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# Effects of Sodium Methyldithiocarbamate-Induced Oxidative Stress on Nf-Kappa B Activation

Monica Cherie Gadson

# EFFECTS OF SODIUM METHYLDITHIOCARBAMATE-INDUCED OXIDATIVE STRESS ON NF-KAPPA B ACTIVATION

By

Monica Cherie Gadson

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EFFECTS OF SODIUM METHYLDITHIOCARBAMATE-INDUCED

OXIDATIVE STRESS ON NF-KAPPA B ACTIVATION

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Approved:

| | |
|---|---|
| Stephen B. Pruett<br>Professor<br>Head of Department<br>Department of Basic Sciences<br>(Major Professor) | G. Todd Pharr<br>Associate Professor<br>Department of Basic Sciences<br>(Committee Member) |
| Russell L. Carr<br>Associate Professor<br>Department of Basic Sciences<br>(Committee Member) | Larry A. Hanson<br>Professor<br>Graduate Coordinator<br>Department of Basic Sciences |
| Kent H. Hoblet<br>Dean<br>College of Veterinary Medicine | |

Name: Monica Cherie Gadson

Date of Degree: May 11, 2012

Institution: Mississippi State University

Major Field: Veterinary Medical Science (Immuno-toxicology)

Major Professor: Dr. Stephen B. Pruett

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Candidate for Degree of Master of Science

Sodium methyldithiocarbamate (SMD) is commonly reported to cause health risks in humans. Previous reports indicate SMD causes oxidative stress, which can contribute to the activation of NF-κB and cause other characteristics of inflammatory responses to be altered. Almost all pro-inflammatory cytokines require NF-κB activation for full expression and development of an innate immune or inflammatory response.

This study evaluated NF-κB activation, providing new information regarding reactive oxygen in macrophages from SMD-treated mice. Studies were conducted in which NF-κB reporter mice were treated with lipopolysaccharide (LPS), SMD, buthionine sulfoximine (BSO), and N-acetyl cysteine (NAC). BSO depletes glutathione (GSH) and increases oxidative stress, whereas NAC spares GSH by acting as a precursor for rapid synthesis to replace oxidized GSH.

The work here indicates that NF-κB is not affected directly by increased or decreased reactive oxygen species (ROS), and oxidative stress is not the major mechanism by which SMD inhibits inflammatory responses.

DEDICATION

To my parents, *Nathan & Kathleen*, for the strength, love, and support in every aspect of my life. Thank you.

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. This report is one of my greatest accomplishments. When things felt like they weren't going right within my experiments, I can always count on Dr. Stephen B. Pruett for encouragement. He has given me an opportunity of a lifetime to be knowledgeable in areas of science that I never knew existed. He was always there to inspire me and to help me learn and be prepared for anything.

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TABLE OF CONTENTS

LIST OF FIGURES

# CHAPTER I

# INTRODUCTION

Sodium methyldithiocarbamate (metam sodium or SMD) is an abundantly used soil fumigant. SMD is used to control the growth of fungi, nematodes, and weeds. According to the Environmental Protection Agency (EPA), SMD is the 3rd most abundantly used conventional agricultural pesticide (by weight) in the U.S [1]. The EPA documented that in 1995, there were over 50 million pounds of SMD used in the U.S. alone.

Humans can become exposed to SMD by an accident leading to a major spill or near sites of recent or recurring agricultural applications. High levels of exposure can occur occupationally in people involved in transport, mixing, and applications. The acute toxicity of SMD is relatively low and the LD50 in mice is 750 mg/kg [2]. SMD breaks down to methyl isothiocyanate (MITC), an extremely potent irritant [1]. There was an incident in which there was a spill of 19,000 gallons of metam sodium into the Sacramento River in 1991 due to a train derailment. Uncontrolled, spontaneous spills as well as occupational or environmental exposure can result in adverse ecological and human health problems. Much of the SMD is used in the U.S. is used in California [3, 4]. SMD use has been restricted to certified applicators [5], and there is a ban on entering the site of application for 48 hours by certain decrees [6]. However, it is likely that non-compliance occurs frequently.

Commercial preparations for metam sodium contains 32.7% SMD in an aqueous solution at a self-buffered pH of approximately 10 [7]. When sprayed on or injected into soil (in which the pH is much lower), SMD decomposes quickly to MITC and hydrogen sulfide [8]. Under more acidic conditions in some soils, SMD is broken down into carbon disulfide and methylamine [9]. These two reactions also occur in pure solutions. These reactions are energetically favorable and do not require enzymatic catalysis [9]. The high vapor pressure and low affinity for adsorption on soil of MITC suggests that volatilization is the most important environmental route of dissipation [10]. MITC can remain in the soil for 12 or more days depending on the soil composition and the moisture in the soil [8].

Metam sodium has been reported to be photolytic in the water and MITC has been reported to be photolytic in the atmosphere [11, 12]. MITC and 1,3 dimethylthiourea are found in water after photolysis. Once MITC volatilizes into the atmosphere, it degrades very quickly forming multiple compounds. These compounds include sulfur dioxide, N-methylformamide, methylamine, carbonyl sulfide, methyl isocyanide, and methyl isocyanate (more toxic than MITC). Because MITC is also soluble in water and has low adsorption in soil, it can potentially seep into ground water and transport to surface water via runoff under a flooded condition [13].

There have been previous studies examining the metabolism of metam sodium in rats and mice [15, 16, 2, 18] and MITC is the foremost initial breakdown product of SMD. The leading major metabolic and excretion pathway for MITC in rats initiates with conjugation to glutathione (GSH). After this, conversion of a portion of the conjugate to the mercapturate occurs, and the mercapturate is excreted in the urine [2]. In

addition, metam sodium can be conjugated to GSH [7] and also metabolized by S-methylation [17] and by sulfoxidation [16], particularly in the liver.

When a train derailed spilling metam sodium into the Sacramento River in 1991, the residents of the nearby town reported strong sulfur/rotten egg odors. Symptoms of people who had been exposed included headache, eye irritation, blurred vision, nausea, dizziness, shortness of breath and diarrhea [1]. Other symptoms include irritant-induced dermatitis in some individuals who were cleaning up the dead fish in the river. There was also a report of induction and/or exacerbation of asthma [39]. This latter observation suggested that SMD may affect the immune system and was the initial rationale for evaluating its immunological effects. Without proper personal protective equipment, people are subject to have adverse effects from the levels of MITC that are present either after a major release or agricultural application. Dermal exposure is also one of the routes of exposure of metam sodium, and in mice, this can cause immunological effects very similar to those caused by oral administration [37].

Rodents can become immunosuppressed by acute exposure to metam sodium [18, 19, 20]. The immunosuppressive affects of oral exposure to metam sodium targets lymphocytes in the spleen, peripheral blood, thymus, and natural killer cell (NK) functions [18, 19, 20]. Although there is a percentage decrease of lymphocytes in the spleen and blood, there is a minute to no change in total leukocyte counts with an increase in spleen cellularity. Also, there is a notable amplification in the total bone marrow cells, macrophage colony forming units in the bone marrow, and splenic non-T and non-B nucleated cells [7] suggesting an amplification in hematopoiesis [20].

MITC is reported to reduce pregnancy success [40]. Metam sodium is listed as a probable human carcinogen [41], as well as affecting the cardiovascular system.

## Oxidative Stress

A stressor can affect immune and inflammatory responses in a positive or negative way. Stress is a complex response of an organism that is not completely understood. Stimuli that induce stress responses are stressors. The most common list of stressors includes physiological, physical, and drug or chemical stimuli [21]. Activation of the physiological stress response is linked with activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. This leads to modifications in the concentrations of several stress-related mediators [22].

