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Methionine Sulfoximine: A Novel Anti Inflammatory Agent

Tyler Peters
Wayne State University, tpeters@med.wayne.edu

METHIONINE SULFOXIMINE: A NOVEL ANTI-INFLAMMATORY AGENT

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TYLER J. PETERS

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DEDICATION

This work is dedicated to my family. I wouldn't have made it this far without your unconditional love and support.

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CHAPTER 1

Methionine sulfoximine as an investigational new drug: discovery, known biochemical targets, and uses in treating models of human disease

The work presented here is an expansion of recently published work:

Brusilow WSAB, Peters TJ. Therapeutic effects of methionine sulfoximine in multiple diseases include and extend beyond inhibition of glutamine synthetase. (2017). *Expert Opin Ther Targets.* 21(5):461-469. doi: 10.1080/14728222.2017.1303484.

Glutamate and glutamine facilitate nitrogen metabolism in living organisms

Nitrogen is an essential ingredient for life, comprising a significant portion of macromolecules in organic life forms. Although approximately 78% of Earth's atmosphere consists of nitrogen gas (N_2), most organisms lack the ability to convert inert nitrogen gas and other inorganic nitrogen species into bioavailable forms. The nitrogen cycle begins largely within soil, in a symbiotic niche near the vicinity of plant roots known as the rhizosphere. Certain prokaryotes within the rhizosphere contain specialized enzyme nitrogenases that form ammonium ions by reducing N_2. Plants and other organisms use ammonia to synthesize amino acids, which serve as building blocks of protein and nucleic acids. Therefore, most organisms rely on nitrogen acquired through the diet.

In humans, dietary nitrogen exists in the form of amino acids, protein, and free ammonia generated from gastrointestinal (GI) bacteria. Dietary nitrogen compounds are centralized through the reactions of glutamine synthetase, glutamate dehydrogenase, and glutaminase, which enzymatically interconvert glutamate with glutamine. Glutamate and glutamine can both act as nitrogen donors, and glutamate can also act as a nitrogen acceptor. Unlike carbon, which can be stored for energy in the forms of glycogen and triglycerides, the human body is not equipped to

store excess nitrogen that is not already incorporated into functional molecules such as protein, DNA, and RNA. Additionally, ammonium ions are slightly basic, with a pKa of 9.24. Therefore, glutamate and glutamine formation comprise the major pathway of ammonia detoxication in all tissues except for liver, which contains a full set of urea cycle enzymes. Lastly, these amino acids provide a safe and effective system for transporting nitrogen to distal tissues for either utilization or excretion.

In addition to controlling the flow of nitrogen throughout the body, both glutamine and glutamate are centrally involved in many reactions of both intermediary metabolism and neurotransmission. Glutamate is the major excitatory neurotransmitter in the brain and is also the amino acid donor in transamination reactions that move amino groups between carbon skeletons containing α-keto groups. Glutamine is the amino acid present in the highest concentration in the blood and body, except for in the brain where its concentration is second to that of glutamate. Glutamine is a substrate in the first reaction of both purine and pyrimidine synthesis pathways. As keystone molecules of nitrogen and carbon metabolism, deviations from homeostatic glutamate and glutamine concentrations pose significant physiological consequences, which are discussed in subsequent sections. The nitrogen cycle discussed in this section is summarized in **Figure 1.**

Methionine sulfoximine inhibits glutamine synthetase

Methionine sulfoximine (MSO) is an amino acid (MW: 180 grams/mole) that is structurally analogous to methionine, glutamine, and glutamate. In mammals, it is an inhibitor of glutamine synthetase and γ-glutamylcysteine synthetase, the first step in glutathione synthesis. MSO has been best characterized as a mechanistic based inhibitor of glutamine synthetase(1-3). The reaction carried out by glutamine synthetase, as well as the mechanism of MSO inhibition is depicted in **Figure 2.** Glutamate and ATP bind to specific positions within the active site. ATP is hydrolyzed

by the enzyme, and the gamma phosphate molecule is transferred to the epsilon oxygen of

glutamate to form γ-glutamyl phosphate. γ-glutamyl phosphate is a high energy intermediate; the

energy stored in the phosphate bond is harnessed by the enzyme to form glutamine by condensing

ammonia with the intermediate.
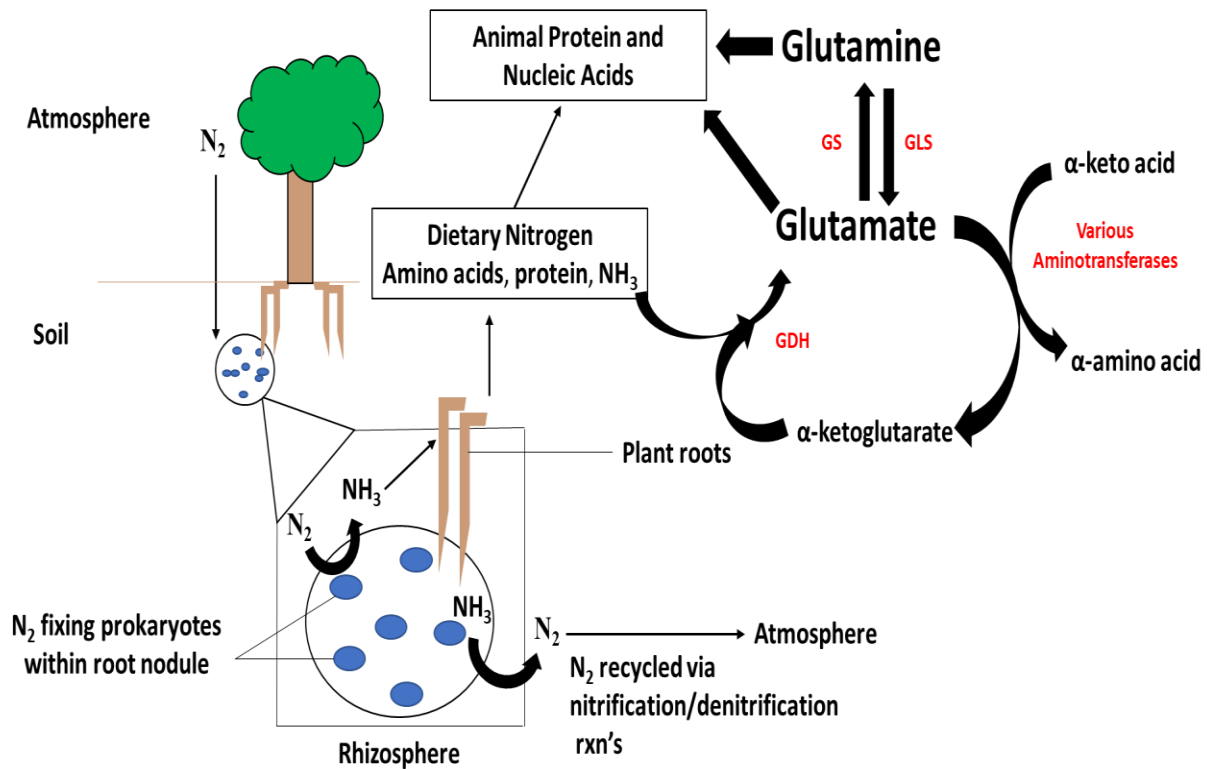
Figure 1. Overview of the nitrogen cycle. Atmospheric nitrogen gas diffuses into soil where it encounters various prokaryotes within the vicinity of plant roots. Nitrogen fixing bacteria contain nitrogenase enzymes that reduce atmospheric nitrogen to ammonia. Nitrifying and denitrifying bacteria also act on ammonia to create other nitrogen species such as nitrate and, nitrite, which can be recycled back into nitrogen gas. NH_3 is taken up by plants and other organisms and incorporated into the amino acids glutamate and glutamine, which serve as precursor molecules for biochemical reactions involving nitrogen.
GDH = glutamate dehydrogenase; GLS = glutaminase; GS = glutamine synthetase; N_2 = nitrogen gas; NH_3 = ammonia; rxn = reaction.

MSO competes with glutamate for binding to the active site of glutamine synthetase. Upon

binding of MSO and ATP, the enzyme transfers the gamma phosphate of ATP to the imine nitrogen

of MSO, forming MSO phosphate. MSO phosphate binds very tightly to the enzyme and cannot

be released from the active site, causing irreversible inhibition. Therefore, MSO may be referred

to as a "suicide inhibitor" because inhibition is mediated through enzyme catalysis. Typical of mechanistic based inhibitors, the cell must synthesize new enzyme to regain glutamine synthetase activity. Rats treated with MSO take 7-10 days to fully regain glutamine synthetase activity(4).

The Ki for MSO inhibition of glutamine synthetase differs from species to species, and the activity of tissue specific isoforms also show varied responses to MSO. This value has been shown to range approximately from 1 µM in *E. coli* to 1 mM in humans, with most reported values in the 1-200 µM range(3). It takes approximately 8 moles each of MSO phosphate and ADP to achieve 100% inhibition per mole of enzyme(1). MSO exists as four stereoisomers, but only the L,*S* configuration inhibits glutamine synthetase. The same diastereomer inhibits γ-glutamyl cysteine synthetase, in a similar ATP-driven fashion.
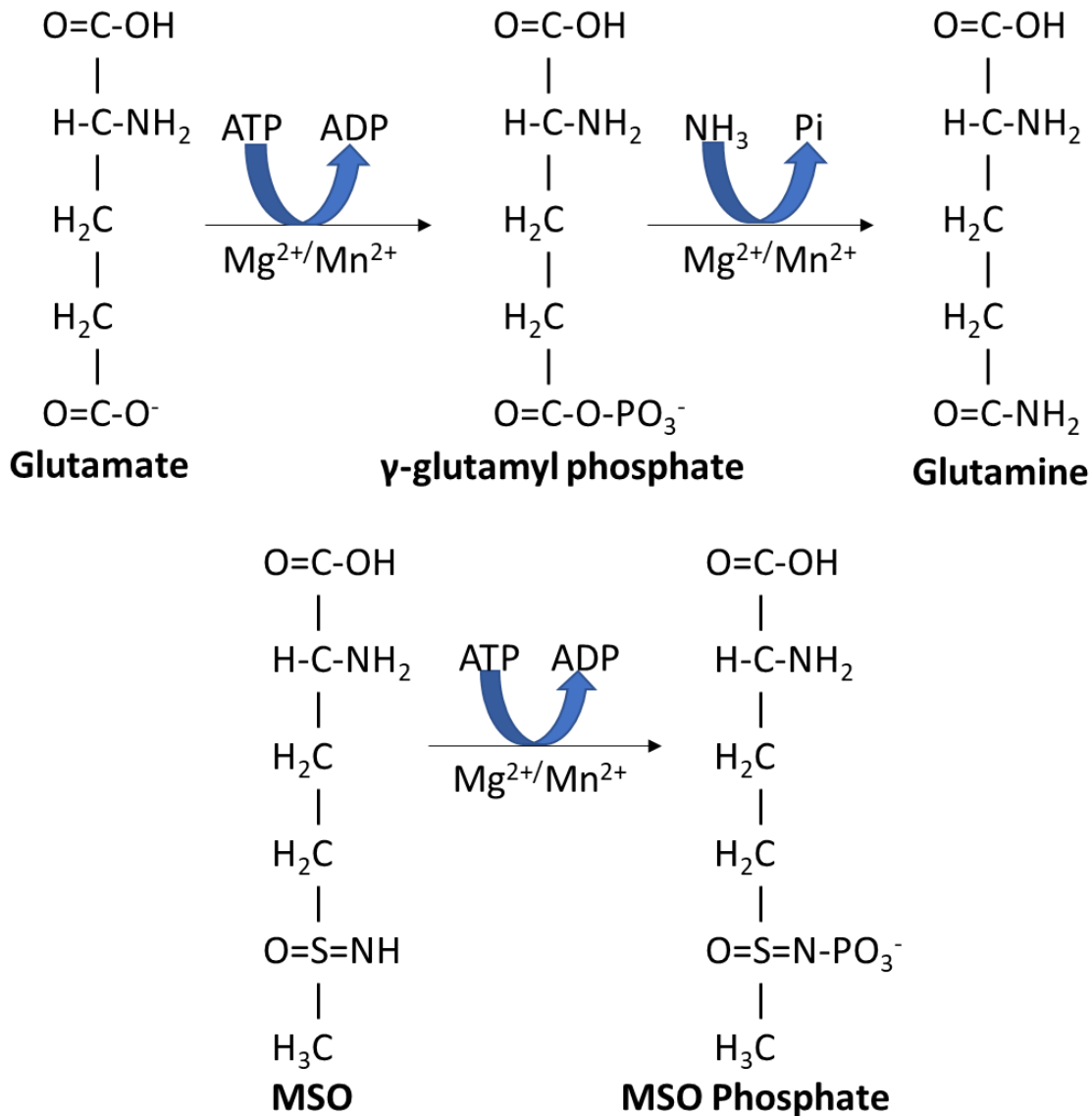
Figure 2. Methionine sulfoximine inhibits glutamine synthetase. *Above:* the reaction carried about by glutamine synthetase. Glutamate and ATP bind to the active site and ATP is hydrolyzed in the presence of magnesium and manganese ions to form gamma-glutamyl phosphate, a high-energy intermediate, and ADP. The enzyme reacts NH_3 with the intermediate to form glutamine, which is released from the enzyme along with inorganic phosphate. *Below:* the mechanism of glutamine synthetase inhibition by MSO. MSO competes with glutamate for binding to the active site. During the ATP hydrolysis step, the gamma phosphate of ATP is transferred to the imine nitrogen of MSO. The resulting molecule, MSO phosphate binds tightly to the enzyme and is not released, causing irreversible inhibition.

ADP = adenosine diphosphate; ATP = adenosine triphosphate; Mg^{2+} = magnesium ion; Mn^{2+} = manganese ion; MSO = methionine sulfoximine; NH_3 = ammonia; Pi = inorganic phosphate

The history of MSO

MSO was first identified by Bentley et al in 1950 as a byproduct of the reaction that used trichloramine (NCl_3), also called agene, to bleach flour(5). Throughout the previous decade, several investigators demonstrated that canines fed high levels of agenized flour exhibited running fits and convulsions, prompting a scientific pursuit of the "toxic factor" within the flour. Fearing that human consumption of agenized flour could potentially cause neurological diseases, such as epilepsy, the agene process was banned by regulatory agencies worldwide by l955, even though there was no evidence for toxicity in humans. The first ever publication describing the molecular structure of MSO labeled it as a "toxic factor" within the title, and shortly after its discovery and isolation, several investigators began to use MSO as a tool to study biochemical events leading to epileptic seizures. To test the effects of agenized flour on humans, Newell et al performed a study where a group of 19 children and adults consumed diets containing 22 to 30 times the amount of agenized protein in a normal American diet. The test population also included 5 epileptics. The diet was continued for up to 7 months, and no physical, neurological or electro-encephalographic changes were observed(5), refuting the belief that MSO was toxic.

Although there is no evidence that MSO is toxic to humans: 1) the historical context in which MSO was discovered 2) the fact that early studies focused solely on MSO toxicity, and 3) its prevalent use as a tool to study epilepsy, have diminished interest within the scientific community to develop MSO as a therapeutic. However, aberrant glutamine synthetase activity is implicated in several neurological diseases, and MSO has been shown to effectively treat a variety of such conditions in experimental animal models. In addition, since glutamine synthetase is essential for nitrogen metabolism and growth in Mycobacterium tuberculosis, and because MSO can inhibit all four isoforms of GS expressed by the pathogen, MSO has been described as a promising treatment

strategy for tuberculosis infection(6). Despite its ability to inhibit enzymes that affect levels of such important molecules, and despite, as will be described below, its activity in animal models of hepatic encephalopathy (HE), amyotrophic lateral sclerosis (ALS), and inflammation leading to liver failure, preconceived negative opinions of MSO pose a significant hurdle in developing this drug for human use.

Glutamine synthetase triggers cerebral edema resulting from hyperammonemia

Acute liver failure (ALF) can result from an inflammatory immune response to molecules found in various medications, bacteria, or viruses (e.g. hepatitis), from toxins, from an acetaminophen overdose, or from preexisting medical conditions. One of the most serious clinical consequences of ALF is overt HE, accompanied by cerebral edema. Death of liver tissue results in hyperammonemia, which causes HE and leads to a dangerous elevation of intracranial pressure(7). HE severity is graded with the West Haven criteria, which are comprised of four grades, ranked by the severity of edema. Signs and symptoms of HE may be mild with little or no clinical signs of cerebral edema, as seen in patients with chronic liver disease (cirrhosis), or very severe with cerebral edema seen in overt HE resulting from ALF(8).

A growing body of research has implicated elevated glutamine as central to the more serious forms of ALF and HE. Increases in blood and brain glutamine are increased in HE patients; it was shown in 1959 that cerebrospinal fluid glutamine levels correlate positively with the degree of HE severity(9). Post mortem analysis of HE patients revealed elevated levels of brain glutamine(10), and proton magnetic resonance studies showed both HE and ALF patients displayed increased brain glutamine measurements that declined with clinical improvement(11,12). Plasma ammonia traverses the blood brain barrier and increases the rate of

the glutamine synthetase reaction, due to the law of mass action. In brain, glutamine synthetase is located primarily within astrocytes, the site of pathology in HE patients(13,14).

The Osmotic Gliopathy hypothesis

Persistent arterial hyperammonemia is associated with increased micro dialysis measurements of brain glutamine(15). Considering the relationship between hyperammonemia and increased brain glutamine observed in HE patients, in 1986 Brusilow et al. proposed a biochemical basis of ammonia intoxication. The model, known as "osmotic gliopathy", postulates that brain ammonia stimulates astrocyte glutamine synthesis activity, causing glutamine accumulation. Glutamine acts as an osmolyte, creating a hypertonic environment where the cell compensates by increasing influxes of water, causing cell swelling and brain edema(16). Osmotic stress and cell swelling caused by elevated glutamine not only impairs astrocyte function, but also compromises the functional integrity of glial, neuronal, and vascular units.

The osmotic gliopathy hypothesis and the role of glutamine in the pathophysiology of HE is supported by the observation that inhibiting astrocyte glutamine synthetase with methionine sulfoximine protected mice from ammonia toxicity(17). In this particular study, the LD_{50} of ammonia was 50% higher in the MSO treated mice than in controls. Interestingly, the peak brain concentration of ammonia in the MSO treated mice remained significantly higher than controls for up to 24 hours after the initial ammonium chloride injection, due to an increased base-line brain ammonia concentration from MSO pretreatment. Importantly, MSO protected 100% of the mice from death even though levels of both brain and blood ammonia remained higher than controls. In conclusion, ammonia intoxication does not depend merely on the presence of ammonia, but rather it is mediated by its major detoxification product, glutamine. This finding was corroborated in humans by a case study that showed encephalopathy was correlated with elevated brain glutamine

levels, but not with elevated ammonia levels(18). The results of this case study are shown in

Figure 3.
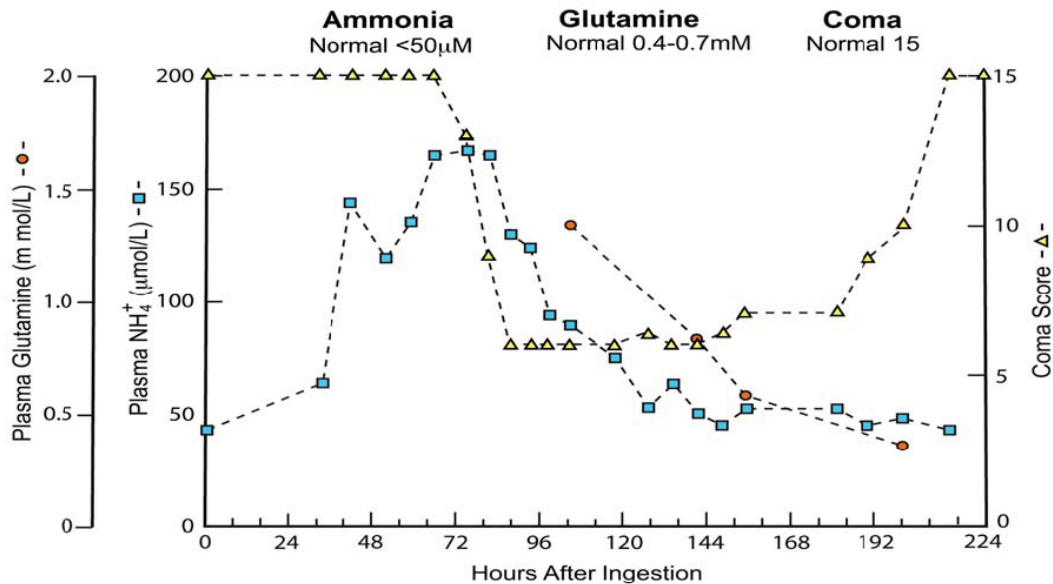
Figure 3. Evidence supporting osmotic gliopathy in a human patient. Results from a case study by Brusilow et al.(18) of a 22 year old female with acetaminophen induced liver failure. Coma score (based on the Glasgow coma scale) and the plasma concentrations of ammonia and glutamine are plotted against time after acetaminophen ingestion. Although ammonia levels return to normal, the patient remained comatose until plasma glutamine levels decreased, highlighting the relationship between hepatic encephalopathy and plasma glutamine concentration.

