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Use of methionine sulfoximine to dissect the role of glutamine synthetase and glutamine in progression of acute liver failure

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USE OF METHIONINE SULFOXIMINE TO DISSECT THE ROLE OF GLUTAMINE SYNTHETASE AND GLUTAMINE IN PROGRESSION OF ACUTE LIVER FAILURE

by

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DEDICATION

This work is dedicated to my parents. I would never have attained this degree without their unconditional love, support and encouragement.

Aai and baba, I love you.

गुरुर्ब्रह्मा गुरुर्विष्णुः गुरुर्देवो महेश्वरः।
गुरुः साक्षात्परब्रह्म तस्मै श्री गुरवेनमः॥

Translation: Guru Brahma, Guru Vishnu Guru Devo Maheshwara, Guru sakshath Parabrahma, Tasmai Shri Gurave Namah

The Guru (Teacher) is the creator Brahma, The Guru is the preserver Vishnu and The Guru is the destroyer Shiva. Guru is the supreme spirit and I bow to such a Guru (Teacher).

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CHAPTER 1

Understanding liver, disease pathogenesis and basic immunology involved in acute liver failure

Liver

The liver carries out a wide range of metabolic and biochemical functions. It is responsible for detoxification, protein synthesis and production of digestive juices. Its other functions include glycogen storage, gluconeogensis, cholesterol synthesis, drug metabolism, and ammonia detoxification, synthesis of blood clotting factors, complement factors and decomposition of red blood cells. Thus, it is an essential organ for survival.

Liver is the largest organ in vertebrates. It is made up of four unequal lobes and is supplied blood by the hepatic portal vein and the hepatic arteries. It is the only organ capable of regeneration in adults; after 2/3 hepatectomy normal function of liver is restored within 2-3 weeks.

Liver is functionally heterogeneous. It has a simple and uniform morphology. Liver can be structurally divided into 1) the parenchyma, and 2) the lymphoid system. The liver parenchyma is structured such that different and opposing functions can be carried out by the liver cells (hepatocytes). There is a distinct metabolic zonation within the parenchyma (Gebhardt, Baldysiak-Figiel et al. 2007). The hepatocytes are referred to as periportal (PP) or pericentral (PC) in the compartment model of the liver. PP hepatocytes are also termed as upstream - away from the blood capillary system - and the PC cells are called downstream hepatocytes – these are the cells closest to the lymphoid system. These two regions are functionally distinct. Another way to compartmentalize liver is by dividing it into three zones. The first zone is the periportal

zone. The second zone consists of midzonal hepatocytes and the third zone is called the pericentral region. Again, each zone consists of hepatocytes with distinct gene expression patterns which lead to expression of proteins and enzymes for specific metabolic functions (Gebhardt, Baldysiak-Figiel et al. 2007). Thus, liver functions are compartmentalized and carried out by distinct subpopulation of the parenchyma (Sasse, Spornitz et al. 1992).

The lymphoid system of liver is made up of 'sinusoids' – the thin walled blood vessels. The capillary network in liver consists of blood vessels delivering blood via the arterial circulatory system and merging with portal venous blood arriving from the gut and the intestine. The sinusoids contain a diverse population of resident immune cells such as macrophages and dendritic cells. Other resident immune cells in liver are T cells, natural killer (NK) cells and natural killer T (NKT) cells. The liver macrophages are called Kupffer cells (KC). KC's are the most abundant immune cell type in liver and are primarily responsible for removal of all antigenic and microbial products present in intestinal blood. These immune cells are continuously exposed to the bacterial toxins from gut. They are in a state of 'active tolerance', which can be quickly changed to 'active immunity' during metabolic imbalance or in presence of unknown pathogens (Crispe 2009).

Varieties of signals are secreted by the resident immune cells to attract humoral (B and T cells) immune cells. These signals – cytokines and chemokines – attract other immune cells to site of infection and initiate the inflammatory immune processes. Thus, the liver is a lymphoid organ with a diverse immune cell population to maintain innate immunity.

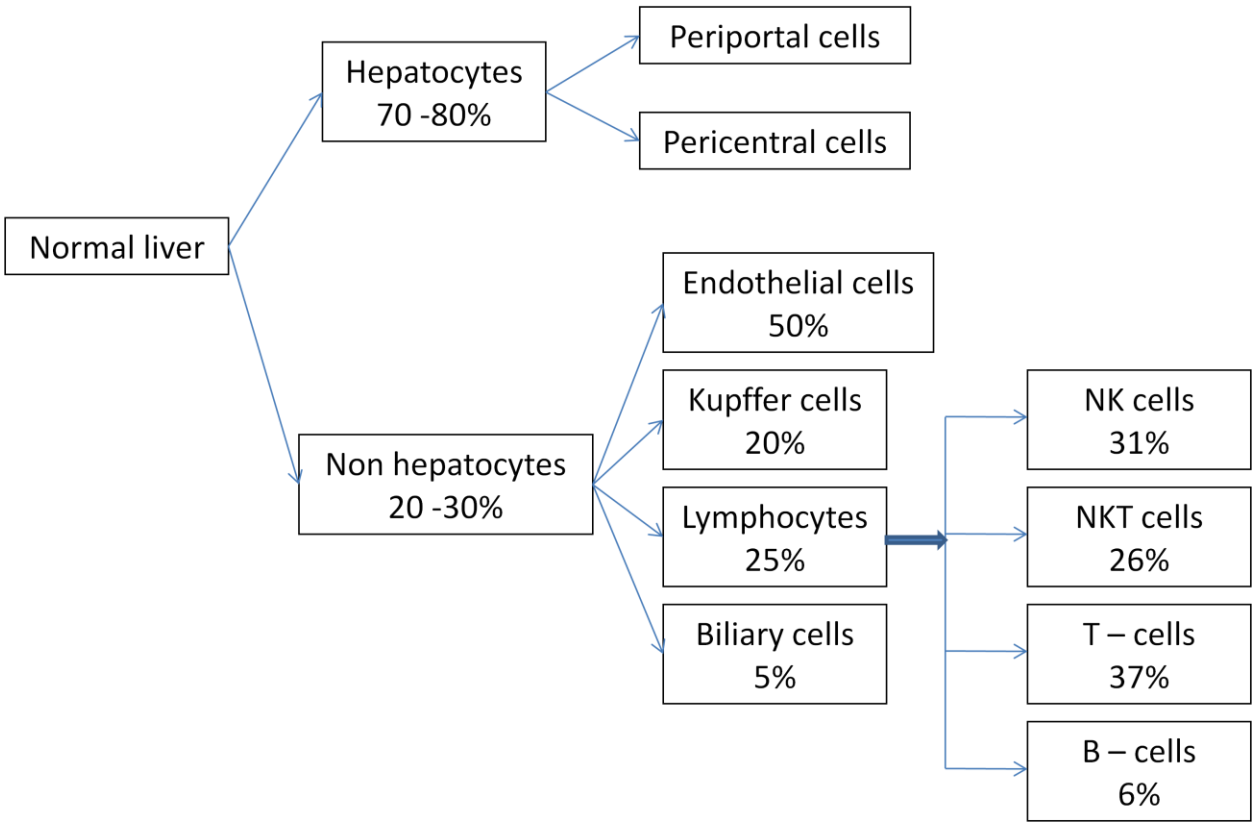

Figure 1.1 Cellular classification of liver.

Acute liver failure (ALF)

Introduction

Acute liver failure (ALF) is a condition characterized by development of coagulopathy and hepatic encephalopathy in a previously healthy individual. Children are also affected by this disease. 2000 cases are reported in US every year and 25% of the patients recover spontaneously. However, for remaining patients the prognosis is extremely poor with death and coma being the most frequent outcome. Treatment is aimed at removing the underlying cause of the disease while reducing disease symptoms such as intracranial pressure. Intensive care is provided to

patients and metabolic parameters are monitored. Liver transplantation is the only treatment for numerous people whose liver continues to deteriorate. Mortality of ALF patients is very high. However, with proper treatment and liver transplant short term survival of patients has increased to 65% from 20%.

Common causes of ALF are acetaminophen overdose, viral hepatitis (A, B and E), autoimmune hepatitis, severe alcohol intake and idiosyncratic reaction to drugs such as tetracycline. Rapid deterioration of liver functions occurs within weeks of disease onset. Clinical symptoms manifest over a very short period of time and correspond to the extent of liver injury. Ultimately complete cessation of liver function occurs.

Classification:

This disease was first documented by Lucke and Mallory in 1946, when they reported rare occurrence of fatal hepatitis (Lucke and Mallory 1946). The patients had very poor prognosis and they often died. Lucke and Mallory classified the disease as fulminant liver failure and acute liver failure. If liver disease development and progression occurred quickly it was termed as fulminant liver failure and if disease development was slow then it was classified as acute liver failure. In 1970, Trey and Davidson defined FHF as "a potentially reversible condition, the consequence of severe liver injury, with the onset of hepatic encephalopathy within eight weeks of the first symptoms and in the absence of pre-existing liver disease" (Trey and Davidson 1970). In 1986 Gimson *et al*, defined acute liver failure and described its onset to be within 8-24 weeks after appearance of first symptom associated with liver injury (Gimson, O'Grady et al. 1986).

Old nomenclature	Classification	Onset of hepatic encephalopathy
	Fulminant hepatic failure	8 weeks
New nomenclature	Fulminant hepatic failure	2 weeks
	Sub – fulminant hepatic failure	2 -12 weeks

Old nomenclature	Classification	Onset of hepatic encephalopathy
	Acute liver failure	8 -24 weeks
New nomenclature	Hyper acute liver failure	7 days
	Acute liver failure	7 -28 days
	Sub – acute liver failure	5 – 26 weeks

Table 1.1. Classification terms used to characterize various forms of liver failure.

Today, FHF term is used for patients who exhibit hepatic encephalopathy within 2 weeks of disease onset. Sub-fulminant hepatic failure is characterized by encephalopathy appearance within 2-12 weeks of liver injury. These days ALF is used as an umbrella term to cover the groups hyper-acute, acute and sub-acute liver failure (O'Grady and Williams 1993). Time and etiology are important factors in disease prognosis and hence these factors are used to classify acute failure. If the encephalopathy develops within 7 days of jaundice onset it's termed as hyper-acute liver failure. The majority of these patients suffer liver injury due to acetaminophen overdose. Some patients also suffer from non-A, non-B hepatitis. If the symptoms appear within 7-28 days after jaundice onset it's categorized as acute liver failure. If the disease development is

very slow and occurs over time of 5 -26 weeks it's categorized as sub-acute liver failure. Viral hepatitis is th leading cause of acute liver failure, whereas, non-A, non-B hepatitis (with no known viral cause) is causative of sub-acute liver failure (Gimson, O'Grady et al. 1986; O'Grady, Schalm et al. 1993). **Table 1.1** shows the current ALF classification based on the onset of hepatic encephalopathy.

Pathology:

During ALF, hepatocyte necrosis and apoptosis are observed within the liver parenchyma (Lee 1993). The hepatic parenchyma collapses and numbers of viable hepatocytes gradually decline. Regeneration is inhibited and areas of regeneration are destroyed. Portal ductules collapse and further sites of inflammation develop. Liver cirrhosis is also observed in alcoholic patients. In all types of ALF, inflammation is followed by massive immune cell infiltration and subsequent release of cytokines at the site of necrotic/ apoptotic death (Lemasters 1999; Luedde, Liedtke et al. 2002).

Symptoms:

It is important to remember that all the symptoms are interconnected. The primary cause of all the symptoms is the failing liver and diminishing liver function.

Hepatic encephalopathy and brain edema:

The yardstick of this disease classification and prognosis is the unique symptom hepatic encephalopathy. It develops in all patients; 75 – 80% of these patients also develop cerebral edema. The exact mechanism for hepatic encephalopathy is not agreed upon, but it is believed that it occurs due to one or more of the following: 1) increased ammonia leading to a) increased astrocyte glutamine and osmotic swelling and b) dysregulation of glutamate leading to

excitotoxicity, 2) a toxic interaction between ammonia, mercaptans and fatty acids, and 3) production of false neurotransmitters.

To elaborate on these possible mechanisms, we need to understand ammonia homeostasis. Gut derived ammonia is detoxified by liver. It is converted into urea via glutamine by glutamine synthetase. Ammonia incorporation into glutamine opens an amino acid formation pathway in the body. During liver failure ammonia accumulates in the arterial blood. The ammonia is not eliminated by liver and hence it reaches brain, where it can cross the blood brain barrier. Ammonia concentrations in neurons and astrocytes increase and this is believed to be responsible for neurotoxicity and convulsions (Zieve 1981). Ammonia can stimulate glutamine synthetase in astrocytes to produce more glutamine, a powerful osmolyte that attracts water to produce astrocyte swelling. Astrocytes in the brain try to soak up the ammonia by converting it to glutamine via glutamine synthetase, which is present only in astrocytes. More and more glutamine accumulates in astrocytes, leading to changes in osmolarity which is not compensated with loss of other organic compounds. At the same time dysfunction of Na+ K+ ATPase pump leads to water accumulation and ultimately astrocyte swelling(Poso and Pegg 1982; Blei and Cordoba 2001; Jayakumar, Rao et al. 2006). Astrocytes are the most the abundant cell type in brain and one-third volume of the cerebral cortex is occupied by them. Hence, the massive swelling of astrocytes leads to brain edema and brain stem compression due to increased intracranial pressure. Thus, pre – and post – synaptic neurons and astrocytes are responsible for encephalopathy. However, astrocytes alone are responsible for causing brain edema (Takahashi, Koehler et al. 1991; Blei 2001; Brusilow 2002).

Excessive ammonia also causes dysregulation in the glutamate neurotransmitter system of brain. Glutamate is a major excitatory neurotransmitter in the mammalian brain. It is released

by pre – synaptic neurons and it binds to its cognate receptors on the post – synaptic neurons (NMDA receptors) and astrocytes (AMPA/ AMKA receptors). Astrocytes sit in the synaptic cleft and are responsible for quenching excess glutamate. Astrocytes take up glutamate via GLT-1 transporter and convert it to glutamine in presence of ammonia. High levels of ammonia down regulate the GLT-1 receptor on the astrocyte cell surface leading to prolonged exposure of post – synaptic neurons to glutamate. Thus, the glutamate uptake is reduced and glutamate receptors on astrocyte surface are altered by elevated peripheral ammonia, and the resulting alteration in glutamate binding and function is considered to be one of the causes of encephalopathy (Norenberg and Bender 1994; Brusilow 2002).

The altered mental status of ALF patients can also be attributed to toxic synergistic interaction between mercaptans (products of enteric bacteria, which accumulate in peripheral blood when liver fails) and ammonia along with short and medium chain fatty acids (Zieve 1981). All three alone and/ or together are considered to be responsible for convulsions and the same has been shown by experiments done in various animal models. It is believed that during liver failure there is a decrease in branched chain amino acids (such as leucine, isoleucine, valine) in plasma (Fischer and Baldessarini 1976). At the same time, the other amino acids increase in plasma, producing an imbalance in the ratio of branched chain amino acids and aromatic amino acids (such as tryptophan, tyrosine and phenylalanine). The amino acid imbalance coupled with increased brain glutamine leads to an increased blood flow and aromatic amino acid deposition in brain. Aromatic amino acids stimulate the synthesis of serotonin and the false neurotransmitter octopamine, which in turn leads to a decrease in the synthesis of adrenaline and noradrenaline (James, Freund et al. 1979; James, Ziparo et al. 1979; Fischer 1982; Hoyumpa and Schenker 1982). Octopamine acts as false weak neurotransmitter and along with

inhibitory serotonin prevents actions of excitatory neurotransmitters adrenaline and noradrenaline, thus causing neural inhibition and encephalopathy (James, Ziparo et al. 1979). The presence of benzodiazepine agonists and altered levels of GABA also contribute towards encephalopathy.

Metabolic complications:

One of the important hepatic functions is mobilization of glycogen stores and gluconeogensis. Thus, the liver is a major site for insulin metabolism. During liver failure these functions are impaired and the resulting high serum insulin levels with decreased glycogen mobilization leads to hypoglycemia. Hypoglycemia contributes towards mental complications observed in the ALF patients. Liver dysfunction might also cause hypophosphatemia, and acid – base imbalance (Astrup, Prytz et al. 1980).

Acidosis is not frequently seen in ALF. However, due to hypoxia, lactate production increases (Bihari, Gimson et al. 1985). Oxygen uptake by cells is impaired due to their inability to extract oxygen from circulating blood. This 'micro-circulatory dysfunction' is responsible for acidosis (Bihari, Gimson et al. 1985). In contrast, alkalosis is frequently observed in ALF patients (Bihari, Gimson et al. 1985). The inability of hepatocytes to make urea from ammonia leads to excessive ammonia and bicarbonate accumulation in systemic circulation. Also, Na+ K+ ATPase dysfunction can cause an increase in sodium reabsorption and result in hypokalemia (Astrup, Prytz et al. 1980).

Coagulopathy:

Coagulopathy is a characteristic symptom of ALF and is used diagnostically to determine severity of this disease. The term coagulopathy broadly encompasses two complications

associated with ALF 1) reduction in coagulation factors and therefore delayed coagulation and increased bleeding, and 2) reduction of fibrinolysis inhibitors.

Since the liver is responsible for production and metabolism of coagulation factors – I, II, V, VII, IX, and X – coagulation time and bleeding is one of the easily identifiable effects of failing liver. There is a marked prolongation of prothrombin time. Factor V has shortest half life amongst the clotting factors and hence its synthesis is often measured to identify the extent of impairment of coagulation factor synthesis. Platelet structure and function is altered. Platelet numbers are also frequently reduced which results into decreased platelet aggregation and increased adhesiveness and thrombocytopenia (Lewis, Bontempo et al. 1985). Disseminated intravascular coagulation further exacerbates the situation (O'Grady and Williams 1986) . Anti – thrombin III synthesis is reduced (Hallen and Nilsson 1964). All these factors lead to bleeding, and hence, gastrointestinal and intrapulmonary hemorrhages and sepsis are common complications observed in ALF patients.

Cardiac and pulmonary abnormalities:

Cardiac complications are rare and are dependent on other factors such as acidosis and alkalosis, capillary leak, low osmotic pressure, arterial hypoxemia, ventilation/ perfusion mismatch and sepsis. In ALF patients there is an elevated cardiac output and lowered systemic vascular resistance index which causes tachycardia. Patients might also suffer from hypotension or hypertension (especially seen in patients with encephalopathy) (Trewby and Williams 1977). Liver and the corresponding cardiac failure lead to intrapulmonal vasodilatation and pulmonary edema. Intrapulmonary bleeding causes further complications, such as hypoxia (Haupt, Gilbert et al. 1985). These symptoms are similar to the abnormalities observed in sepsis and trauma patients.

Hepatorenal syndrome:

Kidney failure is more common in patients with drug poisoning. Acetaminophen and amanita overdose has a direct toxic effect on renal system (Gimson, O'Grady et al. 1986). Since renal function is associated with liver function, this liver associated syndrome is termed as 'hepatorenal failure'. It is characterized by reduced renal perfusion. There is a reduction in urine output and an increased excretion of serum creatinine. Decrease in renal blood flow and circulating immune complexes harm the nephrons and cause kidney failure (Ring-Larsen 1977). The hepatorenal failure is completely reversible and improves spontaneously once the patient's liver function improves or after liver transplantation. However, sepsis or long periods of hypotension can lead to permanent kidney damage due to necrosis of renal tubules.

Infection risk:

Risk of infection is the single biggest threat to an ALF patient under treatment. Enteric bacteria can easily translocate from gut to systemic circulation during chronic inflammation. Moreover, due to dysfunctional neutrophils, kupffer cells and opsonins, bacterial (staphylococcus aureus) and fungal (candida albicans) infections are common (Rolando, Harvey et al. 1990; Rolando, Harvey et al. 1991).

The liver houses a large number of resident innate immune cells and produces proteins of the complement immune system (complement factor C3 and C5 are synthesized in the liver). During ALF, KC's are activated and they recruit additional immune cells. Multiple and excessive cytokine secretion by the immune cells leads to immune network failure which ultimately contributes towards increased risk of infection for ALF patients. Moreover, KC toxicity due to its tendency to secrete tumor necrosis factor – alpha (TNF-α) has been implicated

in various models of hepatotoxicity (Weiss 1989; Thurman, Bunzendahl et al. 1993; Leist, Gantner et al. 1995; Rosser and Gores 1995).

Disease mechanism:

ALF is characterized by severe systemic inflammation irrespective of the presence or absence of infection. Disease development and related complications occur due to two consequences of failing liver. 1) Liver failure has adverse metabolic effects – all liver functions are affected. Urea metabolism, glycogen metabolism and protein synthesis are disrupted. The resulting effects are responsible for disease pathogenesis. 2) Liver failure is cytotoxic. Toxic substances are not cleared from the systemic circulation and dying hepatocytes release more toxic substances. The resultant multi – organ failure exacerbates the situation. **Figure 1.2** describes the mechanism of disease progression and complications.

Endotoxemia activates numerous immune cells. Immune cell infiltration, activation of KC and other macrophages, neutrophils, NK cells, NKT cells, dendritic cells, B and T cells leads to secretion of different cytokines. Macrophages and lymphocytes are activated and a 'cytokine storm' is generated causing inflammation. These cytokines activate multiple cytokine networks and signal transduction pathways which are deleterious to the cells. Many of these cytokines are capable of death pathway activation via STAT proteins or caspases. Activation of death effector molecules with concurrent tissue and organ damage worsens liver condition. The rate of hepatocyte death is much higher than the rate of liver regeneration and this imbalance is responsible for failing liver (Leist, Gantner et al. 1998; Luedde, Liedtke et al. 2002). Thus, the disease development and progression is due to inflammation.

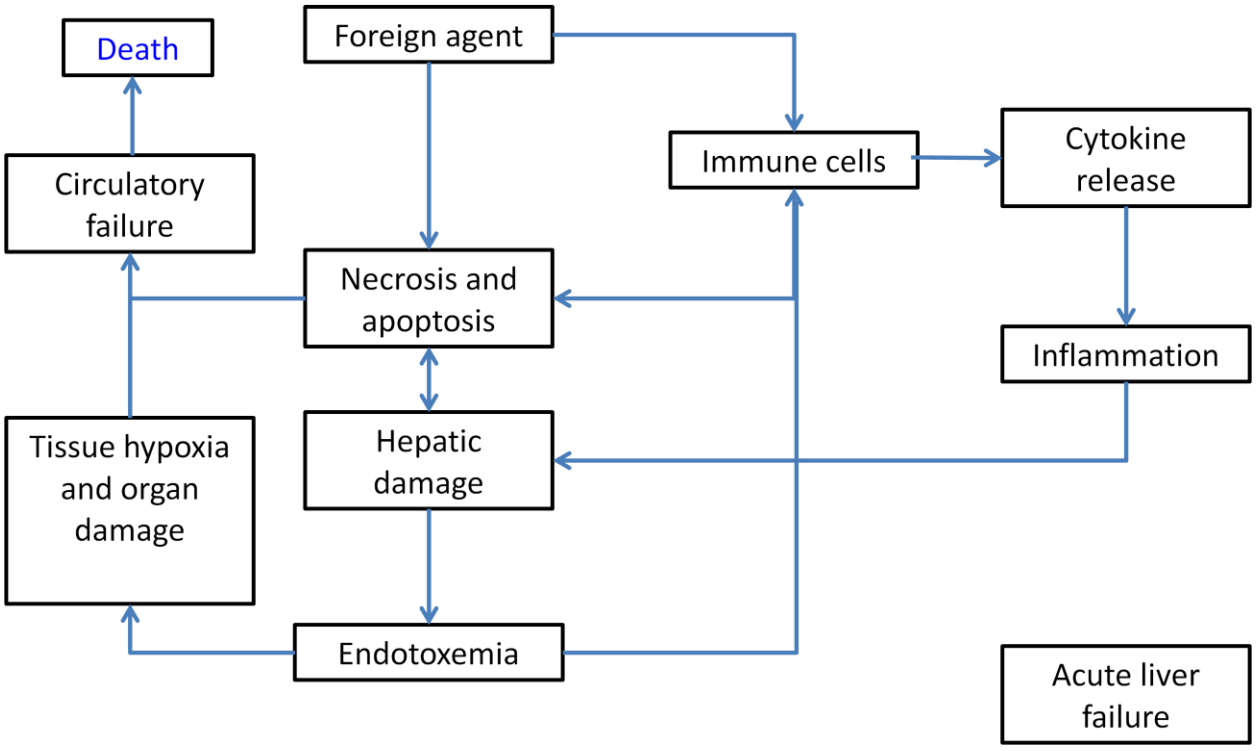

Figure 1.2. Flow chart showing disease mechanisms, symptoms and complications associated with acute liver failure. Foreign agents cause activation of immune cells which leads to inflammation. Inflammatory pathways in turn activate apoptotic and necrotic pathways in hepatocytes. The resulting hepatic damage causes other complications associated with acute liver failure. Other complications consist of circulatory failure, hepatorenal failure, tissue and organ hypoxia and encephalopathy.

Immune reaction and inflammation

Introduction

As mentioned above, the massive inflammatory response mounted against the liver is responsible for disease progression. Inflammation is the body's physiologic response to any kind of stress. Stress can be an infection, a chemical or biological antigen, trauma, tumor, burn, cold or an imbalance in organ function. First, inflammation occurs at the site of injury and thereafter spreads via the systemic circulation. Inflammation attracts various immune cells to the site of

injury for antigen removal, and thereafter healing is initiated. The inflammatory response is supposed to subside once systemic immune cells are involved. Thus, inflammation is supposed to be a rapid and a temporary response – it is a method for stressed cells or tissues to attract systemic attention and heal. However, during chronic inflammation various immune cells are recruited which in turn secrete various cytokines. The resulting uncontrolled, persistent and prolonged cytokine release – called a cytokine storm – causes irreversible damage to organs.

In the liver, any metabolic imbalance coupled with disturbances in liver function homeostasis lead to the induction of 'acute phase response' genes within liver. Acute phase response is a very characteristic response of the liver to infections and tissue injury. Acute phase response is supposed to be a beneficial process. During this response, the body aims to reduce liver metabolism. Drug metabolism is decreased and gluconeogenesis is increased. Glycogen stores are mobilized. Lipid storage is inhibited. Essential proteins are synthesized – complement factors, cytokines, fibrinogen are synthesized and albumin production is inhibited. Thus, metabolism is prioritized and stringently regulated.