The previously listed stress responses in mammals are distinguished by activation of the HPA axis leading to elevated levels of adrenal hormones. These adrenal hormones include epinephrine, norepinephrine, and glucocorticoids [23]. In addition, the sympathetic nervous system is activated leading to the release of norepinephrine from adrenergic nerve terminals, which are in the spleen, thymus, and other lymphoid tissues [24]. As a result, there are many other neuroendocrine mediators that are elevated or reduced in the peripheral circulation during most stress responses. This is due to the cells of the immune system having receptors for many hormones and neurotransmitters and their ability to respond to these elevated or reduced concentrations of stress mediators. An example would be the concentrations of catecholamines, endogenous opiates, ACTH, bombesin [25], prolactin (PRL), and glucocorticoids. These are examples that increase in response to many stressors. Among these, glucocorticoids are the most extensively studied and are uniformly reported to be immunosuppressive and anti-inflammatory.

There are several cytokines that can activate the HPA axis and create effects on the nervous system such as the sleep stimulation and elevated body temperature. The main pro-inflammatory cytokines that could do this are IL-1, TNF-α, and IL-6. In

response to immunological stimuli, these cytokines are frequently produced profusely and contribute to resistance to infection.

Stressors can have either a positive or negative effect on the immune system. This is dependent upon the intensity, duration, the type of stressor, the immunological function that is evaluated, the timing of the stressor, the timing of evaluation of the immunological end points, and several other factors [21].

Oxidative stress is defined as "any of various pathologic changes seen in living organisms in response to excessive levels of cytotoxic oxidants and free radicals in the environment" [26]. Oxidative stress is thought to be linked with the inhibition of inflammation and decreased resistance to infection caused by exposure of mice to SMD. A previous study confirmed that SMD significantly decreases the intracellular concentration of reduced GSH, which suggests oxidative stress [27]. Although there are many reports indication that some degree of oxidative stress can induce or enhance inflammatory responses, there are also reports indicating the opposite effect, so the present study was conducted with the assumption that either oxidative stress could explain or diminish the anti-inflammatory effects of SMD.

**Glutathione**

Glutathione (GSH) is the most abundant thiol (SH) compound in animal tissues, plant tissues, bacteria, and yeast. GSH is a major antioxidant that is produced in humans and other mammals. The immune system depends on a steady supply of GSH in order to maintain homeostasis. If GSH is depleted, the immune system can be modulated, and both enhancement and suppression have been reported, depending on the immunological parameter and experimental system. GSH plays significant roles in organisms such as

protection against reactive oxygen species (ROS) [28] and maintenance of protein SH groups. During these reactions, GSH is converted into glutathione disulfide (GSSG). GSSG is the oxidized form of GSH. GSH is the dominant form found in most living organisms due to the fact that GSSG is enzymatically reduced by glutathione reductase.

### Lipopolysaccharide

Lipopolysaccharide (LPS) is a compound in which a lipid molecule is covalently bound to a polysaccharide. It is a natural constituent of the outer membrane of gram negative bacteria, and responses to LPS through toll-like receptor 4 (TLR4) account for a substantial portion of the inflammatory response induced by gram negative bacteria. LPS is one of the more potent inflammatory stimuli and induces the production of endogenous pyrogen (IL-1) and tumor necrosis factor (TNF). LPS is a potent activator of macrophages, causing them to produce a wide variety of pro-inflammatory mediators [42].

### Buthionine Sulfoximine

Buthionine sulfoximine (BSO) is a potent and specific inhibitor of γ-glutamylcysteine synthetase. When it is administered to animals or incorporated into tissue culture media, it inhibits glutathione synthesis and increases oxidative stress.

### *N*-acetyl Cysteine

*N*-acteyl cysteine (NAC) is an antioxidant also and it has many uses. For the significance of this study, NAC preserves GSH concentrations. NAC is converted enzymatically to cysteine, which is the rate limiting, precursor for GSH synthesis. When GSH levels are normal, NAC does not tend to increase them substantially, but it maintains nearly normal GSH levels in the fact of oxidative stress.

**NF-kappa B (NF-κB)**

NF-κB is a transcription factor that plays a substantial role in controlling biological processes. These processes include immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. In response to the various stimuli, such as stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral components, NF-κB is activated and translocated from cytoplasm to nucleus. In the nucleus, NF-κB binds to its response element in the promoter region and regulates a wide spectrum of gene expression [29]. Nuclear NF-κB is either a homodimer or heterodimer composed of one or two of five different proteins (p65, p50, p52, or c-Rel). Some of these combinations can inhibit transcription, but most induce it [43].

Previous studies indicate that SMD alters signaling and induction of cytokine production through Toll-like receptor (TLR) 4, decreasing IL-12 production and increasing IL-10 production induced by lipopolysaccharide (LPS) in mice [37]. These cytokines are representative of anti-inflammatory and pro-inflammatory mediators, respectively. In addition, a number of other cytokines (mostly pro-inflammatory) were examined to determine if the effects noted were generally applicable or unique to a few cytokines. For Experiment 1 (BSO), IL-10, IP-10, LIF, and MIG were examined. For Experiment 2 (NAC), GM-CSF, IL-5, IL-15, IL-10, MCP-1, RANTES, IL-13, and M-CSF were examined. These cytokines were chosen because when analyzed, the cytokines and chemokines showed significant differences statistically.

SMD causes oxidative stress, which can affect the activation of NF-κB and other characteristics of inflammatory responses. NF-κB reporter mice are transgenic for a luciferase gene with an NF-κB driven promoter, allowing detection of any activating form of NF-κB by catalysis of the reaction converting luciferin to a luminescent product

that can be imaged by a sensitive camera in the IVIS imager. Almost all pro-inflammatory cytokines require NF-κB activation, so one possible mechanism by which SMD decreases production of pro-inflammatory cytokines would be decreasing NF-κB activation.

This study evaluated NF-κB activation, providing new information about the role of reactive oxygen in the activation of NF-κB. The hypothesis tested here is: (1) Characterize the effects of SMD on a wide range of pro-inflammatory cytokines and chemokines as well as IL-10, and (2) determine the role of oxidative stress in the mechanism by which SMD increases LPS-induced IL-10 production and decreases LPS-induced production of multiple pro-inflammatory cytokines by peritoneal macrophages.

CHAPTER II

MATERIALS AND METHODS

## Mice

Male and female NF-κB Reporter mice (8-12 weeks old) were obtained commercially, and they were originally produced by Blomhoff and colleagues [44]. They were used at 8-12 weeks of age and were housed and treated according to the NIH guide, MS State University policies, and the American Assosociation for Accreditation of Laboratory Animal Care (AAALAC) guidelines. Mice were separated into the experimental groups based on age, gender, and signal strength of NF-κB activation. Initial efforts to group mice using PCR to identify offspring with the reporter construct were not successful due to occasional mice with the construct in which the reporter function was inactive. However, grouping by functional assessment was effective.

All of the mice were housed in filter-top shoebox cages with 3-5 mice per cage in a temperature (70-80˚F) and humidity (40-60%) controlled environment. They were given free access to food (Purina Lab Chow) and water and maintained on a 12 h light/dark cycle.

## Experimental Design

This study was designed with a group size of 23 mice each for both experiments 1 and 2. Experiment 1 was designed to evaluate the effects of GSH depletion by BSO, and Experiment 2 was designed to evaluate the effect of sparing GSH from depletion by SMD by treating mice with NAC.