MSO prevents hyperammonemic brain swelling by inhibiting glutamine synthetase

MSO was shown to prevent astrocyte swelling and reduce cerebral edema in rats by reducing brain glutamine, although ammonia levels were elevated(19). Similar experiments have replicated the finding that MSO shows a protective effect against glutamine-mediated brain swelling. Blei et al. confirmed this effect in a liver failure model(20), while Hawkins et al. showed that MSO was able to prevent several metabolic symptoms of HE such as: the decrease in brain glucose consumption, the increase in blood-brain barrier permeability, and the increase in brain glutamine(21)

It has also been proposed that glutamine produced during hyperammonemia is transported to the mitochondria, where is it is converted to glutamate, releasing ammonia(22). The ammonia released within the mitochondria stimulates NADPH oxidase activity, which generates reactive oxygen and nitrogen species (RONS). RONS activate p38 MAPK, which phosphorylates p53 on serine 392 and causes p53 accumulation within the nucleus, where it regulates the expression of several genes that promote cell cycle withdrawal. Senescence is believed to play a role in causing cognitive impairment in HE patients. Astrocytes treated with ammonium chloride display several phenotypic hallmarks of p53 induced senescence, including decreased cell proliferation and DNA synthesis, and increased nuclear p53 accumulation and β-galactosidase expression(23). Additionally, cultured rat astrocytes treated with ammonium chloride showed increased mRNA and protein expression of the Mrp4 transporter, which exports several molecules believed to contribute to the pathogenesis of HE(24). In both studies, MSO treatment completely abolished all deleterious effects of ammonia intoxication. This "Trojan horse" hypothesis (25) also proposes that glutamine induces toxic effects leading to HE, but through a different mechanism than the osmotic gliopathy hypothesis.

MSO as a treatment for excitotoxicity, stroke, and amyotrophic lateral sclerosis

Several neurodegenerative diseases are believed to involve a component of glutamate excitotoxicity. Excitotoxicity refers to the overstimulation of neuronal glutamate receptors, caused by excess background glutamate, leading to neuronal damage and cell death(26). Examples of these diseases include: Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), Huntington's disease, stroke, and Parkinson's disease. During neurotransmission, glutamate is secreted into the synaptic cleft by the presynaptic neuron and binds to both ionotropic and metabotropic receptors on the surface of the post-synaptic neuron. Glutamatergic receptors

initiate downstream cell signaling events either through electrical (eg, ionotropic) or chemical (eg. metabotropic) mechanisms, both resulting in the accumulation of intracellular calcium.

Normally, the level of extra-neuronal glutamate is kept very low to prevent overstimulation of these receptors, which leads to aberrant calcium signaling and apoptosis. To prevent the presence of excess glutamate within the synaptic cleft, the brain utilizes a glutamate-glutamine cycle, described in **Figure 4.** After neurotransmission, glutamate reuptake occurs rapidly through high-affinity transport proteins found in the membranes of surrounding astrocytes. Glutamate may be oxidized by mitochondrial metabolism or recycled as a neurotransmitter. Regarding the latter, glutamine synthetase converts glutamate into glutamine within the astrocyte(27). Glutamine, acting as an inactive precursor, can then be shuttled from the astrocyte to the presynaptic neuron, where glutaminase converts glutamine back into glutamate(28).

Excitotoxicity is believed to involve some defect within this glutamate-glutamine cycle. Due to the cyclical nature of the glutamine synthetase and glutaminase reactions, and neuron dependence on astrocyte glutamine to supply glutamate, it has been shown that inhibiting brain glutamine synthetase leads to decreases in both glutamine and glutamate concentrations(29,30). Using MRI quantitation in different brain regions, Ghoddoussi et al. confirmed that MSO treatment reduces levels of both amino acids in a mouse model of ALS(31).

Brain damage resulting from excitotoxicity is a prevalent aftereffect of stroke, created by the release of glutamate by damaged brain cells, as well as by the activity of glutaminase on glutamine released by these cells. In addition to hypoxic cell death that occurs at the site of the initial occlusion, extensive cell damage permeates throughout the area surrounding the ischemic core, known as the penumbra. The final volume of penumbra that is salvaged from injury is highly correlated with improved clinical outcomes(32). Swanson et al. showed that after middle cerebral

artery occlusion in rats, 52% of cortical gray matter, ipsilateral to the occlusion, became infarcted.

Injecting MSO into the animals 24 hours prior to stroke decreased the mean infarct volume by

33%, indicating the involvement of glutamine synthetase in the pathology of stroke, and that

inhibiting the enzyme with MSO could provide a viable treatment.
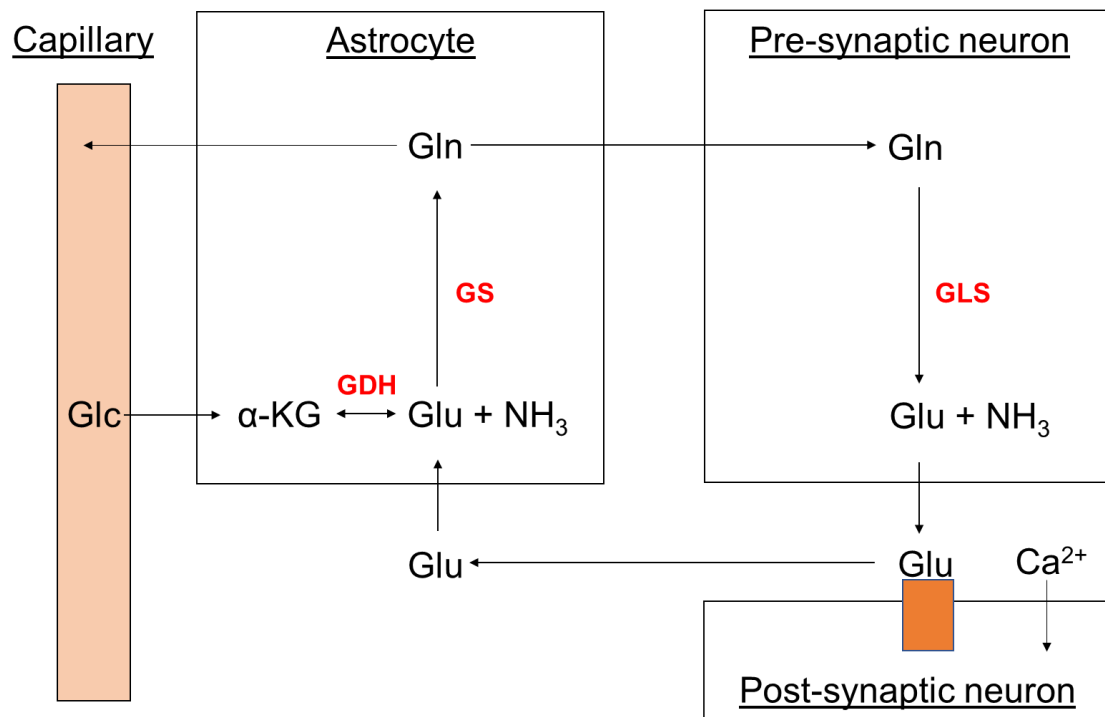
Figure 4. Brain glutamine/glutamate cycle. Blood glucose enters the brain and is metabolized via glycolysis or oxidative phosphorylation, where the latter produces α-KG. Through the reaction catalyzed by GDH, α-KG is conjugated with NH_3 (not shown for clarity) to produce glutamate. GS expressed in astrocytes creates glutamine from glutamate and NH_3, which is shuttled to the presynaptic neuron and reconverted back into glutamate and NH_3 by GLS. Glutamate is packaged into synaptic vesicles and secreted into the synapse during neurotransmission. Glutamatergic receptors initiate downstream cell signaling pathways that lead to increases in intracellular Ca^{2+}. Glutamate is taken up by high-affinity transport proteins in the astrocyte membrane, where it is either metabolized, or recycled.
α-KG = alpha-ketoglutarate; Ca^{2+} = calcium ions; GDH = glutamate dehydrogenase; Glc = glucose; Gln = glutamine; GLS = glutaminase; Glu = glutamate; GS = glutamine synthetase; NH_3 = ammonia.

Excitotoxicity is also believed to play a significant role in the pathogenesis of ALS. Two

inhibitors of glutamatergic neurotransmission, riluzole and gabapentin, were able to extend the

lifespan the G93A superoxide dismutase 1 (SOD1) mouse model of a familial form of ALS(33).

SOD1 exists within the cytosol and mitochondrial inner membrane space, catalyzing the

dismutation of superoxide to hydrogen peroxide and molecular oxygen. Our lab has demonstrated that MSO treatment extends the lifespan of the SOD1 G93A mouse up to 8%, and also restores the plasma amino acid signature observed in diseased animals towards the profile observed in wild-type control animals(31,34).

MSO as an anti-inflammatory for treating acute liver failure

Research in our lab has demonstrated that MSO can prevent death in a mouse model of acute liver failure(35). Injection of bacterial lipopolysaccharides (LPS) and D-galactosamine induces inflammatory liver failure and death by stimulating the uncontrolled release of pro-inflammatory proteins by immune cells, a "cytokine storm." When mice were pretreated with MSO at a dose 50 mg/kg, the overall survival of the mice increased from 20% to almost 80%. As will be described in subsequent chapters, this therapeutic effect is due to an anti-inflammatory mechanism of MSO on cells of innate immunity. MSO treatment resulted in decreased plasma and liver tissue levels of the major pro-inflammatory cytokines TNFα, IL-6, and IFNγ, among several other proteins. By reducing the plasma IL-6 concentration, MSO reduced activation of STAT3, a downstream transcription factor that controls inflammatory gene expression. MSO also prevented caspase 3 cleavage, which is involved in both extrinsic and intrinsic apoptosis. Therefore, MSO treatment reduces inflammation, prevents apoptosis, and affects several pathways involved in the immune response. The effects of MSO on immune cells is largely the focus of this manuscript, to be described in greater detail in the chapters to follow.

Studies on MSO toxicity

As mentioned previously, the discovery of MSO emerged from canine studies, where these animals were shown to be extremely sensitive and displayed various psychomotor abnormalities after being exposed to the amino acid. In 1951, Gershoff and Elvehjem tested the toxicity of MSO

in several animal species, where it became apparent that MSO toxicity is species-specific. Rats were shown to tolerate an MSO dose of 150 mg/kg with no observed abnormalities, although convulsions were observed at higher doses. For non-human primates it was established that MSO is safe at doses ranging from 50-100 mg/kg. However, dogs exhibited toxic effects at MSO doses as low as 3-5 mg/kg(36).

Studies on glutamine synthetase enzymology from different species have concluded differences in kinetic characteristics. Mice can be selected for resistance to different dosages of MSO, and it has been demonstrated that MSO sensitivity is related to differences in the level of glutamine synthetase activity between strains(37). Additionally, administration of a well-tolerated MSO dose of 2.5 mg/kg to non-human primates produced the same degree of inhibition of brain glutamine synthetase as 100 mg/kg produced in rats(7). Lastly mice injected with 20 mg/kg MSO, three times per week for 10 weeks, showed no signs of impairment of cognition and very minimal side effects, even though this dose inhibited brain glutamine synthetase by 80% compared to control mice(38). In summary, animal studies have concluded that MSO sensitivity is species-specific, suggest that MSO can be tolerated for extended periods of time, and provide evidence that MSO may be safer and more effective in primates.

The only human study of MSO was performed in 1961 by Krakoff in seven patients with terminal cancer(39). Patients were given a variety of uncharacterized dosages of MSO over periods of time ranging from 2 to 8 days, with no description of the purity and exact composition of the MSO preparations. There is no way of knowing exactly what compounds those patients were being injected with. The enzymology of MSO was not available at the time, and the dosing schedule described in the narrative was inappropriate for a long-lasting inhibitor. Four patients described having hallucinations which subsided after treatment was discontinued, but no serious adverse

events were reported. Therefore, no data exist indicating MSO is toxic to humans, and available data suggest that primate glutamine synthetase is inhibited at doses that are not neurotoxic and do not produce convulsions. **Table 1** summarizes the various toxicity studies on MSO and lists the doses of MSO discovered to be toxic or tolerable for each species.

Author	Dog	Rat	Mouse	Rabbit	Cat	Primate
Bentley(40)	2.0			2.0		
Gershoff(36)	3.0	(150)				100[b]
Krakoff(39)						(0.2-8)[c]
Proler(41)					18-20	
Warren(17)			(50)			
Wada(42)		(100)			7.5	
Apostolakis(43)				3-9[a]		
Blin(38)			(chronic)[d]			
Brusilow(7)						(2.5)[b]

Table 1. MSO toxicity is species-specific. This table lists the authors of studies on MSO toxicity versus the species tested, and the doses of MSO (mg/kg) that were shown to be toxic or tolerated (in parentheses) by each of the tested species in each study.
a: Developed spastic diplegia
b: Non-human primate
c: In humans – varying doses, estimates assuming 50 kg body weight
d: Ten weeks of MSO: 20 mg/kg, 3 times/week

CHAPTER 2

Macrophage physiology in innate immunity and inflammation

PART 1: Macrophages are a major component of innate immune response to infection

Macrophages

Macrophages are specialized white blood cells that detect and eliminate dangerous pathogens and remove other undesirable particles from the blood and tissues. Macrophages are the largest of the leukocytes, ranging in size from 20-30 microns in diameter. Originally described by Nobel Laureate Élie Metchnikoff in 1882, macrophages are often referred to non-specifically as phagocytes, a translation of Greek origin meaning "devouring cell" which reflects their ability to engulf microorganisms in a process known as phagocytosis. Macrophage populations are found in essentially every tissue of the body, comprising heterogeneous sub-populations that have niche-specific functions. **Table 2** highlights distinct macrophage populations known as the tissue-resident macrophages.

It was originally thought that all macrophages originate from the bone marrow, as hematopoietic stem cells (HSCs), and proceed to differentiate into myeloid precursors of increasingly committed cellular fates before reaching a terminal point of differentiation as immature leukocytes of the plasma known as monocytes. After receiving an environmental stimulus, monocytes are attracted towards the site of a local infection, extravasate from the blood vessels into tissues, and mature into inflammatory macrophages and myeloid lineage dendritic cells. However, recent data from lineage tracing and fate-mapping experiments support a prenatal origin of tissue-resident macrophages, which have been shown to maintain local populations through self-renewal, with very little contribution in number from blood-derived monocyte precursors(44). Albeit the enormous genetic and functional complexity being unearthed in recent

years, in the context of innate immunity all macrophage populations share the common features of initiating, maintaining, and resolving inflammatory responses to infection.

In addition to orchestrating the inflammatory response, macrophages aid in homeostatic processes such as wound healing, tissue regeneration, and tissue homeostasis. Tissue injury involves multiple phases and macrophages have been shown to be essential mediators during each phase. Injury such as a traumatic insult results in necrotic cell death and the release of damage-associated molecular patterns (DAMPS) that act as endogenous danger signals. Highly characterized DAMPS include serum amyloid A, high mobility group box-1, calgranulin A and B, and ATP(45). DAMPS bind to extracellular receptors on macrophages and act to induce, amplify, or perpetuate inflammatory responses.

During the inflammatory phase of injury, macrophages regulate inflammation, clear apoptotic cell debris, and promote epithelial cell proliferation(46). During the formation of new tissue, macrophages are a major source of growth factors essential for cell proliferation, differentiation, and the generation of extracellular matrix. Macrophage depleted mice show impaired angiogenesis, decreased cell proliferation, and a delay in cell repopulation at the wound site after injury(47). Macrophages also resolve inflammatory responses during wound healing by secreting anti-inflammatory cytokines and ingesting apoptotic neutrophils. Macrophages increase arginase expression, which converts arginine to ornithine. Ornithine is used to synthesize hydroxy-proline and polyamines, which are essential for extracellular matrix formation and cell proliferation, respectively. Lastly, during tissue remodeling, a dynamic process that involves the reorganization of wounded tissue, macrophages remove cell debris and excess extracellular matrix fragments around the wound.

Macrophage Type	Tissue of residence	Function	Macrophage Type	Tissue of residence	Function
Peritoneal macrophage	Lining of the abdominal cavity	Ingest apoptotic cells and bacteria	Osteoclast	Bone	Bone reabsorption
Alveolar macrophage	Lung alveoli	Remove small particles, dead cells and respiratory pathogens	Red-pulp macrophage	Spleen	Clearance of red blood cells, iron metabolism
Microglia	Brain and spinal cord	Eliminate dead neurons, synaptic remodeling	White-pulp macrophage	Spleen	Removal of apoptotic cells from germinal centers
Kupffer cell	Liver	Clear portal blood of pathogens, hepatic remodeling	Intestinal macrophage	Gastrointestinal tract	Maintain homeostasis with gut micrbiota
Adipose-associated macrophage	Adipose tissue	Control of insulin sensitivity	Langerhans cell	Skin	Migrate to lymph node to present antigen

Table 2. Location and function of distinct macrophage subpopulations. Tissue resident macrophages arise from distinct lineages and perform tissue-specific functions.

Macrophages initiate the immune response to infection

Macrophages fall under a broad classification of immune cells known as antigen presenting cells (APCs) which specialize in the recognition, processing, and presentation of non-self antigens to cells of the adaptive immune system known as T-lymphocytes. It is through the process of antigen presentation that an acquired, or "adaptive" immune response can be generated against a specific epitope. Although almost all cell types can exhibit some form of antigen presentation, macrophages, along with dendritic cells and B-lymphocytes express MHC class II receptors, pattern recognition receptors, and co-stimulatory molecules that make them especially proficient for the task, and as such these cells are sometimes referred to as "professional APCs." Following this distinction, macrophages are biological sentinels that serve as a first line of defense in recognizing both intracellular and extracellular pathogens.

As innate immune cells, macrophages have evolved to possess receptors that recognize conserved structural elements contained in several bacterial and viral species known as pathogen-associated molecular patterns (PAMPS). Several PAMPS have been identified and the germline-encoded receptors that recognize these motifs are known as pattern recognition receptors (PRRs). The binding of a PAMP to its cognate PRR leads to activation of the receptor, and information is transmitted from the plasma membrane to the nucleus through an array of cell-signaling pathways. These pathways usually overlap, and converge in the activation of one or more major transcription factors, such as NF-κB and the MAP kinases, which bind to promoter regions and activate the transcription of genes involved in pro-inflammatory and anti-microbial responses(48). Broadly, pattern recognition receptors can be divided into two classes based on the types of pathogens they recognize: extracellular PRRs and intracellular PRRs. The most widely studied intracellular and extracellular PRR families are the NOD-like receptors, and the Toll-like receptors, respectively.

NOD-like receptors

The nucleotide oligomerization domain (Nod)-like receptors (NLRs), are cytosolic pattern recognition receptors that are involved mainly in the recognition of bacteria that have entered the cell via infection or phagocytosis. There are 23 NLR members found in humans, which are expressed in immune tissue and epithelial cells, but the expression of certain NLRs is restricted to phagocytes(49). The most widely studied NLRs are NOD1 and NOD2, which recognize distinct structural motifs within peptidoglycan, a major cell wall constituent found in almost all bacteria. NOD1 and NOD2 contain 1) an N-terminal caspase recruitment (CARD) domain involved in downstream cell signaling and effector functions, 2) a nucleotide oligomerization domain that is critical for activation, and 3) a leucine-rich repeat containing C-terminal domain which recognizes peptidoglycan. Proper NLR function is critical for priming rapid innate immune responses, and

NLR signaling initiated inside of the cell can exhibit cross-talk with other innate receptors(50). In addition, NOD1$^{-/-}$ mice were shown to be more susceptible than wild-type mice to early pneumococcal sepsis(51). NLR signaling has also been shown to play a role in development and apoptosis, and abnormal NLR signaling is associated with increased susceptibility to an array of inflammatory diseases such Crohn's disease, gout, and cancer(52).