The acute phase response involves modulation of proteins released by the liver such that synthesis of some proteins is decreased and some proteins are elevated. These proteins are synthesized and secreted by the liver and they in turn function to modulate the liver metabolism. These proteins are called acute phase proteins and are solely responsible for mounting the acute phase response. The acute phase proteins are induced during endotoxemia, acute inflammation, trauma and stress.

In ALF patients the acute phase response leads to uncontrolled inflammation, and some of the acute phase proteins (esp. cytokines) harm the host system. Acute phase protein synthesis and secretion is under the influence of cytokines TNF-α, IL-6 and IL-1 – which are amongst the

first cytokines to be secreted by Kupffer cells and hepatocytes. These cytokines are acute phase proteins and their production increases during tissue injury. The cytokines trigger synthesis of molecules such as C – reactive protein and mannose binding lecithin. These are functionally similar to antibodies but are non specific and thus, act against a broad spectrum of irritants. These proteins function to activate the complement pathway and opsonize pathogens for phagocytosis by macrophages. This process gives the host system some time to mount an elaborate and specific immune response. To describe inflammation in the context of liver failure we need to understand 1) the immune cells, and 2) the cytokines.

Liver resident immune cells

Kupffer cells (KC)

Kupffer cells are also known as Browicz – Kupffer cells. KC's are star shaped or pyramidal cells consisting of a large oval nucleus and a prominent nucleolus. They originate in bone marrow and then migrate to the liver where they develop with liver – specific – specialized functions. These cells are functionally distinct from the alveolar, splenic and peritoneal macrophages. These specialized macrophages are present in the lining of the liver sinusoid walls.

KC forms a bed of cells in sinusoid such that all blood passing through the liver comes in contact with these phagocytes. They are present in between or on top of the endothelial lining of the sinusoid capillaries with their star shaped extensions reaching out to liver parenchyma. Thus, they are in contact with the parenchyma as well as the sinusoid system of liver (Wisse, Braet et al. 1996). The liver is the most abundant source of macrophages and KCs form 20% of non-parenchymal cells (Naito, Hasegawa et al. 1997).

The main function of liver macrophages is phagocytosis of gut derived antigens. They help in red blood cell recycling. They are also responsible for maintenance of liver functions under normal physiological and pathological conditions. Kupffer cells aid in repair of tissue during liver injury. Their immune response includes migration to the site of inflammation, modulation of immune response, T cell activation and proliferation, and immune surveillance (Sun, Wada et al. 2003). They interact with neutrophils. They secrete cytokines and chemokines which attract other immune cells to a site of infection and mount an acute phase reaction. They can activate T cells via antigen presentation and at the same time also suppress T cell functions by secretion of various interleukins to down regulate immune response. The cytokines secreted by KC's are capable of modulating hepatocyte metabolism (i.e. down regulating drug metabolizing enzymes) during stress conditions (Monshouwer, Witkamp et al. 1996).

As mentioned earlier KC is the first line of defense against endotoxins present in the portal blood. These cells phagocytose toxins and eliminate them. In presence of foreign antigens or drug – induced toxins these cells go from a state of active tolerance to active immunity and produce cytokines such as TNF-α, IL-6, oxygen – derived free radicals and nitric oxides (Decker 1990; Laskin and Pendino 1995; Wang, Wang et al. 1999). These molecules are capable of initiating cell survival or cell death pathways.

Neutrophils (PMN)

Neutrophils are granulocytes (also referred to as polymorphonucelar neutrophils (PMNs)). PMNs are the most important part of the innate immune response. This cell type is most the abundant type of white blood cell (60% -70% of leukocyte (WBC) count). It has a characteristic three – five lobular nucleus.

PMNs are generated from various precursor cells in the blood. These precursor cells are generated in bone marrow. Mature PMNs have a very short life of 8 -12 hours, whereas non – active PMNs are viable in the peripheral systemic circulation for an average of 5.4 days (Pillay, den Braber et al. 2010). Active PMNs reach various tissues and can take up specialized functions in response to the type of infection.

PMNs are the first white blood cell response toward an infection. They are recruited within minutes to the site of inflammation and are considered to be hallmarks of an acute immune reaction. These cells are capable of rapid migration through the blood vessels and the interstitial tissue in response to chemotactic signals created by chemokines such as complement factor 5a (C5a) and leukotrine B4. PMNs arrive at the site of inflammation, engulf microbes, kill them by production of acids (such as HClO) and superoxides – this process is called respiratory burst – and finally the cell itself undergoes apoptosis, destroying pathogens within (Savill 1997; Kobayashi, Yamamoto et al. 2001). Hence, neutrophils are phagocytes with extremely toxic effects towards foreign as well host system. The programmed cell death at end of their function is an effective way of reducing their toxic effects. Another method for PMNs to destroy microbes/ infected cell is by secretion of granulocytes. The granulocytes consist of proteins such as collagenase, gelatinase, lysozyme, lactoferrin, myeloperoxidase, serine protease, elastase and cathepsins (Borregaard and Cowland 1997; Galligan and Yoshimura 2003). These granules aid in microbe death during phogocyotsis and when released can initiate apoptosis or attract more immune cells towards infection (Gregory and Wing 2002). Neutrophils are also capable of secreting various cytokines that activate the macrophages and attract other immune cells.

Natural Killer (NK) cells

NK cells are part of the innate immune system. Bone marrow derived lymphoid progenitor cells are capable of dividing into T cells or NK cells. These lymphocytes are also called large granular lymphocytes. NK cells are extremely cytotoxic, they can destroy tumor cells and virus infected cells by apoptosis. Since, they are toxic to host cells; their activation is very tightly regulated.

NK cells are activated by cytokines, Fc receptors and self expressing receptors. These cells are mainly activated by interferons and macrophage derived cytokines. The Fc part of antibody can bind to the Fc receptor expressed on surface of NK cell activating it (Trinchieri 1989). Other activating and inhibitory receptors are also expressed by NK cells which bind to various endogenous and exogenous ligands of target cells. Amongst the inhibitory receptors, major histocompatibility complex (MHC) class I plays a very important role. MHC I molecules are expressed on the surface of host cells and they are capable of inhibiting the cytolytic activity of NK cells.

NK cells function by inducing either apoptosis or osmotic cell lysis in target cells. These cells secrete granzymes – which consist of proteins perforins and proteases. Perforins make pores in cell membranes of target cells. The remaining enzymes and proteins of granzymes enter the cell through the aqueous channels formed and then induce apoptosis or cell lysis. NK cells also secrete cytokines and chemokines. Their response is modulated by the presence of interleukins such as IL-2, IL-4, IL-12, IL-15 and IL-18 (Peritt, Robertson et al. 1998; Deniz, Akdis et al. 2002). The interleukin-activated cells are called ‘mature NK cells’ and are classified as NK1, NK2 or cells resembling Th1 or Th2 (T helper subsets) cell type. This differentiation is based on the cytokines secreted by mature NK cell in response to inflammation.

Natural Killer T (NKT) cells

NKT cells are distinct from NK cells and T cells. They share the properties of both cell types and hence the name. They were first indentified in mice as a subset of T cells. These cells express the natural killer cell associated marker 1.1 and the α chain of T cell receptor (in mice) and the β chain of T cell receptor (in humans) (Lantz and Bendelac 1994). Hence, they are also referred to as NK 1.1 T cells in mice.

The receptors on NKT cells are capable of recognizing only the CD1d molecule (Bendelac, Lantz et al. 1995). The CD1d molecule belongs to the CD1 antigen-presenting family molecule and is presented by foreign lipids and glycolipids. NKT cells are not diverse but mainly function to secrete various cytokines – predominantly IFN-γ, TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF) and interleukins IL-2 and IL-4 (Bendelac 1995; Arase, Arase et al. 1996; Gombert, Tancrede-Bohin et al. 1996; Mendiratta, Martin et al. 1997) – to promote and propagate or inhibit the ongoing immune response.

Major cytokines involved in ALF

Introduction

Cytokines are small proteins, peptides or glycoproteins secreted by immune cells. These cell signaling molecules play an important role in intercellular communication. Multiple cytokines are secreted by immune cells. Cytokines can be secreted simultaneously or sequentially. Also, with presence of multiple immune cells, there is a diverse range of cytokines present at the site of inflammation. These molecules are critical modulators of inflammation. Their action is 'local' – i.e. they act at the site of production and, unlike immune cells, they do not travel very far. But, although these proteins are local and are produced in extremely minute

amounts, their effects are systemic. They are capable of forming a gradient such that other immune cells in the vicinity can be attracted and activated.

Cytokines act as ligands. They bind to their respective cell – surface receptors on other immune cells or target cells to produce an effect. These chemicals need to occupy only a fraction of their receptors to produce a massive biological effect. The cytokines perform multiple actions which are very specific. The cytokines can also activate gene transcription. Some of the gene products are pro – inflammatory and some lead to 'feedback inhibition' of the cytokine. Thus, the cytokines act synergistically or in an inhibitory manner. Some of the cytokines are functionally redundant – and hence their actions are additive. At the same time, functionally synergistic cytokines are limited by their receptors – the receptors of these cytokines are present in minute amounts, and hence receptor unavailability can have a negative effect on cytokine action. Cytokines are pleiotropic and this property plays an important role in their functional regulation.

These small molecules function by 1) attracting other immune cells to the site of infection (they are termed as chemokines), and 2) initiating signal transduction pathways in the target cells to produce a "cell fate" signal which can activate either cell survival pathways or cell death pathways.

Tumor Necrosis Factor -α (TNF-α)

Only TNF-α is discussed (other members of TNF super family are not included) here. TNF-α is the most pleiotropic cytokine. It affects all cells. It can cause growth, differentiation, survival, death and/or functional change. It is one of the first cytokines to be produced by cells. It is secreted by all hematopoietic cells (i.e. cells of immune system). It is also produced by other cell types – endothelial cells and some tissue cells. However, macrophages are believed to be the

main producers of TNF-α. Similarly, TNF receptors (TNFR) are present on all immune cell types and on cells of almost every tissue. TNF-α production is stimulated by pathogens, antigens and other cytokines. TNF-α can stimulate its own production from macrophages and other tissue cells. Its secretion and action are transient.

TNF-α is a non – glycosylated monomer of 17KDa. It is synthesized as a precursor protein of 233 amino acid residues. A 76 amino acid sequence is cleaved from amino terminus of the protein to activate it. The protein sequence also consists of a trans – membrane sequence of 18 residues which is 30 residues away from the amino terminus. After cleavage, the 157 amino acid peptide is secreted and it oligomerizes to form a trimer of 51KDa. A trimer of TNF-α then interacts with three receptors on a target cell surface, and this in turn leads to trimerization of the receptor complex. Ligand binding leads to aggregation and cross – linking of the receptor. Cross – linking of the receptor complex leads to its activation and initiates a downstream signaling cascade. There are two types of TNF receptors (TNFR) called TNFR1 and TNFR2. Signaling pathways initiated by TNFR1 and TNFR2 are shown in **figure 1.3 and figure 1.4**.

TNF-α is a cytotoxic and a cytostatic molecule for tumor cells (Sugarman, Aggarwal et al. 1985). TNF-α modulates a wide range of immune functions directly – by signal transduction and transcription of various genes – or indirectly – by promoting production and synthesis of other cytokines which in turn lead to activation of various signal transduction pathways. It induces synthesis of various chemoattractant molecules such as IP-10, KC and JE in specific cell types and tissues. It can stimulate the production of IL-6, IL-1, GM-CSF, M-CSF, G-CSF (other cytokines) and small pro – inflammatory molecules such as nitric oxide and prostaglandin E2 (PGE2) (Kilbourn and Belloni 1990). For the purpose of ALF we can categorize its functions as

1) effect of TNF-α on endothelium, 2) effect of TNF-α on PMN's and 3) effect of TNF-α on hepatocytes.

TNF-α effect on endothelium

Endothelium is a continuous monolayer of cells lining the inner wall of blood vessels. It is impermeable to solids and cells under normal physiologic conditions. Thus, it forms a barrier and is also the first cell type to respond to an inflammatory signal. The endothelial cells express high affinity TNFR's. TNF-α binding to its cognate receptors leads to changes in the actin cytoskeleton of cells. Cells contract and change morphologically. TNF-α stimulates endothelial cell growth and motility. Intracellular gaps are formed due to above mentioned changes. These gaps allow immune cells to leak into the intravascular space (Beutler and Cerami 1986; Tracey, Lowry et al. 1986). Secondly, TNF-α promotes local thrombosis. It causes a reduction in coagulation (Tracey, Beutler et al. 1986). Endothelium surface undergoes a change after TNF-α binding to its receptor, and as a result of which coagulation proteins are unable to assemble of its surface (Stern, Nawroth et al. 1986). Lastly, leukocyte adherence on the endothelial surface increases in the presence of TNF-α. Adhesion of white blood cells to the endothelium at the site of inflammation aids in increasing the diversity of immune response. Many of TNF-α effects on endothelium are enhanced by another cytokine IL-1.

TNF-α affects endothelial cell phenotype but does not affect its viability. These changes lead to modulation of permeability and coagulation. Thus, in ALF, TNF-α would exacerbate the coagulation defects and attract more white blood cells at the site of inflammation.

Effect of TNF-α on PMN's

As mentioned earlier, neutrophil recruitment is the first response to inflammation and it the predominant type of white blood cell. PMN's have many TNFRs on their surface.

Neutrophils migrate to site of inflammation and then they adhere to the endothelial surface. Diapedesis – i.e. movement of cells from blood to tissue – occurs through the endothelial membrane into the extracellular space. PMN's are activated upon contact with antigens. Thereafter, they remove the antigen and destroy themselves.

Neutrophil adhesion is increased by TNF-α. Adherence of neutrophils to the endothelium is directly proportional to the amount of TNF-α bound to its receptor (Gamble, Harlan et al. 1985). TNF-α alone cannot cause PMN migration. Other chemokines and cytokines (such as IL-8) required for chemotaxis are secreted by cells under the influence of TNF-α (Smith, Gamble et al. 1991). TNF-α activates neutrophils and can induce the respiratory burst leading to production of reactive oxygen species (Klebanoff, Vadas et al. 1986; Nathan, Srimal et al. 1989). It increases phagocytosis and primes them for 'antibody dependent cell mediated cytotoxicity' (Shalaby, Aggarwal et al. 1985). However, the above two effects are also modulated by the active oxygen species produced by respiratory burst. And so, these effects can be directly and indirectly attributed to TNF-α.

Thus, TNF-α is involved in all stages – from neutrophil recruitment to function. TNF-α in conjunction with G-CSF and GM-CSF has an effect on hematopoietic progenitor cells. It can directly modulate B-cell and T-cell production in the bone marrow and thymus. In ALF, the effects of TNF-α on neutrophils can be held responsible for necro-apoptotic death of hepatocytes.

Effect of TNF-α on liver

Since liver has the largest resident population of macrophages – kupffer cells – it produces abundant amounts of TNF-α. Small amounts of TNF-α transcript are detectable in normal hepatocytes due to pathogens present in the systemic circulation (Tovey, Content et al.

1988). Transcript levels increase sharply in response to tissue trauma. Since liver cells also produce acute phase proteins, hepatocytes can also produce TNF-α (which is an acute phase protein). In turn, TNF-α increases production of acute phase proteins. Thus, liver is a source as well as a target of TNF-α.

TNF-α's main effect on liver is to increase the synthesis of acute phase proteins (Mortensen, Shapiro et al. 1988). These effects are mediated directly and indirectly by it. TNF-α binding to its receptor induces transcription of genes which lead to synthesis of pro – inflammatory molecules. It leads to IL-6, IL-1, and complement C3, fibrinogen and fibronectin synthesis (Andus, Geiger et al. 1988; Bertini, Bianchi et al. 1988). The acute phase proteins and TNF-α in particular, reduce drug metabolism (Renton 1986). During stress, the capacity of liver to metabolize drug and certain substances is changed. Synthesis of proteins such as albumin is decreased and lipid metabolism changes. TNF-α reduces cytochrome P450 and dependent enzymes, thus directly decreasing drug metabolism (Ghezzi, Saccardo et al. 1986). TNF-α increases acetyl CoA carboxylase and fatty acid synthetase thereby directly increasing de novo fatty acid synthesis (Grunfeld, Verdier et al. 1988; Feingold, Serio et al. 1989). At the same time it inhibits lipoprotein lipase and decreases lipid storage (Enerback, Semb et al. 1988).

TNF-α binding to its receptors causes cytolysis of infected/ damaged liver cells. Cytolysis aims to prevent release of toxic liver products into circulation. TNF-α is a key regulator of leukocyte migration and adhesion to liver endothelial cells. It induces IL-8 secretion which increases neutrophil and leukocyte adhesion. TNF-α stimulates secretion of IL-1 and IL-6 and many of its effects are mediated via these two cytokines. Lastly, TNF-α is the most important factor in septic shock and is one of the cytokines capable of hepatotoxicity – i.e. hepatocyte death via necrosis and/ or apoptosis.

Interleukin – 6 (IL-6)

IL-6 belongs to a big group of cytokines (IL-6 super family) and only IL-6 has been described in this introduction. IL-6 is another pleiotropic cytokine produced by almost all immune cells and by some tissue cells. IL-1, TNF-α and bacterial endotoxin – LPS – can stimulate IL-6 production (Gauldie, Richards et al. 1987). It is not constitutively expressed and hence, stimulation leads to an increase in its transcript levels and protein synthesis. Macrophages and monocytes are the first and primary producers of this cytokine. Other cells, such as epithelial cells, endothelial cells, B – and T – cells, astrocytes and microglia secrete IL-6 under the influence of appropriate stimuli (i.e. in presence of IL-1/ TNF-α) (Frei, Malipiero et al. 1989; Luger, Schwarz et al. 1989; Hirano, Taga et al. 1990).

IL-6 is a 212 amino acid protein. It is glycosylated in two places and has four cysteine residues. It is synthesized as a precursor protein with a 22 amino acid leader sequence. The active form of IL-6 is a 185 residue peptide and has a mass of 21KDa. Monocytes are capable of secreting five isoforms of this cytokine with differing glycosylation sites and different molecular mass. IL-6 receptor is expressed in numerous tissues and immune cells, but very few or none are present on macrophages. Its receptor is a trans – membrane protein with an extracellular and an intracytosolic domain. The receptor itself has no kinase activity – i.e. it has no tyrosine kinase or serine/ threonine kinase activity. It associated with glycoprotein 130 (gp130) protein. gp130 is also a trans – membrane protein and it is present in all tissue and organs. Ligand binding causes receptor homo dimerization. gp130 dimerization activates tyrosine kinase, and subsequent phosphorylation of tyrosine residues occurs. The tyrosine residues are present on the intracellular domain of the protein and their phosphorylation is responsible for activation of downstream signaling cascade. The soluble IL-6 receptor is formed by cleavage of extracellular portion of IL-

6 receptor. The soluble receptor is capable of ligand binding and gp130 association. The effects of soluble receptor are also mediated by gp130. The soluble receptor has a regulatory function and is important for migration of IL-6 signaling. The biological functions of IL-6 are listed below.

Effect of IL-6 on immune response

IL-6 has an immunomodulatory effect on B cells, T cells, cytotoxic T lymphocytes (CTL) and NK cells. It promotes B cell maturation and differentiation (Kishimoto 1989). It does not affect B cell proliferation but increases IgM, IgG and IgA antibody production (Taga, Kawanishi et al. 1987). IL-6 induces differentiation of mature and naïve T – cells into CTL with the aid of IL-2. It also induces maturation of T – cells and CTL (Okano, Suzuki et al. 1989). IL-6, like TNF-α, primes PMN's for respiratory burst and increases NK activity (Luger, Schwarz et al. 1989). IL-6 is an inhibitor of TNF-α and IL-1 secretion (Schindler, Mancilla et al. 1990). Lastly, GM-CSF belongs to IL-6 super family. IL-6 is homologous to GM-CSF and can perform similar functions, as a result of which it can modulate hematopoiesis (Leary, Ikebuchi et al. 1988).

Effect of IL-6 on liver

IL-6 is an acute phase protein and it increases production of other acute phase proteins (Andus, Geiger et al. 1988). Like TNF-α, it reduces production of albumin and transferrin during inflammation (Andus, Geiger et al. 1988). It reduces drug metabolism and is a modulator of glycosylation of liver secreted acute phase proteins. One of the key impacts of IL-6 is its ability to modulate extracellular matrix integrity. It is known to affect matrix metalloproteinase (MMP) which degrades collagen. IL-6 increases TIMP 1 – a MMP inhibitor – production and causes an imbalance in MMP and TIMP 1 levels (Sato, Ito et al. 1990). IL-6 also increases α1 – antitrypsin – a protease inhibitor of neutrophil elastase. The signal transduction pathway of gp130 (**figure**

1.5) leads to activation of various cell survival and anti – inflammatory genes (Tilg, Dinarello et al. 1997). IL-6 is considered to be hepato – protective because of its above mentioned effects. However, increasing evidence of its toxicity is emerging.

Interferon – γ (IFN-γ)

IFN-γ is a potent cytokine. Its expression is very tightly regulated. It is produced by only T – cells and NK cells. It is produced by both CD8+ and CD4+ T cell types (Th0 and Th1 – T helper cells can produce the cytokine) (Celis, Miller et al. 1986; Mosmann, Cherwinski et al. 1986; Yamada, Meager et al. 1986). Antigen presenting cells bearing major histocompatibility complex - MHC class II bind to CD4+ T lymphocytes to stimulate IFN-γ. Secondly, MHC class I bearing target cells can initiate IFN – γ expression by binding to CD8+ CTL cells. Lastly, activated macrophages secrete TNF-α which activates NK cells and cause IFN-γ production. It has a minor antiviral activity. Its main function is immunomodulation.

IFN-γ is synthesized as 166 amino acid precursor protein with a 23 amino acid secretory signal. The cleaved 146 amino acid protein is glycosylated at 2 places. It forms a dimer and each subunit is of 20KDa or 25KDa. IFN-γ is distinct from IFN-α and IFN-β. This cytokine is secreted immediately after its synthesis.

IFN-γ receptor is present on all cell types except mature erythrocytes. The receptor consists of α and β chain and are identified as IFN – γR1 (α chain receptor) and IFN – γR2 (β chain receptor). R1 is glycosylated and is essential for ligand binding. It is a trans – membrane protein and it dimerizes in presence of IFN-γ dimer. Thereafter, R2 binds to R1. R2 is essential for receptor function. Ligand binding activates tyrosine kinases and recruits latent cytosolic transcription factors. IFN-γ mediates its effects by signal transduction via the above mentioned complex as shown in **figure 1.6**. IFN-γ signaling pathway leads to activation of genes that

ultimately determine the cell's fate. Additionally, transcription of early response and late response pro – inflammatory genes is initiated to propagate and sustain the immune response. Following sections describe the immunomodulatory effects of IFN-γ in detail.

Effect of IFN – γ on B and T – cell

IFN-γ inhibits B – cell growth. It specifically increases IgG2a production and decreases IgG2b, IgG1, IgG3 and IgG E production (Snapper and Paul 1987; Bossie and Vitetta 1991). It modulates T – cell growth and differentiation. Its growth-promoting effects on T cells are responsible for its cytotoxicity. IFN-γ is the only cytokine with an ability to regulate MHC class II genes (Blanar, Boettger et al. 1988; Amaldi, Reith et al. 1989). MHC class II genes need several hours for activation and are therefore involved in the late response of IFN-γ. MHC class II molecules are essential for functions of T cells, T helper cells, B cells and macrophages. Thus, this cytokine has long lasting effects. IFN-γ also stimulates MHC class I gene transcription. MHC class I and II molecules are essential for antigen presentation and subsequent activation of T cells.

Effect of IFN-γ on macrophages and NK cells

IFN-γ activates resting macrophages and NK cells. IFN-γ induced stimulation of macrophages elicits higher amount of TNF-α production – there is a fivefold increase in TNF-α production. Thus, the production of IFN-γ by NK cells leads to a positive feedback loop for TNF-α production (Unanue 1997). Macrophages secrete small amounts of TNF-α on antigen exposure. TNF-α activates NK cells in the vicinity. NK cells being part of innate immune system are capable of generating large amounts of IFN-γ which activate other macrophages to secrete large amounts of TNF-α and this initiates a cascade of TNF-α and IFN-γ. The resulting huge

amounts of TNF-α are responsible for cell toxicity and can cause TNF-α induced shock (e.g. sepsis and toxic shock).

TNF-α and IFN-γ synergistically act to increase the expression of adhesion molecules on endothelial cell surface to aid cell migration and amplify immune response. IFN-γ causes secretion of reactive oxygen species and hydrogen peroxide from macrophages, thereby destroying antigens. It also induces the production of nitric oxide synthetase (iNOS) in macrophages. iNOS is responsible for production of nitric oxide (NO). The anti – tumor, anti – viral and anti – parasitic actions of IFN-γ can be attributed to NO (Karupiah, Xie et al. 1993).

Signal transduction pathways

Signal transduction pathway initiated by TNF-α

TNF-α mediated signaling is not initiated by the ligand binding to its cognate receptors but it is initiated by receptor trimerization which follows ligand binding. Also as mentioned earlier there are two types of TNF-α receptors – TNFR1 and TNFR2. These receptors function together or individually for activation of different pathways. Receptor activation leads to activation of cell death or cell survival pathway.

Death signaling and caspase cascade

TNFR1 and Fas receptor (which belongs to TNF receptor super family) are the only TNF family receptors capable of initiating apoptosis. The cytoplasmic domain of these trans – membrane receptors consists of a 80 amino acid sequence called ‘death domain (DD)’. This sequence of amino acid has an interface for protein – protein interaction. Fas associated death domain (FADD) and TNFR1 associated death domain (TRADD) also contain ‘death domains’ which interact with receptor DD. TRADD binds to DD of TNFR1. Thereafter, FADD binds to

the DD of TRADD or it binds to DD of Fas receptor and the resulting molecular complex recruits various caspases. FADD has a death effector domain (DED). Caspases with DED motifs are cleaved by FADD triggering their activation. Caspase-8 has two intrinsic DED which leads to cleavage of pro-caspase-8 and its subsequent activation.