In Experiment 1, the following groups were included: Naive (untreated); LPS (60µg/mouse, intravenous) + vehicle for SMD; LPS (60µg/mouse) + SMD (200 mg/kg oral); LPS (60µg/mouse) + SMD (200 mg/kg oral) + BSO (see section below for details of BSO dosing); and LPS (60µg/mouse) + BSO. Mice were treated with SMD or vehicle (water) by gavage 30 min before LPS.

In Experiment 2, the following groups were included: Naive (untreated); LPS (60µg/mouse, intravenous) + vehicle for SMD; LPS (60µg/mouse) + SMD (200 mg/kg, oral); LPS (60µg/mouse) + SMD (200 mg/kg, oral) + NAC (see below for NAC dosing detail); and LPS + NAC.

**Administration of BSO (Experiment 1)**

BSO was dissolved in the drinking water at 1333 mg/l (to yield and approximate dosage of 400mg/kg/day). This was provided to the groups of mice as the sole source of water for 16 days prior to administration of LPS.

**Administration of NAC (Experiment 2)**

N-acetyl cysteine (NAC) was administered by oral gavage at 1 g/kg for 3 days prior to experimentation 1 h after the last dose.

**Administration and Preparation of Luciferin, Imaging of Mice**

Luciferin is a chemical substance found in the cells of various bioluminescent organisms (such as fireflies). When luciferin is oxidized under the catalytic effects of luciferase and ATP, a bluish-green light is produced. Because the reaction is dependent on ATP, it allows researchers to determine the presence of metabolic energy or life. Luciferin can be used in a variety of *in vitro* assays as well as *in vivo*. Reporter mice used in this study express luciferase in all cells in which NF-κB has been activated.

When used *in vivo*, this system can be used to evaluate NF-κB activation, which is visualized and quantified with a Xenogen IVIS Imaging System [30].

A previous experiment demonstrated that the peak time for luciferase expression after LPS administration was 2 h. Thus, mice were imaged 2 hours after the LPS injection, and samples of blood and peritoneal fluid and cells were collected.

Mice were injected intraperitoneally with a luciferin solution at a dosage of 30mg/kg, in phosphate buffered saline (PBS). The mice were then transferred into a clear plexiglass anesthesia box that allows unimpeded visual monitoring of the mice, and they were anesthetized with a mixture of isoflurane (2.5-3.0%) and oxygen. After the mice were fully anesthetized, that were transferred from the box to the nose cones attached to the manifold in the imaging chamber where the images can be captured using a computer. The nose cones were used for administration of a lower concentration of isoflurane and oxygen to keep the mice anesthetized during the duration of the imaging procedure. Mice were imaged at a peak time from 10-15 minutes after luciferin injection.

**Sampling of peritoneal lavage**

Mice were euthanized before recovering from anesthesia by inhalation of carbon dioxide. After euthanasia, peritoneal lavage fluid was performed by injection of 1ml of PBS diluted with 10% fetal bovine serum (FBS) from each mouse for both Experiment 1 and Experiment 2. The abdominal cavity was massaged to distribute the fluid and to mix the contents into the peritoneal cavity of the animal. The skin was dissected away to reveal the peritoneum and a sample from the peritoneal cavity was taken using a syringe with a 25-gauge needle. Samples of this fluid were used to quantify glutathione. The fluid was equally divided into tubes where to prepare for centrifugation. The pellets were

resuspended and used for absolute cell counts using a Coulter Z1 particle counter. The rest of the samples were stored in a -80˚C freezer until used to determine GSH.

### Cytokine Assays

Cytokine and chemokine concentrations in the peritoneal lavage fluid were obtained and then analyzed using commercially available ELISA kits (BD Biosciences) and using a mouse multiplexed bead array cytokine/chemokine kit analyzed with a Luminex instrument.

### Statistical Analysis

Cytokine and GSH concentrations were compared using one-way ANOVA followed by Newman-Keul's post hoc test by Prism 5.0 software. Results shown are generally values that significantly differ from the positive control (LPS only). Values of P less than 0.05 were regarded to be significant.

# CHAPTER III

## RESULTS

Results shown in Figure 1 indicate that LPS + BSO did not have a significant effect on peritoneal cell counts when compared with LPS alone. In addition, LPS + SMD did not alter the peritoneal cell counts when compared with LPS + SMD + BSO.

Peritoneal cell counts shown in Figure 2 suggest that NAC tended to decrease cell counts, but SMD had no significant effect.

BSO increases oxidative stress, so if oxidative stress enhances NF-κB activation, as some investigators have reported, and increase in NF-κB should be observed in SMD-treated mice. Analysis of Figures 3C and 3D show that BSO did not increase the activation of NF-κB. Figure 3E shows that BSO also had no effect when given in the absence of SMD. However, SMD non-significantly decreased the activation of NF-κB induced by LPS. However, BSO plus SMD significantly inhibited activation of NF-κB by LPS. Significance was determined after analysis of the images to quantify the results (see subsequent graphed results).

As stated previously, NAC is an antioxidant and it helps to protect and repair cells from damage caused by free radicals. In Figures 4C and 4D, it is shown that NAC also did not have an effect on the activation of NF-κB by LPS in reporter mice. SMD significantly inhibited the activation of NF-κB and this effect was not altered by NAC.

Total Flux is the radiance (photons/sec) in each pixel summed or integrated over the ROI area indicated by the fluorescence in Figure 3A-E and Figure 4A-E. The total

flux is then calculated and the results are shown in Figure 5 and Figure 6. Values significantly different from the control group that received LPS only are indicated by * ($P < 0.05$). In Figure 5, it is shown that the LPS + SMD and the LPS + SMD + BSO groups were not significantly different from each other but the LPS + SMD + BSO group had a decreased total flux as compared to the LPS group. This demonstrates that BSO did not prevent the SMD-induced decrease in NF-κB activation. In fact, there was a tendency for exacerbation of the decreased NF-κB activation, but it was not statistically significant in comparison to the LPS + SMD group. In Figure 6, similar results are shown for mice treated with NAC, except that there was no tendency toward either exacerbation or prevention of NF-κB activation. Thus, activation of NF-κB was not included, enhanced, or inhibited by the agents that increase or decrease oxidative stress.

Figure 7 indicates the GSH concentration in peritoneal macrophages from mice treated with SMD and/or BSO prior to LPS administration. Figure 8 indicates the concentration of GSSG in peritoneal macrophages. These two figures make it evident that both SMD and BSO deplete GSH. This demonstrates that the BSO used in this study was active and that it depleted GSH, even though it did not alter the effects of SMD on most parameters measured in this study.

The concentration of GSH and GSSG was examined in mice treated with NAC can be shown in Figure 9 and Figure 10. The effect of NAC on cytokines in this study (particularly IL-10) were similar to those reported previously from this lab [34], indicating that the biological effects were consistent.

Figure 11A-G shows the effects of BSO pretreatment on SMD-mediated changes in cytokine and chemokine production in the peritoneal cavity. IL-10 (Figure 11D)

production was increased by SMD, but decreased production of the other cytokines (Figure 11A-C, E-F). This pattern was not altered consistently by SMD or BSO.

The effects of NAC pretreatment on SMD-mediated changes in the cytokine and chemokine production can be seen in Figure 12A-H. Significant differences between the group treated with LPS, SMD, and NAC and the group with LPS and NAC are indicated by connecting lines with the significance level printed above. There are similar trends in the cytokines listed. The main cytokines with the similar trends are GM-CSF, RANTES, M-CSF, and MCP-1. This suggests that SMD inhibits the production of these cytokines, but in contrast, NAC has an effect and increases the production of these specific cytokines in the LPS + NAC groups.