Toll-like receptors

Toll-like receptors (TLRs) are pattern recognition receptors expressed within the plasma membranes of immune cells, epithelial cells, endothelial cells, and fibroblasts, and serve to recognize extracellular PAMPS. The *Toll* gene was first described in *D. melanogaster* as an important regulator of dorsoventral polarity during larvae development(53), as well being essential for antifungal responses(54). The toll-like receptor family includes 10 members in humans, and three additional members in mice. **Table 3** describes human members of the TLR family and cognate ligands. TLR activation leads to the recruitment of adapter proteins and the activation of protein kinases that phosphorylate transcription factors that promote the expression of genes involved in pro-inflammatory and anti-microbial processes. Such antigen-induced processes include the promotion of cell proliferation, the expression of pro-inflammatory mediators known as cytokines, and increased survival. Cytokines produced through TLR signaling orchestrate the immune response to infection, binding to receptors on various target tissues and enhancing both innate and adaptive immune cell functions. Cytokine signaling is discussed in greater detail towards the end of Part 1 of this chapter.

In addition, the binding of bacterial constituents to TLRs mediates phagocytosis, where microbes are ingested, destroyed, and peptide antigens are packaged into MHC class II molecules, and presented to cells of adaptive immunity. Therefore, TLR signaling produces a link between

innate functions and the development of an acquired immune response. Several mutations in various members of the TLR family have been described in humans and have been linked to increased susceptibility of developing various inflammatory diseases(55).

Toll Like Receptor (TLR)	Cognate Ligand
TLR1	Triacyl lipopeptides, lipopolysaccharide
TLR2	Lipoteichoic acid, bacterial lipopeptides
TLR3	Viral dsRNA
TLR4	Lipopolysaccharide
TLR5	Flagellin protein
TLR6	Diacyl lipoprotein, other lipopeptides
TLR7	ssRNA in endosomes
TLR8	G-rich oligonucleotides, ssRNA
TLR9	Unmethylated CpG DNA
TLR10	Unknown

Table 3. Human Toll-like receptors and cognate ligands. Toll-like receptors present on cells of innate immunity recognize a variety of pathogen associated molecular patterns.
Toll-like receptor 4 recognizes lipopolysaccharide

A critical advancement of contemporary understanding of innate immunity occurred in 1998, when Nobel Laureate, Bruce Beutler and colleagues discovered that lipopolysaccharide (LPS), the major constituent of the outer membrane of gram-negative bacteria, is recognized by Toll-like receptor family member 4 (TLR-4), and that mice with mutations in TLR-4 were unable to generate an immune response to LPS(56). LPS is a PAMP that induces systemic inflammation and septic shock in animal models when administered in excessive doses. The work of Beutler's group triggered an explosion of research in the field of TLR signaling, and as a result the LPS-induced TLR-4 signaling pathway has been characterized to appreciable detail. **Figure 5** provides a schematic of one arm of the highly complex TLR-4 signaling pathway

involved in the expression of pro-inflammatory cytokines, known as the MyD88 dependent pathway.

TLR-4 signaling begins with the recruitment of LPS to the receptor binding site. This is facilitated by a series of proteins including lipopolysaccharide binding protein (LBP), CD14, myeloid differentiation factor 2 (MD-2) and TLR-4. LBP is a soluble protein found in the plasma that binds to LPS and complexes with CD14. CD14, a glycophosphatidylinositol-linked membrane protein, directs the transfer of LPS to the TLR-4 receptor. TLR-4 cannot recognize LPS alone and therefore it is a requirement for signaling that the receptor non-covalently interacts with MD-2. Upon the formation of the LPS binding complex, monomers of the TLR-4 complex oligomerize, and the receptor assumes an activated conformation, exposing a toll/interleukin-1 receptor (TIR) domain on the cytoplasmic face of the membrane which recruits TIR-domain containing adapter proteins. Five TIR-domain containing proteins that interact with TLR-4 have been identified, but only two of them are involved in the pathway that leads to gene expression of pro-inflammatory cytokines. This signaling pathway is known as the myeloid differentiation primary response gene 88 (MyD88) dependent pathway, and the adapter proteins that facilitate this pathway are MyD88, and TIR domain-containing adaptor protein (TIRAP)(57).

After activation of the receptor, TIRAP associates with the cytoplasmic tail of TLR-4 through TIR domain interactions. TIRAP then recruits MyD88, which also binds to TLR-4 and initiates MyD88 dependent signaling. MyD88 contains a death domain (DD) which recruits a DD-containing kinase known as IL-1 receptor-associated kinase-4 (IRAK-4). IRAK-4 becomes activated through this interaction and from here a series of kinase mediated signaling events occur. IRAK-4 activates IRAK-1, which phosphorylates another adapter protein known as TNF receptor-associated factor 6 (TRAF6). TRAF6 associates with two ubiquitin conjugating enzymes and

collectively this protein complex activates transforming growth factor-b-activated kinase (TAK1). TAK1 kinase activates the mitogen activated protein kinases (MAPK) as well as IκB kinase (IKK).

IKK phosphorylates IκB, an inhibitor bound to the transcription factor NFκB that regulates the expression of pro-inflammatory cytokines, genes involved in antigen presentation, immunoreceptors, adhesion molecules, and other miscellaneous genes involved in the immune response. Phosphorylated IκB dissociates from NFκB and is marked for proteasomal degradation, leaving the free form of NFκB which transports to the nucleus and activates transcription. An additional transcription factor that is involved in the gene expression of pro-inflammatory cytokines, AP-1 is also phosphorylated through MAPK signaling.
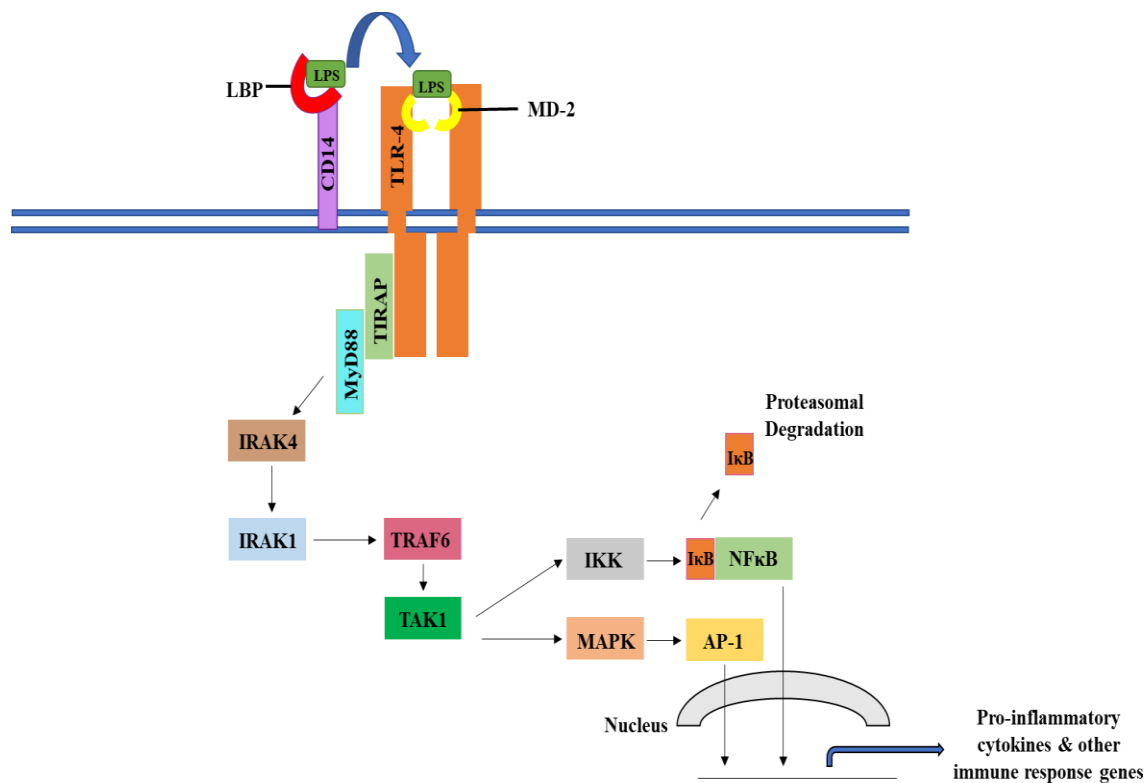
Figure 5. Rudimentary outline of the MyD88-dependent TLR-4 signaling pathway.
Macrophage mediated maintenance of immune responses

Macrophages initiate the immune response to infection through receptor-antigen interactions. Upon activation, macrophages provide critical support functions necessary to

establish a local, non-adaptive immune response, but as mentioned earlier they also present antigens to adaptive immune cells and secrete factors that influence the functional differentiation of these cells. Broadly, macrophages have three major effector functions during the generation and maintenance of an inflammatory response. These functions are 1) phagocytosis, 2) antigen presentation, and 3) immunomodulation.

Phagocytosis

Phagocytosis describes the process by which phagocytes including macrophages, dendritic cells, and neutrophils engulf microbes and absorb them into the cell. Phagocytosis occurs after specific antigens are recognized by phagocytic receptors on the cell surface, where after the cell forms membranous protrusions that surround the microbe and draw it into the cell, forming vesicular structures known as phagosomes. Phagocytosis is the first step of the trapping and killing of microbes within infected tissue and is also necessary for providing the cell with antigenic molecules to be packaged and presented to T-lymphocytes on the cell surface, as the first step of generating an acquired immune response. Macrophages possess an array of phagocytic receptors that can be classified based on ligand specificity(58). **Table 4** highlights some of the more characterized phagocytic receptors and their ligands.

Once absorbed into the cell, microbes within the phagosome are immediately exposed to reactive oxygen species generated by NADPH oxidase, which reduces atmospheric oxygen to superoxide (O_2^-)(59). The phagosome fuses with the lysosome to form a structure known as the phagolysosome. Proton ATPases within the membrane of the phagolysosome lower the pH and this acidification, along with the action of glycosidases and proteases within the phagolysosome forms an effective sterilization system (60).

Phagocytic Receptor	Molecule/Species Recognized
TLR-4	Lipopolysaccharide
Mannose receptor	Mannosylated fungal antigens
Dectin-1	B-glucans and carbohydrates in cell walls of fungi, bacteria, and plants
Scavenger Receptor A (SR-A)	Various bacterial cell wall components
Macrophage Receptor with collagenous structure (MARCO)	Lipopolysaccharide and whole bacteria
Fragment crystallizable γ receptor (FcγR)	Fc region of IgG antibody

Table 4. Macrophage phagocytic receptors and their ligands.
Antigen Presentation

Antigen presentation is a complex process by which APC's such as macrophages, dendritic cells, and B lymphocytes present antigens to T lymphocytes in the form of short peptide fragments. Microbes internalized through phagocytosis are degraded and their proteins are digested into short peptide chains by acidic proteases such as cathepsins B, D, and L(61). The processed peptides generated from extracellular pathogens are packaged into receptors known as major histocompatibility complex, class II (MHC class II). To fully appreciate the complexity of antigen presentation, it is essential to possess a fundamental understanding of the MHC system, which is the vehicle the human body uses to distinguish between self and non-self antigens.

The major histocompatibility complex (MHC) is a large polygenic locus of 4 million base pairs that contains at least 50 genes. In addition, the MHC genes are the most polymorphic genes known, with each gene having multiple alleles. In humans the MHC is referred to as the human leukocyte antigen system (HLA). HLA genes are divided into two classes, each with 3 pairs of genes which are expressed differentially on cells. MHC class I molecules present antigens from the cytosol whereas MHC class II molecules present extracellular derived antigens. Macrophages and B lymphocytes express MHC class II molecules, which are loaded with antigenic peptides and

displayed to a class of T lymphocytes known as T-helper cells. The T cell receptor on these cells recognizes the antigen within the MHC class II receptor, and through this interaction and other costimulatory mechanisms the T helper cells become activated. The main function of T helper cells is to activate other cells of the immune system, including B lymphocytes which are stimulated to secrete antibodies against the antigen recognized by the T cell. In summary, the generation of an acquired immune response depends on antigen presentation by macrophages.

Immunomodulation

In addition to performing the processes of phagocytosis and antigen presentation, macrophages contribute significantly to the inflammatory response by changing the functions of other cells. By influencing the activities of other cells, macrophages globally regulate immune responses. The process of increasing or decreasing the activity of the immune system is referred to as immunomodulation. Activated macrophages influence the immune response 1) directly, by forming cell-cell contacts facilitated by receptor/ligand interactions and 2) indirectly, by secreting various agents that act upon cells of the immune system. By secreting these various factors, macrophages provide cells with a molecular language and a system of communication that spans the level of the entire organism.

Immunomodulatory agents secreted by macrophages include cytokines, chemokines, bradykinins, leukotrienes, and prostaglandins. Importantly, immunomodulation by macrophages can either suppress, or promote the inflammatory response, and such processes are tightly controlled under normal circumstance. Likewise, defects in macrophage-mediated immunomodulation are linked to human disease, largely through an excessive production of the pro-inflammatory signaling proteins known as cytokines.

Pro-inflammatory Cytokines

In response to antigen, activated macrophages provide a first line of defense by secreting a variety of factors that orchestrate inflammation and direct the development of the humoral immune response. These factors, known as cytokines, are a class of soluble, or membrane-bound messenger proteins involved in autocrine, paracrine, and endocrine signaling networks(62). Cytokines bind to receptors on a variety of target tissues, exerting pleiotropic effects that influence cell to cell communication, functional maturation, and cell behavior. More than 200 cytokines have been identified and these proteins are grouped broadly as being either pro-inflammatory or anti-inflammatory. Cytokines are further divided into subgroups that share similar biological functions. These include the interleukins, growth factors, chemokines, interferons, and hematopoietic factors(63).

Many cytokines have redundant or overlapping functions, acting in concert to regulate several homeostatic processes such as growth and development, wound healing, cell division, chemotaxis, lymphocyte maturation, and inflammation collectively(64). An imbalance between pro-inflammatory and anti-inflammatory functions mediated by cytokines is observed in several human diseases(65). Macrophages are capable of producing large quantities of several pro-inflammatory cytokines, and excessive levels of these proteins in circulation can progress the development of acute and chronic inflammatory syndromes. The best studied pro-inflammatory cytokines produced by macrophages, and perhaps the most clinically relevant, include: Tumor necrosis factor alpha (TNFα), Interleukin 6 (IL-6), Interleukin 1 beta (IL-1β), and Interleukin 12 (IL-12).

Tumor Necrosis Factor Alpha (TNFα)

TNFα is a pro-inflammatory cytokine produced predominantly by activated macrophages and acts as a key regulator of the immune response and is therefore one of the most clinically relevant and actively investigated cytokines. Those investigations have dissected both distinct and overlapping signal transduction pathways mediated through TNFα, which influence a range of cellular functions including cell death, survival, differentiation, migration, and proliferation(66). Excessive TNFα production is a consequence of several diseases including auto-immune and chronic inflammatory syndromes. TNFα acts specifically on endothelial cells, stimulating the upregulation of cell-surface adhesion proteins that bind to neutrophils and facilitate cell migration from blood into tissue during local infection(67). Additionally, morphological changes are exhibited by endothelial cells upon interaction with TNFα, such as the disruption of adherens junctions resulting in increased vascular permeability(68,69). Four monoclonal anti-TNFα antibodies and one recombinant TNFα receptor fusion protein have been approved by the FDA to treat rheumatoid arthritis, ankylosing spondylitis, and Crohn's disease(66).

TNFα acts directly and in concert with other cytokines and chemokines to increase neutrophil phagocytosis and respiratory burst capacity used by these to cells capture and eliminate pathogens(70,71). Furthermore, TNFα, along with IL-6 and IL-1β, stimulate hepatocytes to produce acute phase response proteins, constituting yet another critical arm of the innate immune response. TNFα acts in both an autocrine and paracrine fashion, binding to cell surface receptors on macrophages to initiate its own synthesis(72). In this manner an abundance of TNFα is produced and in severe cases of atypical immune responses TNFα can travel systemically through circulation, acting in a hormonal fashion on distal organs. TNFα has been confirmed as the principal mediator of lethality from E. coli endotoxin, evidenced by the fact that baboons passively

immunized with a neutralizing anti-TNFα antibody and subsequently infused with a lethal dose of live E. coli are protected against shock, vital organ failure and death(73). Insofar as clinical relevance, adalimumab, a monoclonal antibody to TNFα marketed under the tradename Humira®, is widely advertised as a treatment for arthritis, psoriasis, Crohn's syndrome, and other inflammatory disorders, generating approximately $16B/year in sales.

Interleukin 6 (IL-6)

IL-6 is a keystone cytokine, exerting both pro-inflammatory and anti-inflammatory effects on a diverse range of cell types depending on its mode of signaling(74). Perhaps the most significant pro-inflammatory role of IL-6 is triggering the acute phase response. Castell et. al concluded that IL-6 controls the acute phase response in human liver cells through demonstration that IL-6 was both necessary and sufficient to induce the full spectrum of acute phase response proteins. In primary human hepatocytes, recombinant human IL-6 was capable of inducing C-reactive protein, serum amyloid A, alpha 1-antichymotrypsin, and fibrinogen synthesis in a dose and time dependent manner. Also, a significant decrease in fibronectin, albumin, and transferrin was observed compared to controls(75). Plasma IL-6 concentration increases 10-100 fold during infection and it has been proposed that IL-6 could be used as a highly accurate diagnostic marker of sepsis(76).

Additionally, IL-6 has a principal role in bridging innate and adaptive immunity during prolonged infection. IL-6 was initially discovered as a factor secreted by T cells that stimulated activated B cells to differentiate into antibody secreting plasma cells without stimulating B cell proliferation(77,78). IL-6 was also characterized as a B cell stimulating growth factor in B cell hybridoma cell lines. IL-6 has been shown to be necessary for T lymphocyte recruitment(79) and rescues these cells from entering apoptosis(80). The overproduction of IL-6 is implicated in the

pathology of inflammatory and auto-immune disorders including rheumatoid arthritis, where IL-6 is over-expressed in synovial tissue. Tocilizumab, a humanized anti-human IL-6 receptor antibody has been approved by the FDA to treat rheumatoid arthritis and is also a primary treatment for life-threatening inflammatory immune response that sometimes occurs in anti-cancer immunotherapy.

Interleukin 1 Beta (IL-1β)

IL-1β is one of seven pro-inflammatory cytokines of the interleukin 1 family. IL-1β secreted by macrophages establishes an axis between the immune and neuro-endocrine systems. IL-1β stimulates the hypothalamus to increase gene expression of cyclooxygenase-2 (COX-2), which plays a key role in the synthesis of prostaglandin E2 (PGE$_2$). PGE$_2$ is an endogenous pyrogen that induces fever, vasodilation, and pain hypersensitivity. Patients injected with IL-1 experience fever, headache, and pain in muscles and joints, all mediated through COX-2 activity(81). In many scenarios IL-1β synergizes with TNFα, mediating the adhesion of neutrophils to the surface of the endothelium, and inducing nitric oxide production(63). The significance of IL-1β in mediating the inflammatory response is highlighted by the fact that mutations in genes that regulate the production of IL-1β cause Cryopyrin-associated periodic syndrome (CAPS), a group of systemic autoinflammatory syndromes(82).

Interleukin 12 (IL-12)

IL-12 is an integral cytokine that brings innate and adaptive immune responses together. This is achieved by the ability of IL-12 to induce functional maturation of T lymphocytes(83). Specifically, IL-12 secreted by activated macrophages provides a co-stimulatory signal that directs the development of the Th1 subset of T-helper cells. Th1 effector cells initiate host defense against bacteria and protozoa as major producers of interferon gamma (IFNγ), a cytokine that enhances

antimicrobial functions of neutrophils and macrophages. Additionally, IL-12 stimulates the proliferation of T cells and natural killer cells, and enhances cytolytic functions of these cells(84).