Active caspase-8 can directly activate the caspase cascade. Also, caspase-8 can activate mitochondrial death pathway by cleavage of protein called p22 Bid to form p15 Bid. p15 Bid which translocates to the mitochondrial membrane and induces release of cytochrome C (*Cyt C*). *Cyt C*, apoptotic protease activating factor – 1 (Apaf -1) and pro-caspase 9 form a complex called "apoptosome" which cleaves pro-caspase 9 to form caspase 9. Active caspase 9 cleaves other pro-caspases and initiates the caspase cascade. By either method, activation of death effector caspases such as caspase-3 occurs which induces cellular apoptosis. TNFR1 and Fas receptor stimulated death pathways are shown in **figure 1.3**.

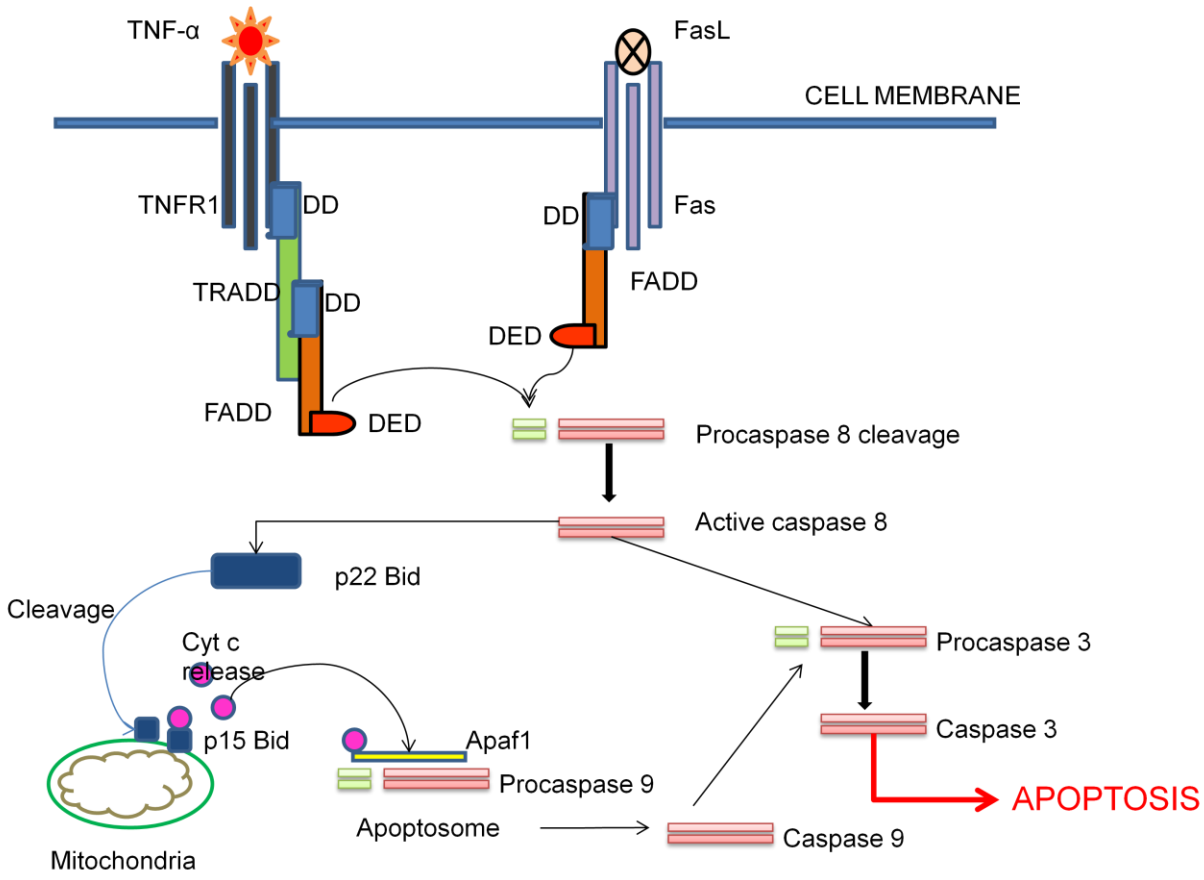

Figure 1.3. Cell death pathway activated by TNF. The signal transduction pathway leading to cell death are activated via TNFR1 and FasL. Death domains of recruited Fas associated death domain (FADD) and TNFR1 associated death domain (TRADD) directly activates caspases or via mitochondrial death pathway to initiate apoptosis.

Cell survival signaling

TNF-α induces cell survival via activation of the NF-κB pathway as shown in **figure 1.4**. TNFR1 and TNFR2 are involved in activation of cell survival pathway. Receptors trimerize in presence of TNF-α. TRADD binds to active TNFR1 complex and thereafter, TNF receptor associated factor (TRAF2) binds to TRADD. TRAF2 can bind directly to TNFR2. The receptor and TRAF2 complex (with or without TRADD) phosphorylates NF-κB inducing kinase (NIK) and activates NF-κB (nuclear factor kappa light – chain – enhancer of activated B cells). Inactive NF-κB is associated with the inhibitory protein IκB kinase (IKK) as a heterodimer complex. IKK

is made up of IKKα and IKKβ subunits. Active NIK phosphorylates IκB – α. This leads to its proteosomal degradation. Remaining IKK complex dissociated from NF-κB and it becomes free to translocate to the nucleus and initiate transcription of cell survival genes.

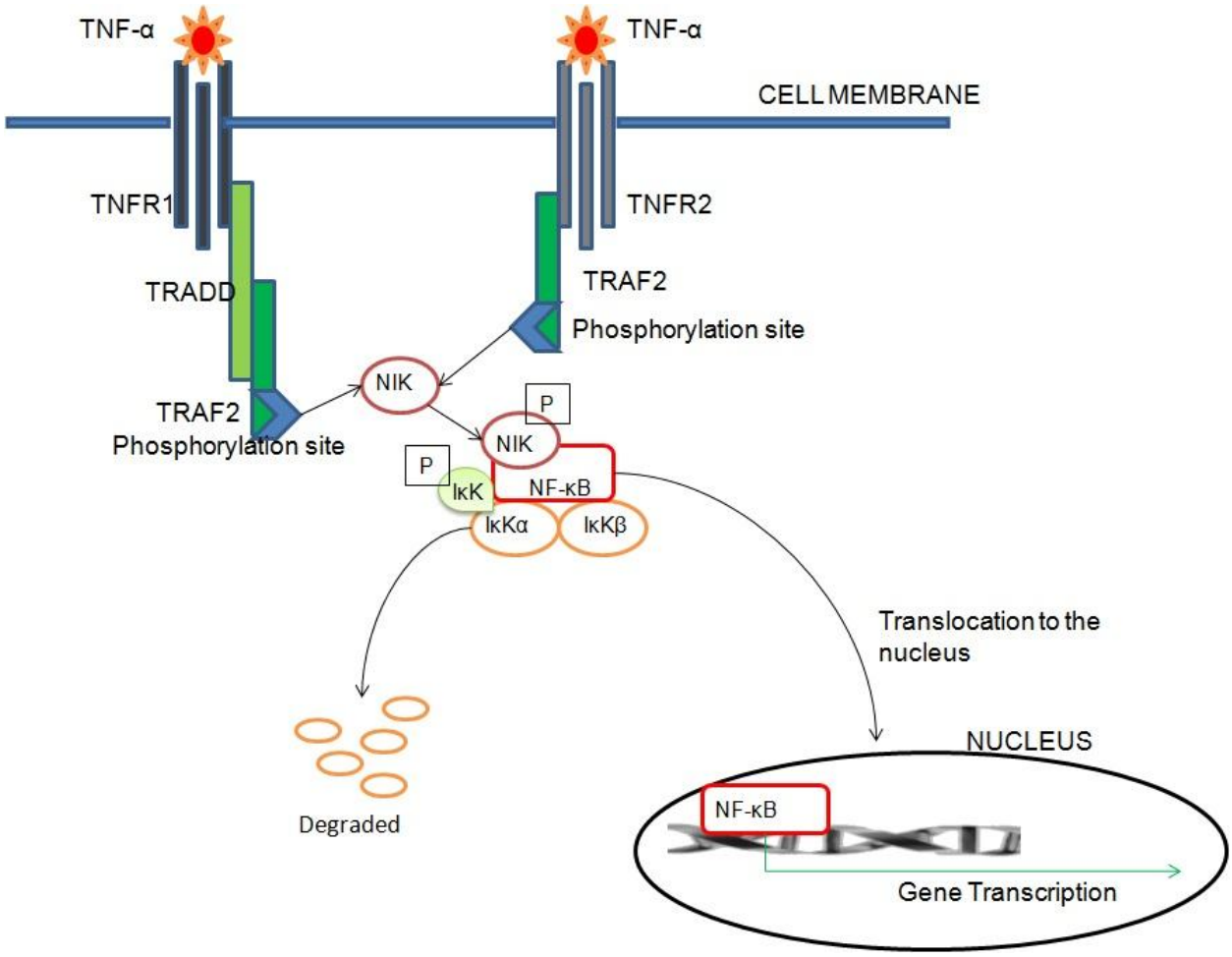

Figure 1.4. The signal transduction pathways leading to cell survival are activated via TNFR1 and TNFR2. TNF receptor associated factor (TRAF2) poshporylates proteins of NF-κB pathway. Activated NF-κB translocates to the nucleus and activates gene transcription of cell survival and anti-inflammatory genes.

Signal transduction pathway initiated by IL-6

The signaling pathways of IL-6 are activated via the gp130 molecule's association with IL-6 receptor. gp130 is capable of recruiting Janus kinase family members (JAK). The tyrosine

kinases of JAK family (JAK1, JAK2 and TYK) phosphorylate various tyrosine residues on gp130. Thus, activated domain of gp130 is responsible for activation of transcription factors – the STAT proteins. STAT's in turn are responsible for transcription of IL-6 response element genes. STAT – signal transducers and activators of transcription – are transcription factors which regulate various aspects of cell cycle. STATs bind to promoters of cytokine inducible genes and initiate transcription of cytokines/ proteins that lead to cell survival, death, differentiation or proliferation.

gp130 homodimerizes, and its interaction with JAK leads to its association with STAT family members. The tyrosine residues on latent cytosolic STATs are phosphorylated. Activated STAT isoforms form homodimers or heterodimers. STAT dimers translocate to the nucleus, bind to target DNA and activate transcription. STAT's are well characterized initiators of apoptosis. However, IL-6 response element binding protein causes the recruitment of acute phase response factor STAT3. STAT3 homodimerization or heterodimerization with STAT1 activates genes that are predominantly anti-apoptotic. The mechanism of IL-6 associated signal transduction pathway is shown below in **figure 1.5**.

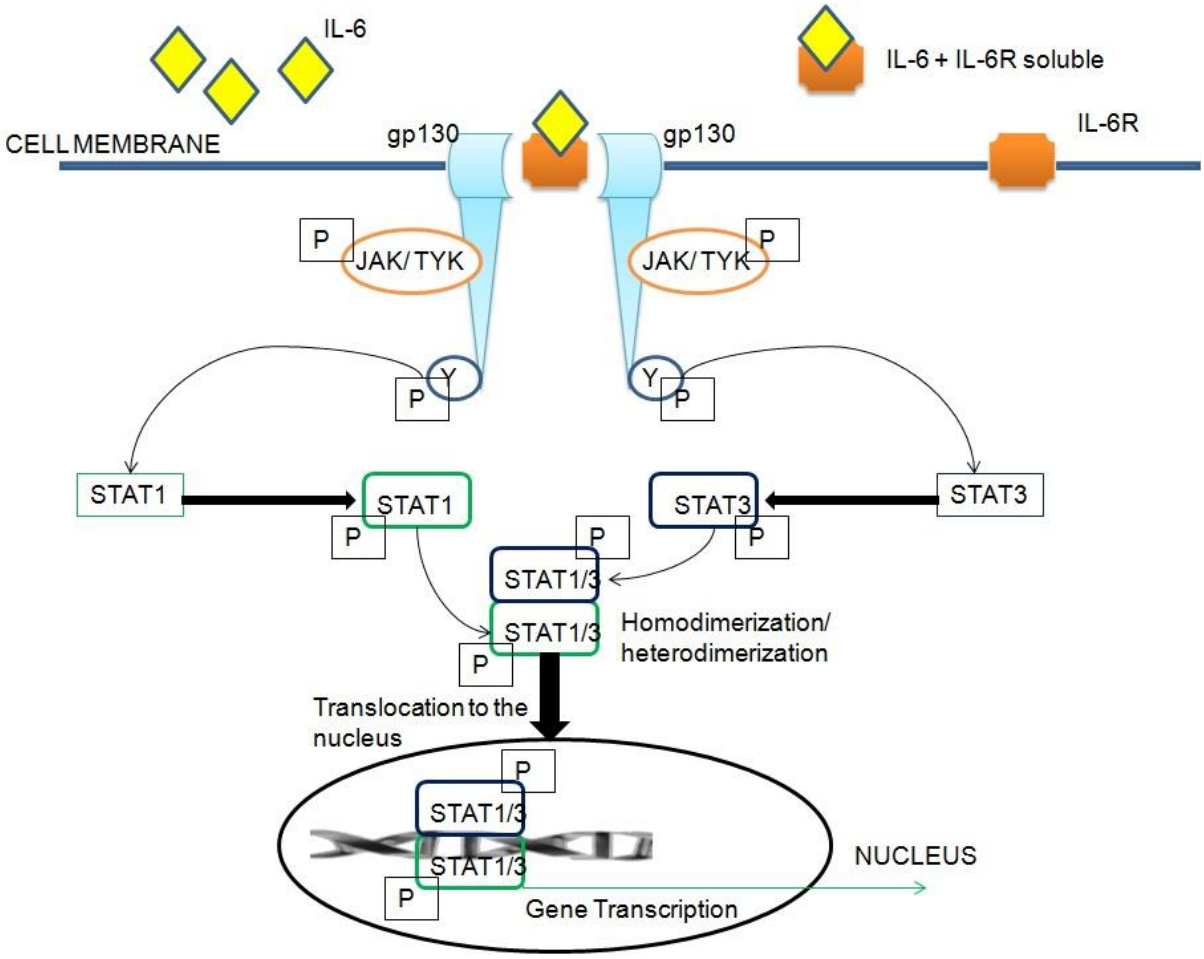

Figure 1.5. Signal transduction pathways activated by IL-6. IL-6 binds to gp-130 receptors. gp-130 phosphorylates Jak proteins and thus activates STAT1 and STAT3. Homodimers and heterodimers of STAT1 and 3 translocate to nucleus and initiate gene transcription.

Signal transduction pathway initiated by IFN-γ

IFN-γ binding to its receptor activates two tyrosine kinases – JAK1 and JAK2. This receptor is unique because it requires both the α and β chain of receptor for function. The α chain receptors bind to IFN-γ and dimerizes. JAK1 is recruited near the receptor – one for each subunit of the α chain of receptor dimer. JAK2 binds the β chain receptor and gets associated with JAK1.

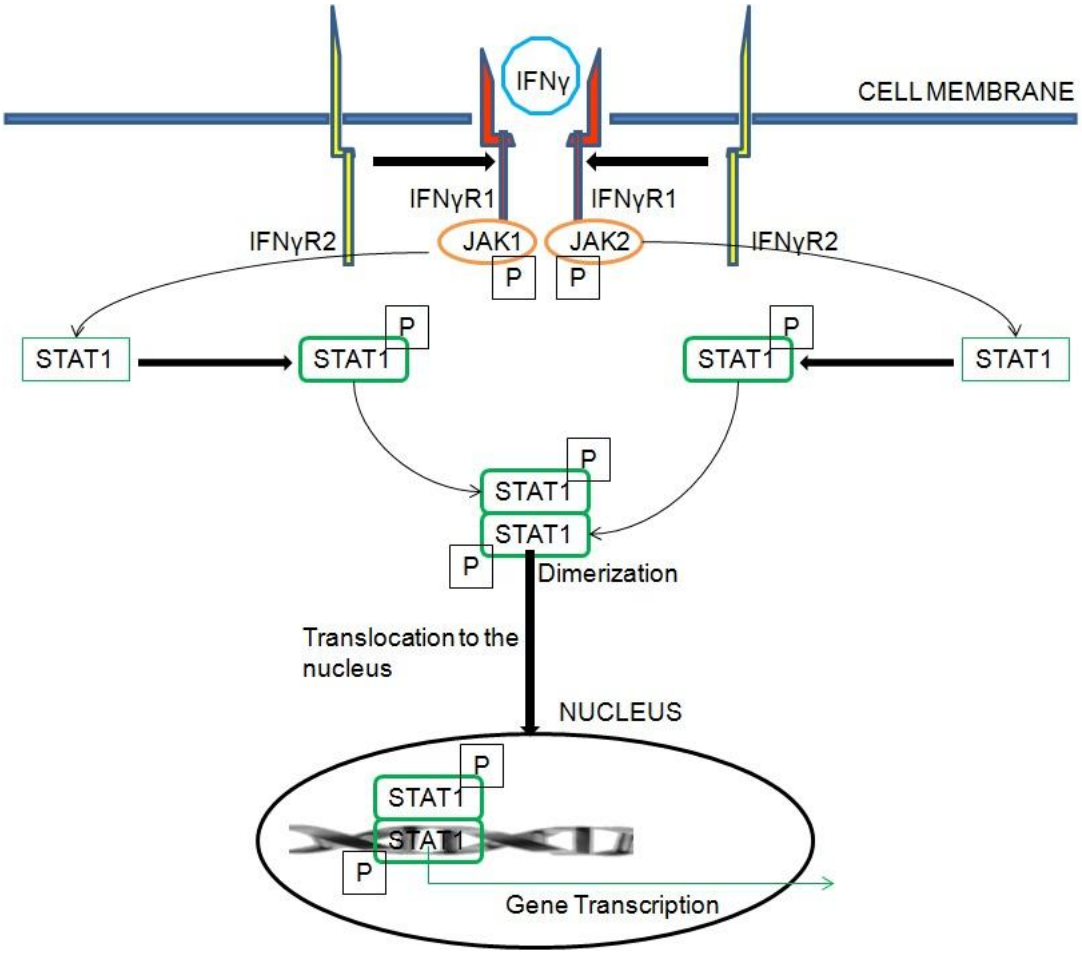

Figure 1.6. IFN-γ activated signal transduction pathways leads to activation of STAT1. IFN-γ binding to receptor IFNγR1 leads to recruitment of JAK1. Then IFNγR2 then associated with IFNγR1 and JAK2 associated with IFNγR2. JAK activate STAT1 and STAT1 homodimers translocate to cell nucleus and bind to Interferon-γ activated sites (GAS) promoters on DNA. GAS promoter initiate transcription of IFN-γ induced genes, several of which induce apoptosis.

Once the αβ receptor chains are in close proximity JAK1 and JAK2 form an active complex which is capable of activating STAT's. IFN-γ is known to specifically activate STAT1.

Two cytosolic STAT1 molecules dock at receptor associated JAK phosphorylation site. The JAKs subsequently phosphorylate STAT1 proteins. STAT1 then dissociates from JAK complex and forms homodimers which translocate to the nucleus. A schematic representation of

IFN-γ and STAT1 pathway is shown in **figure 1.6**. STAT1 binds to interferon-γ activated sites (GAS) on DNA. The Interferon-γ activated sites (GAS) are promoter elements on DNA that initiate transcription of IFN-γ induced genes. Amongst different gene products, STAT1 functions to increase transcription of apoptosis inducing proteins.

Conclusion

ALF – acute liver failure – is a condition wherein multi organ failure follows liver injury. Medical conditions such as encephalopathy, hepatorenal failure, cardiovascular overload and pulmonary complications result from failing liver and are responsible for ALF associated mortality. The main reason for deterioration of patient condition is sepsis and generation of an uncontrolled immune response. Inflammation coupled with acute phase reaction leads to generation of cytokines which reduce liver function, cause a metabolic overload and harm liver and other tissues by activating apoptosis in cells.

Immune response is central to disease progression in ALF. Resident immune cells – KC, NK and NKT cells – recruit other immune cells (such as PMN's, B – cells and T – cells) during inflammation. KC, NK, NKT and PMN's phagocytose pathogens and thereby remove antigens from liver. They secrete cytokines and chemokines to attract other immune cells. These cells secrete various cytokines, key amongst which are TNF-α (secreted by KC, NK and hepatocytes); IL-6 (secreted by KC) and IFN-γ (secreted by NK, NKT and T – cells). Thereafter, cytokines activate gene transcription of cell survival or cell death proteins via various signaling transduction pathways.

It is important to understand the immune mechanisms involved in acute liver failure. Disease pathology is the direct result of complicated immune network reactions in ALF. Solving

this network to identify targets which could be used therapeutically is essential to developing treatments for ALF. The results of these studies might also be applied to other diseases where the immune system harms host organs by excessive and uncontrolled cytokine production.

CHAPTER 2

Glutamine synthetase as a potential therapeutic target and use of methionine sulfoximine to understand mechanisms involved in progression of acute liver failure

The work presented in this chapter was first published as and in:

Methionine sulfoximine, an inhibitor of glutamine synthetase, lowers brain glutamine and glutamate in a mouse model of ALS.

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Introduction

Acute liver failure (ALF) mouse model

Liver injury resembling acute liver failure (ALF) can be induced in animals by administration of low doses of *E. coli* lipopolysaccharides (LPS) with the sugar D-galactosamine (D-GalN; 2-amino-2-deoxy-D-galactose). This model mimics immunological and biochemical

symptoms of ALF. D-GalN was initially administered as six sequential intraperitoneal, intravenous and/or subcutaneous injections (Decker and Keppler 1972). Later, a single injection of high dose of D-GalN was found to be equally effective in causing liver damage (Scharnbeck, Schaffner et al. 1972). Injection of low dose of LPS along with D-GalN is essential for the injury to be liver specific.

Keppler and Decker initiated a detailed study on the toxicity of galactosamine on liver. Morphologic and biochemical changes occurring due to non-lethal doses of D-GalN showed that liver functions are impaired and protein synthesis was inhibited. Hepatic glycogen stores decreased and various hepatic function marker enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST) were elevated. Total billirubin values were also higher than normal (Decker and Keppler 1972). These markers are indicators of liver health and liver functions. Enzyme changes observed in the animal model closely resembled the liver injury symptoms of ALF patients.

D-GalN is a hepatotoxic agent. LPS administration along with D-GalN causes generation of an immune response characteristic of this disease. Thirty minutes post disease induction, nucleolar fragmentation was observed. Amino acid uptake was unaffected but protein synthesis was inhibited. Changes occurred in the polyribosomal profile (Shinozuka, Farber et al. 1973). Liver readily takes up D-GalN and metabolizes it. During metabolism of D-GalN, UDP – GalN is formed by the non-specific enzyme galactose - 1 – phosphate: UDP – glucose uridyltransferase. UDP – GalN cannot serve as an uridylate donor during translation. Continuous formation of UDP – GalN traps uridine phosphates and depletes the supply of uridilylate donors such as UTP, UDP and UMP. Thus, RNA synthesis was inhibited. Lactate – pyruvate and ATP – ADP ratios were found to be normal. However, adenine nucleotide levels were reduced (Decker

and Keppler 1974; Konishi, Shinozuka et al. 1974). Further elucidation of this model has indicated that morphologic liver changes occur after RNA synthesis inhibition.

High doses of LPS alone (in rodents) cause massive inflammation and release of cytotoxic molecules leading to severe sepsis and multi – organ failure. However, the use of a low dosage of LPS with D-GalN leads to the generation of an immune response which is predominantly directed towards liver. Initially, other organs are not affected. Later, multi organ failure occurs. The damage is limited to liver and can be completely reversed by administrating uridine within 3 hours of disease induction (Galanos, Freudenberg et al. 1979). Thus, LPS/ D-GalN model of liver failure mimics the hepatotoxicity observed during liver injury. This model is not specific to any type of drug overdose, but its pathogenesis and symptoms closely resemble those of broad – spectrum liver diseases.

Glutamine Synthetase

Glutamine synthetase (GS, also known as γ-glutamyl: ammonia ligase; EC 6.3.1.2) is an important enzyme in nitrogen metabolism. This enzyme binds glutamate and ammonia in presence of divalent cations (such as magnesium and manganese) and adenosine triphosphate (ATP), and converts it to glutamine (Eisenberg, Gill et al. 2000). The reaction is shown in **figure 2.1**. GS is the only enzyme in body for synthesizing glutamine.

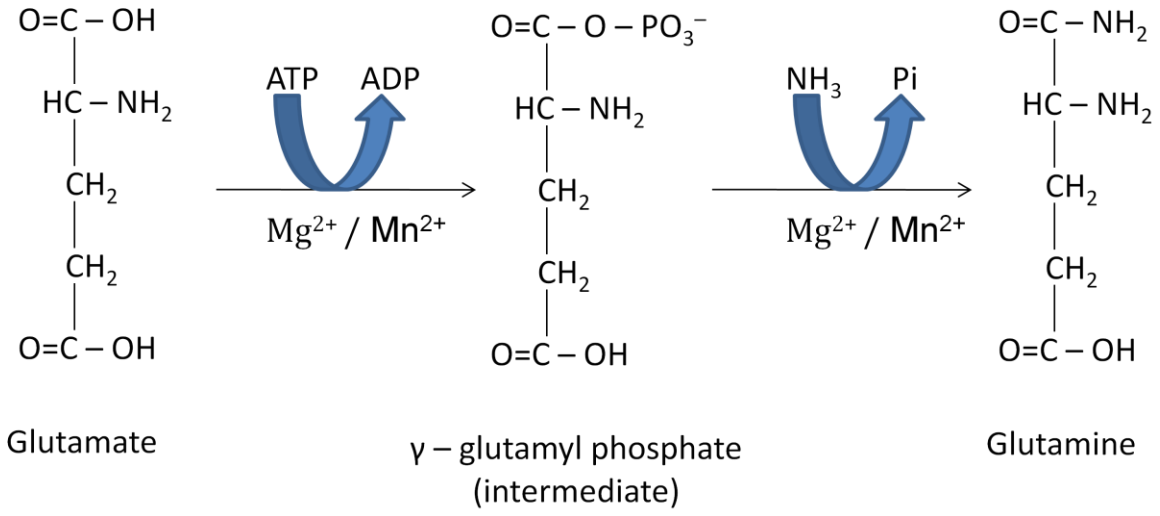

Figure 2.1. Glutamine synthetase catalyses reaction between glutamate and ammonia to form glutamine. γ-glutamyl phosphate intermediate is formed during the reaction. ATP and magnesium ions are required for this reaction to proceed.