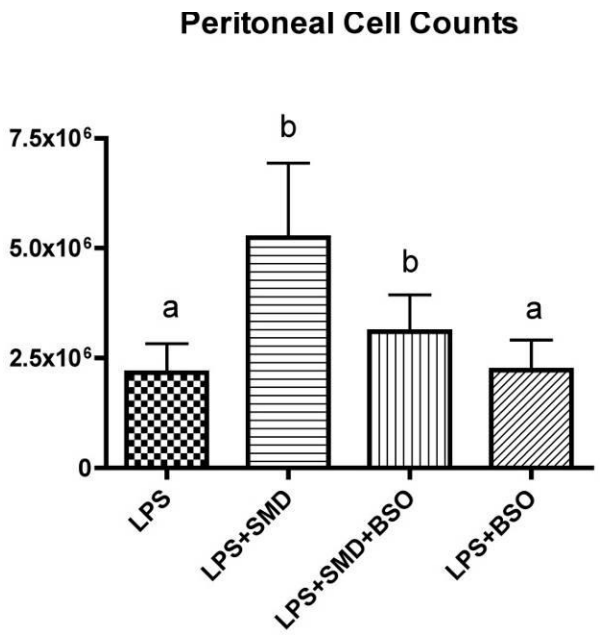



Figure 1 Cell counts in peritoneal lavage fluid (Experiment 1 – BSO).

Peritoneal lavage fluid samples were collected and centrifuged from Experiment 1 (BSO). Cells were washed with 1 ml PBS and 100 µl was obtained for cell count. Values that share any letter in common are significantly different ($P<0.05$) and values that do not share a letter in common are not significantly different ($P>0.05$). The value for naive mice was within the expected range.

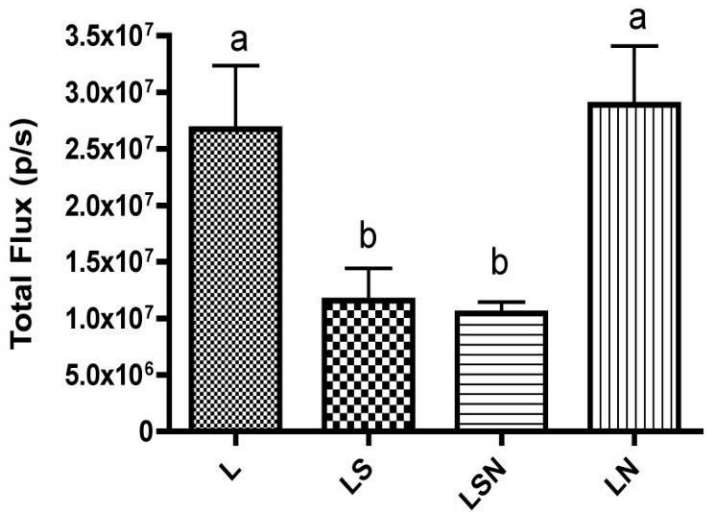
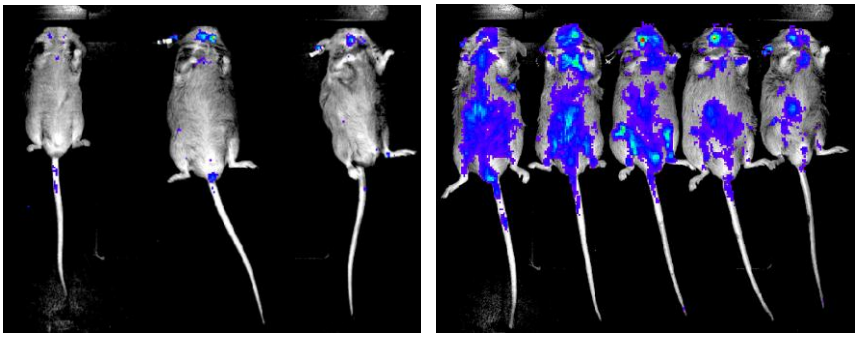



Figure 2 Cell counts in peritoneal lavage fluid (Experiment 2 – NAC).

Peritoneal lavage fluid samples were collected and centrifuged from Experiment 2 (NAC). Cells were washed with 1 ml PBS and 100 µl was obtained for cell count. Values that share any letter in common are significantly different ($P<0.05$) and values that do not share a letter in common are not significantly different ($P>0.05$). The value for naive mice was within the expected range.



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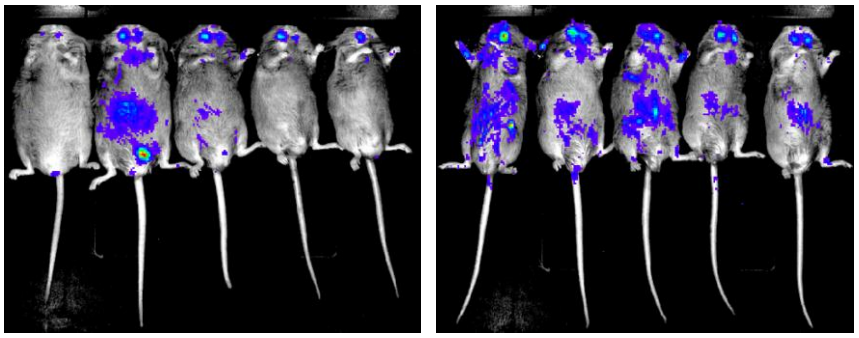
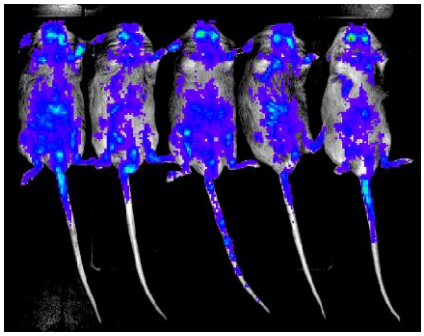


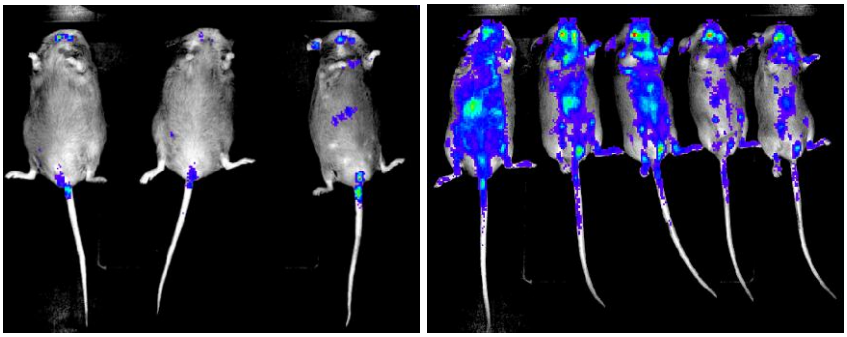
[C] [D]



[E]

Figure 3 Images of Light Emission after Luciferin Injection (Experiment 1 – BSO)

Mice were treated with BSO (1333 mg/l in drinking water for 16 days; 3-D and 3-E), then with SMD (200 mg/kg, by oral gavage; 3-C and 3-D) then challenged with LPS (60 µg/mouse, iv injection). Mice were injected with Luciferin (150 mg/kg, intraperitoneal injection) 2 hours after LPS administration and imaged 10 minutes after that.
[3-A: NAIVE, 3-B: LPS only, 3-C: LPS+SMD, 3-D: LPS+SMD+BSO, 3-E: LPS+BSO]