Chemokines

During the immune response, leukocytes are recruited from various regions of the body to the site of a local infection. Cell migration is accomplished through a system based on chemical attraction, known as chemotaxis. Activated macrophages secrete several chemoattractant proteins called chemokines into the blood which are recognized by chemoreceptors on neutrophils, natural killer cells, and T lymphocytes. Chemoreceptors are embedded uniformly within the plasma membrane of immune cells, which permits the recognition of chemical concentration gradients and subsequent migration towards the source of the chemokine.

Chemokines have been classified into four subfamilies based on the relative position of cysteine residues (CXC, CC, C and CX3C)(85). In addition to inducing cell motility, chemokines also affect angiogenesis and cell proliferation. Certain chemokines are secreted constitutively to promote basal leukocyte migration, whereas others are only synthesized during inflammatory conditions. Pro-inflammatory chemokines secreted by macrophages include CCL2, CCL3, CCL5, CXCL1, CXCL2 and CXCL8. In addition to chemokines, macrophages secrete other chemotactic small molecules, including prostaglandin D2, leukotrienes, and other eicosanoid lipid mediators.

Macrophages resolve inflammatory responses

As immunomodulating cells, macrophages mediate the direction and the duration of the immune response to infection. Inflammation is a taxing stress response that requires significant metabolic resources, and prolonged bouts of inflammation generally causes damage to healthy tissue. Therefore, macrophages possess tightly controlled mechanisms to control the spatiotemporal sequence of initiation, maintenance, and resolution of the immune response. Defects in molecular switches that regulate macrophage responses can have severe acute consequences, and if these situations become chronic, may lead to inflammatory disease.

Macrophage activation is controlled through metabolic shifts, and impinging upon metabolic signaling pathways impacts the function of these cells. Nutrient deprivation starves immune cells of ATP and activates AMP-activated protein kinase (AMPK) signaling, which suppresses several anabolic pathways(86). Additionally, various metabolites and vitamins have been discovered to promote anti-inflammatory genetic programs in isolated macrophages. In mouse peritoneal macrophages, vitamin A was shown to activate the transcription factor GATA6, which promotes the expression of anti-inflammatory genes(87). Macrophages produce the major anti-inflammatory cytokine, interleukin 10 (IL-10) which activates AMPK signaling and turns off the expression of the pro-inflammatory cytokines.

The destructive potential of uncontrolled macrophage activation is also prevented through a type of activation induced cell death (AICD) that is developmentally regulated. In human monocyte cultures, it was discovered that cells exposed to macrophage colony-stimulating factor, a maturation promoting cytokine, displayed decreased sensitivity to AICD. However, IFNγ-primed cells showed increased sensitivity to AICD. IFNγ-primed monocytes that were

differentiated into activated macrophages in vitro retained this sensitivity to AICD, showing that macrophage AICD is developmentally regulated by cytokines(88).

PART II: Macrophage polarization states in the context of inflammatory disease

Macrophage Polarization Model

As biological sentinels, macrophages constantly survey the surrounding environment and receive combinations of sensory input from a variety of stimuli. In addition, macrophages are dynamic, participating in diverse homeostatic processes. Therefore, diversity and plasticity are functional hallmarks of macrophages; these cells can assume a spectrum of activation states contextual to the microenvironment(89). Research on the mechanisms of bacterial killing by macrophages gradually led to the development of a model that accounts for macrophage heterogeneity and functional plasticity, known as macrophage polarization.

Background

It was well established by the 1960's that diversity in structure and function existed between classes of immunoglobulins secreted by B lymphocytes, and a system was put in place to characterize the basic proteins of the antibody response(90). However, it remained unclear how distinct effector functions mediated by antibodies were coupled with cell mediated immunity. In 1986 a dichotomy of T lymphocyte subclasses was hypothesized by Mossman and Coffman, who proposed that these 2 populations of cells, termed Th1 and Th2, could be characterized by which cytokines they secrete(91). The Th1 subset produce high levels of IFNγ and are involved in pro-inflammatory processes, whereas the Th2 subset produces IL-4 and IL-13 and promotes allergic responses.

Data supporting heterogeneity within T helper cell functions already existed at that time, with additional evidence to come. In 1983 it was revealed that IFNγ secreted by T lymphocytes

caused resting macrophages to transform into active cells with enhanced antimicrobial capacities(92). This finding identified IFNγ as the first molecule to induce "classical activation", a term introduced 20 years prior by Mackaness who noted an enhanced but non-specific inflammatory response by macrophages upon re-exposure to pathogen(93). In 1990 it was discovered that macrophages incubated with IL-4 assumed an activated state that differed from cells activated with IFNγ (94). Hence the term "alternative activation" was subsequently coined to describe macrophages stimulated with IL-4.

M1/M2 Macrophage Paradigm

Echoing the classification system created for characterizing T helper cell subsets, a similar concept was originally proposed for macrophages. The proinflammatory phenotype assumed by classically activated macrophages was coined "M1." Along with IFNγ, LPS and TNFα can also induce the M1 phenotype. These cells express pro-inflammatory cytokines, exhibit enhanced production of reactive oxygen and nitrogen species, and have strong anti-microbial and anti-tumor activity. The alternatively activated macrophage was initially referred to as M2. Compared to M1 cells, M2 cells have upregulated MHC-II and mannose receptor expression and decreased respiratory burst capacity. IL-4 and IL-13 can induce the M2 state, and these cells function in parasite infection, tissue remodeling, and tumor progression. The M2 state has been expanded into four groups, M2a-d, that differ by the induction stimulus, surface/expression markers, and secreted factors.

Despite providing a useful framework for understanding select in vitro responses to independent stimuli, the M1/M2 model does not accurately reflect macrophage biology, does not account for heterogeneity among macrophage subpopulations, and lacks comprehensiveness. The M1 and M2 signatures do not always exclude one another and often overlap. Expression markers

traditionally used to distinguish M1 from M2 macrophages are lacking. Furthermore, stimulatory signals received by macrophages are dynamic, pleiotropic, and coexisting in vivo. Expanding upon this, the M1/M2 paradigm neglects to consider that these inputs have combinatory, concentration-dependent effects, have diverse actions at different stages of cell development, and often integrate intracellular signaling pathways that implement novel genetic programs. In vivo, macrophages are exposed to combinations and concentrations of cytokines, chemokines, adhesion molecules, cells, and other factors. Therefore, contemporary literature has called for the reassessment of the traditional bipolar model of macrophage polarization(94).

The current, favored view of macrophage polarization involves a spectrum of activation states with M1 and M2 serving as poles(95). Functional skewing of macrophage polarization states has been shown to occur under normal conditions, as well as during pathology. The biomedical impact of this hypothesis of heterogenous activation states is that macrophages could potentially be swayed from phenotypes that contribute to disease and attracted toward a more favorable state through macrophage-centered therapies(89).

Macrophage polarization in inflammatory disease

Acute Liver Failure

Acute liver failure (ALF) is defined as a loss of liver function that occurs rapidly in patients who usually have no history of liver disease. ALF is relatively rare, but shares a commonality with several more prevalent diseases in that its cause and progression involves severe, systemic inflammation. 20 percent of the non-parenchymal mass of liver is composed of resident macrophages known as Kupffer cells. ALF develops when Kupffer cells become polarized towards a pro-inflammatory state and secrete a bolus of proinflammatory cytokines into systemic circulation, creating what is referred to in the clinic as a "cytokine storm".

Kupffer cells secrete TNF-α, IL-6 and IL-1 which promotes the synthesis and secretion of acute phase proteins by hepatocytes. These proteins include C-reactive protein and mannose binding lecithin, which function similarly to antibodies but lack specificity. The combined actions of acute phase proteins and cytokines trigger tissue injury, exacerbating the inflammatory response further. Hepatocyte cell death pathways are activated, leading to metabolic complications, coagulopathy, brain swelling, and potentially death. Since ALF can be initiated and progressed by macrophage mediated inflammation, skewing macrophage polarization from pro-inflammatory to anti-inflammatory would provide a viable treatment option for patients that currently have few available options.

COPD and Asthma.

COPD and asthma are major global health problems, with COPD predicted to become the third largest cause of death globally by 2020(96), and asthma remains widespread in both developed and developing countries. Chronic, low grade inflammation of the lungs contributes substantially to the pathogenesis of COPD and asthma. In COPD, chronic activation of innate immune cells and increased leukocyte migration leads to airway remodeling and thickening which can lead to irreversible damage(97,98). Alveolar macrophages (AMs) comprise 90 percent of the pulmonary macrophage population and are implicated in the progression of COPD and asthma(99). These resident macrophages are constantly exposed to inhaled substances, functioning as the first line of host defense against inhaled irritants. Clinical research implicates that macrophages are inappropriately activated during the development of these disorders.

CHAPTER 3

In vitro suppression of inflammatory cytokine response by methionine sulfoximine.

The work presented here is an expansion of recently published work:

Peters TJ, Jambekar AA, Brusilow WSAB. In vitro suppression of inflammatory cytokine response by methionine sulfoximine. (2018). *The Journal of Inflammation*.

Abstract

The glutamine synthetase inhibitor methionine sulfoximine (MSO), shown previously to prevent death caused by an inflammatory liver response in mice, was tested on in vitro production of cytokines by mouse peritoneal macrophages triggered with lipopolysaccharide (LPS). MSO significantly reduced the production of Interleukin 6 (IL-6) and Tumor Necrosis Factor Alpha (TNFα) at 4 and 6 hours after LPS-treatment. This reduction did not result from decreased transcription of IL-6 and TNFα genes, and therefore appeared to result from post-transcriptional inhibition of synthesis of these cytokines. MSO treatment did not inhibit total protein synthesis and did not reduce the production of a third LPS-triggered cytokine CXCL1, so the effect was not a toxic or global downregulation of the LPS response.

The anti-inflammatory effects of a glutamine synthetase inhibitor were seen even though the medium contained abundant (2 mM) glutamine, suggesting that the target for this activity was not glutamine synthetase. In agreement with this hypothesis, the L,*R* isomer of MSO, which does not inhibit glutamine synthetase and was previously thought to be inert, both significantly reduced IL-6 secretion in isolated macrophages and increased survival in a mouse model for inflammatory liver failure. Our findings provide evidence for a novel target of MSO. Future attempts to identify the additional target would therefore also provide a target for therapies to treat diseases involving damaging cytokine responses.

Background

Methionine sulfoximine (MSO) is an amino acid inhibitor of glutamine synthetase (GS)(1,100,101). In addition to its historical use in dissecting the mechanism of GS, this molecule has also been shown to have therapeutic effects in animal models of hepatic encephalopathy, amyotrophic lateral sclerosis (ALS), and inflammatory liver failure(102). For hepatic encephalopathy, MSO treatment reduces glutamine in astrocytes, preventing the osmotic swelling that results from ammonia-driven synthesis of glutamine from glutamate(7). For ALS, it appears that MSO reduces excitotoxicity by reducing the levels of both glutamine and glutamate in the brain(31,103). Additionally, MSO appears to have anti-inflammatory effects, since it increases survival in a mouse model for inflammatory liver failure resulting from exposure to lipopolysaccharide (LPS)(35). MSO reduces the LPS-induced cytokine response for several pro-inflammatory cytokines, most notably tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6), two cytokines strongly associated with an array of inflammatory diseases(104,105). It was previously unclear to what extent these effects are mediated by glutamine synthetase inhibition, or if there is another target for this molecule.

In order to determine the anti-inflammatory target(s) for MSO, we measured its effects on the cytokine response of isolated macrophages to LPS treatment, its effects on transcription of cytokine genes, the role of medium glutamine in MSO-inhibition of the cytokine response, and the effects of both the L,*R* and L,*S* stereoisomers of MSO on this anti-inflammatory action in vivo and in vitro.

Methods

Ethics

All animal experiments complied with Institutional Animal Care and Use Committee (IACUC) guidelines for animal welfare at Wayne State University. Male CD1 mice were purchased from Charles River (USA). All animals used for experiments were 6-10 weeks old.

Rat Kupffer Cell Culture

Cryopreserved Rat Kupffer cells (ThermoFisher Scientific, USA) were thawed in a 37°C water bath before being gently pipetted into 8 mL of ice cold Advanced DMEM Medium (ThermoFisher Scientific) containing 5% FBS (Gibco, USA) and 4% Thawing and Plating Cocktail A (Catalog No. CM3000, ThermoFisher Scientific). Cells were centrifuged at 500 x g for 10 minutes at 4°C. The supernatant was discarded, and the cells were resuspended in 2 mL of medium and counted using a hemocytometer. Cells were seeded into 12-well plates at 4×10^5 cells per well and placed into a humidified incubator with 95% air, 5% CO_2 at 37°C. 4 hours later the media was aspirated and cells were washed 3 times with warm PBS before 1 mL of RPMI 1640 with GlutaMAX^{TM} supplement and HEPES (ThermoFisher Scientific), containing 10% FBS and a 1% mixture of penicillin-streptomycin (ThermoFisher Scientific) was added to each well. Cells were incubated overnight (20-24 hours) before any experiments were performed.

Mouse Peritoneal Macrophage Culture

Peritoneal macrophages were isolated as previously described by Zhang et al. (2008) with minor modifications(106). Two mice were used for each experiment. After euthanasia, 10 mL of cold phosphate buffered saline (4°C) was injected into the peritoneal cavities of each mouse. The abdomens were gently massaged before a small incision was made within the abdominal wall and the exudate was aspirated using a disposable transfer pipette and transferred into a 15 mL conical

tube. Cell suspensions were centrifuged at 500 x g for 10 minutes at 4º C. The supernatant was discarded, and the pellet was gently resuspended in 2 mL of Advanced DMEM Medium containing 5% FBS and 4% Thawing and Plating Cocktail A on ice. Approximately 2 x 10^5 cells were added to each well of a 12-well tissue culture plate which was placed in a humidified incubator with 5% CO_2 for 2 hours at 37° C. The media was aspirated, and cells were washed three times with warmed PBS before 1 mL of RPMI 1640 with GlutaMAX^{TM} supplement and HEPES, containing 10% FBS and a 1% mixture of penicillin-streptomycin was added to each well. Cells were incubated overnight (20-24 hours) before any experiments were performed.

Macrophage Activation

All media and reagents were sterile filtered through a 0.22 micron syringe filter prior to use. L Methionine R,S-sulfoximine (Sigma, USA), and Lipopolysaccharides from Escherichia coli O111:B4 (Catalog No. L6230, Sigma), were dissolved in sterile PBS. Purified L,*S* and L,*R* MSO were purchased from Toronto Research Chemicals (Canada). Cells were divided into four treatments each with at least two biological replicates. Treatments consisted of LPS only, MSO + LPS, MSO only, and untreated. All cells in culture were washed 2-3 times with warm, serum-free RPMI 1640 with GlutaMAX^{TM} supplement and HEPES before 0.5 mL of media, with or without MSO, was added to each well. After incubation for one hour, LPS (1 µg/mL) was added to the appropriate wells, and media samples were collected at different times for the measurements described in the different experiments.

Intracellular IL-6 measurement

For intracellular protein quantification, the medium was removed, and cells were lysed directly within wells of the cell culture plate in 20 mM Tris buffer containing 100 mM NaCl, 1 mM EDTA, and 0.2% Triton X-100 (pH 7.4). After 20 minutes of incubation on ice, cells were scraped vigorously and observed under the microscope to ensure complete lysis. IL-6 within cell lysates was measured using the Mouse IL-6 Ready-SET-Go® ELISA kit (eBioscience, USA).

IL-6 Immunofluorescence

Cells were cultured on 8-well glass chamber slides (Millicell® EZ SLIDE 8-well glass, sterile, Catalog No. PEZGS0816, Millipore, USA) in the same manner as described for the experiments performed in tissue culture plates. Cells were pre-treated with 9 mM MSO for one hour prior to the addition of 1 µg/kg LPS. Cells treated with LPS alone served as positive controls, whereas cells treated with MSO alone or left untreated served as negative controls. After 6 hours of LPS stimulation, the medium was aspirated, and cells were washed three times with PBS before being fixed to the chamber slides with a 4% paraformaldehyde solution in PBS for 10 minutes. Cells were made permeable by the addition of a 0.1% Triton X-100 solution in PBS for 20 minutes, followed by an overnight incubation with a monoclonal antibody specific for mouse IL-6 (Cell signaling technology, USA, Catalog No. D5W4V) at 4 degrees. Cells were washed before incubation with an Alexa Fluor-conjugated secondary antibody (Abcam, USA, Catalog No. ab15007) in the presence of 10% goat serum (R&D systems, USA). A mounting medium containing the nuclear stain DAPI (Electron Microscopy Sciences, USA, Catalog No. 17985-50) was applied to a cover slip, and fluorescence was visualized under a confocal microscope.

Glutamine depletion experiments

Cells were isolated and plated in 12-well plates in the conditions listed above. After overnight incubation, cells were washed 3 times with RPMI either containing glutamine or RPMI without glutamine. 0.5 mL of fresh, serum-free medium, with or without glutamine and MSO was added to each well one hour prior to the addition of LPS. Experiments were limited to 6 hours in consideration of the possibility that cell death could occur due to prolonged glutamine depletion.

Enzyme Linked Immunosorbent Assay (ELISA)

Mouse and Rat TNF-alpha Ready-SET-Go® ELISA kits, and Mouse IL-6 Ready-SET-Go® ELISA kits (eBioscience, USA) were used to assess the levels of TNFα and IL-6 in the macrophage activation experiments. Mouse CXCL1/KC DuoSet ELISA (R&D systems, USA) was used to measure mouse CXCL1 in all macrophage activation experiments. IL-10 Mouse Uncoated ELISA kit (ThermoFisher Scientific) was used to quantify IL-10. The procedures were carried out per the manufacturer's instructions, and all data were analyzed using the Epoch microplate spectrophotometer and Gen5 data analysis software (Biotek, USA).

Nitric Oxide (NO) Assay

Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, USA) was used to assess the concentration of NO secreted by macrophages into the culture medium. The procedures were carried out per the manufacturer's instructions.

Lactate Dehydrogenase (LDH) Assay

LDH release from peritoneal macrophages was assessed with the Cytotoxicity Detection Kit (LDH) (Sigma Aldrich, USA) per the instructions provided by the manufacturer. For this assay, LDH activity is proportional to colorimetric reduction of tetrazolium salt measured at 490 nm. To serve as a positive control, a subset of cells was exposed to a Triton X-100 solution for 45 minutes,

and the average OD 490 nm measurement from these cells was considered as the maximum LDH activity (100%) and served to normalize LDH values obtained from cells treated with MSO. An additional subset of cells treated with dH2O, served as a negative control.

RNA isolation and Reverse Transcription PCR (RT-PCR)

Approximately 2 x 10^6 cells per well were plated in 6-well plates after peritoneal exudate cells were isolated in the media conditions (with or without MSO) and incubation times listed above. At 1, 3, or 5 hours after LPS addition cells were lysed and RNA was isolated using the contents provided within the RNeasy mini kit (Qiagen, Germany). The RNA concentration in each sample was measured with a Qubit® fluorometer, using the RNA high sensitivity kit (Thermofisher Scientific Catalog No. Q32852). Reverse transcription reaction was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). 250 ng of total RNA were used in each 20 uL reaction, with equal amounts of total RNA used for each sample.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using a ready-to-use PowerUp™ SYBR® Green Master Mix (ThermoFisher Scientific). SYBR green fluorescence was detected by the 7500 Real Time PCR System (Applied Biosystems), using 25ng of cDNA per reaction. Gene expression was normalized to the expression of the reference gene, beta-actin (β-actin). Oligonucleotide primers for TNFα and IL-6 were previously designed and used by Simpson, Tomkins and Cooper (1997)(107). Primer sequences were purchased as lyophilized powder (Invitrogen, USA) and are as follows: IL-6 (forward: 5'-GAC AAA GCC AGA GTC CTT CAG AGA G-3' and reverse: 5'-CTA GGT TTG CCG AGT AGA TCT C-3'), TNFα (forward: 5'-ATG AGC ACA GAA AGC ATG ATC-3' and reverse: 5'-TAC AGG CTT GTC ACT CGA ATT-3'), β-actin (forward: 5'-GTG GGC CGC TCT AGG CAC CA-3' and reverse: 5'-CGG TTG GCC TTA GGG TTC AGG GGG G-3'). The

quality of each PCR product was confirmed by melting curve analysis. Relative quantification of fold induction of TNFα and IL-6 mRNA was assessed using the ΔΔCt method described by Livak and Schmittgen (2001)(108).