There are three types of GS and each type is structurally different. GS type I and III are found in prokaryotes, but eukaryotes mainly express type II GS (Eisenberg, Gill et al. 2000). Each type of GS consists of two active sites – one for an amino acid and the second for nucleotide (Hibi, Nii et al. 2004). The amino acid binding site is highly preserved, whereas the nucleotide binding site varies across organisms. Additionally, there are three metal ion binding sites for enzyme stability and catalytic activity (Pesole, Gissi et al. 1995). Mammalian GS is homologous across species in terms of amino acid composition, structure and molecular weight. One key feature of this enzyme in mammals is that it is not modified post-transationally, as is the case for the *E. coli* glutamine synthetase (Stadtman, Shapiro et al. 1968; Stadtman 2001).

In mammals, GS is made up of 12 identical subunits of 50KDa each (Meister 1985). These subunits are arranged in two identical hexagonal structures that face each other. The rings are composed such that a channel is formed in each subunit. The wall of this bi – funnel (one ring in each subunit) is made up of six four – stranded β sheets. The β sheets are arranged to

form anti – parallel loops within the subunit structure. Hydrogen bonding and hydrophobic interactions hold the subunits together such that the C-terminus of GS is inserted into the hydrophobic core of the ring shaped structure. The hydrophilic N-terminus remains exposed to solvent. A cartoon of enzyme structure and its substrate binding sites is shown in **figure 2.2**.

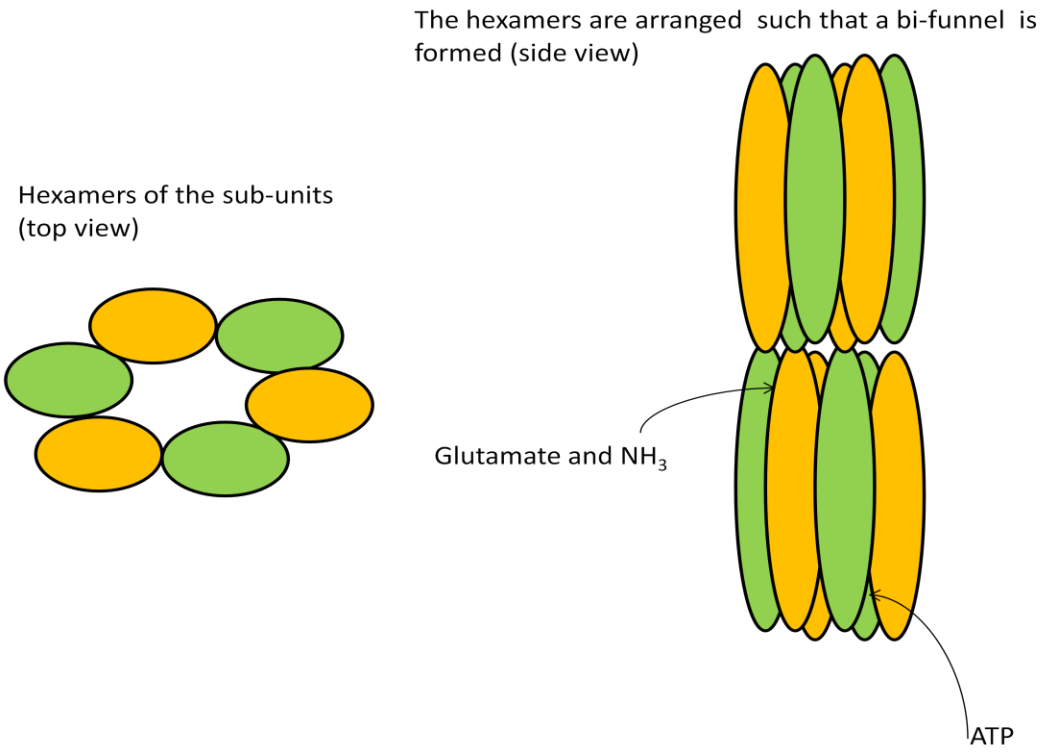

Figure 2.2. A cartoon of glutamine synthetase structure is shown above. GS is made up of 12 identical sub units arranged as two hexamers. The hexagonal structure is made up of anti parallel β – sheets. A bi-funnel is formed within the hexagon. The active site of enzyme is in the bi-funnel and substrate binding site is within it. ATP binds to the active site on the top and glutamate and ammonia bind to the active site on the bottom of the bi-funnel.

Each active site is in the bi – funnel. The active site consists of distinct substrate binding pockets. ATP binds to the top of the funnel structure and is on the external surface of GS. Glutamate and ammonium bind at the bottom of the active site. Two sites for binding of divalent cations are present inside the funnel. These cations stabilize the structure and carry out transfer of phosphate from ATP to glutamate. One cation binding site is in proximity to each of substrate

binding site. The reaction takes place via formation of ADP – γ glutamyl phosphate complex. First ATP is hydrolyzed and phosphate transferred to glutamate to form an acyl intermediate of ADP and glutamate. ADP and Pi do not dissociate from the enzyme until ammonia binds to the carboxy group of glutamate. ADP stabilizes the γ glutamyl phosphate bound form of enzyme via conformational changes. This promotes ammonium ion binding to the polar solvent exposed binding site of the enzyme. Thereafter, ammonium attacks the ADP – γ glutamyl phosphate complex to form glutamine. The resulting ADP and glutamine then leave the complex.

GS can also carry out other reactions. If hydroxyl amine binds to GS instead of ammonium, γ – glutamylhydroxamate is formed. This reaction is exploited to assay GS activity in tissue extracts. The concentration of ammonium determines the fate of the acyl intermediate. There is a substrate binding competition between ammonium and water molecule. If water enters the active site, the hydroxyl group attacks the intermediate to reform glutamate. This ω-amidation reaction is reversible and the enzyme is tightly regulated by its substrates, products and other amino acids which are synthesized from glutamine. GS exhibits cumulative inhibition. i.e. each inhibitor reduces the activity of the enzyme, even when other inhibitors are bound to the enzyme at saturating levels . Glutamine formed by GS is a source of nitrogen in the biosynthesis of tryptophan, histidine, carbamoyl phosphate, glucosamine-6-phosphate, cytidine triphosphate and AMP. GS is negatively regulated by all the above mentioned products and also by alanine and glycine.

GS is present in various tissues – mainly liver, brain and kidneys. Brain and liver isoforms of GS can be octamers. Muscle, lung and immune cells also express GS to fulfill their glutamine requirements and to convert ammonia into a non – toxic form for transport to liver.

GS and liver

The distribution of GS in liver is unique. GS is a cytoplasmic enzyme in liver. It has homogenous distribution within a cell which expresses it. Glutamine Synthetase is expressed exclusively in a small sub population of pericentral hepatocytes (PC). GS is present only in the PCs which surround the central veins in the liver – hence, it is termed as a perivenous enzyme (see **figure 2.3**). Urogenesis (urea formation or ureagenesis) and glutamine synthesis - i.e. ammonia detoxification pathways are compartmentalized in liver. These two functions are done by upstream periportal (PP) and downstream pericentral (PC) hepatocytes respectively (Gebhardt, Baldysiak-Figiel et al. 2007). GS zonation leads to an unequal distribution of function within the hepatocytes. 94% of zone 1, 2 and 3 hepatocytes express enzymes involved in urea formation. 8% of cells function to synthesize glutamine and express GS. 2% overlapping cells can perform both functions (Gebhardt, Lindros et al. 1991). This segregation of enzymes and function enables the liver to efficiently remove ammonia from portal circulation. At the same time, liver plays a major role in glutamine homeostasis by removing or adding glutamine to the blood circulation (Watford, Chellaraj et al. 2002).

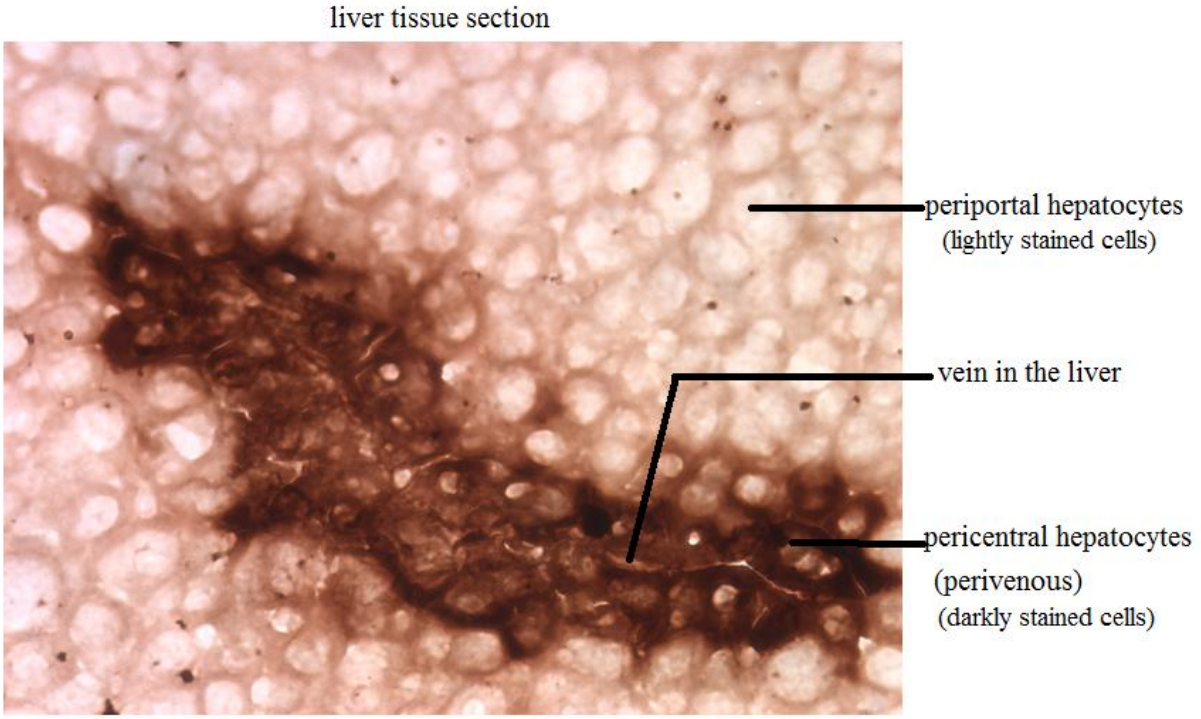

Figure 2.3. Glutamine synthetase distribution in liver tissue. Immunohistochemically stained tissue for liver glutamine synthetase is seen here. The tissue section shows distinct cells. The lightly stained cells are periportal hepatocytes (upstream cells) which do not express GS. In the center of this section a small venule is observed. Cells surrounding the vein are pericentral hepatocytes (downstream cells). The downstream cells express GS and these are called perivenous hepatocytes.

The liver is not a major producer of glutamine, but liver GS has the highest specific activity amongst all organs due to a high glutamine turnover (Cooper, Nieves et al. 1987; Gebhardt and Reichen 1994). This high GS specificity coupled to excessive glutamine (i.e. substrate) turnover occurs due to the "feed – forward – activation" of the glutaminase enzyme. In PP cells, glutaminase breaks down glutamine (Haussinger 1990., Low et al., 1993) and glutamate is released into the blood. Simultaneously, ammonia is continuously fixed into carbamyl compounds by upstream hepatocytes. As blood passes downstream, the portal ammonia concentration declines such that urea formation (urogenesis) can no longer occur effectively. At

this point, the downstream PCs scavenge the remaining ammonium ions to synthesize glutamine. The schematic of glutamine – glutamate cycle occurring in hepatocytes is shown in **figure 2.4**. This newly synthesized glutamine overcomes the loss occurred during glutamine breakdown by PPs, thus, maintaining plasma glutamine levels of 0.8mM in the portal circulation. This hypothesis was supported by the work of Geerts et al. They showed that glutamate concentration decreases in blood collected from upstream to downstream sections of liver but is restored to its normal levels at the end point of portal circulation (Geerts, Jonker et al. 1997). Thus, the nitrogen metabolism and glutamine homeostasis is closely interrelated by GS in liver.

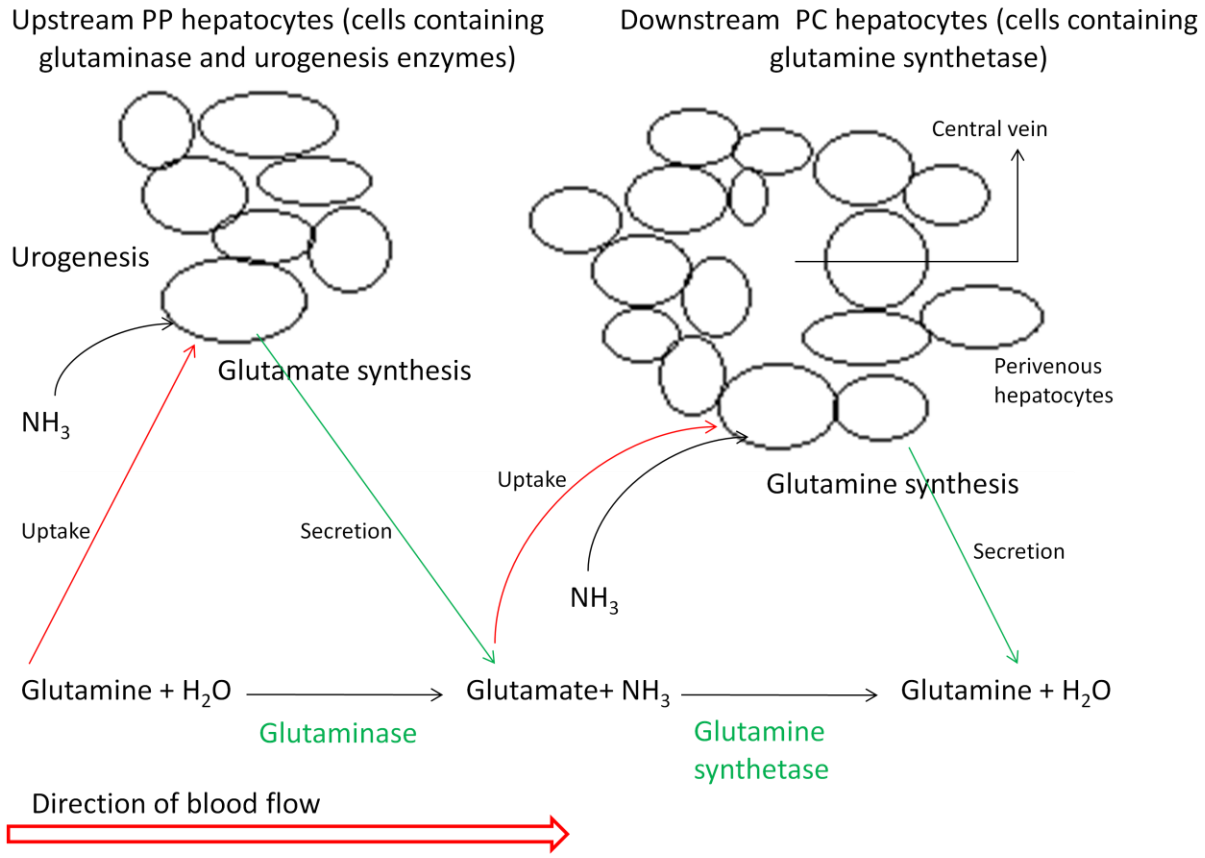

Figure 2.4. A schematic diagram of glutamine – glutamate cycle in liver is shown above. Upstream hepatocytes contain enzymes for urea formation and glutamine breakdown. They take up glutamine, break it down to form glutamate and release it into the blood stream. After urea formation, residual ammonia is scavenged by downstream hepatocytes containing glutamine synthetase. Glutamate from blood stream is also taken up by these downstream pericentral

hepatocytes and is combined with ammonia to form glutamine. Glutamine formed is released back into blood stream.

Glutamine Synthetase as a potential drug target:

GS is being increasingly studied in immunology due to the immune cell's requirement of glutamine for cell proliferation, differentiation and cytokine production (Karinch, Pan et al. 2001; Newsholme 2001). It is well established that glutamine levels influence macrophage function and cytokine release in tissue culture, and long-term dietary changes can modify immune function in living animals (Newsholme 2001; Li, Yin et al. 2007; Roth 2007). A detailed study of the effects of glutamine concentrations on T cell differentiation has divided the response into an early glutamine-independent phase and a later glutamine-dependent phase (Hörig, Spagnoli et al. 1993). Also, dietary glutamine has been known to enhance cytokine production in macrophages and increases T cell responsiveness (Kew, Wells et al. 1999; Rogero, Tirapegui et al. 2008).

Interestingly, only 5% of the hepatocyte population contains glutamine synthetase (Gebhardt and Mecke 1983; Häussinger, Stoll et al. 1989). 9% of the liver GS activity is localized in kupffer cells. Also, kupffer cells constitutively express GS and possess higher GS mRNA levels as compared to parenchymal liver cells (Bode, Peters-Regehr et al. 2000).

Lastly, as mentioned in the introduction, hyperamonemia is observed in ALF patients. Due to failing liver and dying hepatocytes, imbalance in ammonia metabolism occurs. Excess ammonia produced in liver is taken up by astrocytes and converted to glutamine by GS enzyme. Astrocytes then swell up due to glutamine accumulation. This causes an increase in intracranial pressure in brain stem leading to brain edema (Takahashi, Koehler et al. 1991). The hyperammonaemia resulting brain edema in rats is similar to brain swelling during hepatic

encephalopathy of ALF patients (Groflin and Tholen 1978). In a rat model of hyperammonaemic encephalopathy, brain swelling is completely prevented by GS inhibition (Takahashi, Koehler et al. 1991; Brusilow 2002; Tanigami, Rebel et al. 2005).

MSO

L-Methionine Sulfoximine (MSO) is an irreversible inhibitor of Glutamine Synthetase (GS). MSO is one of the most potent GS inhibitors with as Ki of 1.1µM. The ability of MSO to inhibit GS was first reported in 1952 (Pace and Mc 1952). It also inhibits γ-glutamylcysteinesynthetase, the first step in glutathione synthesis. MSO inhibits γ-glutamylcysteinesynthetase at concentration of 0.2-2.0 mM.

MSO is a modified form of the amino acid methionine **(figure 2.5)**. Mechanistically, MSO competes with glutamate for binding in the amino acid pocket of GS. In the presence of ADP and Mg^{2+}, enzyme phosphorylates MSO. The phosphorylated form of MSO – methionine sulfoximine phosphate binds irreversibly to the enzyme and inhibits it (Ronzio, Rowe et al. 1969). Phosphorylated MSO **(figure 2.5)** stabilizes the flexible loop structure in the active site of the enzyme and blocks glutamate entry. Thus, the non-covalently bound form of MSO phosphate forms a transition state mimic for the tetrahedral adduct of glutamate which is formed during glutamine biosynthesis. Phosphate ion, ammonia and glutamate in high quantities compete with MSO for the enzyme binding site (Rowe, Ronzio et al. 1969). Close to eight moles each of MSO and ADP are required for GS inhibition (Ronzio, Rowe et al. 1969). Also, only the L-S-isomer of MSO is able to inhibit the enzyme and is phosphorylated by GS (Manning, Moore et al. 1969; Ronzio, Rowe et al. 1969; Rowe, Ronzio et al. 1969; Meister 1985).

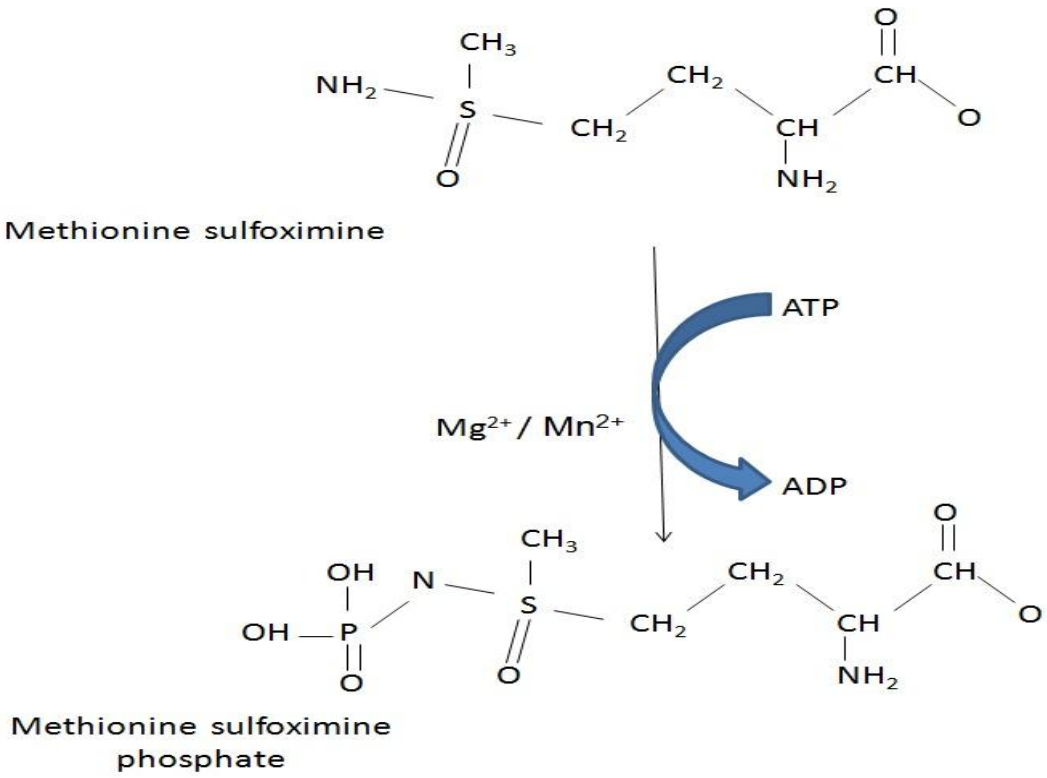

Figure 2.5. Structure of methionine sulfoximine and methionine sulfoximine phosphate. Methionine sulfoximine (MSO) is an irreversible inhibitor of glutamine synthetase (GS). GS in presence of ATP and magnesium binds to MSO and phosphorylates it. Methionine sulfoximine phosphate binds GS non-covalently to inhibit it.

Methionine Sulfoximine as a therapeutic treatment:

There were two reasons for using MSO as an inhibitor of GS to study ALF. 1) MSO is the most potent inhibitor of GS. Being a modified amino acid, it can cross the blood brain barrier – as has been shown by its ability to prevent brain swelling resulting from hyperammonaemia. 2) Liver GS inhibition by MSO has been studied previously – i.e. MSO inhibition of liver GS is maximal at 4 hours. GS activity remains low till 72 hours and thereafter increases again (Lamar 1968). This information enabled us to plan our experiments to explore the therapeutic use of MSO and the effects of GS inhibition on the mouse model of ALF.

As mentioned earlier, GS is predominantly present in astrocytes, and brain oedema is prevented in hyperammonaemic rats by MSO treatment (Takahashi, Koehler et al. 1991). MSO's therapeutic action has been explored in a mouse model for amyotropic lateral sclerosis (Ghoddoussi, Galloway et al. 2010). We found that MSO extends the life span of ALS mouse model (G39A mutant) by 8%. MSO was able to reduce glutamine by 60% and glutamate by 30% in different parts of mouse brain.

My contribution to this study focused on assaying glutamine synthetase activity from brain tissue of ALS mice. The same work has been presented below.

Materials and methods:

Animal groups and tissue collection:

Breeding pairs of the *SOD1* mouse – B6JL (G93A) – were purchased from Jackson Laboratories and offspring containing the human *SOD1*^{G93A} mutation were identified by PCR of tail DNA, using primers described for this strain by the Jackson Laboratories (jax.org). If the mice carried the SOD^{G93A} mutation they were termed as ALS mice. The mice which did not express SOD^{G93A} mutation were termed as wild type. Separate groups of animals were used to assess MSO effects on longevity, and GS activity *in vitro*. All animal experiments were approved by the Animal Investigation Committee of Wayne State University. Methionine sulfoximine (MSO), obtained from Sigma Chemical Company (Cat #5379) was dissolved in 0.9% saline and administered intraperitoneally at a dose of 20mg/kg.

Three experiments were set up to measure the effect of MSO treatment on GS in the mosue model for ALS.

Group 1 mice: SOD^{G93A} mutant mice were injected with MSO (15 mice) or saline (15 mice), three times every week (Monday, Wednesday, Friday), starting at 50 days of age (±2) until they were euthanized. Mice were observed daily and were euthanized when either 1) they lost 20% of their maximum body weight, 2) they were unable to right themselves when put on their back or side, or 3) if, in the opinion of the supervising veterinarians, the mice were in distress or unable to obtain adequate food and water. These were used to obtain MSO's effect on survival and monitor ALS disease progression. (I assisted in maintenance of the mouse colony, genotyping and motor function measurement assay.)

Group 2 mice: The effects of MSO treatment *in vivo* on GS activity *in vitro* were analyzed in brain tissue obtained from SOD^{G93A} mice and their wild type counterparts. Animals were treated with either saline or MSO (20 mg/kg, three time a week – Monday, Wednesday, and Friday – for 3 weeks) starting at 50 days of age (±2 days) and sacrificed at 70 days of age (±2 days).

Group 3 mice: Lastly, in another set of experiments, animals were treated with MSO at a dose of 20 mg/kg. MSO was injected starting at 85 ± 2 days of age. At 85 days, ALS symptoms (such as impaired motor function) appear. Thus, the animals were injected with MSO or saline once a week post symptomatically, till they met the criteria for euthanization (120 – 130 days).

Glutamine synthetase enzyme activity:

Brains were removed, homogenized, and GS activity determined according to the method of Meister (Meister 1985). Tissue was immediately homogenized in ice-cold buffer (0.15 M potassium chloride, 5 mM 2 – mercaptoethanol, and 1 mM EDTA (pH – 7.2)) and centrifuged at 2375 x g for 5 minutes. Tissue supernatant fraction was frozen on dry ice and stored at -80^{o}C.

Glutamine synthetase was assayed as described by Meister (Meister 1985). Brain supernatant (100 µg) was incubated in buffer (0.1 M imidazole – HCl (pH – 7.2), 100 mM L-glutamine, 0.2 mM $MnCl_2$, 62.5 mM hydroxylamine, 10 mM sodium arsenate and 0.4 mM ADP) at 37^{o}C for 10 minutes. The enzyme reaction with the substrates glutamine and hydroxylamine was stopped using ferric chloride stop solution (0.37 M $FeCl_3$, 0.67 M HCl, 0.20 M TCA) and the colorimetric product formed – γ-glutamyl hydroxamate – was measured at 535 nm. The absorbance values were converted to activities by comparison to a standard curve of pure glutamyl hydroxamate (a generous gift from Arthur J. Cooper, Department of Biochemistry and Molecular Biology, New York Medical College).