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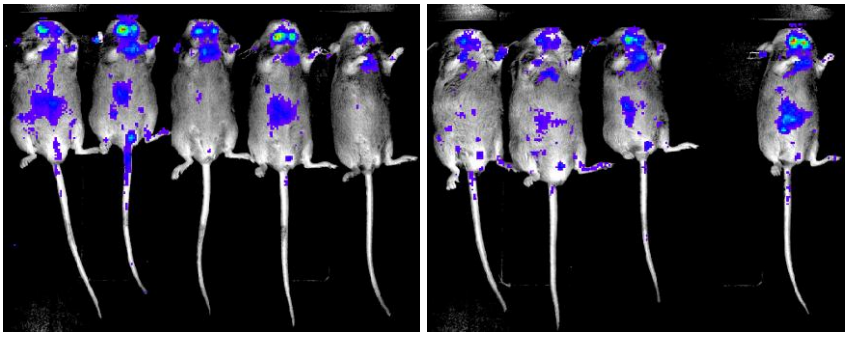
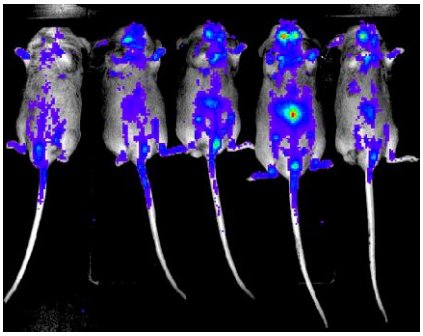


[C] [D]



[E]

Figure 4 [A – E] Images of Light Emission after Luciferin Injection (Experiment 2 – NAC)

Mice were treated with NAC (1 g/kg, oral gavage 3 days; 4-D and 4-E), then with SMD (200 mg/kg, by oral gavage; 4-C and 4-D) then challenged with LPS (60 µg/mouse, iv injection). Mice were injected with Luciferin (150 mg/kg, intraperitoneal injection) 2 hours after LPS administration and imaged 10 min after that.
[4-A: NAIVE, 4-B: LPS only, 4-C: LPS+SMD, 4-D: LPS+SMD+NAC, 4-E: LPS+NAC]

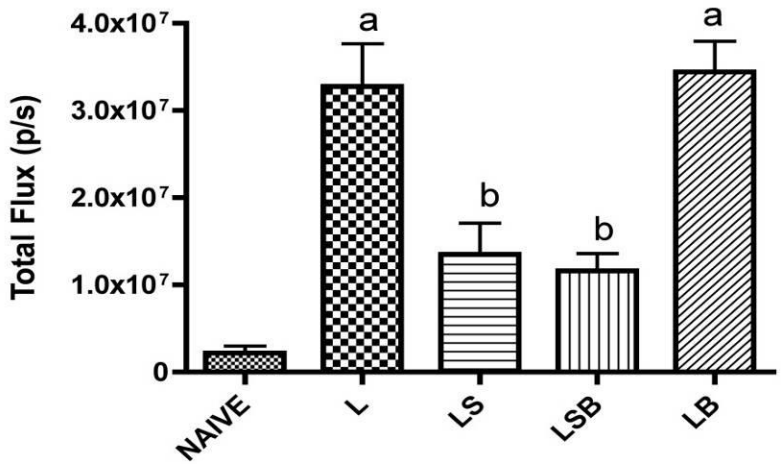



Figure 5 Total Flux Quantification of Experiment 1 (BSO)

Mice were imaged using an IVIS Spectrum 2 hours after LPS injection and Total Flux (photons/sec) was quantitated using Living Image software 3.0.
Values are means ± SEM. Values that share any letter in common are significantly different ($P<0.05$) and values that do not share a letter in common are not significantly different ($P>0.05$).

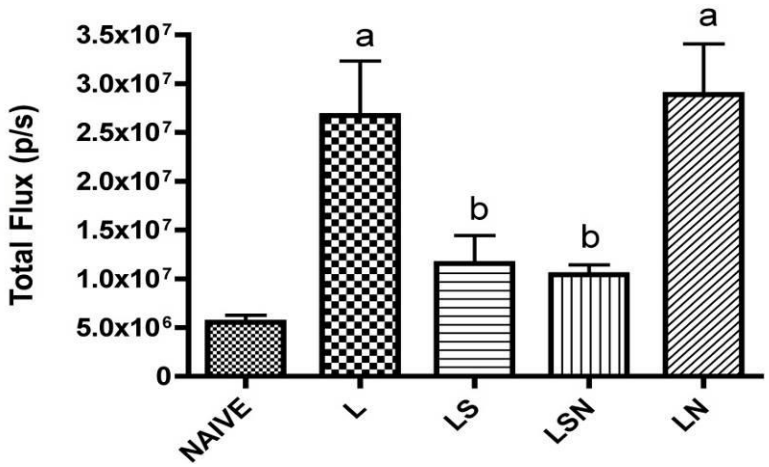



Figure 6 Total Flux Quantification of Experiment 2 (NAC)

Mice were imaged using an IVIS Spectrum 2 hours after LPS injection and Total Flux (photons/sec) was quantitated using Living Image software 3.0.
Values are means ± SEM. Values that share any letter in common are significantly different ($P<0.05$) and values that do not share a letter in common are not significantly different ($P>0.05$).

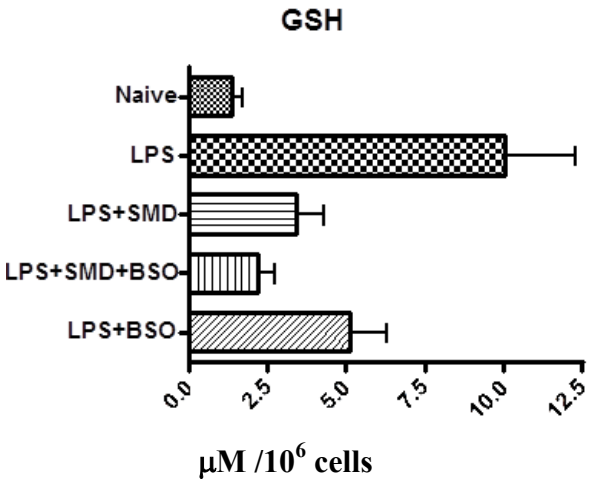



Figure 7 GSH Concentration of Experiment 1 (BSO)

Concentration of GSH is expressed as µM/cell divided by the number of cells in the sample x $10^6$ (to compensate for changes in cell number related to treatment or normal biological variability).

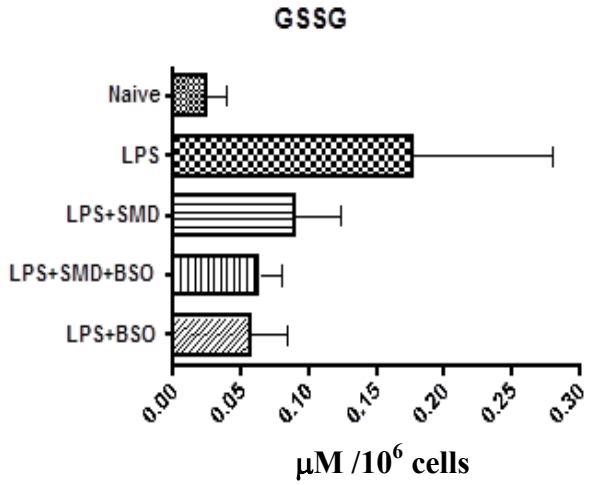



Figure 8 GSSG Concentration of Experiment 1 (BSO)

Glutathione is converted to GSSG during ROS & maintenance of protein thiol group; GSSG is enzymatically reduced by glutathione reductase.