Acute Liver Failure Mouse Model

We used an accepted method to induce acute liver failure into mice by co-injecting LPS (20 µg/kg) and D-galactosamine (800 mg/kg, Sigma, USA) intraperitoneally (IP). 3 hours prior to injecting LPS/D-Gal, mice were injected IP with either saline (negative control), L,*S* MSO (25 mg/kg), or L,*R* MSO (25 mg/kg). Food was removed from the cages prior to any injections, but mice were allowed access to water throughout the duration of the experiment. Mice that failed to respond to prodding and right themselves were euthanized. Survival was monitored for 24 hours.

Statistical analysis

Values are expressed as mean ± standard error ($s \pm$ SEM). All experiments were repeated at least three times. T-tests were performed for analyses of statistical significance in cell culture experiments. For the mouse experiments, differences in survival between groups were assessed using Fisher's exact test. All statistical analyses were performed using IBM SPSS Statistics version 24 for Windows. Differences between treatments were considered significant when $p < 0.05$.

Results:

We have previously shown that treatment of mice with MSO reduces the LPS-induced production of cytokines TNFα, IL-6, and IFγ in vivo(35). In this report, we describe the effects of MSO on IL-6 and TNFα production by isolated macrophages treated with LPS.

Effect of MSO on IL-6 production

We first determined the minimal effective concentration for inhibiting the LPS-triggered IL-6 response by treating cells cultured in normal medium (2 mM glutamine) with either 9 mM,

4.5 mM, or 1 mM MSO for one hour before the addition of LPS (1 µg/mL) to the culture medium.

Figure 6A shows that in two independent experiments, 9 mM MSO appeared to be the minimum effective concentration required to significantly reduce IL-6 in the culture medium of LPS-stimulated mouse peritoneal macrophages at 4 and 6 hours after LPS exposure. These data suggest that a dose-dependent relationship might exist, with 9 mM MSO being the most effective in reducing IL-6 secretion, 1 mM being ineffective, and 4.5 mM falling in between. IL-6 levels in the culture medium of untreated cells or cells treated with MSO alone were undetectable (not shown).

Effect of MSO on cellular and secreted IL-6

The observed effect of MSO on the production of secreted IL-6 resulted from either decreased synthesis or decreased secretion. We therefore compared IL-6 levels in the culture medium to IL-6 levels measured in cell lysates. IL-6 levels measured in the medium were much higher than in the cell lysates for all samples. 4 hours after LPS treatment, the amount of IL-6 in the supernatant was approximately 3 times greater in the supernatant than in the lysate (3.8X for LPS-only controls and 2.9X for MSO-treated cells). At 6 hours, the IL-6 measured in the supernatant was over 10 times greater than inside the cells (16.6X for LPS-only controls and 11.2X for MSO-treated cells).

Figure 6B shows that, when compared to controls, the % decreases of IL-6 resulting from MSO-treatment were similar in both the medium (secreted) and the cell lysate (intracellular). MSO treatment reduced secreted and intracellular IL-6 concentrations at both 4 and 6 hours after LPS addition. At 4 hours, a mean 33% reduction of IL-6 was observed in the medium of MSO treated cells, compared to a mean 20% reduction of IL-6 in the lysates. At 6 hours, a mean 45% reduction of IL-6 was observed in the medium of MSO treated cells compared to a mean 42% reduction of IL-6 in the lysates. MSO-treatment therefore did not result in cells "filling up" with cytokine.

To further demonstrate that the decrease in medium IL-6 could be attributed to decreased levels of IL-6 within the cells, we used immunofluorescence to visualize IL-6 in MSO-treated and untreated cells 6 hours after LPS treatment (Figure 1C). In agreement with the results shown in Figure 6B, **Figure 6C** demonstrates a clear reduction of cytoplasmic IL-6 with MSO treatment when compared to cells treated with LPS only.
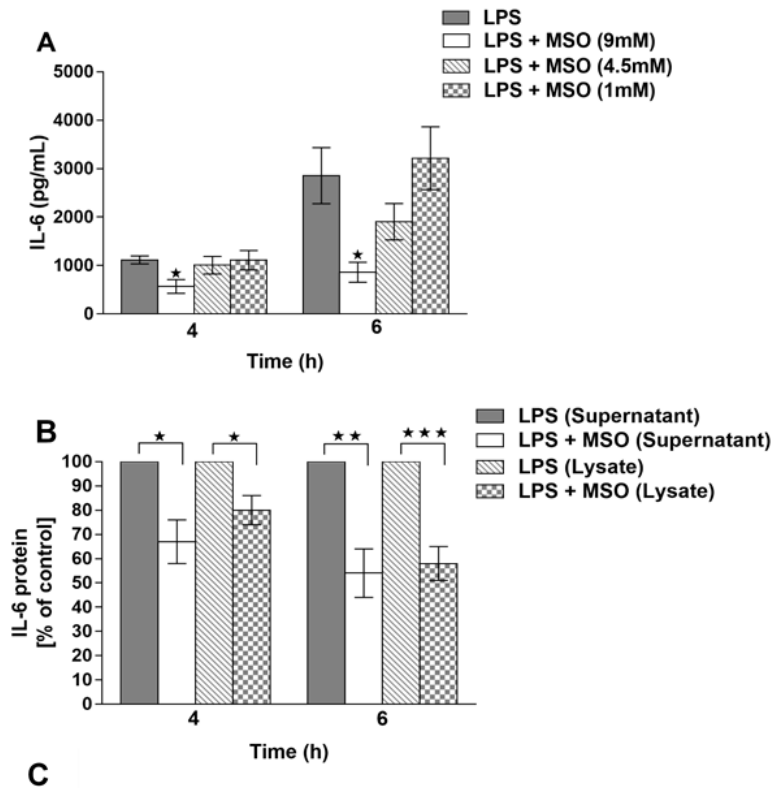
Figure 6. Effects of MSO on IL-6 production. A, dose-dependent effects of MSO on LPS-triggered IL-6 production (s ± SEM). Peritoneal macrophages were treated with three concentrations of MSO one hour prior to the addition of 1 µg/mL LPS, and IL-6 production in the medium was quantitated by ELISA 4 hours and 6 hours after LPS treatment (n=6, *p < 0.05). **B,** effects of MSO on intracellular and extracellular IL-6 production**.** Cells were treated with 9 mM MSO, and in addition to measuring IL-6 in the medium after LPS treatment, cells were washed, lysed, and the amount of IL-6 in the lysate was quantitated as a measure of intracellular IL-6. Untreated cells, or cells treated with MSO alone (minus-LPS) did not produce detectable levels of IL-6. ELISA values obtained from LPS stimulated control (minus-MSO) samples were normalized as 100%, and the average amounts of IL-6 produced in the treated supernatant and lysate samples are indicated as percentages of the controls. These data represent averages from 3 additional macrophage preps different from those represented in A, with three biological replicates for each treatment. *p < 0.05, **p < 0.01, ***p < 0.001. **C**, immunofluorescence detection of intracellular IL-6 protein, 6 hours after LPS treatment**.** The top row shows, from left to right, levels of intracellular IL-6 in untreated cells, cells treated with LPS, treated with LPS and MSO, or treated with just MSO. The same cells were also stained with the nuclear stain DAPI, and the two fluorescent images were merged in the bottom row, showing that the nuclei of treated and untreated cells appear the same.

Effect of MSO on production of TNFα

Peritoneal macrophages also produce TNFα after LPS stimulation. **Figure 7A** shows LPS-stimulated production of TNFα in cells treated with MSO after 4, and 6 hours. We found no significant effects after 2 hours (not shown). Compared to cells treated with LPS alone, cells pretreated with MSO showed significant reductions in TNFα secreted into the culture medium at 4 hours (reduced by 40% ±7%) and 6 hours (reduced by 38% ± 8%).

As an added test of the ability of MSO to inhibit TNF-a production, we carried out these same assays in commercially available rat Kupffer cells (see Methods). Kupffer cells are the resident liver macrophages responsible for inflammatory liver failure(109). They respond to LPS by producing cytokines including IL-6 and TNFα. Rat Kupffer cells are commercially available, but when we tested the effects of MSO on cytokine production by those cells, we found batch-to-batch variability in the LPS-triggered responses. Most of the preparations that we purchased (five out of eight) responded to LPS treatment and produced TNFα, as they should. In those experiments, we could observe a significant decrease in TNFα secretion when these cells were pre-

treated with MSO. **Figure 7B** shows inhibition by MSO of LPS-triggered TNFα production in primary rat Kupffer cell cultures (s ± SEM), with 58% ± 9% and 45% ± 3% mean decreases in TNFα secretion after 4 and 6 hours, respectively.

However, several batches of these commercially-available cells (three out of eight) did not respond to LPS, indicating that these cell preparations were of poor quality. The substantial batch-to-batch variability led us to concentrate instead on the peritoneal macrophage preparations for essentially all of our studies besides those shown in Fig. 2B, which shows the results obtained only from those batches of cells that demonstrated significant production of TNFα when treated with LPS alone.
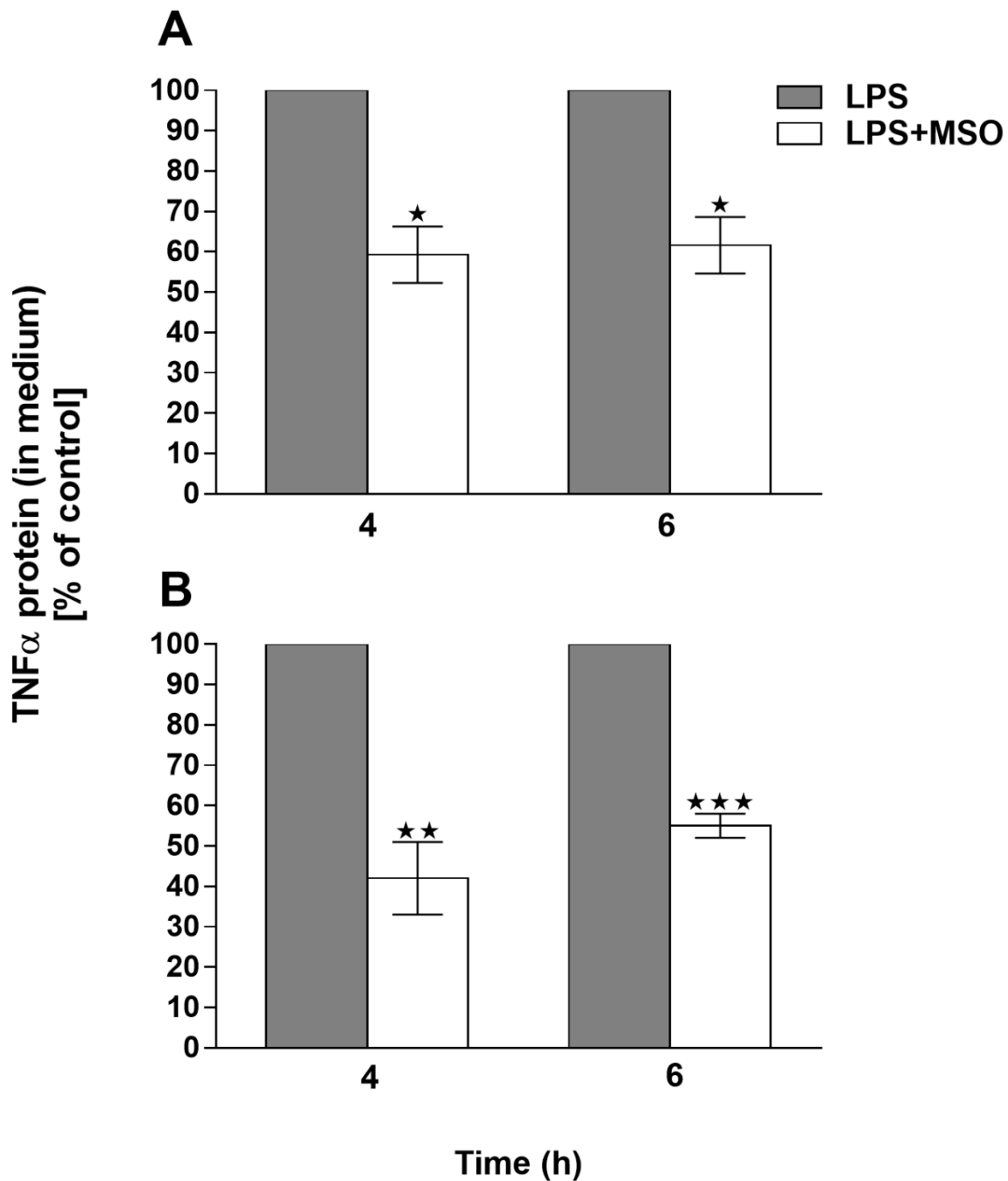
Figure 7..Effect of MSO on TNFα production. TNFα secretion by (**A**) primary mouse peritoneal macrophages and (**B**) primary rat Kupffer cells after incubation in the presence or absence of MSO and LPS. Untreated cells, or cells treated with MSO alone (minus-LPS) did not produce detectable levels of TNFα. The level of TNFα induction by LPS stimulated controls, was normalized to 100%. A, mean TNFα protein concentration in LPS-stimulated controls was 114 pg/mL at 4 hours and 183 pg/mL at 6 hours. B, mean TNFα protein concentration of LPS stimulated controls was

71 pg/mL at 4 hours and 117 pg/mL at 6 hours. These data represent 3 peritoneal macrophage preparations and 5 Kupffer cell preparations. Each preparation contained at least 2 biological replicates for each treatment. **p <0.01, ***p <0.001

Effect of MSO on transcription of TNFα and IL-6

The expression of the genes for TNFα and IL-6 has been shown to peak between 1 and 3 hours after LPS stimulation in mouse peritoneal macrophages(107). To test if MSO affected transcription of TNFα or IL-6, cells were treated with LPS for up to 5 hours, then lysed and the mRNA was isolated, reverse-transcribed into cDNA, and subjected to quantitative PCR, with relative β-actin level serving as an endogenous control. Both IL-6 and TNFα mRNA levels were unaffected by MSO (**Figure 8**), suggesting that the inhibitory action of MSO on the production of these proteins does not occur at the level of gene expression.
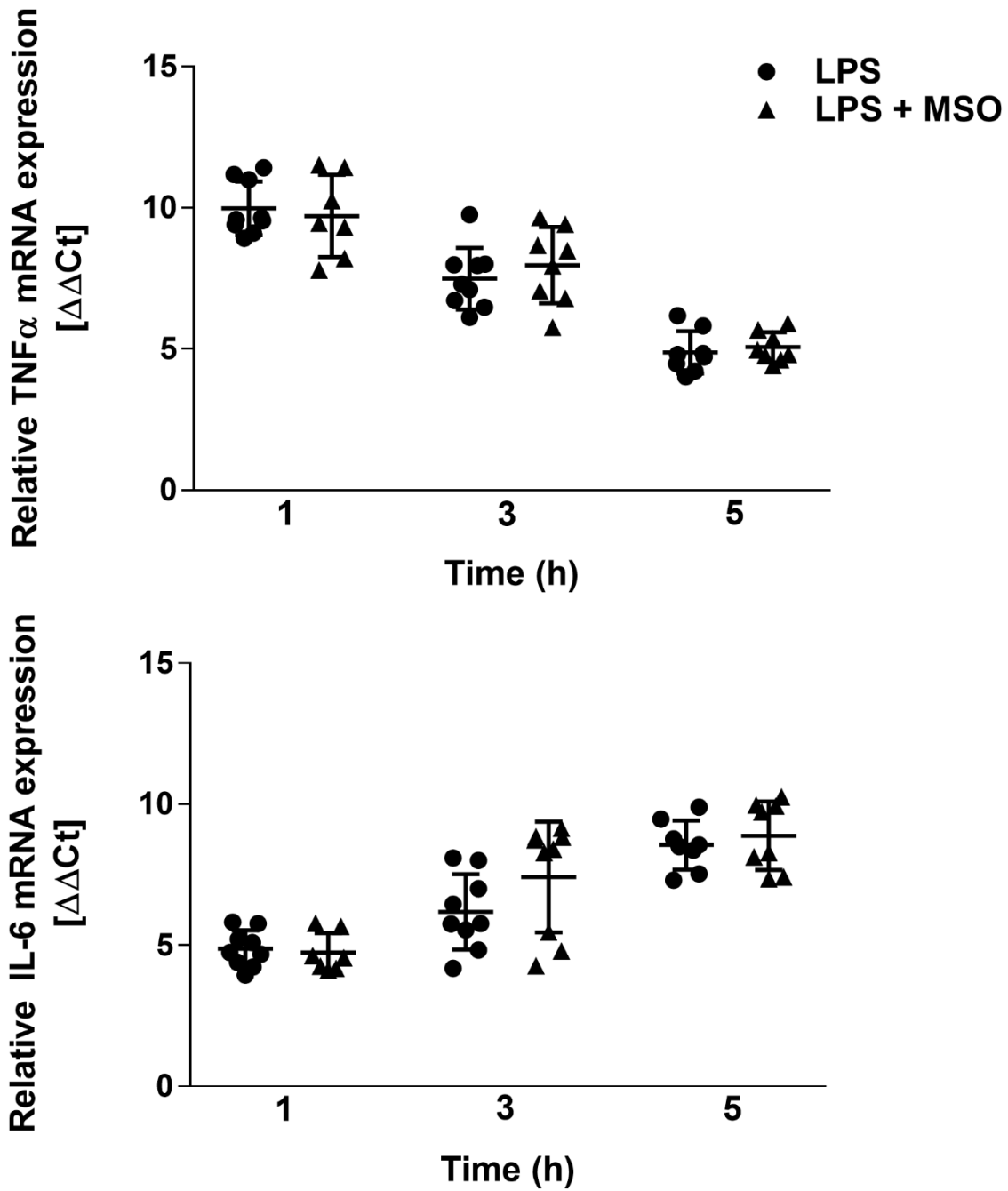
Figure 8. **Effect of MSO on transcription of the cytokine genes**. Mouse peritoneal macrophages were treated with MSO, then LPS as indicated for the experiments in Figure 1 and Figure 2. At 1, 3, or 5 hours after LPS treatment, cells were, harvested, lysed, and mRNA was isolated as described in Methods. qRT-PCR was conducted using primers specific for either TNFα or IL-6. Relative mRNA levels are plotted as ΔΔCt, the $\log_2$ transformed fold increase in mRNA compared

to untreated cells (108). Data from each timepoint were obtained from at least 3 experiments, with all technical replicates shown for clarity.

Effect of MSO on other important macrophage functions

In addition to secreting pro-inflammatory cytokines, macrophages perform other essential functions to initiate, maintain, and resolve immune responses to infection. A central function of macrophages is to phagocytose and digest foreign antigens, namely through the production of reactive nitrogen species(110). LPS stimulated macrophages express an inducible nitric oxide synthase whose expression has been shown to be regulated by other cytokines, namely TNFα and IL-1β(111). Since MSO profoundly reduced TNFα secretion by peritoneal macrophages, we tested to see whether MSO could affect the production of NO by these cells. Nitric oxide is highly reactive; upon production it is quickly reduced to the more stable compounds nitrate and nitrite. Using a commercially available kit, we detected the total level of nitrate and nitrite secreted by macrophages stimulated with LPS for 24 hours, in the presence or absence of MSO. **Figure 9A** shows that MSO did not have any appreciable effect on the level of NO production by these cells, suggesting that MSO treated cells maintain the ability to carry out other functions, and that the effects of MSO appear to be specific

Activated macrophages secrete several chemotactic proteins that recruit neutrophils and other immune cells to an infected area, along with promoting angiogenesis(112). One such protein secreted by macrophages with chemotactic properties is CXCL1(113). As a chemokine, it is also involved in the inflammatory response mediated by macrophages. We used the same type of ELISA assay to measure LPS-induced CXCL1 production in the presence or absence of MSO and found no effect (**Fig. 9B**). LPS was required to induce CXCL1 production; untreated cells or cells treated with MSO alone (minus-LPS) did not produce detectable levels of CXCL1.

In order to resolve inflammation once foreign agents are eliminated, macrophages secrete proteins that are critical in limiting immune mediated responses(114). The major anti-inflammatory cytokine secreted by macrophages is interleukin 10 (IL-10). IL-10 secretion induces T cell hypo-responsiveness, and down regulates TNFα production, and in peritoneal macrophages has been demonstrated to peak approximately 24 hours after LPS stimulation(115). We treated peritoneal macrophages with LPS for 24 hours (+/- MSO) and used ELISA to quantify the concentration of IL-10 in the supernatant (**Figure 9C**). MSO had no effect on the production of IL-10 compared to controls, suggesting that MSO treated cells remain capable of eliciting anti-inflammatory functions.