Results:

MSO's effect on GS from brain tissue of group 2 mice:

Each group consisted of 3 animals. GS activity from the brain tissue was 1.1 ± 0.04 in wild type animals and 0.95 ± 0.05 in ALS animals treated with saline. The activity was drastically reduced to 0.17 ± 0.03 and 0.18 ± 0.02 in wild type and SOD1 animals respectively, with MSO treatment. Activity is expressed as µM γ – glutamyl hydroxamate formed/min of reaction/ mg of protein ± standard error of mean. The results are described in **table 2.1**.

These results affirmed the potent inhibition of glutamine synthetase by MSO. GS activity is reduced by more than 85% in both wild type and SOD1 animals with MSO treatment.

GENOTYPE	TREATMENT	AVERAGE ACTIVITY (μM GGH/min/mg of protein)	SEM
WT (N = 3)	SALINE	1.10	0.04
WT (N = 2)	MSO: 20 mg/ kg body weight of mice	0.17	0.03
ALS (N = 3)	SALINE	0.95	0.05
ALS (N = 3)	MSO: 20 mg/ kg body weight of mice	0.18	0.02

Table 2.1. Glutamine synthetase activity in brains tissue of 70 day wild type (WT) and ALS mice with and without MSO treatment is shown above. WT and SOD1 mice have average GS activity of 1.10 and 0.95 μM γ-glutamyl hydroxamate/ min of reaction/ mg of protein. MSO inhibits GS and a decrease in average GS activity is observed. GS activity in MSO treated WT and SOD1 mouse are 0.17 and 0.18 respectively.

MSO's effect of GS from brain tissue of group 3 mice:

GS activity was 1.2 ± 0.19 in wild type animals (n = 7) and 1.12 ± 0.09 in ALS animals (n = 16) treated with saline. The activity was reduced by more than 80% to 0.25 ± 0.04 and 0.25 ± 0.03 in wild type (n = 6) and ALS (n = 16) animals respectively, with MSO treatment. Activity is expressed as μM γ – glutamyl hydroxamate formed/min of reaction/ mg of protein ± standard error of mean. The results are described in **table 2.2** below.

GENOTYPE	TREATMENT	AVERAGE ACTIVITY (µM GGH/min/mg of protein)	SEM
WT (N = 7)	SALINE	1.19	0.19
WT (N = 6)	MSO: 20mg/ kg body weight of mice	0.25	0.04
ALS (N = 16)	SALINE	1.12	0.09
ALS (N = 16)	MSO: 20mg/ kg body weight of mice	0.25	0.03

Table 2.2. GS activity in brain tissue of 120 day old mice, treated with MSO starting at 85 days in wild type (WT) and ALS mice is shown above. With MSO treatment average GS activity in WT and SOD1 mice decrease from 1.19 to 0.25 and 1.12 to 0.25 µM GGH/min of reaction/ mg of protein respectively.

GS activity seems to remain constant in saline treated animals during the course of disease (comparing group 2 and group 3 mice). Also, long term MSO treatment (one injection / week, starting at 85 days of age till euthanization) is able to maintain low GS activity.

Discussion:

ALS is an adult onset fatal neurodegenerative disease that results in a progressive loss of motor neurons (Bruijn 2002). The cause of neurodegeneration in ALS is unknown, but the onset or progression of ALS is believed to have an excitotoxic component involving glutamate. There

is no known effective treatment for ALS and patients have very poor prognosis (ALS patients die within a few years after disease onset.)

We wanted to determine the effect of limiting glutamine formation in brain. For the same, we treated animals with glutamine synthetase inhibitor MSO, which would affect the glutamine – glutamate cycle in brain. We found that chronic treatment of *SOD1*^{G93A} transgenic mice with MSO over an extended period of time (at least 10–11 weeks) significantly ($p = 0.008$) increased their life span by 8%, as compared to saline-treated animals Survival increased from 138 ± 3 days to 149 ± 4 (SEM) days for *SOD1*^{G93A} transgenic mice (data not shown in this chapter). The survival analysis for MSO-treated and saline-treated groups ($n = 15$ each) show the survival range for saline-treated animals was 119 to 157 days, and the survival range for MSO-treated animals was 128 to 167 days. Corresponding to an increase in survival we saw an 80 - 85% decrease in brain GS activity in brain tissue of the animals. Work done by our collaborators Dr. Farhad Ghoddoussi and Dr. Matthew P. Galloway found that the decrease in GS activity could be responsible for amino acid fluctuations that were seen in brain sections of the animals. Proton magnetic resonance spectroscopy, with magic angle spinning of intact samples of brain tissue, showed that MSO treatment reduced brain levels of glutamine by 60% and of glutamate by 30% in both the motor cortex and the anterior striatum (full text of the paper explains the various changes).

For the purpose of my thesis, these results were indicative of potent GS inhibition by MSO. Glutamine is required by immune cells for cellular proliferation, differentiation and cytokine production (Li, Yin et al. 2007), and so its modulation would have an impact on immune and inflammatory process.

Hence, to investigate the role of glutamine and glutamine synthetase in inflammation and encephalopathy we proposed to study the effects of MSO in acute liver failure (ALF) using the LPS/D-GalN liver injury model.

CHAPTER 3

A Glutamine Synthetase inhibitor increases survival and decreases cytokine response in a mouse model of Acute Liver Failure

The work presented in this chapter was first published as and in:

A glutamine synthetase inhibitor increases survival and decreases cytokine response in a mouse model of acute liver failure

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Introduction

As mentioned earlier, Acute Liver Failure (ALF) is defined as rapid deterioration of liver function in the absence of pre-existing liver disease and is characterized by blood coagulation, multi-organ failure, and altered mental status. ALF can be triggered by diverse agents such as drugs (acetaminophen, thioacetamide, and α-amanitin) and viruses (primarily hepatitis viruses A, B, and E). A critical feature of ALF is the mounting of a massive immune response, with

recruitment of cellular effectors and uncontrolled cytokine release, a so-called cytokine storm, which destroys healthy cells and organs, leading to death. There is currently no effective therapy against ALF, a condition that kills between one and six people per million every year (Bernal, Auzinger et al. 2010).

ALF is relatively rare, but it is one of many conditions resulting from a unrestrained cytokine release, and a pathological cytokine release is also believed to be involved in many other diseases that occur much more frequently, including rheumatoid arthritis (Chen and Goeddel 2002), Parkinson's disease (Park, Yule et al. 2008), acute pancreatitis (Makhija and Kingsnorth 2002), and complications from flu infection (e.g. sudden acute respiratory syndrome (SARS)(Wang, Le et al. 2010).

A well-established mouse model of ALF consists of injecting animals with a combination of low doses of *E. Coli* lipopolysaccharides (LPS, which activates the innate immune response) and D-Galactosamine (D-GalN, which specifically sensitizes the liver towards the cytotoxic effects of LPS through a transcriptional block (Decker and Keppler 1974; Galanos, Freudenberg et al. 1979; Leist, Gantner et al. 1998). This induces a systemic response which is predominantly directed towards liver. As the liver fails, the resultant hyperammonemia produces brain edema similar to the brain swelling seen in hepatic encephalopathy of humans that results from liver disease(Groflin and Tholen 1978; Watanabe, Higashi et al. 1979) .

Brain swelling is prevented in hyperammonemic rats by treatment with methionine sulfoximine (MSO) (Takahashi, Koehler et al. 1991; Tanigami, Rebel et al. 2005). MSO is a modified amino acid that acts as a very strong mechanism-based inhibitor of glutamine synthetase, the ATP-dependent enzyme that converts glutamate and NH_3 into glutamine – a central reaction in cellular nitrogen metabolism (Manning, Moore et al. 1969; Ronzio, Rowe et

al. 1969; Griffith, Anderson et al. 1979). MSO displays multiple additional biological activities; increases survival in a mouse model of amyotrophic lateral sclerosis (Ghoddoussi, Galloway et al. 2010), it inhibits the first step in glutathione synthesis, γ-glutamyl cysteine synthetase (Griffith, Anderson et al. 1979), and it lowers S-adenosyl methionine levels by an unknown mechanism (Schatz and Sellinger 1975).

We have been exploring the therapeutic use of MSO in the LPS/D-GalN mouse model of ALF, by studying if MSO affects the early events that lead to liver failure. We have found that pretreatment with MSO greatly increases the survival of mice challenged with LPS/D-GalN. The mechanism of action of MSO in this system appears to involve a reduction in the early cytokine response of macrophages, indicating that glutamine synthetase activity is required for cytokine synthesis elicited by LPS/D-GalN in inflammatory cells.

Materials and Methods

Animals:

6-8 week old CD1 mice were obtained from Charles River. Animals were housed in Wayne State University. Food was withdrawn the night before the day of the experiment. All experiments were approved by the Animal Investigation Committee of Wayne State University. The universities complies with the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Survival and Analysis:

Animals were divided into two groups. Group one animals consisted of both male and female mice injected with LPS and D-GalN to induce hepatic failure. Survival was monitored

post LPS/D-GalN administration and the time of injection was considered to be zero hour (t=0). Group two animals consisted of mice injected with MSO three hours prior to LPS and D-GalN administration. A mixture of LPS (20 µg/kg body weight, sigma – LPS from *E. Coli* 0111:B4 Sigma #L2630) and D-GalN (800 mg/kg body weight, Sigma cat #G1639) was prepared in saline and administered intraperitoneally (IP). MSO (50 mg/kg body weight, Sigma cat #M5379) was also prepared in saline and administered IP. For analysis of the effect of MSO treatment on the cell death pathway activated by the Fas ligand, mice were pre-treated with MSO as described above, and at time zero were injected with Jo2 antibody (200 µg/kg).

Animals were monitored continuously post LPS/D-GalN or Jo2 administration. Six to ten hours after treatment with LPS/D-GalN animals became very sick, and they were euthanized if their distress increased to the extent that they had difficulty moving, or if they were unresponsive to prodding. The time of death was recorded as hours after LPS/D-GalN injection. Fisher's exact test was used to determine two-tailed *p* values for the statistical between the treated and untreated groups.

Immunoblots of cleaved caspase-3 and phospho-STAT proteins:

Liver homogenates were prepared from male mice five hours after IP administration of LPS/D-GalN. The proteins in liver homogenates were separated on a 10% SDS-PAGE slab mini gels, electro-blotted onto nitrocellulose membranes (Amersham Biosciences Little Chalfont, UK – HybondTM-ECLTM) and the membranes were sequentially immunoblotted with antibodies against cleaved caspase-3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH – Chemicon, Milano, Italy) was used as a loading control). Other antibodies used in these studies included Phospho-STAT antibodies (cell Signaling cat # 9914), including phospho-STAT1 (Tyr701), phospho-STAT2 (Tyr690), phospho-STAT3 (Tyr705), phospho-STAT3 (Ser727), phospho-

STAT5 (Tyr694), and phospho-STAT6 (Tyr641). Anti-rabbit IgG, HRP-linked secondary antibody was used and the blots were developed either using the Immun-Star WesternC Kit (BIO-RAD cat # 170-5070) on a BIO-RAD imager.

Caspase-3 blots of our Italian collaborators were used in the paper. However, the experiment was repeated and the result presented in this chapter was obtained by me.

Isolation of peritoneal macrophages:

Peritoneal macrophages were isolated from the abdomens of CD-1 mice, and $2x10^4$ cells were added to each well of a 6-well cell culture plate. The cells were incubated in RPMI – 1640 (with 2.05 mM glutamine) with 5% fetal calf serum and 1% penicillin/streptomycin (all solutions were from Hyclone) for 4 hours. The medium was then changed to remove any non-adherent cells, and the cells were again incubated in a 37 ^oC - 5% CO_2 incubator for 16 hours. In each plate, one well was a negative control (no treatment), one well was a MSO control (9 mM) and one well was a LPS control (1 µg/ml LPS). The other three wells were treated with 9 mM MSO and 1 µg/ml LPS. One well was treated with MSO for 3 hours, and LPS was then added. In another well, LPS and MSO were added at the same time. Time of LPS addition was considered time zero. Four hours after LPS addition the cell culture medium was collected and 50 µl of the medium was mixed with 50 µl of blocking buffer and used for the TNF-α ELISA assay.

Animal groups for tissue and plasma collection:

Tissue and plasma were collected from male mice, which were divided into two groups. One group was injected with saline three hours prior to LPS/D-GalN injections. The second group was given MSO three hours prior to being injected with LPS/D-GalN. Control mice (t=0) were treated with MSO or saline for 3 hours and then euthanized (not exposed to LPS/D-GalN).

After LPS/D-GalN injection at time zero, tissue and plasma samples were collected at one-hour time intervals from 1 to 5 hours.

Blood collection and plasma preparation:

For experiments analyzing plasma cytokines, the male mice from the groups described above were anesthetized using Avertin (solution of 15.5 ml of tert-amyl alcohol –Sigma #240486 and 25 gm of 2-2-2 tribromoethanol – Sigma #T48402) and blood was collected by cardiac puncture. Plasma was obtained by transferring blood to plasma separator tubes containing lithium heparin (BD Microtainer tubes). Plasma samples were immediately frozen on dry ice and stored at -80^{o}C.

Enzyme activity assay:

To analyze enzyme activities liver homogenates were prepared from liver tissue excised and immediately homogenized in ice-cold buffer consisting of 250 mM sucrose, 10 mM Tris and 0.1 mM EGTA with protease inhibitor cocktail (sigma #P8340). The homogenized tissue was centrifuged at 2375 x g for 5 minutes. The tissue supernatant fraction was frozen on dry ice and stored at -80^{o}C.

Glutamine synthetase was assayed as described by Meister (Meister 1985). Liver supernatant (100 µg) was incubated in buffer (0.1 M imidazole, 100 mM L-glutamine, 0.2 mM $MnCl_2$, 62.5 mM hydroxylamine, 10 mM sodium arsenate and 0.4 mM ADP) at 37^{o}C for 15 min. The reaction with the substrates glutamine and hydroxylamine was stopped using ferric chloride stop solution (0.37 M $FeCl_3$, 0.67 M HCl, 0.20 M TCA) and the colorimetric product formed – γ-glutamyl hydroxamate – was measured at 535 nm. The absorbance values were converted to activities by comparison to a standard curve of pure glutamyl hydroxamate (a generous gift from Arthur J. Cooper, Department of Biochemistry and Molecular Biology, New York Medical

College). Graphpad analysis software was used to obtain two-tailed *p* values using an unpaired *t* test.

Cytokine Blots:

Mouse cytokine array kits (Proteome Profiler Arrays – cat # ARY006) were obtained from R&D Systems and used according to the manufacturer's guidelines. Each array, which consisted of a nitrocellulose membrane spotted with 40 different mouse cytokines, was tested with two hundred microliters of plasma from one animal. Each condition was duplicated – two mice for each group. Control blots were from plasma collected from two untreated mice (not injected with LPS/D-GalN and/or MSO). For untreated and MSO-treated groups, plasma was collected at either 1 hour or 3 hours after LPS/D-GalN administration. The blots were developed using the Immun-Star WesternC Kit (BIO-RAD cat # 170-5070) on a BIO-RAD imager. The corresponding Quantity One software of the BIO-RAD imager was used to quantify the signal obtained on the blots.

ELISA assays:

TNF-α, IFN-γ, and IL-6 ELISA kits were obtained from eBioscience, and experiments were performed using the manufacturer's guidelines for 96-well plate experiments. One hundred microliters of plasma was used per well. The TNF-α ELISA assay had a sensitivity of 8-1000 pg/ml: the IFN-γ ELISA had a sensitivity of 0.75 – 100 pg/ml. The IL-6 ELISA assay had a sensitivity of 0-500 pg/ml, and the combination of ELISA sensitivity and the amounts of IL6 in plasma required us to reduce the amount of plasma tested to 5 µl.

Results

MSO prevents LPS/DGalN induced ALF lethality in mice:

Figure 3.1 shows survival curves for CD1 mice treated with LPS/D-GalN to induce liver failure. Mice were injected with MSO three hours prior to LPS/D-GalN treatment. Without MSO pre-treatment, LPS/D-GalN administration resulted in death 6 - 10 hours after administration. MSO pretreatment increased male survival from 19% to 71% (**Figure 3.1A**) n=21; p<0.002 . MSO pre-treatment increased female survival from 27% to 81% (**Figure 3.1B**) n=14; p<0.005. MSO therefore substantially increases survival in both male and female mice with LPS/D-GalN induced ALF.

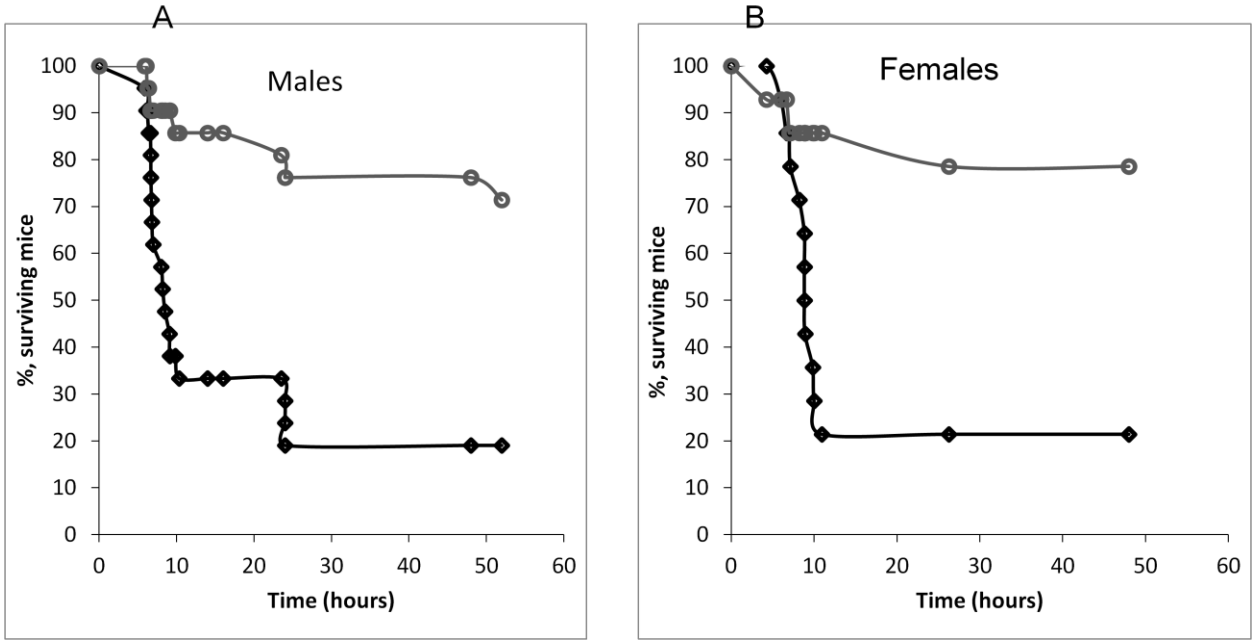


Figure 3.1. Survival of male and female CD1 mice pretreated with either MSO (open circles) (50 mg/kg IP) or saline (open diamonds) three hours prior to injection with LPS and D-Galactosamine (D-GalN) at time zero to induce liver failure. Time of death was recorded as hours after LPS/D-GalN injection (X axis), and percent remaining animals was plotted on the Y axis. For males (left), 4 out of 21 mice injected with LPS/D-GalN survived. Survival increased to 15 mice (out of 21) when animals were injected with MSO. Two tailed p values for these two groups < 0.002. For females (right), 3 out of 14 mice injected with LPS/D-GalN survived. Survival increased to 11 mice (out of14) when animals were pretreated with MSO. Two tailed p values for these two groups < 0.005.

MSO treatment reduces activity of glutamine synthetase:

MSO is a well-characterized inhibitor of glutamine synthetase. We assayed glutamine synthetase activity in liver extracts from LPS/D-GalN treated animals. **Figure 3.2** shows that at all time points, MSO pre-treatment reduced glutamine synthetase activity to less than 10% of that found in animals pre-treated with saline, supporting the conclusion that inhibition of glutamine synthetase activity might be involved in the therapeutic effects of this drug.

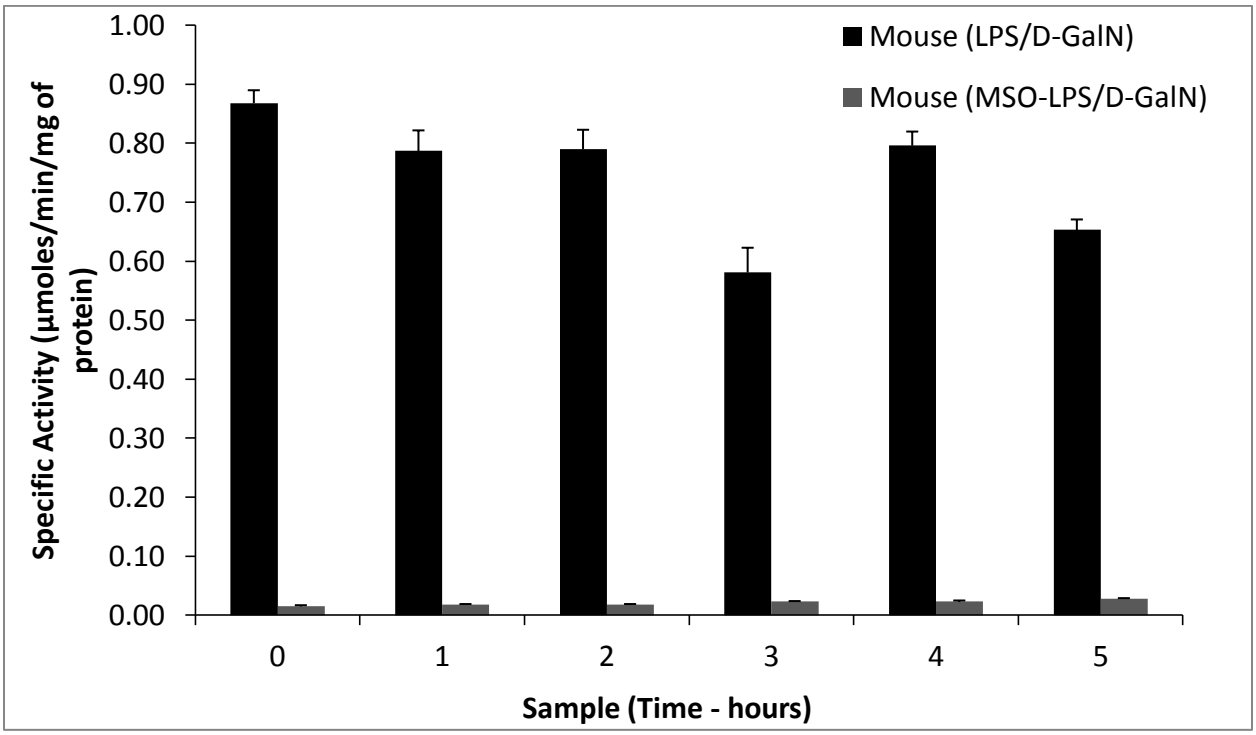


Figure 3.2. Glutamine synthetase activities measured in liver extracts from mice treated with LPS and D-GalN three hours after IP injection of either saline (black bars) or MSO (grey bars). Each time point represents the average glutamine synthetase activity in liver extracts from 15 animals.

MSO inhibits the massive cytokine response seen in LPS/D-GalN treatment:

One of the earliest effects of LPS/D-GalN administration is to trigger the release of pro-inflammatory cytokines(Leist, Gantner et al. 1998). We exploited a mouse cytokine array to

determine how MSO treatment affected plasma levels of 40 different cytokines following treatment of mice with LPS/D-GalN in order to draw a comprehensive picture of how MSO might be affecting the early immune response. We used these arrays to visualize the cytokine response in plasma collected one hour or three hours after LPS/D-GalN administration. **Figure 3.3** shows the cytokines seen in plasma drawn one and three hours after LPS/D-GalN administration with or without MSO treatment. Each blot represents the plasma from a single animal. For **Figure 3.3A (i),** the two control blots, were on plasma isolated from negative controls (overnight-starved animals not treated with MSO or LPS/D-GalN). We analyzed blots of plasma taken from two animals killed three hours after LPS/D-GalN administration, shown in **Figure 3.3A (ii)**. **Figure 3.3A (iii)** shows the two blots of plasma from animals pretreated with MSO three hours prior to LPS/D-GalN injection. For the locations of each of the 40 cytokines detectable in these blots, see http://www.rndsystems.com/pdf/ary006.pdf

The results showed that, as expected, secretion of many of these cytokines is dramatically increased during ALF progression. Three hours after disease induction, 22 of the 40 cytokines were found to be elevated and 20 of these were reduced by MSO pretreatment. The results for all 40 cytokines are summarized in **Figure 3.4A** and **figure 3.4B**, and the major differences are described in the following paragraphs.

Cytokines C5a, sICAM-1, KC, M-CSF, and TIMP-1 were all detected on the control blots. In the one-hour samples from LPS/D-GalN-treated mice (**figure 3.3B (i)**), only IL-6, KC, M-CSF, JE, MIP-2, TNF-α and TIMP-1 were elevated, and only IL-6 and TNF-a were reduced – both by >50% - in plasma from MSO-pretreated animals (**figure 3.3B (ii)**). The signal intensity calculations from one hour blot samples are shown in **figure 3.4C**. Three hours after LPS/D-GalN treatment, both LPS/D-GalN treated animals and MSO-pretreated LPS/D-GalN treated

animals showed a 10-20% reduction in C5a and sICAM. M-CSF values were the same for all three groups. KC and TIMP-1 signals were increased by more than 50% in LPS/D-GalN treated mice. KC values for MSO pre-treated values were same as in LPS/D-GalN animals; however, TIMP-1 values were reduced to approximately control values.

Induction of IFN-γ, IL-1ra, IL-6, IL-16, and IL-23 by LPS/D-GalN was abolished by MSO, whereas IL-1a level was reduced by more than 50%. Neutrophil-produced cytokines such as IP-10, I-TAC and MIG were all detected in plasma from LPS/D-GalN treated mice. IP-10 was reduced by more than 50% with MSO pretreatment and MIG and I-TAC were not detected. Macrophage-produced cytokines such as MIP-1a and MIP-1b, and chemokines such as JE and RANTES, which were elevated with LPS/D-GalN treatment, were either reduced or not detected with MSO pretreatment. The two exceptions were MCP-5 (monocyte chemotactic protein) and MIP-2 (macrophage inflammatory protein -2 gamma), both of which were increased in plasma from MSO-treated animals. Most importantly, TNF-α induction by LPS/D-GalN was abrogated by MSO pretreatment.