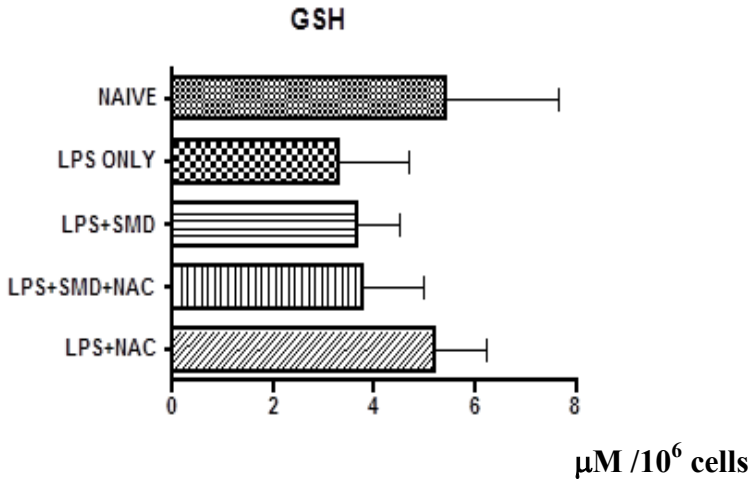



Figure 9 GSH Concentration of Experiment 2 (NAC)

Concentration of GSH is expressed as μM/cell divided by the number of cells in the sample x $10^6$ (to compensate for changes in cell number related to treatment or normal biological variability).

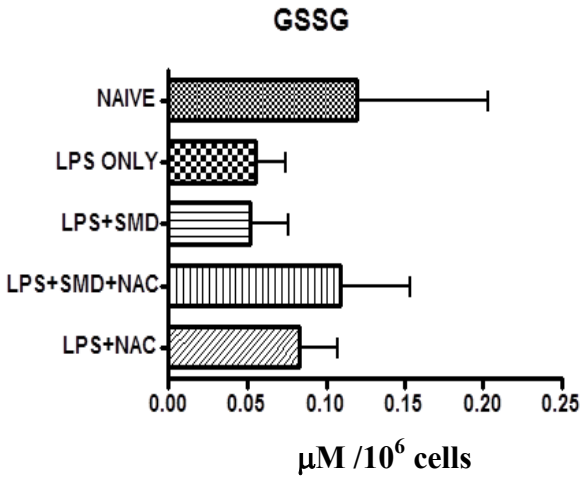



Figure 10 GSSG Concentration of Experiment 2 (NAC)

Glutathione is converted to GSSG during ROS & maintenance of protein thiol group; GSSG is enzymatically reduced by glutathione reductase.

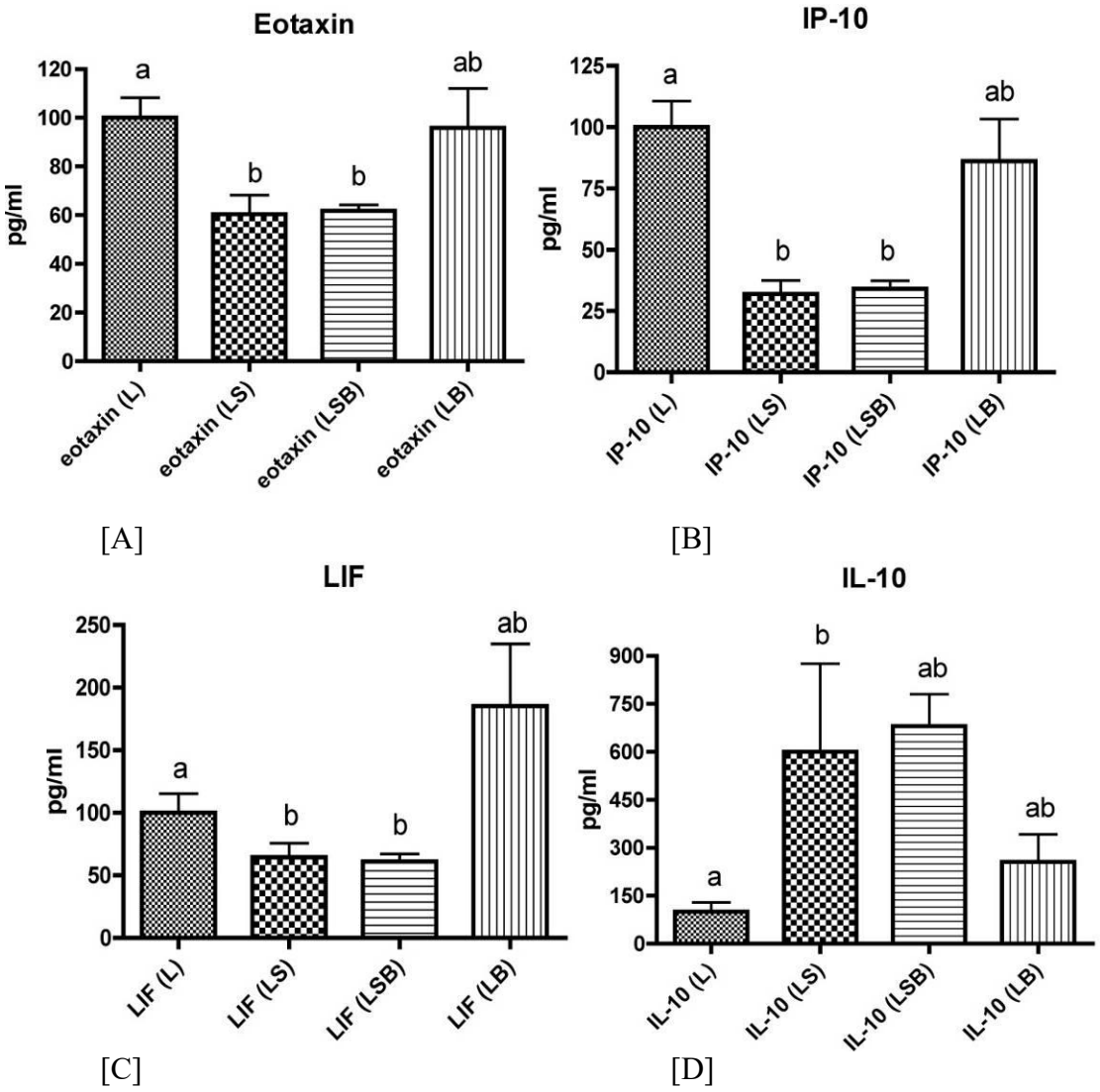
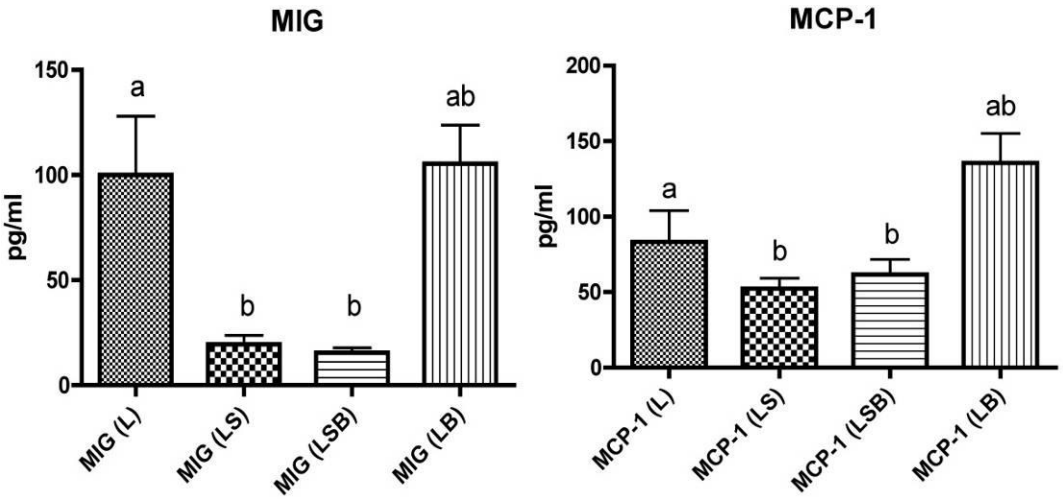



Figure 11 [A – G] Effects of BSO pretreatment on SMD-mediated changes in cytokine and chemokine production.

