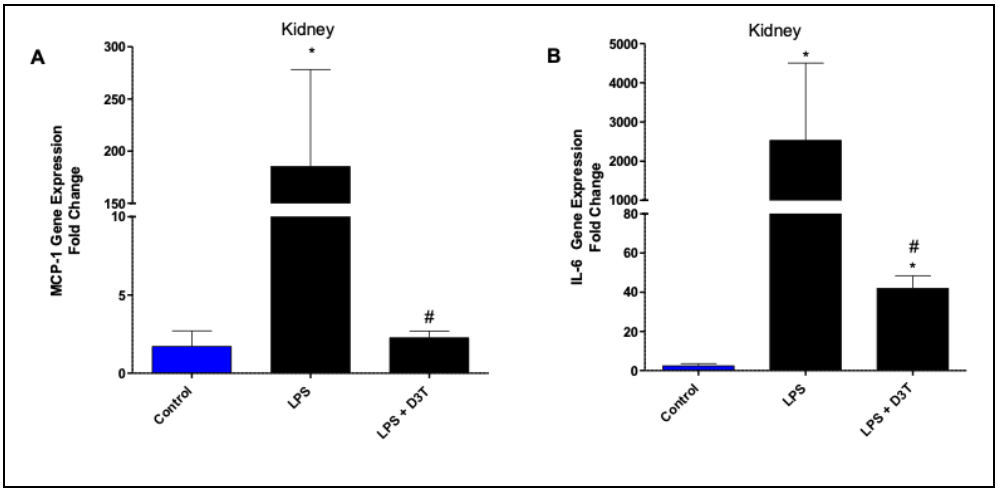
HO-1	5’- CACGCATATACCCGCTACCT-3’	5’-CCAGAGTGTTCATTCGAGCA-3’
CCL2	5’- GTC TCA GCC AGA TGC AGT TAA T-3’	5’- GCT GAA GTC CTT AGG GTT GAT G-3’

CHAPTER III: RESULTS

In vivo studies of D3T and LPS

D3T has previously been shown to provide protection against inflammation in various cancers, but it is unclear if it shares similar properties when used with the endotoxin LPS *in vivo*. The role of D3T was examined in C57/BL6 mice that were treated with LPS, D3T and a cotreatment of LPS and D3T. Both liver and kidney tissue were analyzed via qRT-PCR following RNA isolation and cDNA synthesis of the tissue. There was 185-fold change of MCP-1 pro-inflammatory gene expression in LPS treated mice compared to control ($p < 0.05$) but only a 1-fold change of LPS+D3T treated mice (Figure 1A), suggesting that there was an anti-inflammatory effect of D3T in the *in vivo* treatment. As shown in Figure 1B, the kidney tissue of D3T treated mice showed a statistically significant decrease in LPS-induced IL-6 pro-inflammatory gene expression compared to LPS group ($p < 0.05$).

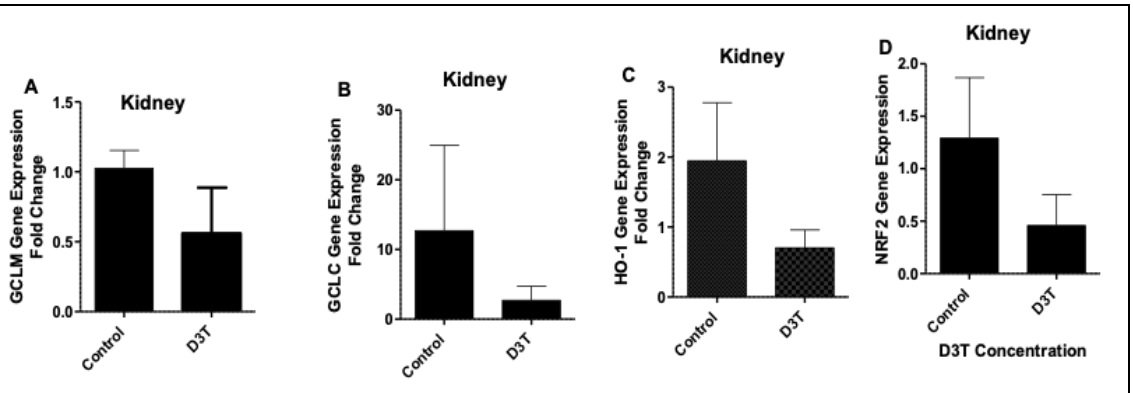
Figure 1. D3T reduced expressions of pro-inflammatory genes (MCP-1 and IL-6) in kidney tissue in LPS treated mice.



Exposure to LPS in the presence and absence of D3T to the C57BL/6 mice was described in Methods. The relative mRNA of each above gene was evaluated by real-time PCR and data were normalized using beta -Actin. Values are mean ± SEM, n=3-4. *, p<0.05 vs. control; #, p<0.05 vs. LPS alone-treated mice.

Antioxidant genes GCLM, GCLC, HO-1 and NRF2 were measured in the mice kidney tissues with no statistically significant changes between control and D3T treated mice ($p < 0.05$) although all gene expressions were shown to have a decrease in D3T treated mice compared to control (Figure 2).