We have recently shown that MSO does not globally down regulate protein synthesis and has negligible effects on the total level of secreted proteins (116). This finding, in agreement with the results shown in Figure 9, suggest that MSO specifically down-regulates TNFα and IL-6, does not globally downregulate cellular function in response to LPS, and is safe to use at a concentration of 9 mM, as was shown in other laboratories(117-119).
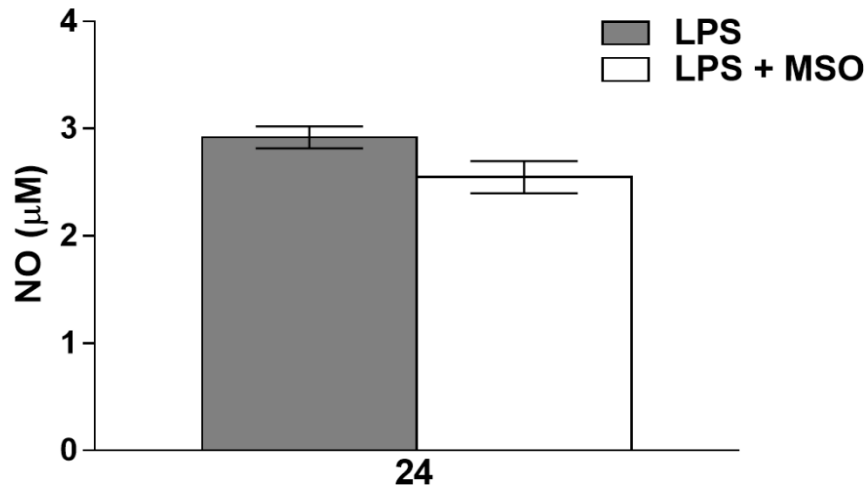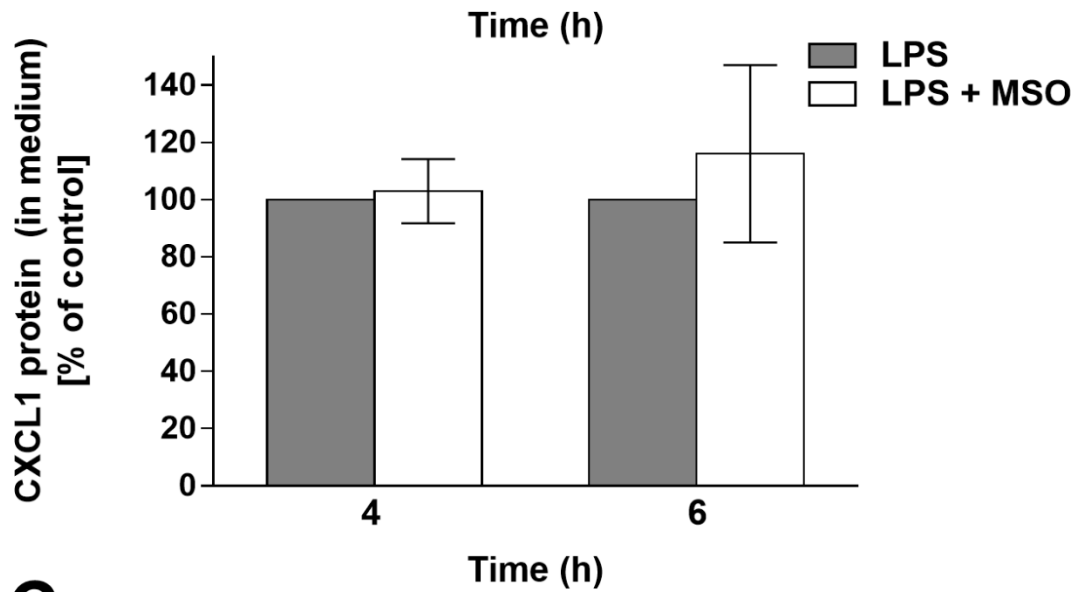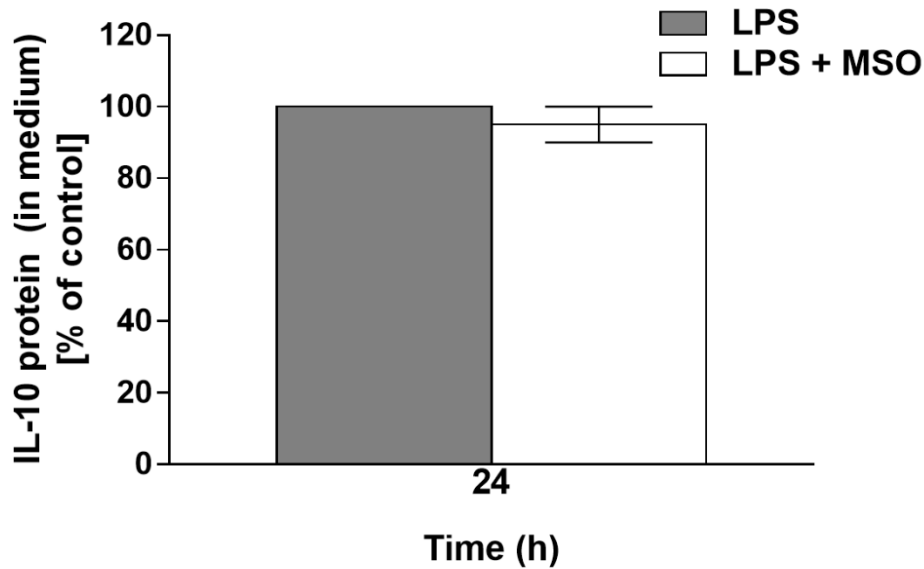
Figure 9. Effects of MSO on NO production, CXCL1 secretion, and IL-10 secretion. **A**, mouse peritoneal macrophages were treated with MSO or left untreated before being stimulated with LPS for 24 hours and the concentration of NO in the culture medium was quantified using a commercially available kit (n=3). The mean NO secreted into the medium after 24 hours (s ± SEM) was 2.92 ± 0.1 for LPS controls, and 2.55 ± 0.15 for MSO treated cells (difference not significant). NO was not detectable in the medium of cells left untreated or treated with MSO alone. **B**, effect of MSO on LPS-triggered production of the cytokine CXCL1 (n=3).   Experiments were carried out as described for Figure 1 and Figure 2. ELISA was used to quantitate LPS-triggered production of CXCL1 in the presence or absence of MSO 4 hours and 6 hours after LPS treatment. CXCL1 was not detectable in the medium of untreated cells, or for cells treated with MSO alone (minus-LPS). The mean CXCL1 protein concentration measured in LPS stimulated controls was 450 pg/mL at 4 hours and 750 pg/mL at 6 hours. Control values were normalized to 100%, and average values from treated samples were plotted in comparison to those controls. **C**, effect of MSO on LPS-triggered production of the anti-inflammatory cytokine IL-10 (n=3). Experiments were carried out as described for Figure 1 and Figure 2. ELISA was used to quantitate LPS-triggered production of IL-10 in the presence or absence of MSO 24 hours after LPS treatment. IL-10 was not detectable in the medium of untreated cells, or for cells treated with MSO alone (minus-LPS). The mean IL-10 protein concentration measured in LPS stimulated controls was 1293 pg/mL. Control values were normalized to 100%, and average values from treated samples were plotted in comparison to those controls

Finally, to rule out other nonspecific effects of MSO on cell viability such as perturbations

in osmotic pressure and plasma membrane integrity, we treated cells with a range of MSO

concentrations from 1 to 100 mM and determined cytotoxicity by measuring lactate dehydrogenase

activity released into the culture medium(120). MSO concentrations of up to 100 mM were non-

toxic in this assay (**Table 5**).

Parameter	Triton X-100	dH$_2$0	1 mM	10 mM	20 mM	50 mM	100 mM
OD (490 nm)	0.8	0.04	0.04	0.05	0.03	0.03	0.03
% Cytotoxicity	100	0	0	0	0	0	0

Table 5. **MSO is non-toxic to cells at high doses.** Lactate dehydrogenase (LDH) activity in the medium of cells treated with 1 to 100 mM MSO. Cells were treated with concentrations of MSO ranging from 1 to 100 mM for 2 hours or left untreated. Afterwards, cells were treated with Triton X-100 (positive control), or dH2O (negative control), and incubated for an additional 45 minutes before LDH activity in the medium was assessed. LDH activity was completely absent in the medium of cells treated with up to 100 mM MSO, indicating that MSO was not cytotoxic. 100 mM MSO had no inhibitory effect on purified, recombinant LDH (not shown). See methods for a more complete description of this assay.

Role of medium glutamine on MSO transport and its effect on IL-6 production

MSO is a glutamine/glutamate/methionine analogue, and it has been demonstrated that glutamine and methionine can compete with MSO for transport into cells in different prokaryotes (121,122). Additionally, glutamine is known to enhance the function of immune cells(123). Levels of medium glutamine and cellular glutamine metabolism are therefore complicating factors for in vitro cell culture experiments involving MSO. The experiments described above were all carried out in medium containing 2 mM glutamine. We therefore attempted to dissect the role of medium glutamine in the observed responses by first measuring LPS-triggered production of IL 6 (+/- MSO) in medium containing different concentrations of glutamine (**Figure 10A**).

When glutamine was removed from the medium, LPS-stimulated IL-6 secretion by isolated peritoneal macrophages was reduced by approximately 50% compared to production in medium containing glutamine, commensurate with observations from other laboratories. More importantly, the effect of 9 mM MSO was more pronounced in the absence of medium glutamine. The effect of MSO on IL-6 production was the same in media containing 1, 2, and 4 mM glutamine. We then tested the effect, in the absence of glutamine, of the lower concentrations of MSO (4.5 mM and 1 mM), that previously had little or no effect on IL-6 production in the presence of glutamine (see Figure 6B). **Figure 10B** shows that when glutamine was absent, all 3 of the tested MSO concentrations yielded significant reductions in medium IL-6, 6 hours after the cells were stimulated with LPS, with no significant difference between the % reduction of IL-6 between any of the concentrations.

This increase in potency was reversed by the addition of 0.5 mM glutamine to the culture medium (data not shown). The fact that MSO potency increases substantially in the absence of glutamine is most likely explained by the presence of a common transport system for MSO and

glutamine, as was seen for S. enterica (121), although competition between MSO and glutamine

for transport has not been studied in murine macrophages.
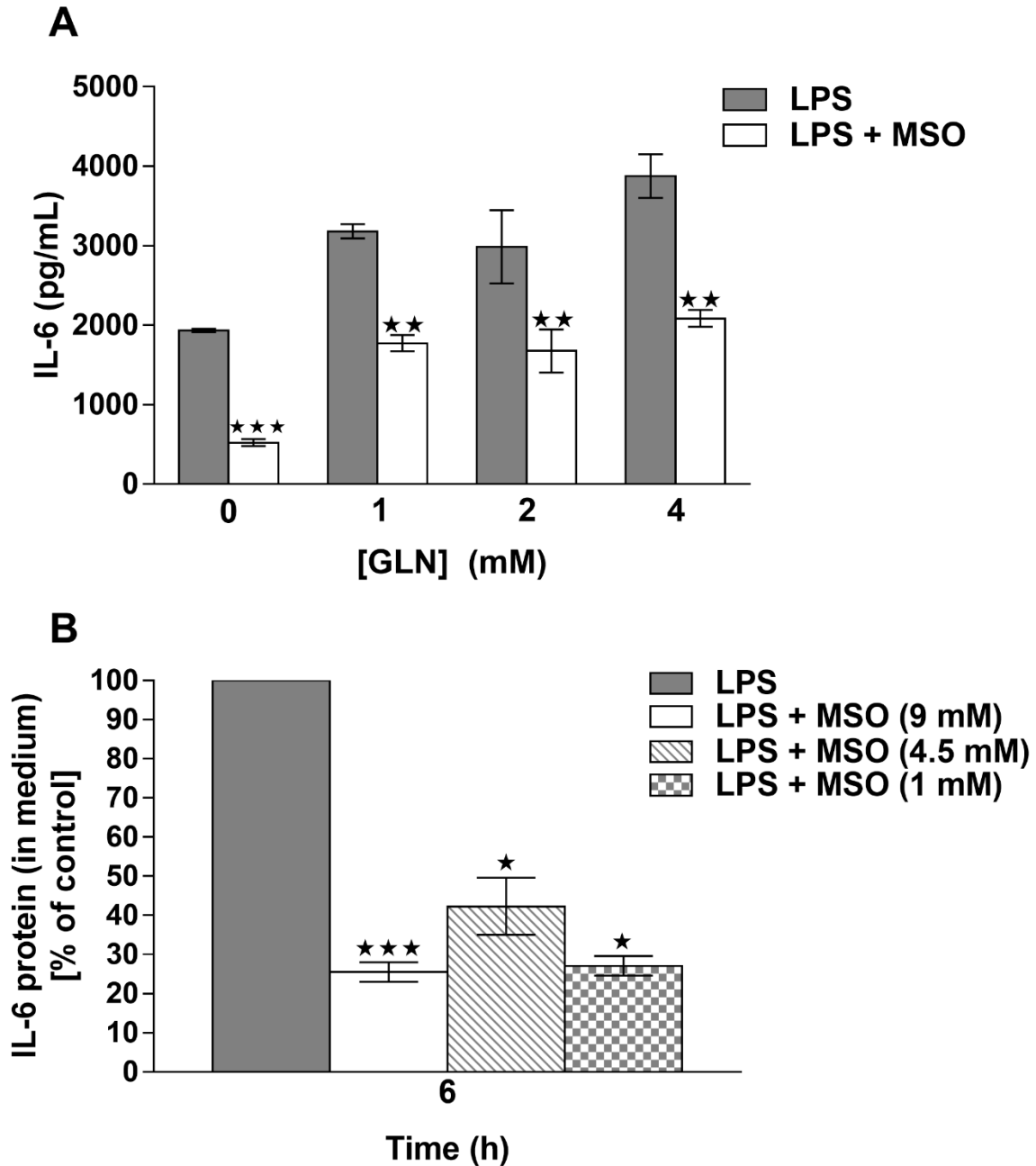
Figure 10. Effect of medium glutamine on IL-6 response to LPS and MSO. (**A**) Peritoneal macrophages were prepared as previously described but were incubated in medium glutamine concentrations ranging from 0 to 4 mM one hour prior to the addition of LPS. As indicated, cells were also treated with 9 mM MSO. At 6 hours after LPS treatment, IL-6 in the culture medium

was assayed by ELISA. (s ± SEM, n=6) **(B)** Peritoneal macrophages were treated with either 1, 4.5, or 9 mM MSO in medium lacking glutamine for 1 hour prior to a 6-hour exposure to LPS. Control values were normalized to 100%, and average values from treated samples were plotted in comparison to those controls. All 3 of the tested MSO concentrations yielded significant reductions in medium IL-6, with no significant difference between the % reduction of IL-6 between any of the concentrations.

Role of glutamine synthetase in MSO mediated cytokine suppression

It is apparent that extracellular glutamine enhances LPS-stimulated IL-6 production in vitro, but it is not clear to what extent, if any, that MSO is acting to inhibit IL-6 secretion through inhibiting the intracellular production of glutamine, because – aside from these experiments measuring dependence on glutamine concentration - in all of our other experiments extracellular glutamine (2 mM) is present in the medium, and MSO concentrations above its Ki for glutamine synthetase still inhibit cytokine production. We feel it is therefore unlikely that the effect depends on MSO targeting glutamine synthetase. Nonetheless, we carried out an additional test of the role of GS inhibition in the observed anti-inflammatory effects of this drug by testing different stereoisomers of MSO. Commercial preparations of MSO contain a racemic mixture of two diastereomers – L,*S* and L,*R*, but only the L,*S* isomer inhibits glutamine synthetase activity (124).

To validate the purity of the isomers and to establish GS inhibition, we tested the effect of purified formulations of both the L,*R* and L,*S* diastereomers of MSO, along with the commercial racemic mixture, on mouse liver GS activity using an established GS enzyme assay(125). We treated mouse liver homogenates with either 1 mM of the racemic mixture, or 0.5 mM of each purified isomer. As was first shown in 1969 (101)we found that the L,*S* isomer inhibited glutamine synthetase, and the L,*R* did not (not shown).

We performed experiments using purified formulations of both L,*R* and L,*S* diastereomers of MSO in normal glutamine-containing culture medium (**Figure 11A** and **11B**). Both isomers showed a similar dose-dependent response, with 9 mM significantly reducing IL-6 at both time

points, whereas 4.5 mM only showed significant IL-6 reduction at 6 hours, and 1 mM was ineffective at both time points. As one would expect if glutamine synthetase were the sole target, the L,*S* isomer significantly reduced IL-6 production at both 4 and 6 hours after cells were stimulated with LPS (Figure 11B). However, this effect could only be achieved using 9 mM of purified isomer, twice the concentration present in a racemic mixture, assuming a 50:50 mix of diastereomers. Strikingly, under normal medium conditions, the L,*R* isomer at 9 mM was also shown to independently reduce IL-6 in the culture medium to a significant degree at both time points tested (Figure 11A). Both isomers therefore inhibit the in vitro cytokine response seen in isolated macrophage preparations.

Figure 11A and 11B illustrate the variability in response from different preparations of macrophages made from different batches of mice. Although the percent inhibition by MSO is consistent across all experiments, the LPS response of the positive controls (minus-MSO) can vary 2-3 fold depending on the batch of mice and the age of the mice. It also varies because the harvesting of macrophages involves peritoneal lavage, which sometimes harvests a significant number of non-macrophage cells. These differences can result in plating different numbers of actual macrophages, even though the number of cells plated is the same.

When glutamine was removed from the culture medium 1 hour prior to the addition of LPS, L,*R*-MSO (**Figure 11C**) and L,*S*-MSO (**Figure 11D**) at a concentration of 1 mM, significantly reduced IL-6 in the culture medium at both 4 and 6 hours after LPS exposure, as was the case with the racemic mixture as seen in Figure 5. Significantly, 4.5 mM of either isomer was ineffective alone in normal culture medium but was effective in reducing IL-6 secretion when combined as a 9 mM racemic mixture. The fact that the L,*R* isomer significantly reduces IL-6 production but does not inhibit GS, combined with the fact that MSO can inhibit cytokine production even in the

presence of substantial medium glutamine, strongly suggests that glutamine synthetase is not the most important functional target that inhibits cytokine production in the presence of MSO, if in fact it is a functional target at all.
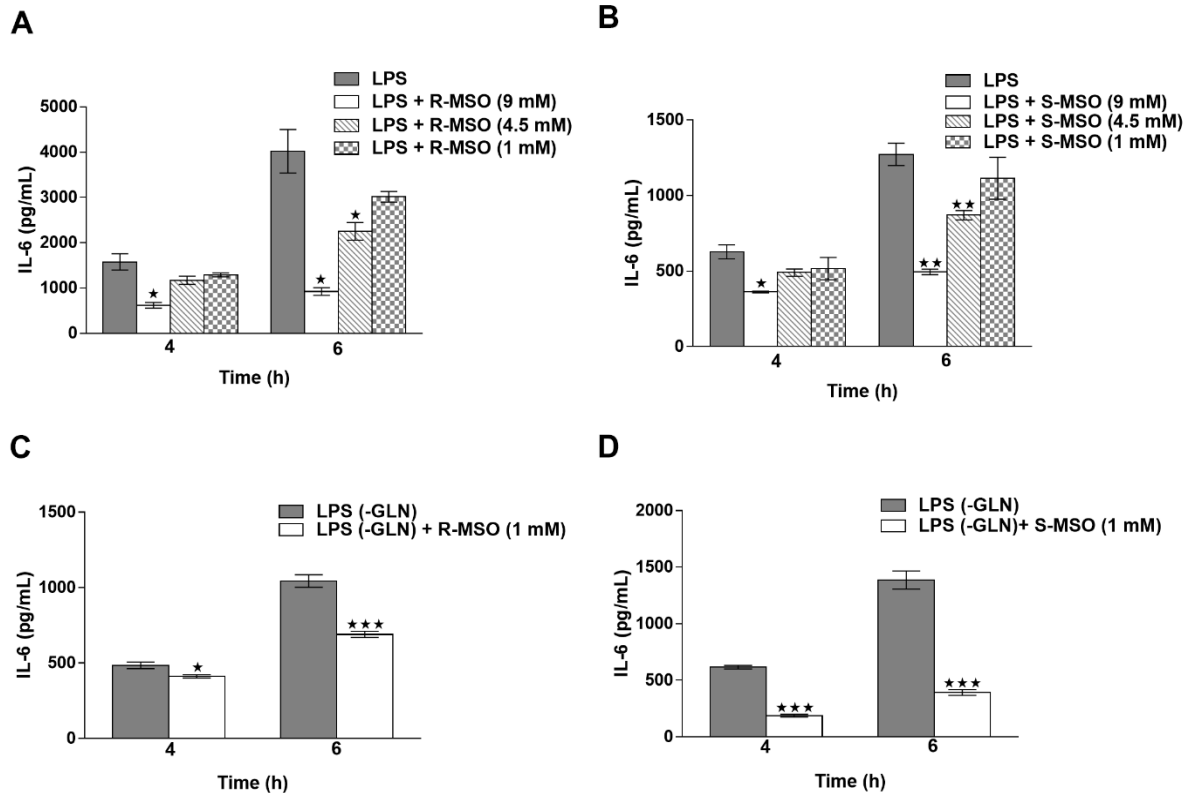
Figure 11. Concentration dependent effect of purified MSO isomers on IL-6 secretion (s ± SEM). Under normal medium conditions (2 mM glutamine), peritoneal macrophages were exposed to either 1, 4.5, or 9 mM of R-MSO **(A)** or S-MSO **(B)** one hour prior to the addition of LPS. At 4 and 6 hours after LPS addition, the medium was harvested and IL-6 was quantified using ELISA. As observed with the racemic mixture, only 9 mM of either isomer caused significant decreases in IL-6 at both time points tested. However, 4.5 mM of either L,R or L,S MSO caused a significant decrease in IL-6 after 6 hours of LPS stimulation. Removing glutamine from the culture medium resulted in an increase of potency of both isomers, as L,R **(C)** and L,S **(D)** MSO at a concentration of 1 mM significantly reduced IL-6 secretion in the culture medium at both time points tested.