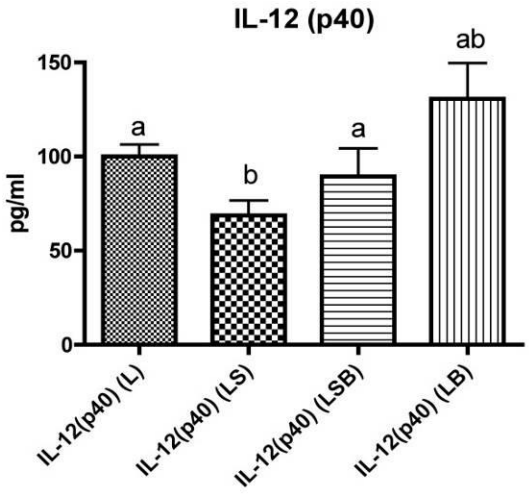
Levels of cytokine and chemokine production are shown in the peritoneal cavity of the BSO groups (pg/ml).Values are means ± SEM. Values that share any letter in common are significantly different ($P<0.05$) and values that do not share a letter in common are not significantly different ($P>0.05$).
Naive is not presented because this would affect the other values.
[L: LPS only, LS: LPS+SMD, LSB: LPS+SMD+BSO, LB: LPS+BSO]




[E]




[F]




[G]

Figure 11 (continued)

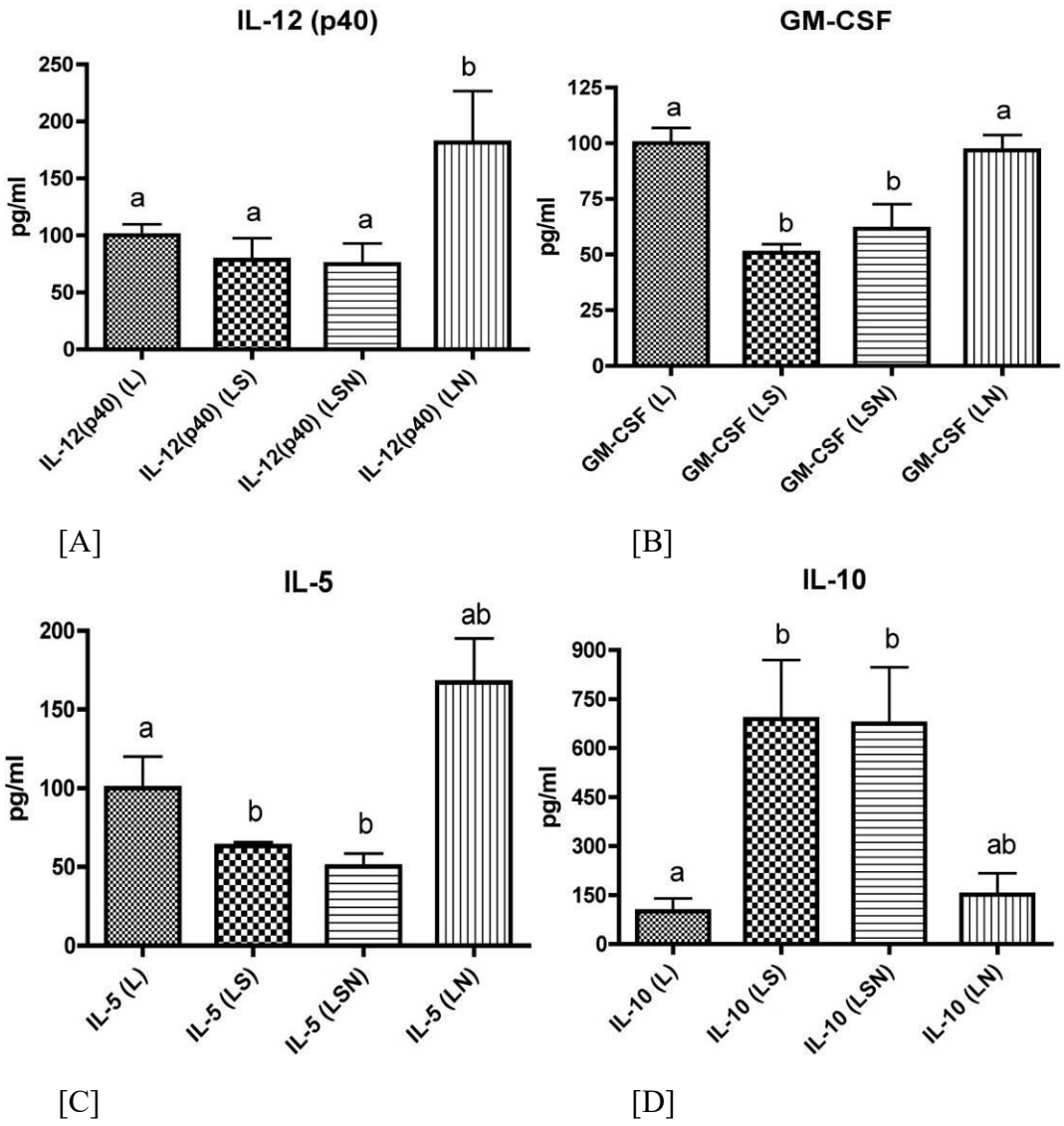
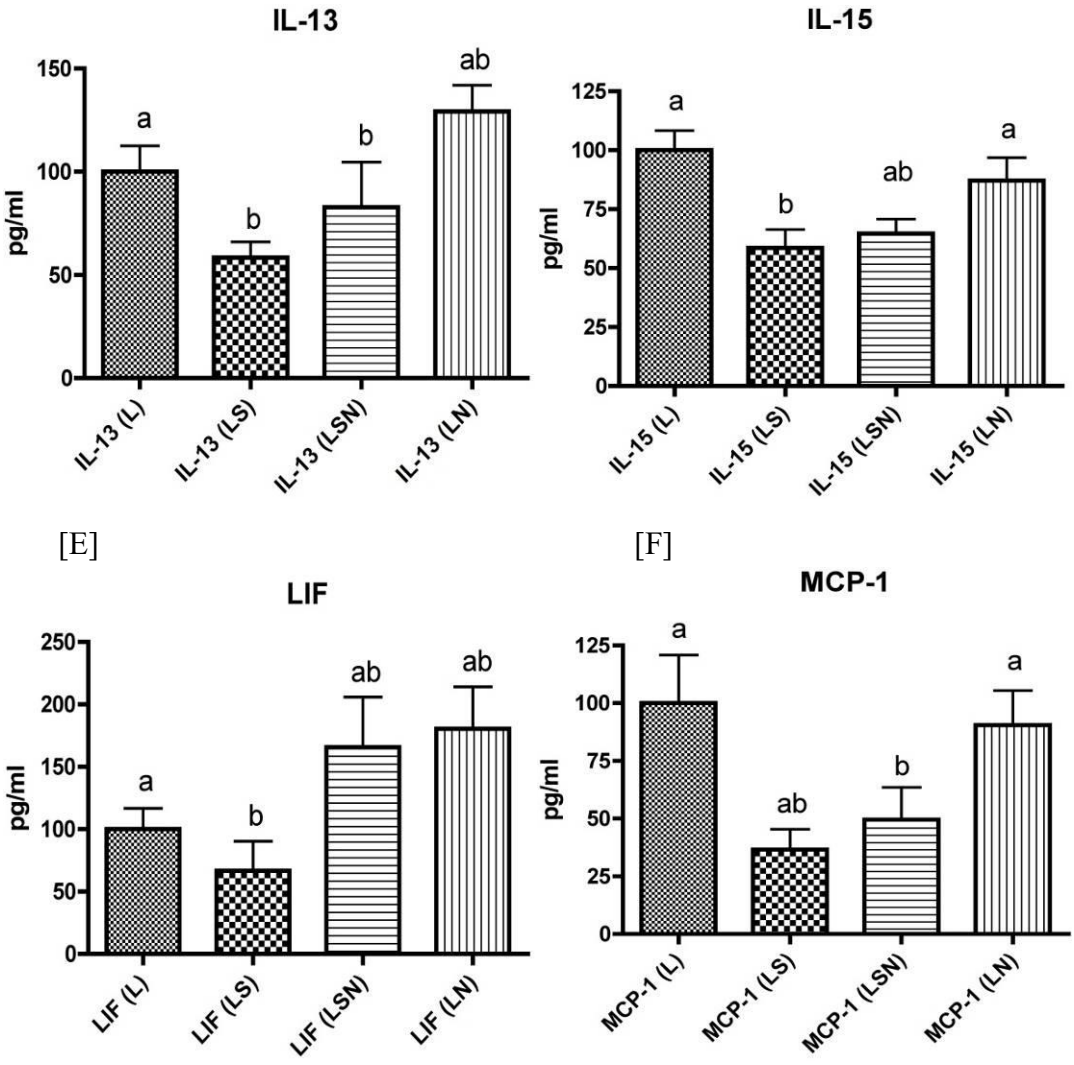