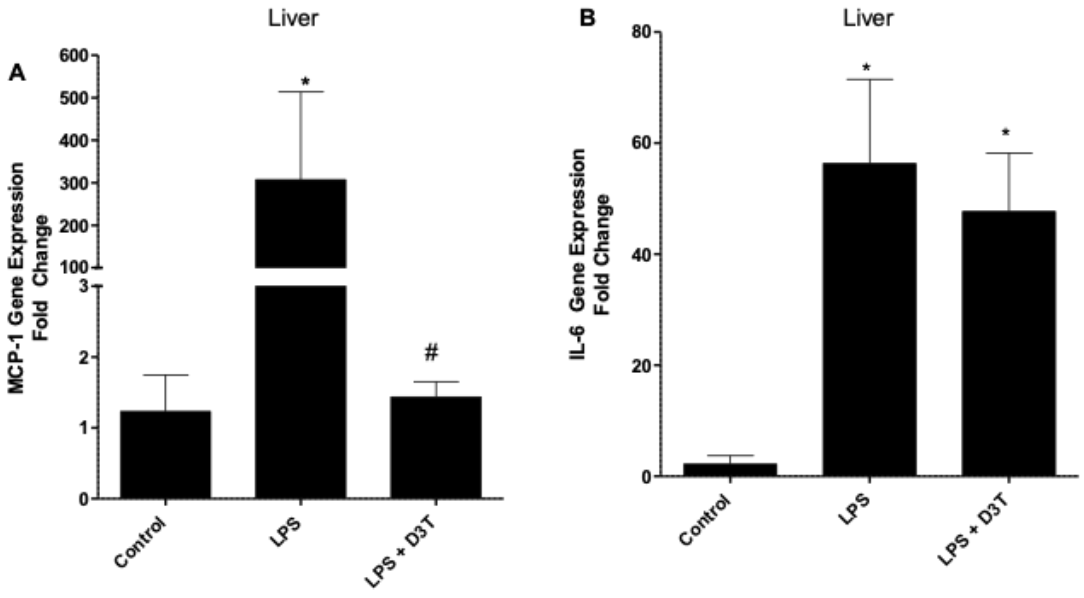
Figure 2. Effect of D3T on expressions of anti-inflammatory/antioxidant genes (GCLM, HO-1, GCLC, NRF2) in kidney tissues



The relative mRNA of each above gene was evaluated by real-time PCR and data were normalized using beta-Actin. The values are shown mean ± SEM, n=4.

As shown in Figure 3A, the liver tissue showed an approximate statistically significant 300-fold change between control and LPS for pro-inflammatory gene expression of MCP-1 ($p < 0.05$). The treatment of D3T caused a significant inhibition of LPS-induced MCP-1 gene expression in liver tissues ($P<0.05$). This is consistent with the inhibitory effects seen on MCP-1 in kidney tissues. However, the addition of D3T did not affect the LPS-induced gene expression of IL-6 in liver tissue (Figure 3B).

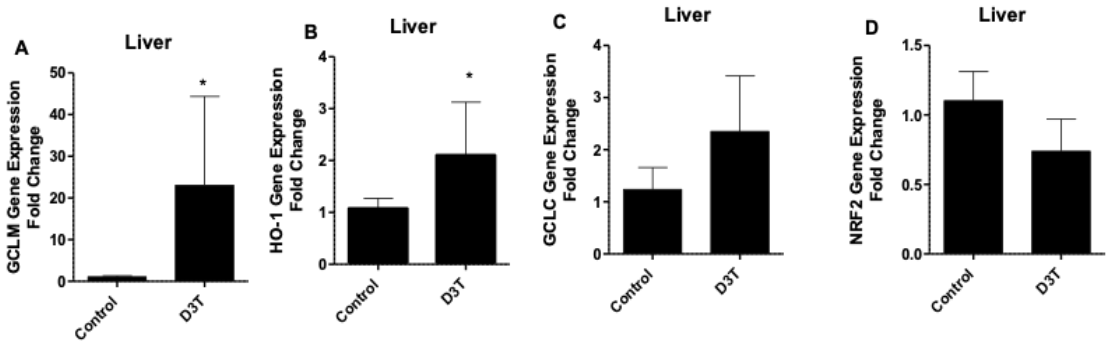
Figure 3. Effect of D3T on expressions of pro-inflammatory genes (MCP-1 and IL-6) in liver tissues of mice treated with LPS.



The relative mRNA of each above gene was evaluated by real-time PCR and data were normalized using beta -Actin. Values are mean ± SEM, n=4. *, $p<0.05$ vs. control; #, $p<0.05$ vs. LPS alone-treated mice.

Antioxidant genes GCLM, GCLC, HO-1 and NRF2 were measured in mice liver tissue. The expression of GCLM and HO-1 show a significant increase when treated with D3T and compared to control (Figure 4A-B). While there was an increase in the gene expression of GCLC (Figure 4C) and a decrease in Nrf2 gene expression (Figure 4D), these changes were not statistically significant ($p < 0.05$) when compared to control.

Figure 4. Effect of D3T on expressions of anti-inflammatory/antioxidant genes (GCLM, HO-1, GCLC, NRF2) in liver tissues.

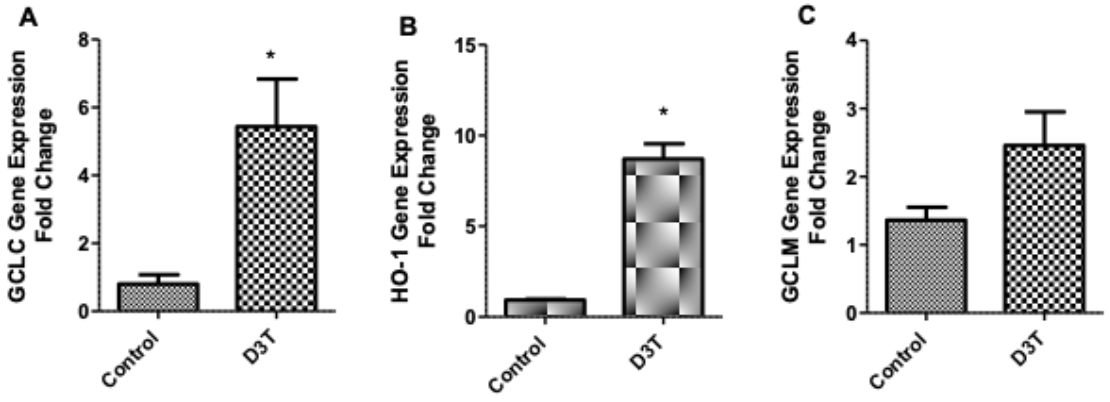


The relative mRNA of each above gene was evaluated by real-time PCR and data were normalized using beta-Actin. Values are mean ± SEM, n=4.