Lastly, **figure 3.3C (i)** shows blot from one female LPS/ D-GalN animal and **figure 3.3C (ii)** was obtained from plasma of one female mouse pre – treated with MSO. Since, the cytokine profile from female mice was similar to those from male mice; densitometry analysis from the blot was not performed and the experiment was not repeated with more animals.

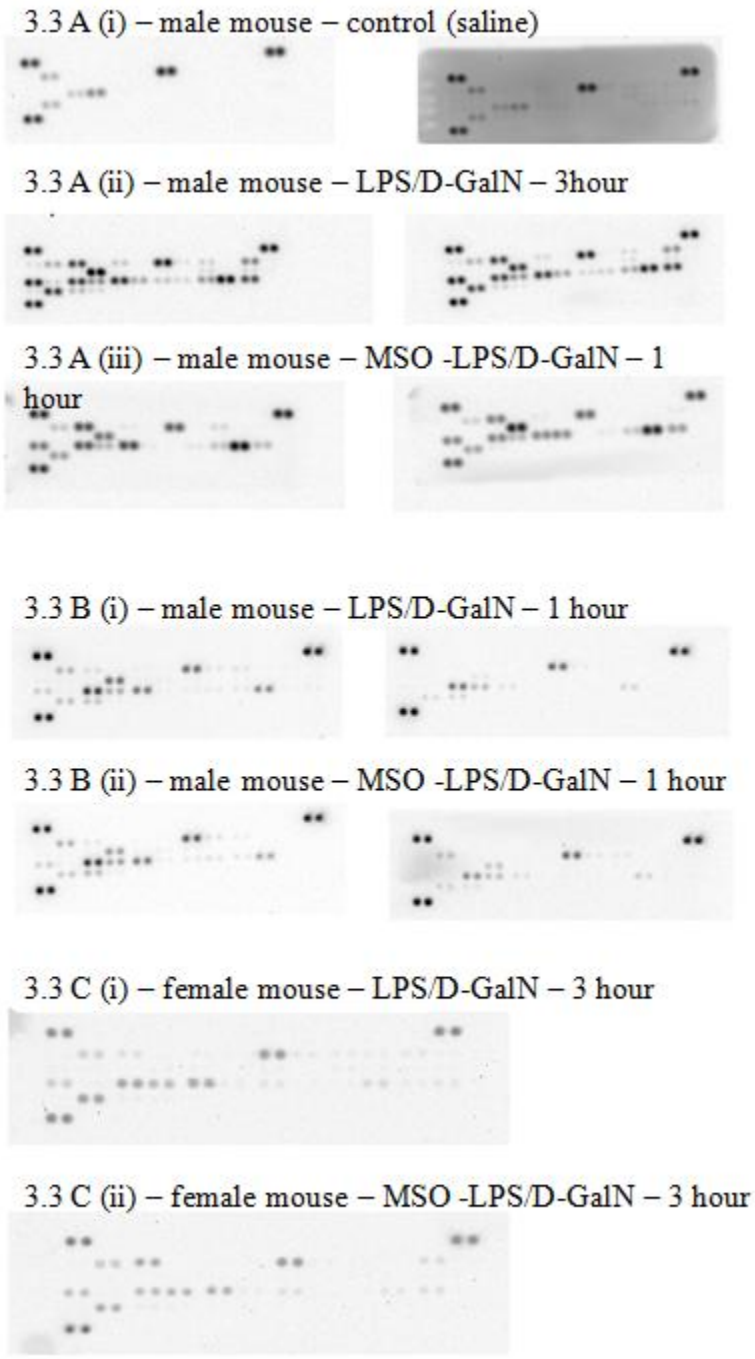


Figure 3.3. Nitrocellulose membrane-based cytokine arrays. Cytokine arrays were obtained from R&D Systems. Each membrane has 40 different mouse cytokines spotted in duplicate. The cytokines were identified using the column and lane number of each spot corresponding to the manufacturers table. Each membrane contained three positive and one negative control, each spotted in duplicate. Each blot represents the plasma cytokine profile from a single mouse. Panel A (i) shows cytokines in plasma from a control mouse (not treated with either MSO or

LPS-DGalN). 3.3. A (ii) show cytokines from plasma of animal treated with LPS/D-GalN 3 hours post disease induction and 3.3. A (iii) shows plasma from MSO pretreated animal. Panel B shows cytokines in plasma from a mice treated with LPS/D-GalN (3.3. B (i) and MSO + LPS/D-GalN (3.3. B (ii))for one hour. Panel C shows cytokines in plasma from female mice treated with LPS/D-GalN (3.3.C (i)) and pretreated with MSO three hours before being treated with LPS/D-GalN (3.3. C (ii)) for three hours. For the locations of each of the 40 cytokines detectable in these blots, see http://www.rndsystems.com/pdf/ary006.pdf

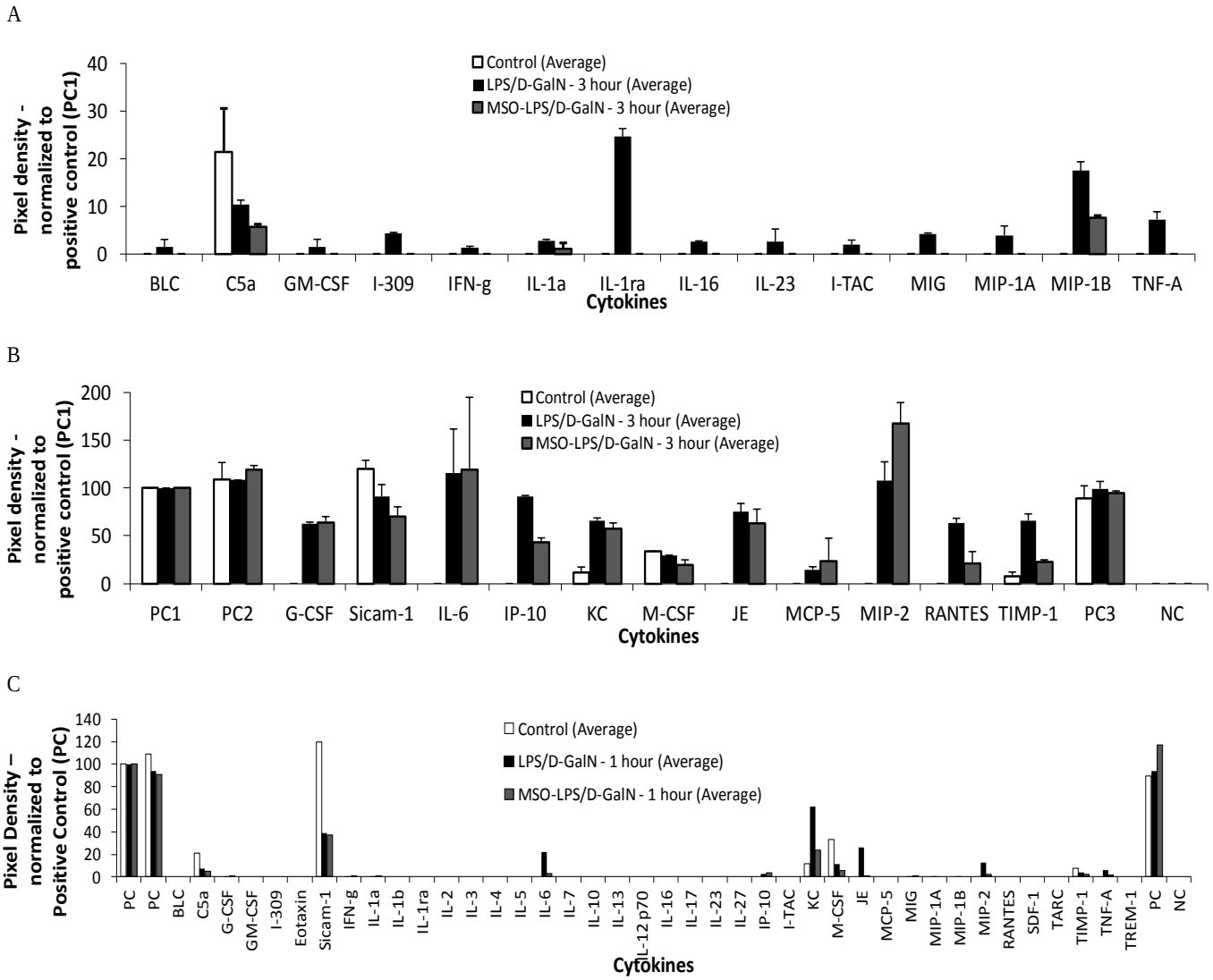


Figure 3.4. Changes in cytokines calculated from cytokine array blots. Each treatment group had two animals in it and the values for the 40 different cytokines were averaged for each group and represented as pixel densities, normalized to the average pixel densities of the positive

controls (PC1), and plotted on this bar graph. Figure A and B show all the cytokines present in control, LPS/D-GalN and MSO – LPS/D-GalN treated animals. The cytokines not detected on the blots are not shown. Open bars represent the cytokine values for untreated negative control mice. Black bars represent the cytokine values for mice treated with LPS/D-GalN. Grey bars represent the cytokine values for mice pretreated with MSO three hours before being injected with LPS/D-GalN. The error bars represent the maximum pixel density for the cytokine. A and B) Shows the signal intensity calculation from cytokine blots of three hour diseased animals – figure 3.3 A i, ii, and iii. C) Shows signal intensity calculation from cytokine blots of one hour diseased animals – figure 3.3. B i, and ii.

ELISA studies on plasma cytokines:

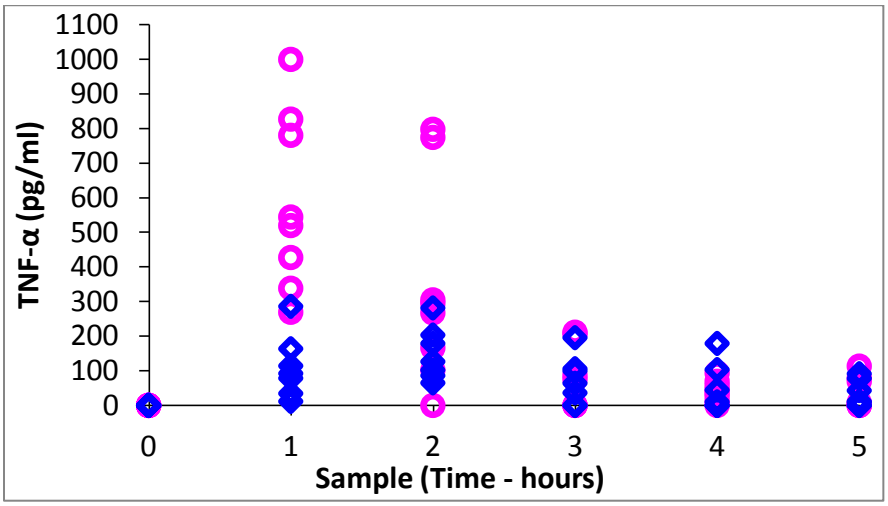
TNF-α and IFN-γ are secreted by different cells as part of the response to LPS administration, and we see both in the cytokine arrays described above. TNF-α and IFN-γ activate separate apoptotic pathways. TNF-α activates cell death via the JNK pathway (Schwabe and Brenner 2006) and IFN-γ activates apoptosis via the Jak-STAT1 pathway (Horvath 2004). To identify the effect of MSO on secretion of these two cytotoxic cytokines, we followed up the cytokine array studies with ELISA analysis of plasma levels of TNF-α and IFN-γ. Figures 6A and 6B show ELISA assays carried out on plasma taken from individual mice killed either at t=0 (control animals – injected with MSO or saline for 3 hours, but not exposed to LPS/D-GalN) and at 1, 2, 3, 4, or 5 hours after LPS/D-GalN treatment. These are the same mice for which glutamine synthetase activities in liver extracts are shown in Figure 3.2.

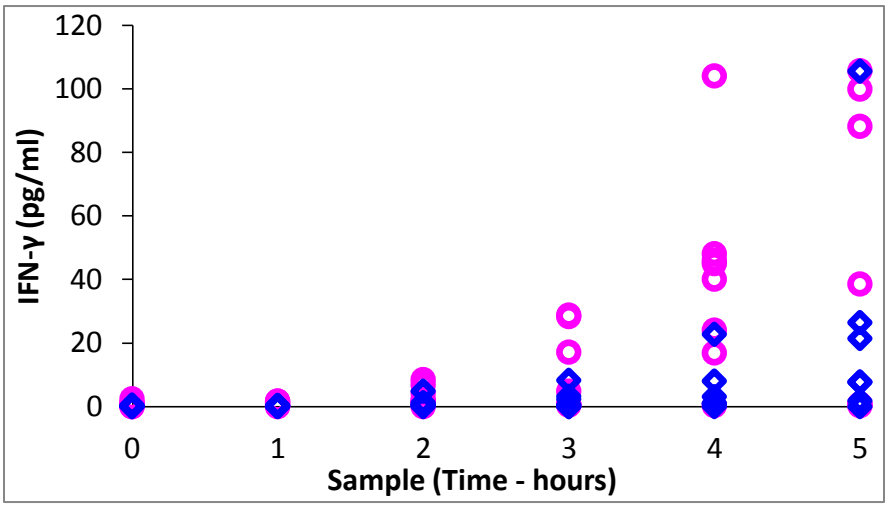
Figure 3.5A shows that TNF-α was not detectable in plasma taken from t=0, control mice injected three hours previously with either MSO (n=7) or saline (n=7). For saline pretreated and LPS/D-GalN-treated animals, TNF-α levels peaked at one hour (mean: 590 ± 91 SEM pg/ml) then dropped at later times. In contrast, MSO-pre-treated animals exhibit low TNF-α levels throughout the time course of experiment, never reaching 200 pg/ml (peak 1 hour time

point mean: 112 ± 35 SEM pg/ml). The *p* value for the difference between the saline-treated and the MSO-treated groups of mice at 1 hour is < 0.001.

Another important cytokine whose expression is elicited by LPS is IFN-γ, which could contribute to apoptosis regulation via activation of the JAK/STAT pathway (Horvath 2004). **Figure 3.5B** shows that LPS/D-GalN-treatment increases IFN-γ cytokine levels from 0.84±0.25 pg/ml at one hour to 41±11 pg/ml at 4 hours, and that MSO markedly inhibits this increase (0.19±0.05 pg/ml at 1 hour; 5.0±3.0 pg/ml at 4 hours). The *p* value for differences between the LPS/D-GalN and MSO treated groups of mice at 4 hour is 0.01.

We also conducted ELISA assays to test the effect of MSO pretreatment on IL-6, another acute phase protein involved in orchestrating the early phases of the inflammatory response (Streetz, Wustefeld et al. 2001).IL-6 is secreted by macrophages in liver and along with TNF-α is a critical early mediator of immune signaling during disease progression. Low levels of IL-6 are hepatoprotective, and the resultant low levels of STAT3 activation are also hepatoprotective. As described above, IL-6, like TNF-α, is seen in the cytokine blots of both the one-hour and three-hour plasma samples from LPS/D-GalN treated mice. **Figure 3.5C** shows that LPS/D-GalN treatment increases IL-6 from 166±41 pg/ml (mean ± SEM) at one hour to 401±14 pg/ml (mean ± SEM) at two hours. Thereafter it decreases gradually till 5 hours. The plasma from MSO pre-treated mice show low levels of IL-6, with a peak of (mean) 325 ± 27 SEM pg/ml at 3 hours. The *p* value for the difference between the saline-treated and the MSO-treated groups of mice at 1 hour is < 0.001.


TNF-α (pg/ml)
1100
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
Sample (Time - hours)


IFN-γ (pg/ml)
120
100
80
60
40
20
0
0
1
2
3
4
5
Sample (Time - hours)

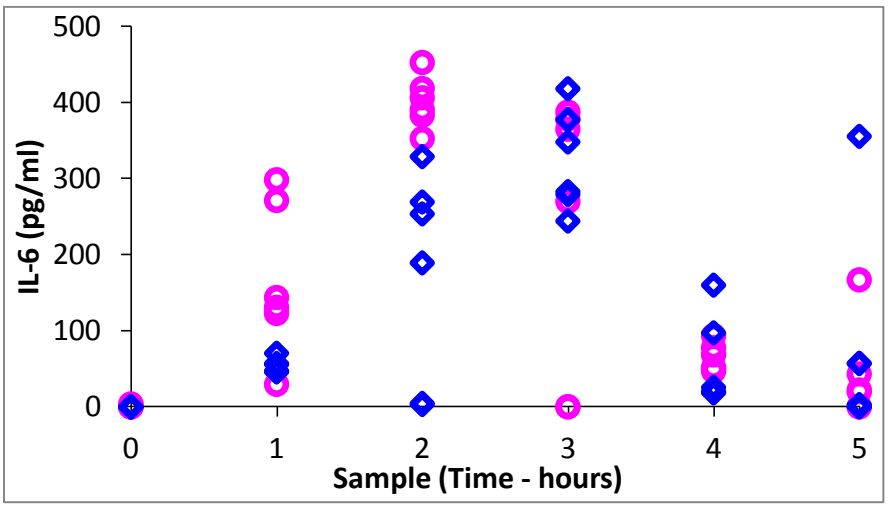

IL-6 (pg/ml)
500
400
300
200
100
0
0
1
2
3
4
5
Sample (Time - hours)

Figure 3.5. ELISA quantitation of cytokine levels in plasma from LPS/D-GalN treated mice. The time zero values show the levels of each cytokine in negative control mice, not treated with LPS/D-GalN but injected with either saline (n=7) or MSO (n=7) three hours prior to be killed. Each of the hour time points shows cytokine levels in 7 or 8 mice killed at that time point after LPS/D-GalN treatment. For each cytokine, open red circles represent cytokine levels in plasma isolated from mice pretreated with saline, and open blue diamond's represent cytokine levels in plasma isolated from mice pretreated with MSO prior to LPS/D-GalN treatment. A) TNF-α levels. B) IFN-γ levels. C) IL-6 levels.

MSO inhibits STAT1 activation and caspase – 3 cleavage:

The effects of IFN-γ as well as other IFNs, are mediated by its activation of the tyrosine kinases Jak1 and Jak2, which phosphorylate and activate the transcription factors of the STAT family (Levy and Darnell 2002). As this is the primary pathway for the activation of IFN-γ. responsive genes we wished to determine if the reduction in IFN-γ seen with MSO was sufficient to affect this pathway. As can be seen in **figure 3.6**, LPS/D-GalN treatment leads to a large increase in STAT1 activation and this increase is eliminated in the presence of MSO. We also tested the effects of MSO treatment on activation of STAT2, 3, 5, and 6. None showed effects as dramatic as those seen with STAT1, but phosphorylated STAT3 is decreased by MSO-treatment in both LPS/D-GalN treated and untreated mice.

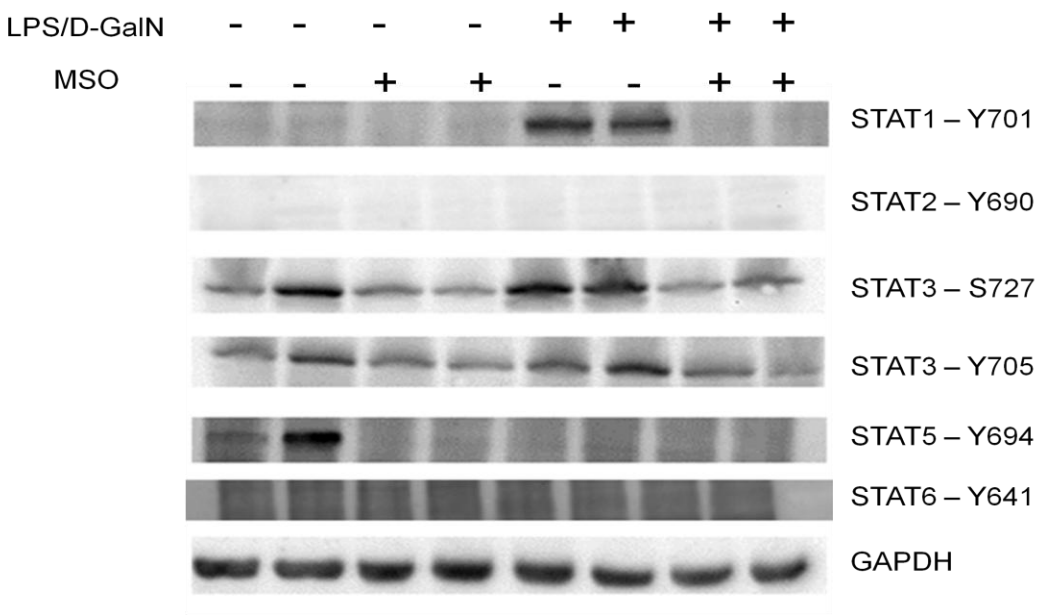


Figure 3.6. Immunoblots of liver tissue homogenates from LPS/D-GalN treated mice probed with anti phospho-STAT's. The top two lines show treatments, either LPS/D-GalN, MSO, neither, or both. The blot shows that LPS/D-GalN treatment phosphorylates and activates STAT1, but not if mice are pretreated with MSO. Glyceraldehyde phosphate dehydrogenase (GAPDH) is shown as a loading control. Phosphorylated STAT2, 5 and 6 was not detected in any treatment group. STAT3 phosphorylated at S727 was present in LPS/D-GalN treated animal and absent with MSO treatment.

Caspase 3 is a key apoptotic effector, both in intrinsic and in extrinsic apoptotic pathways, and it is activated downstream to engagement of TNF or Fas receptors(Taylor, Cullen et al. 2008). We therefore measured caspase-3 activation as a hallmark of apoptosis induction in mouse liver extracts. **Figure 3.7** show that, animal exposure to LPS/D-GalN for 5 hours elicits a marked caspase-3 activation, which is completely prevented by MSO pretreatment. This suggests that MSO acts either downstream to TNF-α binding to its cognate receptor, blunting some intracellular steps of apoptosis signaling; or upstream to TNF-R engagement, e.g. by inhibiting ligand production or delivery.

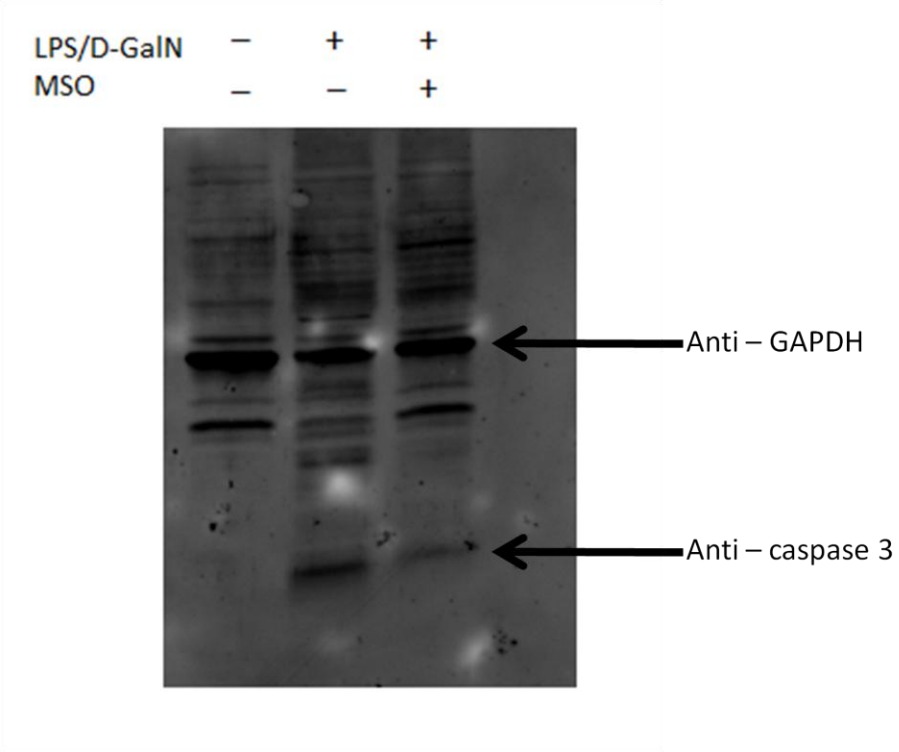


Figure 3.7. Effect of MSO treatment on caspase-3 activation. Western immunoblots of liver extracts were hybridized with anti-cleaved, *i.e.* activated caspase-3. First lane is control – untreated with LPS/D-GalN and MSO. Second lane is LPS/D-GalN sample and third lane is MSO pretreated LPS/D-GalN tissue. MSO treatment reduces formation of active caspase – 3. Three different lanes are shown. Blots were probed with anti-GAPDH as a loading control.

Effects of MSO on in vitro production of TNF-α by peritoneal macrophages

Taken together, our data demonstrate that MSO exerts its protective function prior to apoptosis induction in hepatocytes, as it down-modulates cytokine induction following LPS exposure. Macrophages coordinate the early phases of LPS-induced inflammatory response, through the synthesis of TNF-α and other cytokines. We conducted *in vitro* experiments to directly test if MSO inhibits TNF-α production on LPS-stimulated macrophages obtained from mouse peritoneum. Macrophages were triggered with LPS with or without MSO added either three hours before, or at the same time of LPS. Four hours after LPS addition, TNF-α in the culture supernatant was quantitated by ELISA. In six experiments, we found that MSO

treatment three hours before LPS addition resulted in an average decrease in TNF-α production of 26% with a range of 14% to 55%. MSO treatment at the same time as LPS addition resulted in an average decrease in TNF-α production of 18% with a range of 8% to 37%. Trypan blue testing confirmed that there was no effect of MSO on cell viability over the time course of these experiments. This experiment verified that MSO can act directly on macrophages to inhibit cytokine production, despite the presence of 2 mM glutamine in the culture medium. These results with peritoneal macrophages show that MSO does decrease TNF in the presence of external glutamine, but since Kupffer cells are likely to have the major role in TNF-α production in these experiments, and since the local concentration of glutamine will be different than the plasma levels for these cells, additional experiments will be needed to see if the inhibition of GS is sufficient to account for the diminution of the immune response. The significance of MSO acting even in the presence of external glutamine is discussed below.