Figure 12 [A – K] Effects of NAC pretreatment on SMD-mediated changes in cytokine and chemokine production.

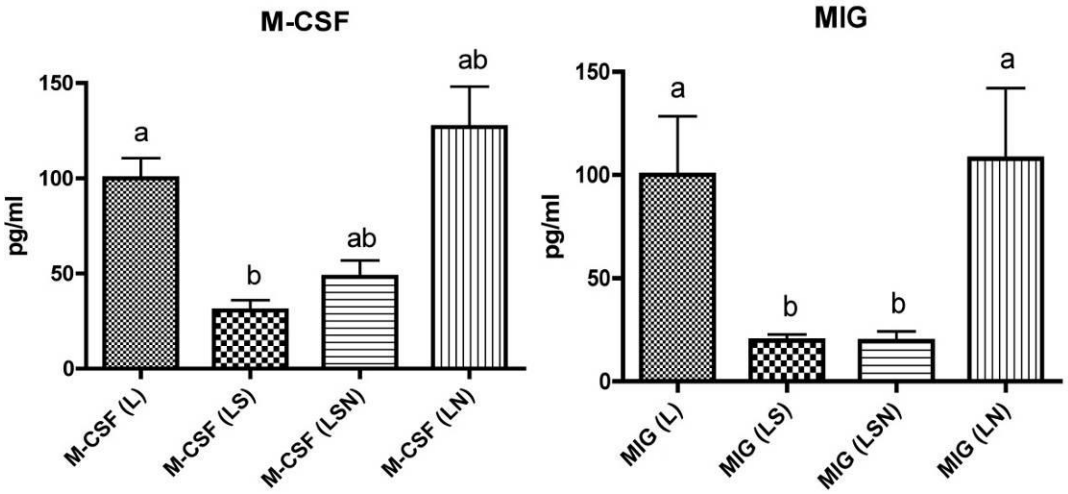
Levels of cytokine and chemokine production are shown in the peritoneal cavity of the NAC groups (pg/ml).Values are means ± SEM. Values that share any letter in common are significantly different ($P<0.05$) and values that do not share a letter in common are not significantly different ($P>0.05$).
Naive is not presented because this would affect the other values.
[L: LPS only, LS: LPS+SMD, LSN: LPS+SMD+NAC, LN: LPS+NAC]




[E] [F]

[G] [H]

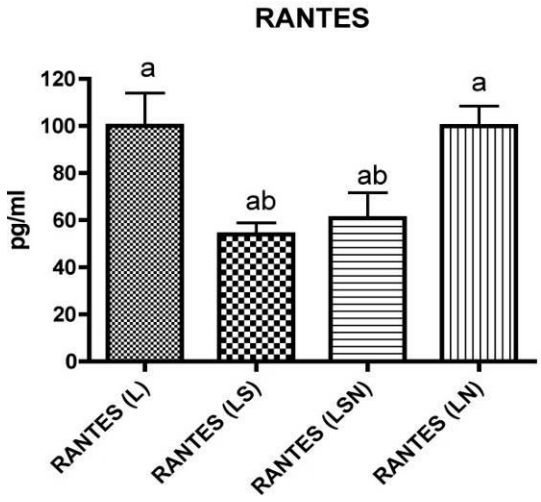
Figure 12 (continued)




[I]




[J]




[K]

Figure 12 (continued)

# CHAPTER IV

# DISCUSSION

Understanding the mechanism by which toxic chemicals affect the immune systems will be necessary to determine if animal models are appropriate to indicate the effects to be expected in humans and to determine if it is possible to ameliorate the immunotoxicity by counteracting the mechanism of toxicity. The purpose of this study was to: 1) Evaluate NF-κB activation, providing new information about the role of reactive oxygen in the activation of NF-κB; 2) Evaluate mechanisms by which SMD and increases LPS-induced IL-10 production and decreases LPS-induced production of IL-12 and other pro-inflammatory cytokines and chemokines by peritoneal macrophages.

There have been previous studies that report that reactive oxygen species positively effects signaling for the production of IL-12 and negatively effects IL-10 production [31, 32, 33]. However, our results with BSO or NAC suggest this. NF-κB reporter mice have not been previously used in any experiment.

The suppression of LPS-induced proinflammatory cytokines was somewhat selective with regard to amount of suppression, but all of the proinflammatory cytokines seemed to not have a change at all when pretreated with BSO or NAC.

In previous studies in our labs, NAC was used to diminish oxidative stress [34] which suggested that oxidative stress modulates IL-12 and IL-6. Treatment of mice with SMD plus NAC in this current study continued to cause a further suppression of IL-12.

Results from this study suggest that there is another mechanism of action in determining the role of reactive oxygen in the activation of NF-κB. Neither BSO nor NAC altered the effect of SMD on NF-κB activation, as well as the production of most of the cytokines and chemokines. With regard to GSH and GSSG, BSO along with SMD depleted GSH demonstrating that it acted as expected, but even though it produced similar depletion of GSH as SMD did, it did not have the same effect on NF-κB activation or cytokine production. This strongly suggests that although SMD depletes GSH and induces a response to oxidative stress at the level of gene expression [27], this oxidative stress is not the major mechanism by which SMD decreases NF-κB activation and alters cytokine and chemokine production. The results obtained with NAC were confirmatory, because this antioxidant and GSH precursor also did not change the effects of SMD on NF-κB activation or cytokine and chemokine production.

On a more general level, it has been reported that oxidative stress can enhance or suppress NF-κB activation [45, 46, 31, 47, 48]. The present study did not reveal either of these effects. The reason for this discrepancy is not clear, but most studies involved depleting GSH drastically, not the moderate depletion noted here with SMD. In addition, many of these studies were conducted in cell culture, which exposes cells to many times the oxygen concentration as compared to those in vivo and may not provide an accurate representation of the role of oxidative stress in regulation of NF-κB.

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