D3T treatment induces antioxidant gene expression in THP-1 cells

The use of THP-1 monocytes have been useful in vitro as models of endotoxin-mediated studies related to sepsis and LPS-induced inflammation[28]. D3T has been previously reported to have antioxidant properties in neurological disease models but has not been studied in THP-1 cells [23]. In order to determine the effect of D3T in THP-1 cells, $9x10^6$ cells were treated with 100 uM D3T for 3 hours and measured in qRT-PCR following RNA isolation and cDNA synthesis GCLM, HO-1 and GCLC are antioxidant genes that were used to analyze the gene expression of D3T treated THP-1 cells. There is an increase in the antioxidant gene expression of all three genes, with statistically significant 8-fold change increase ($p < 0.05$) being shown in HO-1 (Figure 5B) and a 5-fold change increase in GCLM (Figure 5A) when D3T treated mice are compared to control (Figure 5).

Figure 5. Effect of D3T on expressions of anti-inflammatory/antioxidant genes (GCLC, HO-1, and GCLM) in human THP-1 monocytes.



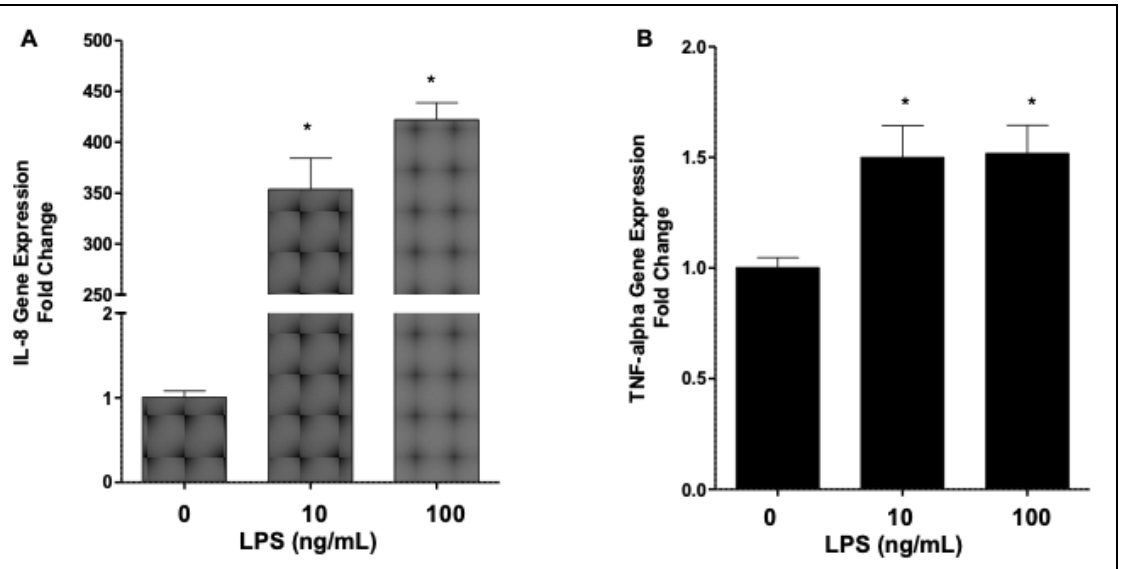
9 x 10^6 cells were treated with 100 µM D3T for 3 hours. After treatment, cells was extracted for RNA and cDNA was synthesized, for qRT-PCR. The relative mRNA of each above gene was evaluated by real-time PCR and data were normalized using beta-Actin. Values are mean ± SEM, n=2. *, $p<0.05$ vs. control

LPS treatment induces pro-inflammatory gene expression in THP-1 cells

LPS is a widely used compound known to induce inflammatory responses in cells. IL-8 and TNF-α are pro-inflammatory cytokines that were used to analyze the gene expression of LPS treated THP-1 cells. 9x10^6 cells were treated with 10 ng/mL and 100 ng/mL LPS for 3 hours and measured in qRT-PCR following RNA isolation and cDNA synthesis. There are statistically significant increases in IL-8 and TNF-α gene expression by LPS at 10 ng/mL and LPS 100 ng/mL when compared to control (Figure 6). Results of this study led to LPS at 100 ng/mL to be chosen for the D3T effect. Figure 7 demonstrates the gene expression of IL-8 and TNF-α in cells treated with LPS 100 ng/mL, LPS 100 ng/mL in the presence of 100 µM D3T, and cells only treated with 100 µM D3T. Interestingly, Figure 7A shows that the co-treatment of 100 µM D3T caused a significant increase in the LPS-mediated gene expression of IL-8. In D3T treated cells

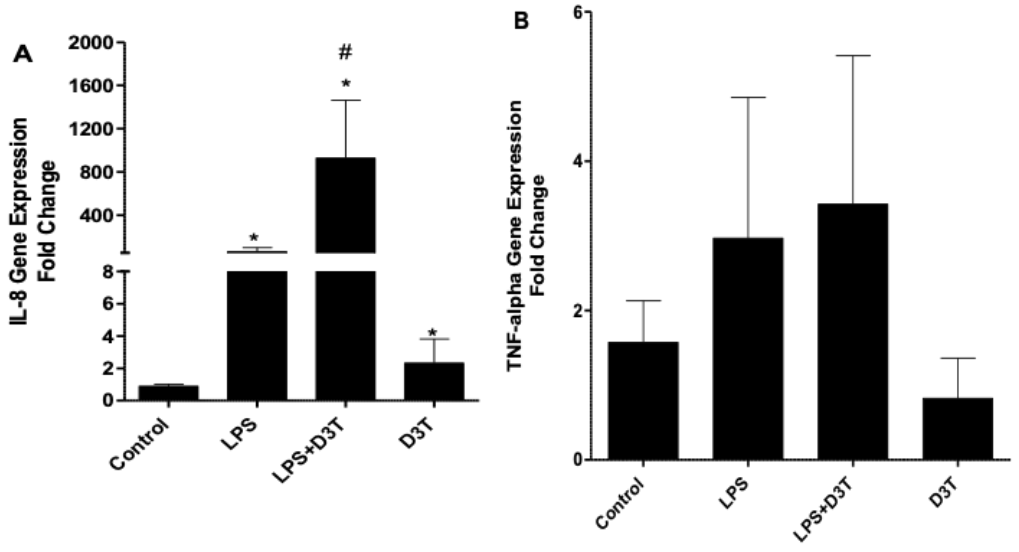
alone, there is a 2-fold increase in IL-8 gene expression compared to control ($p < 0.05$). Figure 7B shows a 2-fold increase in TNF-α gene expression by LPS in presence or absence of D3T when comparing to control but this change is not significant ($p < 0.05$).