Discussion

Methionine sulfoximine is a well characterized inhibitor of glutamine synthetase and gamma-glutamyl cysteine synthetase (Griffith and Meister 1978). It is a mechanism-based (i.e. "suicide") inhibitor for both enzymes, whose mode of action involves binding to the active site and being phosphorylated by ATP. This reaction produces methionine sulfoximine phosphate, which binds very tightly to the active site and inactivates the enzyme. In rats, MSO-treatment and inhibition of glutamine synthetase has been shown to prevent brain swelling caused by hyperammonemia and MSO has been suggested as a possible treatment for hepatic encephalopathy (Takahashi, Koehler et al. 1991; Brusilow 2002; Tanigami, Rebel et al. 2005). Because of its effectiveness in those studies, we had initially included MSO treatment as an

adjunct therapy to other drugs targeting the mitochondrial permeability transition, a mitochondrial channel whose prolonged opening irreversibly commits cells to death, as PTP dysregulation is implicated in ALF and other liver diseases (Rasola and Bernardi 2007). The unexpected effectiveness of MSO by itself led to these studies on MSO alone.

Treatment of mice with MSO alone three hours prior to inducing liver failure with LPS and D-GalN significantly increased survival. Subsequent analyses showed that MSO treatment prevented caspase-3 activation, indicating that this drug was affecting a stage upstream to cell death in the uncontrolled response which is primarily directed against the liver during ALF (Strasser, O'Connor et al. 2000; Leist and Jäättelä 2001; Luan, Zhou et al. 2007). Immunoblot analysis showed that MSO pretreatment reduced the production of several pro-inflammatory cytokines, and more sensitive ELISA studies showed that MSO suppressed the production of TNF-α and IFN-γ. TNF-α is an important acute phase protein whose production by macrophages and binding to the TNF-α receptor on hepatocytes activates a network of signal transduction pathways that regulate gene expression and cell death. When transcription is blocked, e.g. with D-GalN, pro-apoptotic pathways, including aggregation of a death-inducing signaling complex and activation of the stress kinase JNK, prevail and lead to a rapid cell dismantling (Schwabe and Brenner 2006; Wullaert, Heyninck et al. 2006). IFN-γ is produced in the liver by natural killer (NK) and NK T cells and is involved in the immune response against viral and bacterial infections by stimulating macrophages to produce more cytokines(Saha, Jyothi Prasanna et al. 2010). Moreover, IFN-γ is independently capable of initiating cell death by inducing the Jak-STAT pathway (Levy and Darnell 2002), and we see STAT1 activation in the livers of LPS/D-GalN treated mice. MSO treatment affects both the TNF-α and IFN-γ - dependent pathways, but the suppressive effect of MSO treatment on TNF-α and IFN-γ production also involves a larger

suppression of cellular immunity, since MSO treatment affects the levels of other macrophage secreted cytokines such as MIG, MIP-1a, MIP-1b, and MCF in plasma drawn 3 hours after disease induction. Most of these cytokines were either absent or reduced by more than 50% in the plasma from MSO pre-treated mice.

The simplest explanation for the ability of MSO to alter the immune response initiated by LPS/D-GalN is that changes in glutamine concentration, either in the plasma or in immune cells, affect the activation of immune cells, most likely peritoneal macrophages or Kupffer cells, since these cells are the earliest producers of TNF-α after an IP administration of LPS. It is well established that glutamine levels influence macrophage function and cytokine release in tissue culture, and long-term dietary changes can modify immune function in living animals (Newsholme 2001; Li, Yin et al. 2007; Roth 2007). The action of MSO on glutamine synthetase may be lowering plasma glutamine and thereby changing the global anabolic response to infection. *In vitro,* lowered glutamine leads to decreased proliferation in mitogen-stimulated lymphocytes and impairs the differentiation of plasma cells (Crawford and Cohen 1985; Szondy and Newsholme 1989). The reduction in glutamine seen after severe burns may also be responsible for the impaired immune response seen in this and other stress conditions (Kew, Wells et al. 1999). Again *in vitro,* a detailed study of the effect of glutamine concentrations on T cell differentiation divided the response into an early and glutamine-independent phase and a latter glutamine dependent phase (Hörig, Spagnoli et al. 1993). Finally, dietary glutamine enhances cytokine production in macrophages and increases T cell responsiveness (Kew, Wells et al. 1999; Rogero, Tirapegui et al. 2008).

It has been shown that macrophage depletion improves survival in mice challenged with LPS/D-GalN (Emoto, Emoto et al. 2003), and that transfer of macrophages from wild type mice

in TL4R-mutant mice is sufficient to confer susceptibility to LPS/D-GalN – induced lethality (Freudenberg, Keppler et al. 1986). The relative importance of peritoneal and Kupffer cells in the lethality caused by LPS/D-GalN is not known, but the conventional view is that, given the large mass of Kupffer cells and their close proximity to hepatocytes, their secretion of TNF-α and other cytokines is the proximate cause of death (Luster, Germolec et al. 1994; Ide, Kuwamura et al. 2005; Tacke, Luedde et al. 2009). Since inhibiting glutamine synthetase can affect cell death, MSO might be inhibiting Kupffer cell response by either decreasing the external concentration of plasma glutamine in the vicinity of Kupffer cells, or by directly inhibiting cellular glutamine synthetase, as we have shown here for peritoneal macrophages treated with MSO *in vitro.* The fact that treatment of isolated peritoneal macrophages with 9mM MSO in the presence of 2mM glutamine significantly reduces TNF-α indicates that even in the presence of external glutamine, peritoneal macrophage glutamine synthetase is still required for maximal TNF-α synthesis and/or secretion. Since Kupffer cells are likely to have the major role in the production of TNF-α in our experiments, additional and more complex experiments will be needed to determine if the inhibition of glutamine synthetase in these cells is sufficient to account for the striking diminution of the immune response which we observe.

However, studies of the *in vitro* effects of glutamine on isolated systems of immune cells are obviously inadequate for understanding the complex events occurring with LPS/D-GalN stimulation. We have demonstrated that an IP injection of 50 mg/kg MSO lowers plasma glutamine concentrations by about 50 %. Under long term MSO treatment, plasma glutamine levels return to normal even though glutamine synthetase in the liver remains inhibited by 90%, presumably due to the large amounts of glutamine present in the standard mouse diet. In any case, plasma glutamine levels might be of minimal importance in the context of macrophage

activation in the liver, where the local production of glutamine by glutamine synthetase may influence the external concentration seen by Kupffer cells. As blood in the liver flows from the portal to the venous side, glutamine concentrations change. The periportal cells actively transport glutamine, concentrating it by 10 fold (Häussinger, Lang et al. 1990; Labow and Souba 2000). This removal of glutamine is central to liver function since it is converted by liver glutaminase into urea and glutamate, controlling blood ammonia levels through its incorporation into urea. Both glutamate and urea are transported back into the periportal blood. As the glutamate-enriched and glutamine-depleted blood reaches the perivenous side of the liver, glutamate is transported into the perivenous cells and used by glutamine synthetase to make glutamine, which is then available to Kupffer cells associated with the perivenous hepatocytes. Perivenous hepatocytes, while constituting only 5% of the hepatoocyte population are the only liver cells containing glutamine synthetase (Gebhardt and Mecke 1983; Häussinger, Stoll et al. 1989). There are two populations of Kupffer cells as distinguished by morphology, function, and location. The Kupffer cells at the periportal side are more active in phagocytosis while those at the perivenous side respond much more strongly to LPS (Sleyster and Knook 1982; Hoedemakers, Morselt et al. 1995; Armbrust and Ramadori 1996; Bykov, Ylipaasto et al. 2004). As noted above, it is clear that glutamine is required for the activation of immune cells. Our results suggest that the immunologically reactive Kupffer macrophages may be dependent upon the glutamine synthesized specifically by perivenous hepatocytes. The inhibition of glutamine synthetase in this population by MSO could reduce the response to LPS/D-GalN, independent of any changes in plasma levels of glutamine.

An additional mechanism independent of plasma glutamine levels may also be involved. As shown in this report, MSO is also able to reduce TNF-α secretion in isolated peritoneal

macrophages even in the presence of 2mM glutamine, implying that the macrophage glutamine synthetase is directly involved in the synthesis and/or secretion of TNF-α.

Finally, inhibition of glutamine synthetase by MSO may reduce internal glutamine in immune cells and thereby interfere with energy generation or another critical biochemical processes required for activation. For example, mTOR activation requires an internal pool of glutamine and the critical size of this pool may be altered by the inhibition of glutamine synthetase by MSO (Nicklin, Bergman et al. 2009). Liver tumor cells often have high levels of glutamine synthetase and presumably internal glutamine, priming them for proliferation (Apte, Singh et al. 2009; Nicklin, Bergman et al. 2009). In addition, the synthesis of glutamine synthetase is under the control of the Wnt/β-catenin signaling pathway with β-catenin required for three enzymes involved in glutamine metabolism, including glutamine synthetase (Sekine, Lan et al. 2006; Apte, Singh et al. 2009), again suggesting that glutamine plays a central role in hepatocyte metabolism.

In conclusion, MSO, a well-characterized mechanism-based inhibitor of glutamine synthetase and other enzymes, has a striking and unexpected effect of increasing survival in the LPS/D-GalN mouse model for ALF. It prevents the activation of caspase-3 and furthermore significantly reduces the overall inflammatory immune response as measured by reductions in the levels of plasma cytokines, and it prevents the ensuing activation of caspase-3 and apoptosis induction in hepatocytes. Of particular interest is that levels of TNF-α and IL6 are reduced one hour after LPS/D-GalN treatment, indicating that the primary target of MSO is some step very early in the immune response, most likely in the alteration of macrophage/Kupffer cell activation.

Determining the relative importance of these mechanisms in the suppression by MSO of TNF-α and cytokine production will require further studies focusing on macrophage/Kupffer cells and their environment, but it is clear that MSO, with its highly specific target, and at non-toxic doses, can efficiently rescue mice exposed to lethal doses of LPS/D-GalN. Glutamine synthetase may therefore warrant additional attention as a new drug target in liver failure.

CHAPTER-4

Methionine sulfoximine's prevention of acute liver failure: understanding the involvement of glutamine synthetase and glutamine

Introduction

Previous work from this lab has characterized the beneficial role of MSO in the extension of life span in the SOD1 mouse model of amytropic lateral sclerosis (ALS). The studies described in this thesis show that the use of MSO therapeutically in the mouse model for ALF leads to a decrease in plasma cytokines – mainly TNF-α, IFN-γ, and IL-6 - and an increase in survival by 75%. MSO inhibits the enzyme glutamine synthetase (GS). Glutamine, which is produced by GS, is considered essential for the generation of cytokines. It also plays an important role in immune cell proliferation, differentiation and maturation (Newsholme 2001). Hence, the simplest explanation for MSO's effects on ALF mouse model is its ability to inhibit glutamine production and subsequently reduce the immune response. Our studies aim to understand the effects of MSO and by extension the role of GS on inflammation *in vitro* and *in vivo*. In this chapter we initiated experiments aimed at defining the mechanism of action of MSO-induced GS inhibition on our ALF mouse model.

The experiments described in the previous chapter established that MSO prevents LPS/D-GalN induced liver failure via its inhibitory action on inflammation. Two of the key modulators of cell fate - TNF-α and IFN-γ – are reduced by more than 50% with MSO pre-treatment. The plasma TNF-α and IFN-γ values explain the downstream effects of the inflammatory apoptotic pathways seen in this disease (i.e. caspase-3 and STAT1 activation is prevented by MSO pre-treatment). However, these anti-apoptotic effects do not fully explain the cellular mechanism

involved in MSO's prevention of ALF. This chapter describes our subsequent efforts to explore the effects of MSO on GS and TNF-α at early disease stage – i.e. 1 or 2 hours post disease induction. We tested the effects of MSO treatment on TNF-α and GS transcription, on plasma levels of glutamate and glutamine, and on macrophages and GS protein in liver tissue from mice. For disease induction, we continued to use E *coli* lipopolysaccharides (LPS) and sugar D-Galactosamine (D-GalN). We also investigated the cellular implications of glutamine level variation and GS inhibition by MSO on isolated primary peritoneal macrophages. We measured total protein synthesis in LPS-stimulated macrophages in the presence and absence of MSO treatment.

Materials and Methods

Animals:

6 – 8 week old male CD1 animals were obtained from Charles River. Animals were housed in Wayne State University and cared using the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). All experiments were approved by the Animal Investigation Committee of Wayne State University.

Animals were starved overnight before the experiment. Control animals were injected with saline and after 3 hours another saline injection was administered intra peritoneally (IP). Diseased group animals were injected with saline, and after 3 hours, a mixture of LPS (20 µg/kg body weight, sigma – LPS from *E. Coli* 0111:B4 Sigma #L2630) and D-GalN (800 mg/kg body weight, Sigma #G1639) was administered. The treatment group consisted of mice injected with MSO (50 mg/kg body weight, Sigma #M5379) three hours prior to LPS and D-GalN

administration. All solutions were prepared in saline and administered IP. Since our previous work showed TNF-α peaking at 1 hour post LPS/D-GalN administration, we studied samples taken 1 hour or 2 hours after disease induction.

Western Blotting:

Liver homogenates were prepared from male mice either 3 hours after saline or MSO injections (time zero controls) or at one hour time after IP administration of LPS/D-GalN. Liver was homogenized in the presence of protease inhibitors. The homogenization buffer was made up of 150 mM KCl, 5 mM β-mercaptoethanol and 1 mM EDTA. Livers were homogenized by a mechanical rotor and centrifuged for 5 minutes at 10,000 x *g* - 4°C. Coomassie plus protein assay (Thermo Scientific #1856210) was used to determine the protein concentration. 10µg of protein extract was loaded for each sample. The supernatant extracts were boiled for 5 minutes and identical protein amounts were separated on a 12.5% by SDS-PAGE and immunoblotted for glutamine synthetase using anti-GS antibody (Sigma #G2781). Anti- GAPDH antibody (Sigma #G9545) was used as a loading control.

IHC:

Liver was collected and cut into small 5mm sections which were frozen immediately in OCT compound. The frozen tissue was stored at – 80^{o}C when not in use. 10 micron sections were cut in a microtome cryostat and loaded onto poly-lysine coated slides. The cut sections were also stored at – 80^{o}C.

For staining, the tissue sections were brought to room temperature for 30 minutes and then fixed in ice cold acetone for 10 minutes. Thereafter the tissue was blocked in 10% BSA (made up in phosphate buffered saline (PBS – pH 7.4) with 0.5% Tween 20 for an hour. The tissue was incubated overnight in primary antibody at concentration of 1:100 and secondary

antibody at concentration of 1:100 for 1hr. All antibodies were prepared in 1% BSA solution made up in PBS – pH 7.4. Anti-glutamine synthetase antibody (Sigma #G2781) was used to identify hepatocytes expressing GS and anti F4 80 antibody (e-biosceince #14-4801-81) was used to stain for Kupffer cells. Secondary antibody of anti rabbit – HRP (Bio-Rad #170-6515) was used against anti GS and anti rat – HRP (ebiosceince #18-4818-82) was used against F4 80 antibody. Slides were washed in for 5 minutes in PBS 3 – 5 times between each step. Lastly, the tissue was exposed to 3', 3' – D, aminobenzidine tetrahydrocholride hydrate (DAB, Sigma #D5637) solution prepared in 0.1M tris – pH 7.2, and 0.3% hydrogen peroxide. After color development the sections were dehydrated in sequentially increasing concentrations of ethanol and mounted with coverslips using permount (Fisher chemicals #FL-10-0505) mounting medium.

Determination of plasma glutamate and glutamine:

Up to 1ml of blood was collected from anesthetized mice following cardiac puncture and transferred to heparinized tubes (BD Microtainer tubes). Plasma obtained was transferred to ice immediately and treated to remove all proteins. 10% (v/v) perchloric acid was added to plasma at 1:1 concentration and then the solution was centrifuged at 3000Xg for 3 minutes. The protein precipitate was discarded and supernatants were neutralized with 20% (w/v) KOH in the presence of a universal pH indicator. The solution was left on ice for 10 minutes and then centrifuged again at 3000Xg for 3 minutes. The precipitates were again discarded and 250µl of supernatant was used for the glutamine – glutamate determination (method based on protocol from Lund P., 1986). L-glutamine and L-glutamate from protein-free plasma were quantified using the manufacturer's protocol for enzymatic determination of glutamine and glutamate (the GLN-1 kit from Sigma-Aldrich was used)

RNA isolation and RT-PCR:

Liver tissue was obtained from mice and stored in RNAlater solution (Qiagen #76154). RNA was isolated using the manufacturer's protocol for the RNeasy mini kit (Qiagen #74104). The amount of RNA was quantified spectrophotometrically by measuring the absorbance at 260 nm and 280 nm. 1 µg of RNA was used to set up a reverse transcriptase reaction and form cDNA using Quantitect reverse transcriptase kit (Qiagen #205311). 2 ul of cDNA was then used to set up the qRT-PCR reaction using the quantifast SYBR green PCR kit (Qiagen #204054). Each reaction was set up in triplicates. The RT-PCR reaction consisted of melting at 95°C for 15 seconds, annealing at 60°C for 30 sec and extension at 72°C for 15 sec. 40 cycles were run and Ct values were obtained. Mouse gene specific TNF-α, GS, GAPDH and actin primers were obtained from Qiagen (quantitect primer assay, Mn_Tnf1_1_SG # QT00104006; Mm_Actb_2_SG # QT01136772; Mm_Glut_1_SG # QT01062306; Mm_Gapdh_3_SG # QT01658692) . Actin expression was used as a control for GS and GAPDH was used as a control gene for TNF-α.

Cell culture:

All cell culture experiments were done using peritoneal macrophages obtained from male CD1 mice. Mice were euthanized in a CO_2 chamber and transferred to a cell culture hood. The peritoneum was injected with 10ml of cell culture medium RPMI with glutamine (RPMI with glutamine - 2 mM) containing 10% FCS and 1% penicillin and streptomycin solution. The peritoneum was massaged gently for 5 minutes, and the medium was extracted from the peritoneum and centrifuged. The cell pellet obtained was re-suspended in 3 ml of RPMI with glutamine. Cells were counted and 2 ml of cells were plated in 6-well plates at the density of 2 X

10^4 cells/ml. The cells were transferred to 37^{o}C – 5% CO_2 incubator for 4 hours. Thereafter they were washed three times with RPMI, and 2ml of fresh RPMI w or w/o glutamine was added. The cells were again transferred to the 37^{o}C – 5% CO_2 incubator for 16 hours. Cells were washed the next day with RPMI, and thereafter the experiments were started.

After 16 hours of overnight incubation, peritoneal macrophages were incubated in 1ml of RPMI with or without glutamine. The medium was made up of 10% FCS and 1% penicillin/streptomycin. The negative control well was not treated with LPS and/ or MSO. In the positive control well 1µg/ml of LPS was added and in another MSO control well 9mM MSO was added. The treatment wells had both 1mg/ml LPS and 9mM MSO added at the same time. Cells were then transferred to 37^{o}C – 5% CO_2 incubator for the duration of the experiment – i.e. 4 hours. At the end of the experiment the cell culture medium was collected and frozen immediately on dry ice and transferred to -20^{o}C. The same treatment conditions were used for radioactive protein measurements.

TNF-α Elisa:

TNF-α ELISA kits were obtained from ebioscience, and experiments were performed using manufacturer's guidelines for 96-well plate experiments. Each well contained 50µl of cell culture medium mixed with 50 µl of blocking buffer. The TNF-α ELISA assay had a sensitivity of 8-1000 pg/ml.

Protein synthesis measurement in cells and medium:

The peritoneal macrophages were incubated in 1ml of RPMI with glutamine. At the start of the experiment 50µCi of S^{35} labeled cocktail of methionine and cysteine was added to the medium. Samples were collected at 0, 1, 2, 4, 6, and 8 hours after S^{35}, LPS, and/ or MSO addition.

For secreted protein measurements, 50 µl of medium was used. For cellular protein synthesis measurements, PBS was added to cells, and then they were scraped and collected. To cells as well as medium 10 ml of 10% TCA was added and tubes were incubated on ice for 10 minutes. TCA precipitated protein was filtered through glass fiber filter paper. The filter papers were rinsed twice with 10 ml of 10% TCA. Filter papers were dried and TCA-precipitated counts of S^{35} labeled proteins were measured in a scintillation counter.

Results:

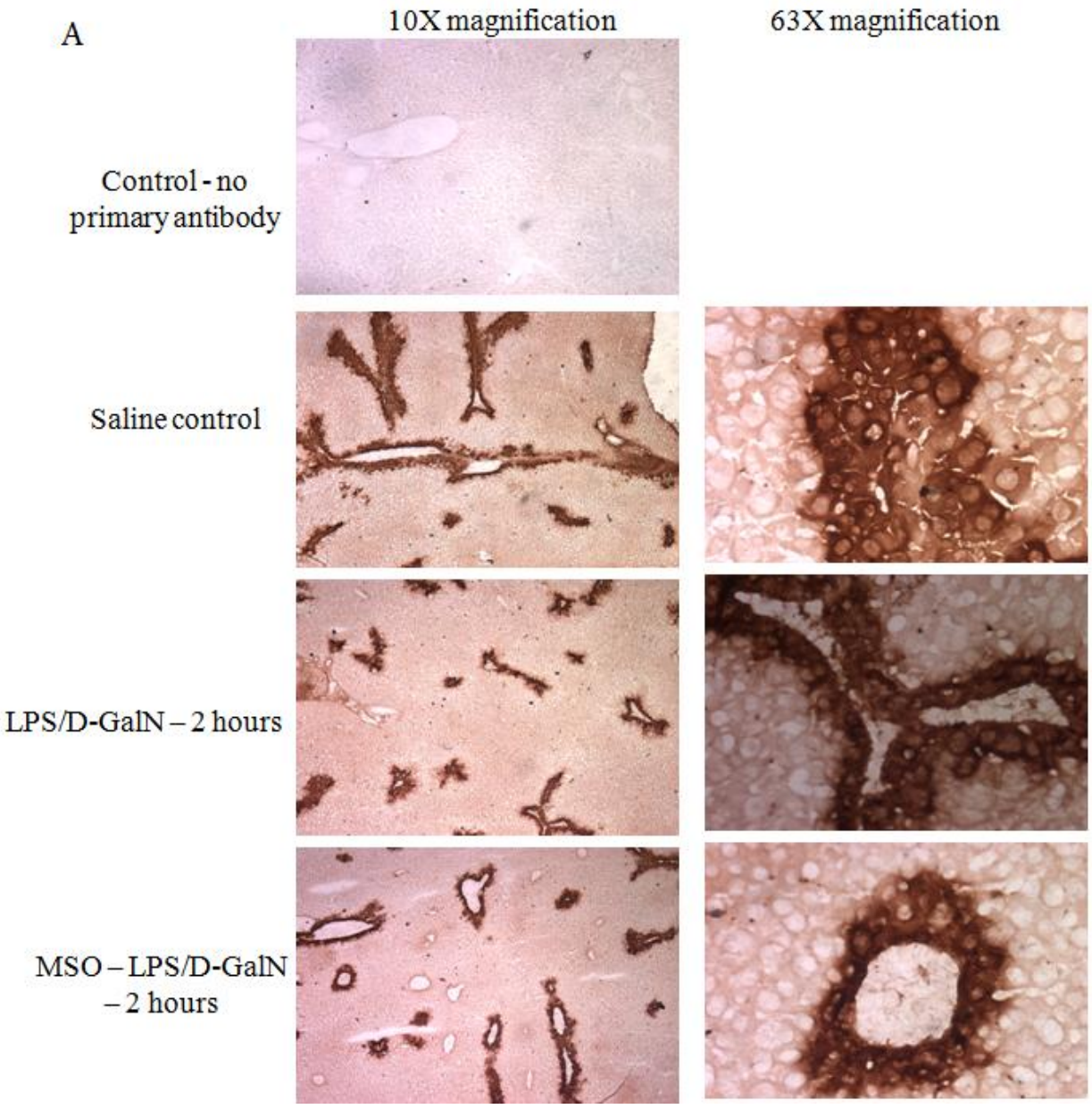
Effect of MSO on Glutamine synthetase:

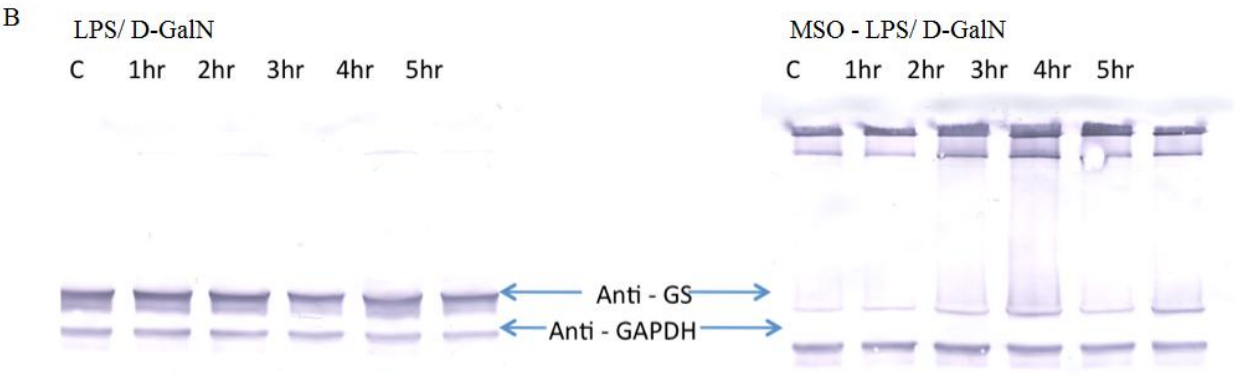
Figure 4.1A shows immunohistochemical staining of liver tissue sections obtained from a control mouse (saline treated), LPS/D-GalN mouse (2 hours post disease induction) and MSO pretreated LPS/D-GalN mouse (2 hours post disease induction). The 10 micron slices observed here under 10X magnification show a section of the stained tissue. The control tissue shows defined staining of perivenous hepatocytes, which is the known location of glutamine synthetase in the liver. As expected, the hepatic parenchyma surrounding veins in the liver are stained dark brown. Similar staining is seen in LPS/D-GalN tissue and MSO – LPS/D-GalN tissue section. The amount of staining is visibly reduced in both MSO treated and untreated disease groups as compared to the saline alone control tissue. However, there is no obvious change in the extent or distribution of GS with and without MSO treatment. The negative control section was exposed to only secondary antibody (no primary antibody exposure) during the staining procedure, and shows no GS staining.