This in vitro activity of the L,R isomer led us to test its activity in vivo, in the same mouse model for liver failure used previously, to show that racemic MSO could protect mice against death from liver failure resulting from an inflammatory immune response(8). We repeated those

experiments using the purified isomers, and the results are shown in **Table 5**. Both isomers were capable of significantly increasing survival in mice injected with LPS and D Galactosamine to induce fatal liver failure caused by an inflammatory immune response.

Treatment	Alive	Dead	Total (*n*)	Percent Survival (%)	*p*[a]
Saline	3	19	22	14	-
L,*S* MSO (25 mg/kg)	19	9	28	68	0.0002
L,*R* MSO (25 mg/kg)	18	12	30	60	0.0013

Table 6. MSO isomers prevent LPS induced lethality in mice. 24 hour survival analysis of mice injected with saline, L,*R* MSO (25 mg/kg), or L,*S* MSO (25 mg/kg) 3 hours prior to injection of LPS (20 µg/kg) and D-Galactosamine (800 mg/kg). a = determined by Fisher's exact test.

Discussion

The production of IL-6 and TNFα is essential in mediating the innate immune response to infection(66,74), as well as regulating other important homeostatic processes such as wound healing(126) and sleep(127,128). However, during acute inflammatory crises, overproduction of these and other cytokines can compromise the self-limiting ability of innate immunity and can result in a damaging and occasionally fatal outcome. As such, serum IL-6 levels were shown to be highly valuable in the diagnosis of infection and the prognosis of critically ill ICU patients (76,129). Macrophages are the main source of these cytokines, which act in concert to initiate the acute phase response by hepatocytes(75), to increase vascular permeability(68), and to amplify inflammatory responses through a variety of immune cell types. TNFα and IL-6 collectively exacerbate the immune response and its effects on multiple organ systems.

MSO is a well-characterized inhibitor of glutamine synthetase(1,3,101). Our previously published studies on the anti-inflammatory effects of this drug in vivo raised the obvious question of how glutamine synthetase inhibition might be responsible for the observed effects. The in vitro

studies described here have enabled us to dissect this process in additional and greater detail. These studies have demonstrated that glutamine plays an important role in both the overall cytokine response –shown previously(130,131) – and that glutamine levels can modulate how well MSO inhibits that cytokine response.

When we substituted glutamine-free medium 1 hour prior to the addition of LPS, we observed a 50% decrease of IL-6 in the culture media compared to cells treated with LPS in medium containing glutamine. The combination of MSO treatment, which decreases glutamine synthetase activity, along with depletion of extracellular glutamine caused the largest decrease in IL-6 secretion that we observed in our experiments (Figure 10A), suggesting that the anti-inflammatory mechanism might involve glutamine deprivation. Further, since those results suggest that glutamine in the medium might compete with MSO for transport into the cell, then the reverse competition – MSO competing with glutamine for uptake, albeit weakly, since it takes 9 mM MSO to show an effect in the presence of 2 mM glutamine – might partially explain the anti-inflammatory effect, if MSO reduces cellular glutamine uptake and therefore reduces the cytokine response.

However, the demonstration that 1) high levels of MSO can inhibit the response even in the presence of 2 mM medium glutamine (conditions where glutamine synthetase would not be needed), and 2) the L,*R* isomer has anti-inflammatory activity on its own, despite not inhibiting glutamine synthetase, means that there is another target for this drug to affect the inflammatory response. MSO may therefore be influencing some aspect of glutamine metabolism important to cytokine production, either by inhibiting glutamine synthetase, inhibiting glutamine transport, or acting on some other unknown target that might or might not involve glutamine metabolism, or some combination of these two potential targets.

As analogues of glutamine, glutamate, and methionine, either or both isomers could be binding to a receptor or transporter for any of these amino acids and exerting their effects through a variety of signaling pathways. These important questions are not addressed by these studies, but answering them could identify a target or targets that could be useful in treating the deleterious side effects of a cytokine storm resulting from an inflammatory immune response.

Conclusions

1) MSO treatment reduces the LPS-stimulated production of IL-6 and TNFα in vitro, in agreement with our previously published in-vivo results. 2) This effect is specific and not the result of toxicity or overall downregulation of the response to LPS, 3) MSO does not act on transcription, but may be acting on translation or on cytokine turnover and 4) the L,*R* isomer of MSO, which does not inhibit glutamine synthetase, has the same anti-inflammatory activity as the L,*S* isomer, and independently increases survival significantly in an established mouse model of acute liver failure.

If glutamine synthesis is the sole limiting factor in IL-6 production, the L,*R* isomer should not have any activity in these assays. Although it appears possible that glutamine and MSO compete for transport, as the concentration needed to reduce IL-6 by both isomers decreased 10-fold in the absence of extracellular glutamine, such competition, if it occurs, cannot account for the effects of both isomers of MSO on the cytokine response in medium lacking glutamine. Preliminary experiments in our lab measuring the IL-6 response have demonstrated EC_{50} values of sub-millimolar concentrations of MSO in medium lacking glutamine (not shown). Therefore, it appears that the anti-inflammatory effects of MSO involve some target besides, or perhaps in addition to, glutamine synthetase or glutamine transport. Future attempts to identify

this target would therefore also provide a target for therapies to treat diseases involving

potentially damaging cytokine responses.

CHAPTER 4

Conclusions, current progress, and future directions

The data presented in Chapter 3 indicate that glutamine synthetase is not the sole target of methionine sulfoximine in reducing pro-inflammatory cytokines secreted by murine macrophages. Additionally, since the L,*R* isomer of MSO can prevent lethality in a mouse model of acute liver failure, without causing neurological side effects associated with glutamine synthetase inhibition, this begs the question of how MSO is acting at the biochemical level. In this Chapter, I will present current directions, unpublished preliminary data, and will propose a mechanism for how MSO might be decreasing the synthesis of pro inflammatory cytokines, based on several key findings that have occurred in the last 40 years.

Methods

Mouse Peritoneal Macrophage Culture

Peritoneal macrophages were isolated as previously described in Chapter 3 with minor modifications. Four mice were used for each experiment. After euthanasia, 4 mL of cold phosphate buffered saline (4°C) was injected into the peritoneal cavities of each mouse. The abdomens were gently massaged before a small incision was made within the abdominal wall and the exudate was aspirated using a disposable transfer pipette and transferred into a 15 mL conical tube. Cell suspensions were centrifuged at 500 x g for 10 minutes at 4º C. The supernatant was discarded, and the pellet was gently resuspended in 6 mL of Advanced DMEM Medium containing 5% FBS and 4% Thawing and Plating Cocktail A on ice. Cells were grown on Poly-D-Lysine coated glass coverslips (Neuvitro Corporation, USA) placed in the bottom of each well of a 6-well tissue culture plate. 1 mL of cell suspension was pipetted onto each cover slip and that culture plate was placed in a humidified incubator with 5% CO_2 for 2 hours at 37° C. The media was aspirated, and cells

were washed three times with warmed PBS before 1 mL of RPMI 1640 with GlutaMAX^{TM} supplement and HEPES, containing 10% FBS and a 1% mixture of penicillin-streptomycin was added to each well. Cells were incubated overnight (20-24 hours) before any experiments were performed.

Radioactive glutamine transport

Glutamine transport was measured on mouse peritoneal macrophages grown on glass coverslips by techniques similar to those described by Oxender et. al.(132). The coverslips were removed with forceps and submerged into a 1 x PBS solution to remove dead cells. The cells were placed into another 6 well plate containing 1 mL of uptake medium which consisted of phosphate buffered saline containing 0.1% glucose a mixture of [^{14}C]-labeled (New England Nuclear, USA) and unlabeled glutamine (ThermoFisher Scientific) and Lipopolysaccharides from Escherichia coli O111:B4 (Catalog No. L6230, Sigma, USA). The total glutamine concentration for these experiments ranged from 500 nm to 4μM. After rinsing, the coverslips containing the cells were placed into uptake media in 5-minute intervals for up to 15 minutes at 25ºC. The incubations were terminated by rinsing the coverslips twice in 1 x PBS before placing them into 0.5 mL of 200 mM NaOH to dissolve the cells. A 200 uL sample was removed for determining the radioactivity and another 200 uL sample was assayed for total protein content by the method of Lowry et al. Uptake of glutamine in cells treated with LPS alone was compared to cells incubated in the presence of 1 mM racemic MSO (Sigma, USA).

Interleukin 12 (IL-12) Enzyme Linked Immunosorbent Assay (ELISA)

Mouse peritoneal macrophages were grown in 6-well tissue culture plates at a density of approximately 1.5 x 10^6 cells per well. Macrophages were treated with MSO or left untreated for 1 hour followed by the addition of LPS (1μg/mL). Samples of culture media were analyzed for the

presence of IL-12 after 24 hours using the IL-12/IL-23 p40 (Total) Mouse Uncoated ELISA (ThermoFisher Scientific). The procedures were carried out per the manufacturer's instructions, and all data were analyzed using the Epoch microplate spectrophotometer and Gen5 data analysis software (Biotek, USA).

Preparation of mouse liver lysate

Liver lysates from 4-8 week old male CD1 mice (Charles River, USA) were prepared to provide a protein mixture for MSO affinity chromatography. 2 mice were euthanized before the livers were removed, placed within a steel strainer and minced into pieces using sterile scissors. The fragmented livers were rinsed with PBS to remove as much blood as possible before being transferred to glass tubes in 3 mL of a PBS lysis buffer, pH 7.4, containing 100 mM NaCl, 0.1 M EDTA, 1% NP-40 and a cocktail of protease inhibitors (Roche, Switzerland). The livers were homogenized using a glass Teflon homogenizer on ice. Liver lysates were centrifuged for 15 minutes at 1,000 x g to sediment cellular debris. The supernatant was aspirated with a syringe and sterile filtered using a 0.22-micron syringe filter. Total protein content was calculated using the Coomassie (Bradford) Protein Assay Kit (ThermoFisher Scientific).

MSO pull-down assay

The N-terminus of racemic MSO, L,*S*-MSO, and L,*R*-MSO was covalently cross-linked to N-hydroxysuccinimide linked beads marketed as Affi-Gel 10 (Bio-Rad), according to the manufacturer's instructions. The following procedure was performed at 4^{0}C. To 5 mL affinity chromatography columns, 3 mL of a 50% Aff-Gel: isopropyl alcohol suspension was added and quickly rinsed with 50 mL of dH2O followed by 50 mL of 50 mM MOPS buffer, pH 8. 100 mM MSO solutions in MOPS buffer, pH 8 were added to the columns. The gel-MSO mixtures were gently shaken overnight. The MSO solutions were eluted, and the columns were rinsed 3 times

with 50 mL MOPS buffer. The gels were incubated with a 1 mg/mL solution of BSA for 1 hour to block potential unreacted sites and to prevent non-specific reactions. The gels were rinsed with 50 mL of MOPS, an equal volume of MOPS was added to the columns to create a 50% suspension, and 500 uL aliquots of these suspensions were pipetted into Eppendorf tubes. The same cross-linking reaction was performed using a 1 M polyethanolamine solution, pH 8 as a negative control. 50 uL samples of 50% Affi-Gel 10 reacted with either MSO or polyethanolamine were added to 0.5 mL PCR tubes and centrifuged at 2000 RPM for 2 minutes. The supernatant was removed, and the gels were incubated overnight with 0.5 mL of 30 mg/mL mouse liver lysate on a laboratory rotator. Samples were centrifuged at 2000 RPM for 2 minutes, the supernatants were discarded, and the gels were resuspended in 600 uL of wash buffer consisting of 1 x PBS, 100 mM NaCl, 1% NP-40, pH 7.4. This process was repeated for a total of 5 washes before the supernatants were removed and 20 uL of 2 x Laemmli sample buffer was added to each sample. The samples were subjected to SDS-PAGE at 100 V using 10% polyacrylamide gels. The gels were stained using the Silver Stain Kit (Bio-Rad) per the manufacturer's instructions.

Results (Preliminary Data)

Effect of MSO on glutamine transport

When glutamine is removed from culture medium one hour prior to stimulating the cells with LPS, the concentration of IL-6 secreted by these cells is about half of what is seen when extracellular glutamine is available. In addition, when glutamine is removed from culture medium, the amount of MSO necessary to significantly reduce IL-6 secretion decreases from 9 mM to sub-micromolar concentrations (a 500-fold reduction on average). Adding glutamine to the medium eliminates this apparent increase in MSO potency. This lead us to test if MSO competes with glutamine for transport into the peritoneal macrophages after LPS stimulation.

We tested the ability of 1 mM MSO to inhibit the transport of [^{14}C] glutamine into mouse peritoneal macrophages stimulated with LPS for up to 15 minutes. For these assays we tested four concentrations of glutamine ranging from 1 to 4 μM in the presence and absence of 1 mM MSO. Figure 12 is a Lineweaver Burke plot of 1 divided by nanomoles of [^{14}C] glutamine transported into cells per minute per microgram of total protein versus 1 divided by the concentration of total glutamine. These preliminary results suggest that 1 mM MSO might competitively inhibit glutamine transport in these cells. In mammals, there are 14 families of amino acid transport proteins capable of transporting glutamine across the plasma membrane(133). Therefore, only apparent Vmax and Km values could be approximated in these preliminary in vitro studies.

The apparent Vmax and Km of glutamine transport in LPS stimulated cells (minus MSO) was approximated to be 0.22 nmol gln/min/μg total protein and 5.9 μM glutamine, respectively. The apparent Vmax and Km of glutamine transport in LPS stimulated cells in the presence of MSO was approximated to be 0.12 nmol gln/min/μg total protein and 16.7 μM glutamine, respectively. MSO treatment produced a plot that resembles competitive inhibition. However, more concentrations of glutamine must be tested to improve the accuracy and ensure reproducibility of these results.
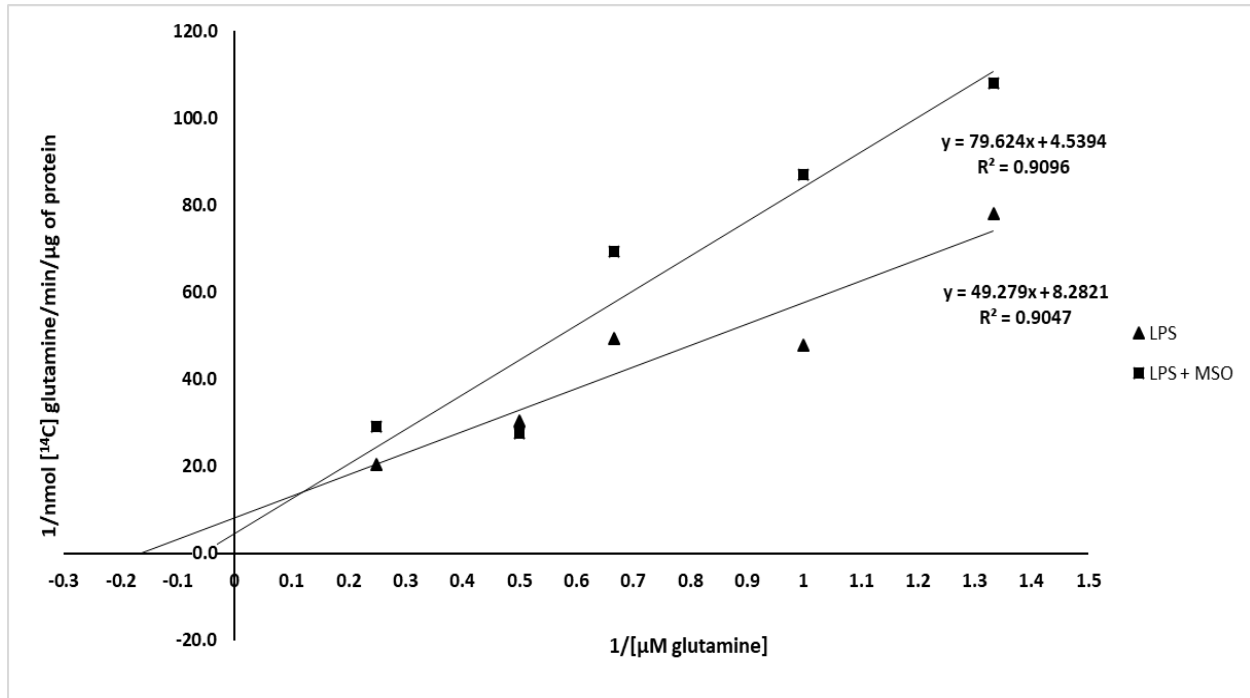
Figure 12. Glutamine transport in LPS stimulated mouse peritoneal macrophages. Mouse peritoneal macrophages were stimulated with LPS in the presence or absence of MSO and the average rate of [^{14}C] glutamine uptake was recorded (n=3). Four concentrations of glutamine were tested, ranging from 1 to 4 μM. MSO treatment appeared to compete with glutamine for transport in these cells.

Effect of MSO on IL-12 secretion

Our lab has previously shown that mice treated with MSO show a significant reduction in plasma IFNγ after being infected with lipopolysaccharide and D-galactosamine(35). Peritoneal macrophages generate IL-12, the major inducer of the Th1 T lymphocyte subclass that produces IFNγ during infection. Therefore, we tested the effects of MSO on IL-12 production in isolated macrophages to assess whether the observed in vivo decrease of IFNγ after MSO treatment could be attributed to a reduction in macrophage-produced IL-12. Preliminary data, shown in **Table 7**, suggest that MSO treatment can reduce IL-12 secretion by mouse peritoneal macrophages after being exposed to LPS for 24 hours. The results describe two independent macrophage populations; replication of the experiment is necessary to determine whether this effect is statistically significant.

IL-12 (pg/mL)			
Sample	**LPS**	**LPS +MSO**	**Untreated**
1	58.96	41.68	0
2	88.60	43.40	0

Table 7. Effect of MSO treatment on IL-12 secretion. In two independent samples, MSO treatment reduced the concentration of IL-12 secreted into the medium by peritoneal macrophages stimulated with LPS for 24 hours (29% for and 52% for Samples 1 and 2, respectively). Untreated cells did not produce measurable levels of IL-12. The effect of MSO alone (minus LPS) was not tested on these cells.