Figure 6: Dose-dependent response of IL-8 and TNF-α gene expression due to LPS exposure.



THP-1 monocytes Cells (9x 10^6) were treated with 0, 10, and 100 ng/mL of LPS for 3 hours. Gene expression levels of) and IL-8 (A) and TNF-α (B)were measured by RT-PCR. Data were normalized using the housekeeping gene GAPDH. (All results are represented as mean ± SEM, n=3, *, $p<0.05$ vs. control).

Figure 7. Pro-inflammatory cytokine gene expression response to LPS/D3T Treatment in THP-1 cells



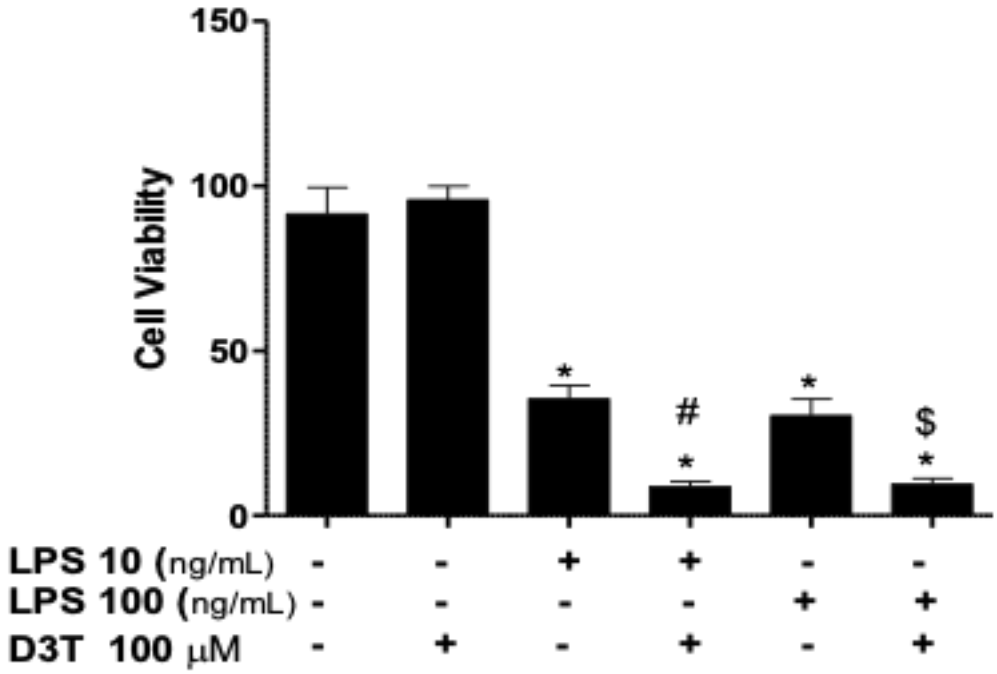
Cells (9x $10^{6)}$ were cotreated with 100 μM D3T and 100 ng/mL LPS for a 3-hour. mRNA level of IL-8 and TNF-α was measured by RT- PCR. Data was normalized using GAPDH as a housekeeping gene. Data represents mean ± SEM (, n = 3, *, P<0.05 vs. Control, #, P<0.05 vs.100 ng/mL LPS).

Effects of D3T on LPS-induced cytotoxicity in THP-1 cells.

LPS is known to cause the expression of pro-inflammatory genes that correspond to lethal toxicity in various cell types. It is not known whether the antioxidant properties of D3T can reduce such toxicity, resulting in higher cell viability. Cell viability was measured following a cotreatment of 100 μM D3T with 100 ng/mL LPS and 1000 ng/mL LPS for 24 hours. The viability of the cells was then measured with the Trypan blue assay. There is a statistically significant decrease in the viability of cells treated with LPS at 100 and 1000 ng/mL compared to control. Surprisingly, the treatment of D3T further enhanced cytotoxicity induced by LPS in co-treatments with both concentrations of LPS (Figure 8), It must be mentioned that while other treatments have 3-6 replicates, D3T

only has two. Hence the uncertainty of D3T alone causing toxicity, which is seen by the expression of pro-inflammatory genes (Figure 7A).

Figure 8: Effect of D3T on LPS-induced toxicity in THP-1 cells.



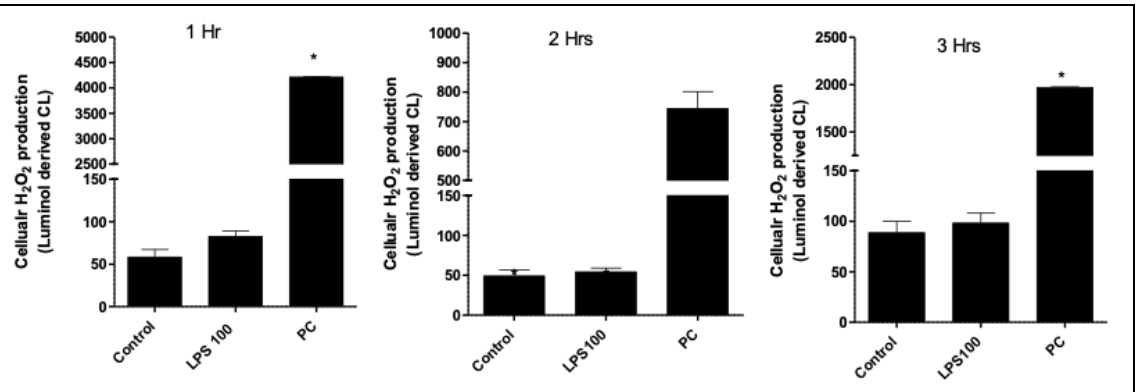
Cells were cotreated with different concentrations of LPS for in the presence or absence of 100 μM D3T for 24h and cell viability was measured using Trypan Blue assay. Data are mean ± SEM (n = 3–6 per group, except for n=2 for D3T alone). *, $p<0.05$ vs. control; #, $p<0.05$ vs. LPS 10 ng/mL alone-treated cells; $, $p<0.05$ vs. LPS 100 ng/mL alone-treated cells.

H_2O_2 gene expression induced by LPS

Reactive oxygen species such as peroxide (H_2O_2) can contribute to inflammatory responses and oxidative stress. To determine whether ROS contributes to the inflammatory effects of LPS, the generation of cellular H_2O_2 was measured in THP-1 cells. Figure 9 demonstrates the effect of LPS on the gene expression of H_2O_2 in THP-1 cells. There is no significant change ($p < 0.05$) in the production of cellular H_2O_2 between

control and LPS at any represented time point (1 hr (figure 9A), 2 hr (Figure 9B), 3 hr (Figure 9C). However, there is a statistically significant increase of the H_2O_2 production of the 9-10-PNQ positive control when compared to control at all given time points ($p < 0.05$). These results indicate that the cellular production of H_2O_2 in THP-1 cells may not contribute to the inflammatory effects of LPS.

Figure 9: Cellular H_2O_2 levels in LPS-treated THP-1 cells.



Production of H_2O_2 was measured using luminol -derived chemiluminescence (CL), for 1 hr (A), 2 hr (B), and 3 hrs (C) as described in Material and Methods. In the positive control (PC), the THP-1 cells were incubated alone with 1 µM of 9 10-phenanthrenequinone (9,10-PNQ) for various hours as indicated above. Data are mean ± SEM (n = 3 per group). *P < 0.05 compared to control group.

CHAPTER IV: DISCUSSION

Lipopolysaccharide (LPS) is an endotoxin found in the walls of gram-negative bacteria. The compound has previously been associated with its capabilities of inducing inflammation by stimulating cells to produce pro-inflammatory cytokines [29, 30]. For this reason, LPS has been a long-standing model to simulate inflammation-mediated sepsis both *in vivo* and *in vitro* [29, 30]. However, there have been no specific studies exploring the effect of D3T on LPS-induced inflammatory injuries. This study aimed to examine the effect of D3T on LPS-induced expressions of anti-oxidative and anti-inflammatory genes *in vivo* and *in vitro.* The *in vivo* mice trials indicated that D3T mitigated LPS-induced gene expressions of two notable pro-inflammatory cytokines, MCP-1 and IL-6, in the kidney tissues. D3T-treated mice showed a decrease in the expression of MCP-1 in the liver tissue, which correlates to the inhibitory effects of the MCP-1 gene expression in kidney tissue. The results are indicative that D3T has an anti-inflammatory effect *in vivo*. Liver tissue showed significant increases in the antioxidant gene expression of GCLM and HO-1 by D3T, but kidney tissue did not.

Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a chemokine known for its potency and recruitment of monocytes. MCP-1 is produced following the induction of oxidative stress and has been seen in the pathologies of many inflammatory diseases such as sepsis, cancer and HIV. MCP-1/CCL2 has long been studied as it was one of the first discovered chemokines [31-33]. The gene for MCP-1 is located on chromosome 17 and can be induced by a variety of factors such as TNF-α. The binding of MCP-1 to coupled G-protein receptors on leukocytes activates cellular reactions, including intracellular

calcium release. MCP-1 recruits monocytes during infection when bound to its receptor CCR2. Infection can cause MCP-1 to promote the migration of inflammatory monocytes from bone marrow to blood in circulation [34]. The present studies show an over 300-fold-increase in MCP-1 gene expression in mice liver tissue treated with LPS and a 185-fold-increase in the mice kidney tissues when compared to control. The treatment of D3T in kidney tissue eradicated the LPS-induced gene expression of MCP-1 by 185 times to the same levels as control. Similarly seen in the liver tissue was a 300 time decrease to the LPS-induced gene expression of MCP-1 with the administration of D3T. Such results indicate that D3T can potentially reduce inflammation by targeting and inhibiting MCP-1 gene expression.

Interleukin-6 (IL-6) is known as a regulator to transit from neutrophil to monocyte recruitment during inflammation [35-38]. This cytokine travels through the bloodstream to the liver following inflammation. This movement induces rapid gene expression of acute phase proteins such as serum amyloid A (SAA). The rapid proliferation of the phase proteins can lead to chronic inflammatory pathologies due to the generation of amyloid A amyloidosis, eventually causing organ failure [35-38]. IL-6 is also known to promote specified differentiation for naïve CD4 T-cells. This works to link the innate to acquired immune response. There is a variety of responses to IL-6 that are found in other cell types besides hepatocytes and lymphocytes that can contribute to chronic inflammatory diseases. Its ultimate function is to work as a mediator of notification when an emergent event occurs, such as infection. The results of this study displayed that IL-6 gene expression in mice liver tissue was increased by more than 50 times with the

administration of LPS compared to control mice. IL-6 gene expression in mice kidney tissue increased by more than 2500 times when compared to control mice. The treatment of D3T inhibited IL-6 gene expression induced by LPS in kidney tissue from 2500 times to 40 times. Despite these findings, there was no effect from D3T on the IL-6 gene expression in liver tissue of mice co-treated with LPS. Such results indicate that inhibition of IL-6 gene expression by D3T may be specific to each organ.