MSO can bind to Eukaryotic elongation factor 1 alpha 1 (eEF1A1)

In order to identify other possible targets of MSO, we covalently linked the N-terminus of racemic MSO, L,*S*-MSO, and L,*R*-MSO to N-hydroxysuccinimide linked beads and performed pull-down assays. **Figure 13** shows a unique protein that was pulled down by all three MSO mixtures but not with the polyethanolamine control. The gel presented in Figure 13 was submitted to the Wayne State Proteomics Core Facility and the protein band was identified as eukaryotic translation elongation factor 1 alpha 1 (eEF1A1). Eukaryotic translation elongation factor 1 alpha 1 has been verified as a functional regulator of IL-6 production in human astrocytes(134). However, the MSO gel suspensions did not bind to glutamine synthetase, the known target of MSO. Furthermore, cross-linking MSO to a solid support eliminates the positive charge on the amino terminus, altering the structure of MSO and potentially altering the binding specificity. Therefore, further investigation must be performed before EEF1A1 can be validated as a functional target of MSO.
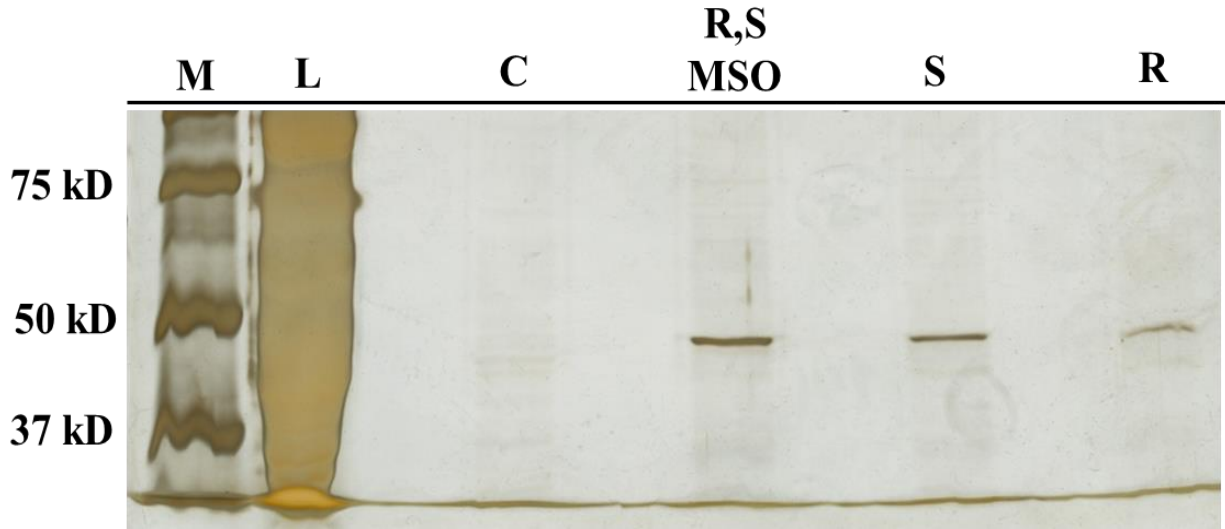
Figure 13. MSO can pull-down eEF1A1. SDS-PAGE gel result of an MSO pull-down experiment. All 3 formulations of MSO pulled down a 50 kD protein, that was not pulled down by a polyethanolamine sample.

M = molecular weight marker; L = rat liver lysate; C = polyethanolamine control; *R,S*-MSO = racemic MSO; S = L,*S*-MSO, R = L,*R*-MSO

Discussion and Conclusions

Preliminary data from our lab have provided insight towards potentially novel functions of MSO and have taught important lessons on the practicality of designing future experiments to elucidate new targets of MSO. These data suggest that 1) MSO might compete with glutamine for transport into peritoneal macrophages, 2) MSO may also inhibit IL-12 along with IL-6 and TNFα, and 3) MSO may bind eEF1A1, a translation factor which could potentially add weight to our hypothesis that MSO acts at the level of translation. However, each one of these experiments must be repeated several times and specific concerns must be addressed in order to validate these results.

As it was eluded to in Chapter 3, MSO appears to compete with glutamine for transport across the plasma membrane of peritoneal macrophages. However, we have only tested this effect with1 mM MSO, a concentration that is significantly higher than the glutamine concentrations used in our experiments, and this competition appears to be very weak. Several concentrations of MSO need to be tested to confirm that this inhibition is significant, that it is indeed competitive,

as well as to calculate an apparent Ki value of inhibition. Although multiple investigators have used liquid chromatography paired with mass spectrometry techniques to quantify the intracellular concentration of glutamine in LPS stimulated peritoneal macrophages(135,136) the rate of overall glutamine transport in peritoneal macrophages has not been effectively characterized, most likely because several confounding variables exist that may significantly affect the results of such transport experiments.

As mentioned previously, there are over a dozen families of amino acid transporters that are capable of transporting glutamine across the plasma membrane of mammalian cells. Furthermore, several factors may influence the rate of glutamine uptake in cell culture systems, such as temperature and the Na^+/K^+ concentrations in the uptake media. Our data suggest that glutamine concentrations exceeding 4 μM are needed to saturate the rate of glutamine transport. Lastly the homeostatic plasma glutamine concentration is in the millimolar range, so it is likely that very high concentrations of glutamine are needed to saturate the transport reaction. We tried to keep the specific activity the same for all of our transport experiments, requiring changes in the amount of isotope added at each glutamine concentration. Therefore, testing large concentrations of glutamine would require using an impractical amount of isotope. Considering these concerns, we feel it would be more practical to perform these experiments using radiolabeled MSO rather than labeled glutamine.

Regarding the effect of MSO on IL-12 secretion, we are confident that this effect is repeatable and noteworthy but that we must increase our sample size for the sake of statistical significance. In two independent samples, MSO treatment resulted in a decrease of IL-12 compared to LPS only controls that ranged from 20-45 percent. When MSO is injected into mice prior to the injection of LPS, these mice show significant decreases in the IFNγ, which is produced

predominately by IL-12 stimulated T lymphocytes during the inflammatory response. We believe this in vivo result can be partly explained by a significant reduction in macrophage generated IL-12. Lastly, since IL-23, another pro-inflammatory cytokine, shares the p40 subunit of IL-12 detected with our ELISA kit, it is possible that MSO could also reduce IL-23 secretion.

In addition to the benefits of using radiolabeled MSO for transport studies, we believe it will also provide a more suitable approach for identifying putative targets of MSO. Since MSO is a small molecule, we assume the probability of this molecule having multiple targets would be higher than what would be expected for a larger, bulkier molecule or protein. Although the literature is quite sparse, often involving a single study, other targets of MSO have been reported in various cell types, such as ornithine decarboxylase(137), histamine N-methyltransferase(138), and the mammalian target of rapamycin(139). We have identified eEF1A1 as a possible binding partner of MSO. However, we have had variable success in reproducing our original results to a satisfactory level. Binding MSO to a solid substrate such as Affi-Gel or other commercially available probes ultimately alters the molecular structure and net charge of MSO. As it is an amino acid, MSO is zwitterionic at physiological pH. Therefore, we believe using radiolabeled MSO would be a more suitable approach since it would not alter the size, structure, nor would it affect the net charge. A hypothetical experiment would involve the following steps: 1) incubating $[^{14}C]$ labeled MSO with a tissue lysate, 2) performing gradient centrifugation to isolate subcellular fractions containing the highest amount of MSO radioactivity, 3) a purification step using size exclusion chromatography, 4) subjecting samples to native 2D gel electrophoresis followed by autoradiography to elucidate bound proteins, and 4) identification of putative targets using mass spectroscopy.

A hypothesis to explain MSO mode of action

MSO increases cerebral transmethylations in vivo

As mentioned in Chapter 1, MSO was initially discovered as a byproduct of methionine in the industrial process that bleached wheat gluten with nitrogen trichloride. In dogs and cats, MSO treatment was shown to induce convulsions that resemble tonic-clonic seizures observed in human epilepsy. Additionally, unlike most epileptogenic agents that induce seizures immediately upon exposure, it takes up to 6 hours for an animal to have a seizure after MSO treatment. Thus, MSO became a favorable model because this pre-convulsive latency period provided researchers with a window of time in which biochemical events associated with the generation of seizures could be investigated.

In 1975, Otto Sellinger and colleagues reported that intraperitoneal injection of MSO into rats resulted in significant decreases in cerebral S-adenosyl methionine (SAM)(140). SAM is the universal methyl group donor molecule in cellular methylation reactions performed by methyltransferase enzymes. MSO-induced decreases in SAM were not attributed to decreased synthesis of SAM, but rather an increase in SAM utilization that occurred halfway through the pre-convulsant period. Injection of adenosine and homocysteine thiolactone into rats, a treatment that produces higher than normal SAH levels, was shown to antagonize cerebral methylations and protect against MSO seizures(141).

Several other studies by Sellinger provide evidence for an increased in vivo transmethylation flux in brain tissue after MSO treatment. In brain, the major catabolic route for the neurotransmitter histamine begins via methylation into 3-methyl histamine. Mice that received MSO prior to the intraventricular injection of [^{3}H] histamine showed increases in [^{3}H] 3-methyl histamine compared to controls that received saline(142). In vitro, MSO was shown to accelerate

N-methyl transferase(138). Additionally, MSO treatment lead to increased methylations of various tRNA species, phospholipids, and increased the total carboxymethylated protein content in rodent brain tissue(138). Therefore, MSO causes a hypermethylation flux within brain after administration in vivo.

As previously mentioned in Chapter 1, the fact that injection of SAH or methionine can decrease the incidences and increase the latency of MSO seizures challenges the notion that MSO seizures are caused by glutamine synthetase inhibition, because neither molecule interferes with MSO binding to GS or affects the level of the product glutamine.

MSO increases polyamine synthesis in rodent brain

Since S-adenosyl homocysteine (SAH), the demethylated product of SAM, was also significantly decreased after MSO treatment, Sellinger also hypothesized that SAM decarboxylation was occurring at high rates in MSO treated mice. MSO-induced decreases in SAM were shown to lead to increased synthesis of the biogenic polyamines spermidine and spermine. In mammals, the three polyamines: putrescine, spermidine, and spermine, can be synthesized via two routes, of which one depends on the enzyme ornithine decarboxylase (ODC). The polyamine biosynthesis pathways are described in **Figure 13**. The ODC dependent pathway begins with the cleavage of arginine into ornithine and urea by the enzyme arginase. Ornithine is decarboxylated by ODC into putrescine. Spermidine is synthesized by spermidine synthase, which combines putrescine with a propyl amine group from the decarboxylated form of S-adenosyl methionine. Spermidine can then be converted to spermine by spermine synthetase, which also uses decarboxylated S-adenosyl methionine. Spermine can be converted back to spermidine through the formation and oxidation of an N-acetyl spermine intermediate. Likewise, spermidine can be converted back to putrescine through an N-acetyl spermidine intermediate. Therefore, spermidine

and spermine can be synthesized from putrescine synthesized through ornithine directly by ODC or from putrescine formed through the recycling pathway involving N-acetyl spermidine.

In MSO-treated mice, injection of [1,4-^{14}C] putrescine did not result in increased levels of [^{14}C] spermidine or spermine. However, injection of [3,4-^{14}C] methionine into MSO treated mice lead to 50% increases in cerebral spermidine and spermine. In accordance with the fact that MSO depletes SAH along with methionine and SAM, this evidence suggests that SAM decarboxylation is increased after MSO treatment. The increase in SAM decarboxylation results in increased cerebral concentrations of spermidine and spermine, which rely on the level of decarboxylated SAM synthesized by the enzyme S-adenosyl methionine decarboxylase. However, it has never been tested if MSO has direct effects on S-adenosyl methionine decarboxylase activity. Although Sellinger showed that injection of putrescine does not result in increased spermidine or spermine levels after MSO treatment, MSO has been shown to increase ODC activity in rat brain. It has not been confirmed if MSO binds to ODC directly or if the accelerated ODC activity is a consequence of a rise in cellular ammonia that results from glutamine synthetase inhibition, since ODC has activity has been shown to increase with increasing ammonia levels.(137). In conclusion, two independent groups have verified that MSO treatment affects polyamine levels, but the exact biochemical mechanism remains unclear.
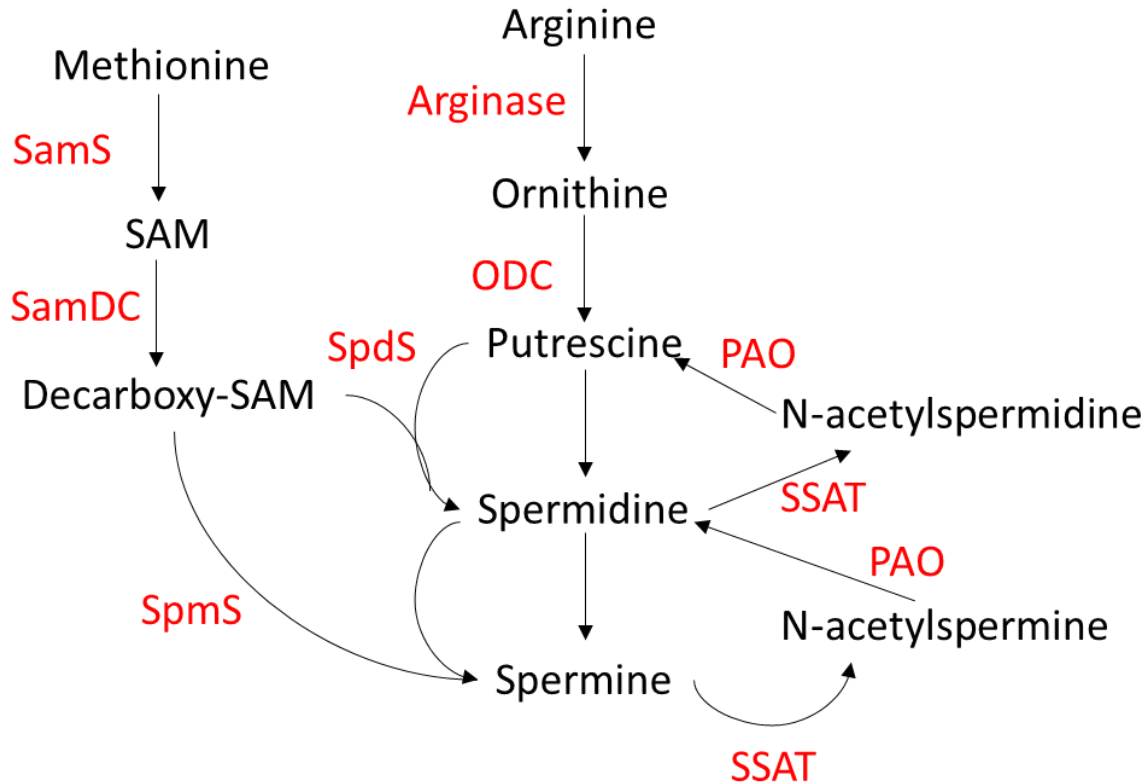
Figure 14. Polyamine synthesis pathways. The polyamines found in animals: putrescine, spermidine, and spermine are synthesized in the cytoplasm from the amino acids arginine and methionine. The non-proteinogenic amino acid L-ornithine is generated from arginine by the enzyme arginase and serves as precursor molecule for the formation of spermidine and spermine. Polyamines are also interconverted through the enzymatic activities of spermidine/spermine acetyl transferase and polyamine oxidase. The generation of spermidine and spermine requires decarboxylated S-adenosyl methionine to provide a propyl amino group.
ODC = ornithine decarboxylase; PAO = polyamine oxidase; SamDC = S-adenosyl methionine decarboxylase; SamS = S-adenosylmethionine synthetase; SpdS = spermidine synthetase; SpmS = spermine synthetase; SSAT = spermine/spermidine acetyltransferase.

Spermine inhibits macrophage activation

Polyamines are essential molecules for life, involved in several basic biological processes such as growth, replication, and differentiation. In addition, the polyamine spermine has been shown to play an important role in resolving immune responses. When infected or dying cells are destroyed, they release spermine into the local tissue environment. Zhang et al. showed that exogenous spermine significantly reduces the production of TNFα and IL-6 by isolated macrophages(143). This inhibition of cytokine synthesis occurred at the post transcriptional level,

because the transcript levels of these proteins were unaffected by spermine treatment. Spermine has also been shown to reduce IL-12 secretion by peritoneal macrophages and IFNγ secretion by Th1 cells(144).

Using metabolomics approaches for future research

The effects of spermine on peritoneal macrophages are strikingly similar to those of MSO. Both molecules inhibit the production of the pro-inflammatory cytokines TNFα, IL-6, and IL-12. In the case of TNFα and IL-6, both spermine and MSO inhibit the production of these proteins at the post-transcriptional level. Spermine is increased in brain after MSO treatment. As previously mentioned, MSO treatment accelerates ornithine decarboxylase activity, although it remains unclear whether MSO and the enzyme interact directly. Additionally, peritoneal macrophages express both ODC and S-adenosyl methionine decarboxylase at appreciable levels. Therefore, it is possible the same phenomenon that occurs in brain after MSO treatment also occurs in these cells. To test this hypothesis, we propose that metabolomic analysis and direct quantification of these small molecules would need to be performed on isolated macrophages treated with LPS (+/-) MSO.

Recent developments in metabolomics and bioinformatics have allowed investigators to perform studies involving untargeted approaches for global identification and quantification of cellular metabolites in LPS stimulated peritoneal macrophages(145). Our data indicate that MSO acts post-transcriptionally to reduce IL-6 and TNFα. Metabolomic approaches would allow us to quantify the intracellular concentrations of several molecules of interest including the polyamines, and could also potentially tell us if specific biochemical pathways are affected by MSO treatment. For example, such metabolomic approaches have discovered that quiescent macrophages primarily use oxidative phosphorylation to generate ATP, whereas LPS stimulated macrophages undergo metabolic reprogramming and rely heavily on aerobic glycolysis due to an overall increase in

metabolic demand. We cannot rule out the possibility that MSO could act as a novel metabolite that interacts or interferes with the metabolic state of the cell.

Metabolomic analyses could also shed light on whether MSO acts indirectly on metabolic pathways. For example, inhibiting glutamine synthetase with the L,*S* isomer of MSO results in increases in cellular ammonia levels. Multiple proteins (including glutamine synthetase) have been shown to have activities that are either increased or decreased by changes in cellular ammonia concentrations, including monoamine oxidase, GABA aminotransferase(146), and catalase(147). We believe such approaches will provide the best opportunity to identify the consequences of MSO treatment on the cellular metabolome.

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ABSTRACT

METHIONINE SULFOXIMINE: A NOVEL ANTI-INFLAMMATORY AGENT

by

TYLER J. PETERS

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Advisor: Dr. William Brusilow

Major: Biochemistry and Molecular Biology

Degree: Doctor of Philosophy

The glutamine synthetase inhibitor methionine sulfoximine (MSO), shown previously to prevent death caused by an inflammatory liver response in mice, was tested on in vitro production of cytokines by mouse peritoneal macrophages triggered with lipopolysaccharide (LPS). MSO significantly reduced the production of Interleukin 6 (IL-6) and Tumor Necrosis Factor Alpha (TNFα) at 4 and 6 hours after LPS-treatment. This reduction did not result from decreased transcription of IL-6 and TNFα genes, and therefore appeared to result from post-transcriptional inhibition of synthesis of these cytokines. MSO treatment did not inhibit total protein synthesis and did not reduce the production of a third LPS-triggered cytokine CXCL1, so the effect was not a toxic or global downregulation of the LPS response. The anti-inflammatory effects of a glutamine synthetase inhibitor were seen even though the medium contained abundant (2 mM) glutamine, suggesting that the target for this activity was not glutamine synthetase. In agreement with this hypothesis, the L,*R* isomer of MSO, which does not inhibit glutamine synthetase and was previously thought to be inert, both significantly reduced IL-6 secretion in isolated macrophages and increased survival in a mouse model for inflammatory liver failure. Our findings provide evidence for a novel target of MSO. Future attempts to identify the additional target would

therefore also provide a target for therapies to treat diseases involving damaging cytokine responses.

AUTOBIOGRAPHICAL STATEMENT

Tyler Peters obtained his B.S. with a major in biology from the University of Detroit Mercy in 2014. While working on his degree he developed an interest in medicinal research and toxicology and began researching the effects of herbicides on crayfish behavior in the lab of Rachelle Belanger, Ph.D. In 2014, Tyler joined the Biochemistry and Molecular Biology Department as a masters student for one year before matriculating into the doctorate program under the mentorship of William Brusilow, Ph.D. in 2015. He graduated with is Ph.D. in Biochemistry and Molecular Biology in 2018.