It is unclear as of now which molecular mechanisms are responsible for the anti-inflammatory effects of D3T on LPS *in vivo.* Nuclear factor-kappa B (NF- κB)'s pathway is highly involved in the expression of pro-inflammatory cytokines and chemokines induced by LPS [14, 39, 40]. When LPS binds to Toll-like receptors (TLRs) on immune cells, it activates the NF- κB transcription factor. When the transcription factor is activated, it begins the transcription and activity of pro-inflammatory cytokines. The increasing accumulation of pro-inflammatory cytokines contributes to the progression of sepsis [41]. In both liver and kidney studies *in vivo*, it has been shown that LPS activates inflammatory signals following interaction with CD14 and TLR-4 to stimulate the transcription factor NF- κB, which regulates inflammatory processes [42]. Once NF-κB enters the nucleus, it induces the gene expression of the previously mentioned pro-inflammatory cytokines, leading to oxidative stress and ultimately organ injury [43]. NF-κB can be inactivated by IκBα, which is an inhibitor of the κB kinase (IKK) . IκBα can be used as an indicator as to whether or not NF-κB is being inhibited by observing the amount of degradation of IκBα in the cytosol. IκBα degradation in the cytosol is indicative that NF- κB is not being inhibited, and thus continuing to express pro-

inflammatory genes [44]. Further studies are needed to determine whether the NF- κB pathway's interaction with D3T in vivo causes the reduction of inflammatory responses. The NF- κB pathway can be evaluated by using the NF- κB RAW264.7 cell line and the use of immunoblot. Coelenterazine is used to detect the presence of the NF-κB response element in the cell line. The response element's presence will be evaluated following treatment with LPS and D3T using the NF-κB reporter cell line. The use of immunoblot is to detect the degradation of I κB α in the cytosol. I κB α is an inhibitor of the κB kinase which inactivates NF- κB. The degradation of I κB α is indicative that NF- κB is not being inhibited and continuing to express pro-inflammatory cytokines.

Nuclear factor erythroid 2-related factor 2 (Nrf2) has been shown in previous studies to have downstream upregulations of anti-inflammatory and anti-oxidative genes such as heme oxygenase-1 (HO-1) and GCL that are pivotal to the downregulation of NF- κB [45, 46]. . It has been shown that Nrf2 can interfere with NF- κB for p-50 translocation to the nucleus and the inhibition of Nrf2 activity can be a target in inflammation therapy [45, 46]. HO-1 contains cytoprotective qualities, such as antioxidant and anti-inflammatory characteristics, which can reduce inflammation, apoptosis and cellular distress [47, 48]. Glutathione (GSH) is a protective antioxidant in various organs and possesses anti-inflammatory properties. GSH synthesis from constituent amino acids requires an ATP-dependent enzyme, γ-glutamylcysteine ligase (GCL) [49, 50]. The heterodimer GCL is a rate limiting enzyme in the pathway and contains a catalytic (GCLC) and modulatory (GCLM) subunit. The previously mentioned Nrf2 acts as a transcription factor in the cell's cytosol and is isolated by keap1. Nrf2

works with the antioxidant response element (ARE) to activate the expression of anti-oxidative and anti-inflammatory genes such as GCLM and GCLC [51, 52]. The results of this study showed that D3T treatment leads to a significant increase in the gene expression of GCLM and HO-1 in liver tissue indicating that activation of Nrf2 maybe be a potential pathway for D3T to protect against LPS-induced inflammation in vivo. Future studies are needed to examine the relationship between NF- κB and Nrf2 to determine whether the activated Nrf2 pathway occurs *in vivo* with the administration of D3T. Such a study could further explain how D3T contributes to the decrease of LPS-induced pro-inflammatory gene expression.

Both the liver and kidney were evaluated in this study as they are both vital organs to be targeted by LPS. The liver can be injured by a variety of factors such as toxins, inflammatory mediators or pathogens. The dysfunction of the liver can often be seen in signs of early sepsis and can ultimately lead to total liver failure. Those with pre-existing chronic liver diseases such as cirrhosis are often more susceptible to higher rates of morbidity. Injuries to the liver shown in sepsis are often confirmed by histological changes in the tissue. The liver is the largest organ in the body and holds many roles in maintaining homeostasis. However, while it is able to clear bacteria endotoxin LPS from the body, it can also cause further inflammation and organ damage [53]. Septic acute kidney injury (AKI) is the most commonly found AKI in ICUs. It is the acute impairment of kidney function and organ damage following a duration of sepsis in the body. Biomarkers have become the main detection method for early kidney stress and damage in order to try and prevent AKI. The kidney is another organ that provides immune

responses to inflammatory reactions, including cell cycle arrest, which may inhibit further injury. Like the liver, septic AKI has been indicated in higher mortality rates [54]. This implicates LPS to be a contributor to liver and kidney injury associated with sepsis and inflammatory responses following LPS exposure. The aforementioned study has shown that LPS-induced sepsis can lead to multiple organ failure as seen *in vivo* and in sepsis patients [3]. Further exploration is needed to discover whether D3T can eradicate the LPS-induced inflammatory injury of other organs such as the heart, lungs, and brain.

The *in vitro* portion of this study demonstrated that 100 μM D3T significantly increased the gene expression of GCLC and HO-1 in THP-1 cells. The results are consistent with the gene expression of anti-oxidative and anti-inflammatory genes of liver tissue *in vivo* following D3T treatment. However, the anti-inflammatory effects of D3T in *in vitro* studies with THP-1 monocytes do not yield similar results. While the study involving differing concentrations of LPS in monocytes shows an increase of pro-inflammatory cytokines, all other cellular studies have shown that D3T was not effective in mediating the gene expression of pro-inflammatory cytokines in combination with LPS. Cell viability studies also indicates that the co-treatment of D3T with 100 ng/mL LPS may further cause toxicity in cells rather than ameliorate it when compared to LPS-alone treated cells. 100 μM D3T treated cells showed an over 2-fold-increase in the gene expression of the previously mentioned pro-inflammatory genes. The results indicate that high concentrations of D3T are capable of pro- and anti-inflammatory properties. Thus, the pro-inflammatory effect of D3T at higher concentrations could cause cell toxicity. Further studies should investigate whether low doses of D3T will have an effect on LPS-

induced pro-inflammatory gene expression. A study by Michael A. Trush, et al looking at the chemoprotective properties against benzene-induced hematoxicities found that D3T was able to induce the gene expression of quinone reductase in HL-60 cells and glutathione S-transferase in mouse liver. The study also found that D3T was able to protect against inhibition by hydroquinones of the differentiation of ML-1 cells to macrophages and monocytes. There was evidence in the present study that showed D3T does have a protective effect on both liver and kidney tissue *in vivo* in concurrence with the previously mentioned study. However, the current study indicates that D3T may not have these same effects in monocytes due to the use of the LPS compound compared to the hydroquinone compound of the study by Trush [55]. Future studies should also study the effects of LPS and D3T on different types of cells such as hepatocytes, as cell signaling processes may yield different results than the ones mentioned above.

In summary, the results of this study demonstrated that D3T mitigated the LPS-induced gene expression of key pro-inflammatory cytokines, MCP-1 and IL-6, in mouse kidney tissue. D3T treated mice also showed a decrease in the LPS-induced gene expression of MCP-1 in mouse liver tissue. The mouse liver tissue showed significant increases in GCLC and HO-1 antioxidant gene expression. These results are indicative that D3T has an anti-inflammatory effect *in vivo.* The results of this study may contribute to the use of D3T being a potential alternative to reduce inflammatory responses caused by endotoxin LPS.

REFERENCES

1. Mervyn Singer, M., FRCP, Clifford S. Deutschman, MD, MS, Christopher Warren Seymour,, et al., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)*. 2016.
2. Faix, *Established and novel biomarkers of sepsis.* Biomarkers in Medicine, 2011: p. 117-130.
3. Prauchner, C.A., *Oxidative stress in sepsis: Pathophysiological implications justifying antioxidant co-therapy.* Burns, 2017. **43**(3): p. 471-485.
4. Dickson, K., & Lehmann, C. , *Inflammatory Response to Different Toxins in Experimental Sepsis Models.* International Journal of Molecular Sciences, 2019: p. 1-13.
5. Hernandez A, P.N., Stothers CL, Luan L, McBride MA, Owen AM, Burelbach KR, Williams DL, Sherwood ER, Bohannon JK, *Immunobiology and Application of Toll-like Receptor 4 Agonists to Augment Host Resistance to Infection.* Pharmacological Research, 2019.
6. Hu, Z., Du, R., Xiu, L., Bian, Z., Ma, C., Sato, N., … Wang, X. , *Protective effect of triterpenes of Ganoderma lucidum on lipopolysaccharide-induced inflammatory responses and acute liver injury.* Cytokine, 2020.
7. Hung, Y.-L., Fang, S.-H., Wang, S.-C., Cheng, W.-C., Liu, P.-L., Su, C.-C., … Li, C.-Y, *Corylin protects LPS-induced sepsis and attenuates LPS-induced inflammatory response.* Scientific Reports, 2017.
8. Li, H.-R., Liu, J., Zhang, S.-L., Luo, T., Wu, F., Dong, J.-H., … Zhao, L, *Corilagin ameliorates the extreme inflammatory status in sepsis through TLR4 signaling pathways.* . BMC Complementary and Alternative Medicine, 2017.
9. Liu, M., Bedouhene, S., Hurtado-Nedelec, M., Pintard, C., Dang, P. M.-C., Yu, S., & El-Benna, J. , *The Prolyl Isomerase Pin1 Controls Lipopolysaccharide-Induced Priming of NADPH Oxidase in Human Neutrophils.* . Frontiers in Immunology, 2019.
10. Nemzek, J.A., K.M.S. Hugunin, and M.R. Opp, *Modeling Sepsis in the Laboratory: Merging Sound Science with Animal Well-Being.* Comparative Medicine, 2008. **58**(2): p. 120-128.
11. Devi Ramnath, R., et al., *Inflammatory mediators in sepsis: Cytokines, chemokines, adhesion molecules and gases.* Journal of Organ Dysfunction, 2006. **2**(2): p. 80-92.
12. Mittal, M., et al., *Reactive oxygen species in inflammation and tissue injury.* Antioxid Redox Signal, 2014. **20**(7): p. 1126-67.
13. Zhang, J.A., J., *Cytokines, Inflammation, and Pain.* International Anesthesiology Clinics, 2007. **45**(2): p. 27-37.
14. Lu, Y.-C., Yeh, W.-C., & Ohashi, P. S. , *LPS/TLR4 signal transduction pathway.* Cytokine, 2008: p. 145-151.
15. JIAN WAN, Y.S., YIBO FAN, CONGHUI FAN, SONG CHEN, JIE SUN, and L.Q. LILI ZHU, MENGJIN YU and ZHAOFEN LIN, *NF-κB inhibition attenuates LPS-induced TLR4 activation in monocyte cells.* Molecular Medicine Reports, 2016.
16. Phungern Khongthong, A.K.R.a.J.E., *The NF-KB pathway and endocrine therapy resistance in breast cancer.* Endocrine-Related Cancer, 2019. **26**(6): p. R369-R380.

17. Sun, S.-C., *The non-canonical NF-κB pathway in immunity and inflammation.* Nat Rev Immunol, 2017. **17**(9): p. 545-558.
18. Luo, C., Liu, Z., & Xu, Y. , *Protective effects of mesenchymal stem cells on acute liver injury via TLR4/NF-kB signaling pathway in sepsis mice.* Int J Clin Exp Med. **12**(1): p. 1196-1205.
19. Ahmed, S.M., et al., *Nrf2 signaling pathway: Pivotal roles in inflammation.* Biochim Biophys Acta Mol Basis Dis, 2017. **1863**(2): p. 585-597.
20. Wible, R.S., et al., *Pharmacogenomics of Chemically Distinct Classes of Keap1-Nrf2 Activators Identify Common and Unique Gene, Protein, and Pathway Responses In Vivo.* Mol Pharmacol, 2018. **93**(4): p. 297-308.
21. Kuo, P.-C., et al., *3H-1,2-Dithiole-3-thione as a novel therapeutic agent for the treatment of experimental autoimmune encephalomyelitis.* The Journal of Immunology, 2016. **196**(1 Supplement): p. 139.1-139.1.
22. Dong, J., Y. Dong, and C. Shao-yu, *Stabilization of Nrf2 Protein by D3T Provides Protection against Ethanol-Induced Apoptosis in PC12 Cells.* PLoS One, 2011. **6**(2).
23. Cui, Y., et al., *Pharmacological activation of the Nrf2 pathway by 3H-1, 2-dithiole-3-thione is neuroprotective in a mouse model of Alzheimer disease.* Behav Brain Res, 2018. **336**: p. 219-226.
24. Gotts, J.E. and M.A. Matthay, *Sepsis: pathophysiology and clinical management.* BMJ, 2016. **353**: p. i1585.
25. Åhlgren, J. and V. Voikar, *Experiments done in Black-6 mice: what does it mean?* Lab Animal, 2019. **48**(6): p. 171-180.
26. Nair, A.B., Jacob, Shery, *A simple practice guide for dose conversion between animals and human.* Journal of Basic and Clinical Pharmacy, 2016. **7**(2): p. 27-31.
27. Aslantürk, Ö.S., *In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages.* Genotoxicity - A Predictable Risk to Our Actual World, 2017.
28. Chanput W, M.J., Wichers HJ, *THP-1 cell line: an in vitro cell model for immune modulation approach.* Int Immunopharmacol, 2014: p. 37-45.
29. Brooks, D., et al., *Human lipopolysaccharide models provide mechanistic and therapeutic insights into systemic and pulmonary inflammation.* Eur Respir J, 2020. **56**(1).
30. Brinkworth, J.F. and N. Valizadegan, *Sepsis and the evolution of human increased sensitivity to lipopolysaccharide.* Evol Anthropol, 2021. **30**(2): p. 141-157.
31. Deshmane, S.L., et al., *Monocyte chemoattractant protein-1 (MCP-1): an overview.* J Interferon Cytokine Res, 2009. **29**(6): p. 313-26.
32. Zhu, S., et al., *The molecular structure and role of CCL2 (MCP-1) and C-C chemokine receptor CCR2 in skeletal biology and diseases.* J Cell Physiol, 2021.
33. Dommel, S. and M. Blüher, *Does C-C Motif Chemokine Ligand 2 (CCL2) Link Obesity to a Pro-Inflammatory State?* Int J Mol Sci, 2021. **22**(3).
34. Yadav, A., V. Saini, and S. Arora, *MCP-1: chemoattractant with a role beyond immunity: a review.* Clin Chim Acta, 2010. **411**(21-22): p. 1570-9.
35. Tanaka, T., M. Narazaki, and T. Kishimoto, *IL-6 in inflammation, immunity, and disease.* Cold Spring Harb Perspect Biol, 2014. **6**(10): p. a016295.
36. Li, L., et al., *Effects of immune cells and cytokines on inflammation and immunosuppression in the tumor microenvironment.* Int Immunopharmacol, 2020. **88**: p. 106939.

37. Koschmieder, S. and N. Chatain, *Role of inflammation in the biology of myeloproliferative neoplasms.* Blood Rev, 2020. **42**: p. 100711.
38. Jahandideh, B., et al., *The pro-Inflammatory cytokines effects on mobilization, self-renewal and differentiation of hematopoietic stem cells.* Hum Immunol, 2020. **81**(5): p. 206-217.
39. De Zoysa, M., et al., *Molecular evidence for the existence of lipopolysaccharide-induced TNF-α factor (LITAF) and Rel/NF-kB pathways in disk abalone (Haliotis discus discus).* Fish & shellfish immunology, 2010. **28**(5-6): p. 754-763.
40. Wijayanti, N., et al., *Role of NF-kB and p38 MAP kinase signaling pathways in the lipopolysaccharide-dependent activation of heme oxygenase-1 gene expression.* Antioxidants & redox signaling, 2004. **6**(5): p. 802-810.
41. Hasturk, H., A. Kantarci, and T.E. Van Dyke, *Oral inflammatory diseases and systemic inflammation: role of the macrophage.* Front Immunol, 2012. **3**: p. 118.
42. Tsai, T.H., et al., *Deletion of caveolin-1 attenuates LPS/GalN-induced acute liver injury in mice.* J Cell Mol Med, 2018. **22**(11): p. 5573-5582.
43. Niu, X., et al., *Harmine mitigates LPS-induced acute kidney injury through inhibition of the TLR4-NF-κB/NLRP3 inflammasome signalling pathway in mice.* Eur J Pharmacol, 2019. **849**: p. 160-169.
44. Chen, J., et al., *IkappaB Kinase Inhibitor Attenuates Sepsis-Induced Cardiac Dysfunction in CKD.* J Am Soc Nephrol, 2017. **28**(1): p. 94-105.
45. El-Shitany, N.A. and B.G. Eid, *Icariin modulates carrageenan-induced acute inflammation through HO-1/Nrf2 and NF-kB signaling pathways.* Biomed Pharmacother, 2019. **120**: p. 109567.
46. Rubio, V., et al., *Different roles of Nrf2 and NFKB in the antioxidant imbalance produced by esculetin or quercetin on NB4 leukemia cells.* Chem Biol Interact, 2018. **294**: p. 158-166.
47. Araujo, J.A., M. Zhang, and F. Yin, *Heme oxygenase-1, oxidation, inflammation, and atherosclerosis.* Front Pharmacol, 2012. **3**: p. 119.
48. Choi, A.M. and J. Alam, *Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury.* Am J Respir Cell Mol Biol, 1996. **15**(1): p. 9-19.
49. Franklin, C.C., et al., *Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase.* Mol Aspects Med, 2009. **30**(1-2): p. 86-98.
50. Chen, Y., et al., *Glutathione defense mechanism in liver injury: insights from animal models.* Food Chem Toxicol, 2013. **60**: p. 38-44.
51. Syed, A.M., et al., *A review on herbal Nrf2 activators with preclinical evidence in cardiovascular diseases.* Phytother Res, 2021.
52. Song, M.Y., et al., *The Role of NRF2/KEAP1 Signaling Pathway in Cancer Metabolism.* Int J Mol Sci, 2021. **22**(9).
53. Yan, J., S. Li, and S. Li, *The role of the liver in sepsis.* Int Rev Immunol, 2014. **33**(6): p. 498-510.
54. Bellomo, R., et al., *Acute kidney injury in sepsis.* Intensive Care Med, 2017. **43**(6): p. 816-828.

55. Trush, M.A., et al., *Analysis of target cell susceptibility as a basis for the development of a chemoprotective strategy against benzene-induced hematotoxicities.* Environ Health Perspect, 1996. **104 Suppl 6**(Suppl 6): p. 1227-34.