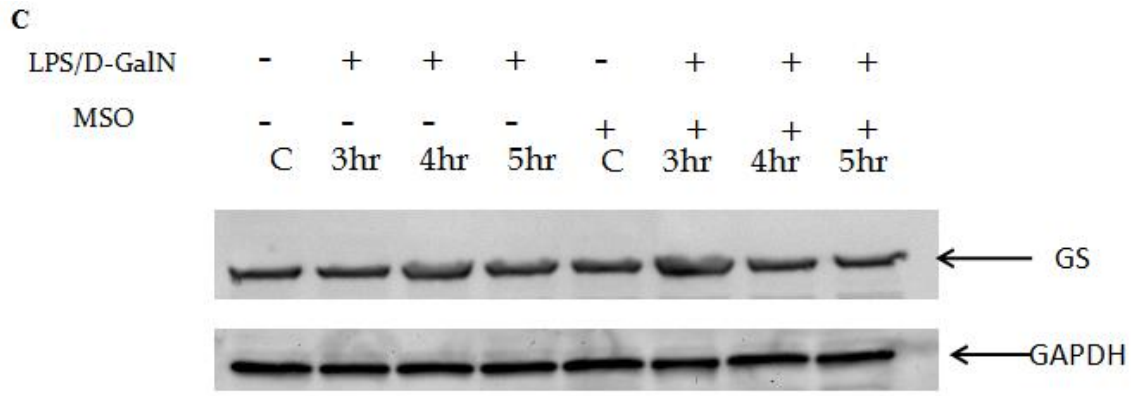
Figure 4.1B shows western blots obtained from liver tissue homogenates of treated and untreated tissue. We have found that glutamine synthetase forms higher molecular weight

aggregates when treated with MSO, which is observed in all non-boiled samples from MSO treated tissue. These aggregates are broken down, and the normal molecular weight GS band is seen when the tissue samples are boiled at 100 ^{o}C for 5 minutes. A western blot with boiled tissue samples from MSO treated and untreated LPS/D-GalN exposed mice is shown in **figure 4.1C.** The blots show that there are no differences in the GS protein levels in treated and untreated disease tissue collected from animals at 3 hours, 4 hours and 5 hours after disease induction. We have not pursued the question of GS aggregation in the presence of MSO, although the same phenomenon has been observed by another laboratory (Rao and Meister 1972).

Figure 4.1D shows the results of using real time – PCR to quantify the transcript levels of GS. The saline control group transcripts were considered baseline, we can see a very small fold change – 1.5 fold increase – in transcript levels of GS for LPS/D-GalN (1 hour post disease induction) and MSO control tissue. There is also a very small change – 0.7 fold reduction – in transcript levels in MSO treated tissue (1 hour post disease induction). Considering the apparent importance of GS in this model, these changes in transcript levels within various groups may have an implication in how MSO prevents liver failure. However, the changes are very small and are not explained by the effects of this drug on enzyme activity.


A
10X magnification
63X magnification
Control - no primary antibody
Saline control
LPS/D-GalN – 2 hours
MSO – LPS/D-GalN – 2 hours


B
LPS/ D-GalN
C 1hr 2hr 3hr 4hr 5hr
MSO - LPS/ D-GalN
C 1hr 2hr 3hr 4hr 5hr
Anti - GS
Anti - GAPDH


C
LPS/D-GalN - + + + - + + +
MSO - - - - + + + +
C 3hr 4hr 5hr C 3hr 4hr 5hr
GS
GAPDH

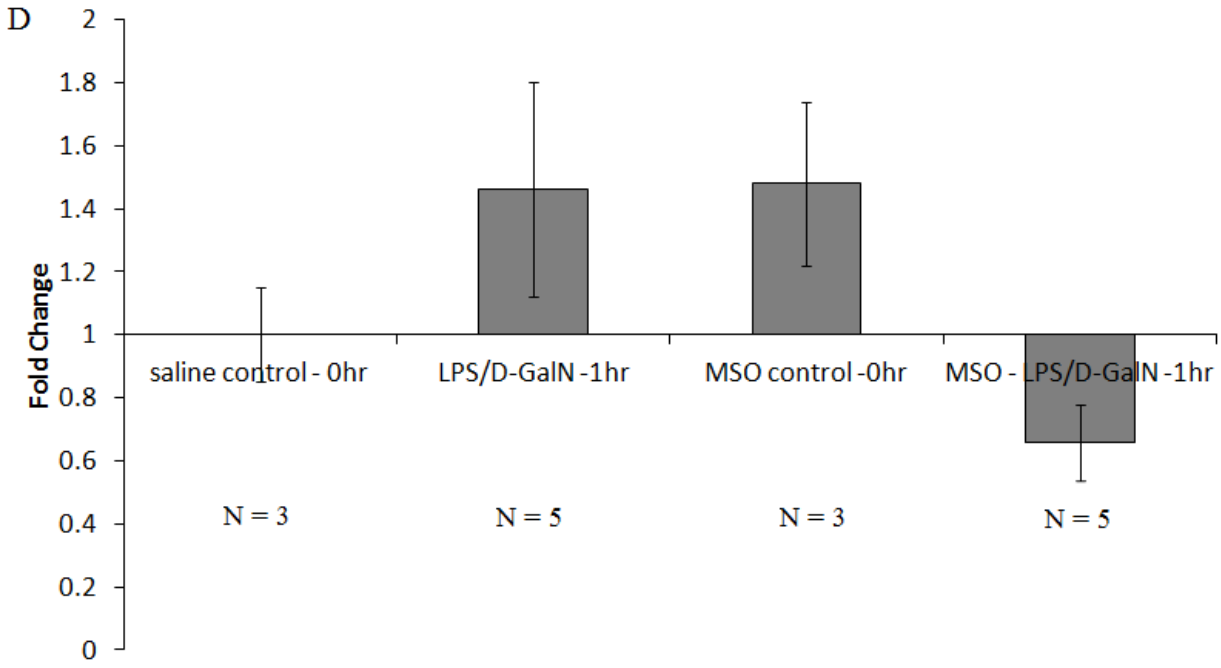

D
Fold Change
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
saline control - 0hr
LPS/D-GalN -1hr
MSO control -0hr
MSO - LPS/D-GalN -1hr
N = 3
N = 5
N = 3
N = 5

Figure 4.1. Effects of MSO treatment on protein and transcript levels of GS. Figure 1A shows immunohistochemical staining of liver tissue sections observed under 10 X and 63 X magnifications. The first panel is a control section stained with secondary antibody. It was not exposed to primary antibody. Tissue sections from saline treated control liver, 2 hours LPS/D-GalN treated liver, and MSO pre-treated 2 hour LPS/D-GalN treated liver show the characteristic perivenous GS stained hepatic cells. The same results were observed in tissue collected from 3 different animals for each group. 6 sections were stained from each tissue sample. **Figure 1B** shows western blots of non-boiled liver homogenate samples blotted for GS. Tissue homogenates were prepared at one hour time intervals after LPS/D-GalN administration. GS protein amounts are similar in all samples. MSO treatment leads to formation of higher molecular weight aggregates of GS. These aggregates are lost upon boiling the samples before running SDS-PAGE. **Figure 1C** shows western blots of liver homogenates blotted for GS. The samples collected at 3hr, 4hr and 5hr after LPS/D-GalN administration were boiled for 5 minutes and thereafter run of SDS-PAGE. The higher molecular weight bands of MSO pre-treated samples are not present in boiled samples. The controls in all the blots consisted of tissue collected from animals treated with saline for 3 hours for LPS/D-GalN exposed mice and animals treated with MSO for 3 hours for MSO-LPS/D-GalN exposed mice. **Figure 1D** shows the transcript levels observed in liver tissue from mice treated with saline alone or MSO alone (controls – first and third bars in the graph) or mice exposed to LPS/D-GalN for 1 hour after saline or MSO administration (second and fourth bars in the graph). Each 1 hour treatment group has 5 animals and each control group had 3 animals. Using the house keeping gene GAPDH, fold change in GS transcripts was calculated. Here, the Ct values obtained for GS were normalized to those of GAPDH (to obtain ΔCt). Thereafter, the sample ΔCt values were normalized to LPS/D-GalN control group (to obtain ΔΔCt). The values thus obtained were converted from log scale to the linear scale (2^-ΔΔCt). The error bars are obtained from Standard error of mean of GS ΔCt values. There is a 1.5 fold increase in GS transcripts in MSO treated control tissue and saline treated LPS/D-GalN 1 hour treated liver. However, a slight decrease of 0.7 fold is observed in MSO pre-treated 1 hour LPS/D-GalN treated liver.

MSO's effect on Kupffer cells:

TNF-α is secreted primarily by macrophages, and in this liver failure model, Kupffer cells are known to play a very important role (Karlmark, Wasmuth et al. 2008; Dong, Zuo et al. 2009; Leber, Mayrhauser et al. 2009). We looked at the Kupffer cells in the treated and untreated tissue at 2 hours post disease induction. By this time, TNF-α level had peaked in plasma and hence any abnormality with Kupffer cell numbers would be visible. In **Figure 4.2**, Kupffer cells stained with F4 80 antibody can be seen in saline control, LPS/D-GalN and MSO – LPS/D-GalN tissue sections. F4 80 is a marker for all types of macrophages. Control tissue exhibits well

defined Kupffer cells which are evenly distributed evenly across the entire section. Treated and untreated tissue shows fuzzy Kupffer cell staining which might be indicative of active Kupffer cells undergoing a morphology change. However, there seems to be no difference within MSO treated and the diseased tissue. Hence, MSO does not appear to affect Kupffer cell activation or numbers in this ALF mouse model.

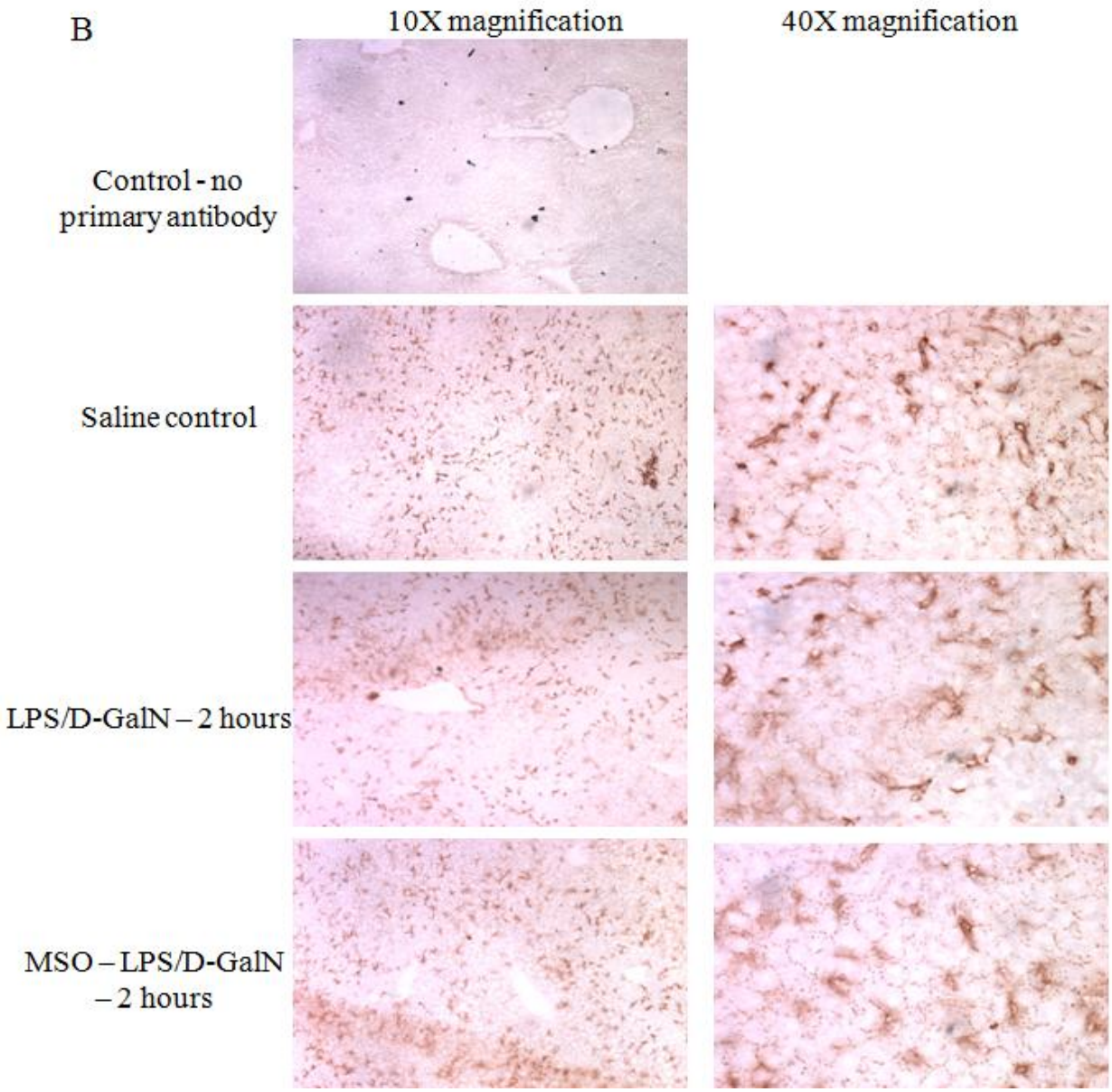


Figure 4.2. Effects of MSO treatment on macrophage staining in liver tissue. This figure shows immunohistochemical staining of liver tissue sections observed under 10 X and 40 X magnifications. Here Kupffer cells were stained with antibody marker called F4/80. This antibody stains for all macrophages. The first panel is a control section stained with secondary antibody. It was not exposed to primary antibody. Tissue sections from saline treated control liver, 2 hours LPS/D-GalN treated liver, and MSO pre-treated 2 hour LPS/D-GalN treated liver show the macrophages present throughout the liver parenchyma. The saline control tissue show distinctly shaped macrophages, whereas with LPS/D-GalN treatment the macrophages seem to be activated and do not show a distinct morphology. The same is seen in MSO pre-treated LPS/D-GalN treated liver section. The same results were observed in tissue collected from 3 different animals for each group. 6 sections were stained from each tissue sample.

MSO treatment reduces plasma glutamine but does not affect plasma glutamate:

Plasma was collected from mice 1 hour post disease induction, and plasma glutamate and glutamine were quantified (**Figure 4.3**). Each group consisted of 4 animals. As shown in **Figure 4.3A**, plasma glutamate values for saline and MSO control animals were 320 ± 46 µM and 240 ± 54 µM respectively. 1 hour post disease induction, these values were elevated to 800µM and 760 ± 57 µM respectively. These changes from control time point (i.e. 0hrs) to 1 hour for both diseased and MSO pre-treated animals is significant ($p=0.001$ for both groups). However, it is interesting to note that MSO does not in any way affect the plasma glutamate levels in these animals. The fluctuations seen in glutamate after disease induction is significant and MSO independent.

Figure 4.3B, shows the changes occurring in plasma glutamine. In control LPS/ D-GalN animals at time zero, glutamine values were 680 ± 264 µM. Glutamine was slightly elevated to 800 ± 162 µM/L at 1 hour after LPS/D-GalN administration. With MSO pre-treatment, animals exhibited much lower plasma glutamine. MSO control animals had plasma glutamine values of 68 ± 48 µM, which increased to 200 ± 57 µM at 1 hour post disease induction. Glutamine changes between saline control and LPS/D-GalN treatment and MSO control and MSO –

LPS/D-GalN treatment group are insignificant. However, the differences seen between control saline and control MSO; and 1 hour saline and 1 hour MSO are significant ($p = 0.019$ and $p = 0.0025$)

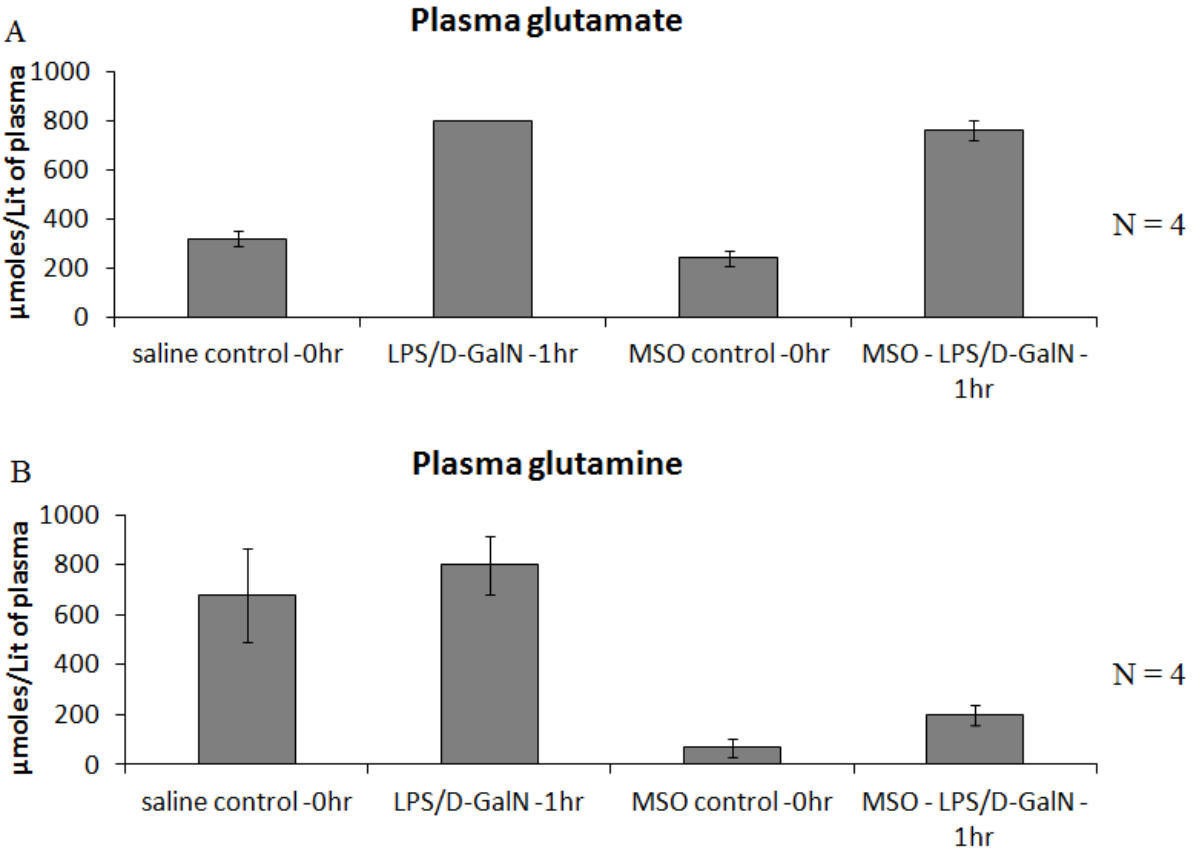


Figure 4.3. Plasma glutamate and glutamine levels in ALF mice. Figure A shows glutamate levels determined in plasma from saline control, MSO control, LPS/D-GalN and MSO pre-treated LPS/D-GalN treated animals. Each groups consisted of 4 animals. Plasma was collected one hour after LPS/D-GalN administration. Plasma glutamate levels increased from 300µM of plasma (saline control) and 240 µM of plasma (MSO control) to 800 µM of plasma (LPS/D-GalN) and 760 µM of plasma (MSO – LPS/D-GalN). This increase in plasma glutamate one hour after liver failure induction is significant for both MSO treated and untreated groups ($p = 0.001$). Figure B shows plasma glutamine levels of the above mentioned four groups of animals. MSO control animals exhibited low levels of glutamine in plasma 68 µM of plasma as compared to saline control animals which had 680 µM of plasma. This reduction due to MSO treatment was statistically significant ($P=0.02$). Similarly plasma collected from animals one hour post LPS/D-GalN administration had 800µM glutamine, compared to MSO pre-treated LPS/D-GalN treated animals (200µM glutamine). This difference was also statistically significant ($p = 0.003$). All the p values were calculated using an unpaired *t* test.

Effect of MSO on TNF-α transcript:

Figure 4.4 shows the fold change in the transcript levels of TNF-α in liver tissue from LPS and MSO-treated mice. The MSO – LPS/D-GalN control group had the same transcript levels as LPS/D-GalN control group. Transcripts increased by 100 ± 0.9 fold for LPS/D-GalN treated animals and 115 ± 0.54 fold for MSO – LPS/D-GalN treated animals at one hour after disease induction. (Values are represented as fold change ± SEM of ΔCt). These results show that the TNF-α transcript increased by more than 100 fold post disease induction. Here we see no significant difference in transcript levels when mice were pre-treated with MSO. Therefore, MSO treatment does not affect TNF-□ □transcription.

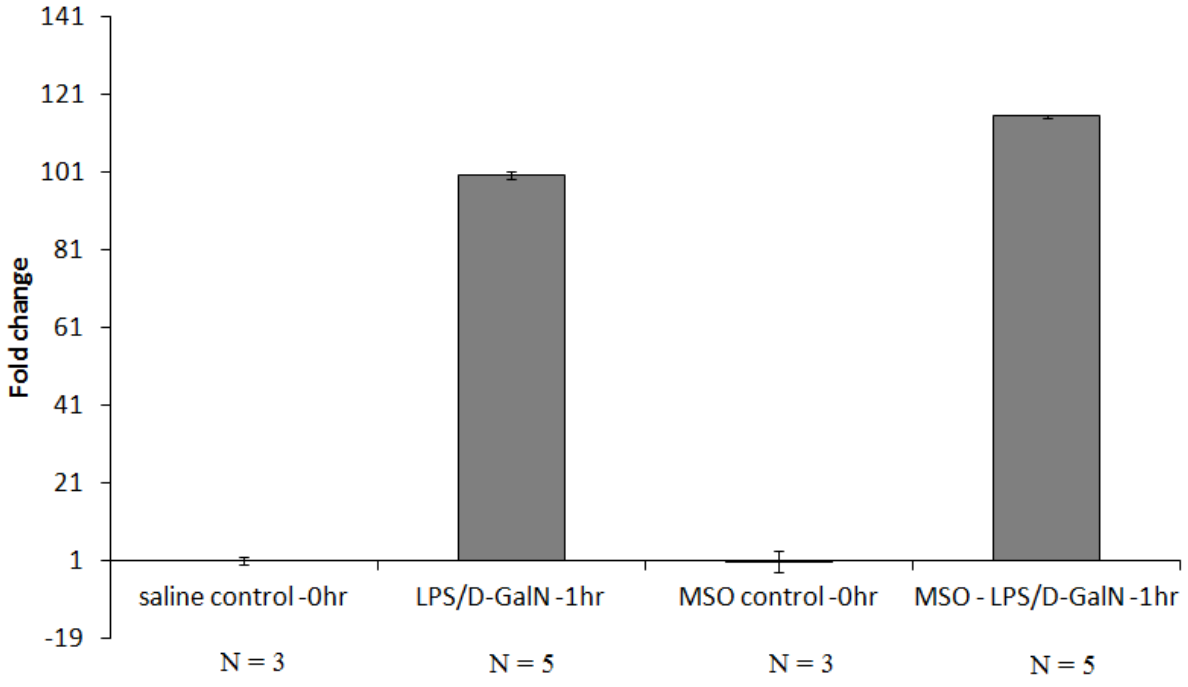


Figure 4.4. Effect of MSO treatment on TNF-α transcript levels. This figure shows the transcript levels observed in liver tissue from mice treated with saline alone or MSO alone (controls) or mice exposed to saline or MSO and treated with LPS/D-GalN for an hour. Each 1 hour treatment group had 5 animals and each control group had 3 animals. We calculated the fold change in TNF-α transcripts relative to the transcript of the housekeeping gene actin, so the

Ct values obtained for TNF-α were normalized to those of actin to obtain ΔCt. Thereafter, the sample ΔCt values were normalized to LPS/D-GalN control group (to obtain ΔΔCt). The values thus obtained were converted from log scale to the linear scale (2^-ΔΔCt). The error bars represent the standard error of mean of TNF-α ΔCt values. There is a 100 and 115 fold increase respectively in TNF-α transcript in tissue from MSO untreated and treated mice exposed to LPS/D-GalN to 1 hour.

Effect of glutamine on TNF-α secretion:

MSO reduces both liver glutamine synthetase activity and plasma glutamine in animals 1 hour post LPS/D-GalN administration, suggesting that the synthesis of glutamine, or the presence of glutamine, plays a role in the production of TNF-α. Chapter 3 shows that MSO treatment at the same time as LPS addition resulted lead to a 18% average decrease in TNF-α production (the decrease ranged from 8% to 37% ($p=0.0017$) in six separate experiments). In these experiments, cells were incubated in glutamine during the overnight incubation phase (16 hours) after cell isolation and during the LPS or LPS+MSO treatment (4 hours) phase. Hence, we conducted an experiment to test the effects of glutamine variation on TNF-α secretion by primary peritoneal macrophages.

Each experiment consisted of two negative controls – treatment 1 consisted of untreated cells and treatment 2 consisted of cells treated with 9mM MSO alone. Treatment 3 consisted of cells exposed to 1 μg/ml of LPS and treatment 4 consisted of cells exposed to both 1 μg/ml of LPS and 9 mM MSO. Cells were exposed to the above mentioned four conditions for 4 hours, with the time of LPS and/or MSO addition being considered as time zero.

Each experiment was repeated six times, and the results are presented in **Table 4.1**. When cells were incubated overnight in the presence of glutamine and then in the absence of glutamine for the 4 hours during LPS treatment (condition 2 in Table 4.1), they exhibited an average decrease in TNF-α production of 10% with a range of 7% to 15% with MSO treatment ($p =$

0.04). When glutamine was absent from the culture medium throughout the experiment (condition 3 in Table 4.1), MSO treatment reduced production of TNF-α by an average of 14% (with a range of 6% to 27% - p=0.0055). Lastly, MSO treatment reduced TNF-α production by 1.8% (average) during **condition 4** – no glutamine in cell culture medium during the 16 hour overnight incubation and 2 mM glutamine present during the 4 hours of LPS treatment. The fourth condition exhibited the most variation. In three out of six experiments, MSO treatment increased TNF-α production by 4%, 2.5% and 7.5% and in three experiments it led to a decrease of 12.5%, 4.3% and 7.5%. Averaging all the values gave a low average of 1.8%. **Appendix 1** shows the TNF-α value in pg/ml for each of the six experiments done for the four glutamine conditions.

Glutamine conditions	4 hours after macrophage isolation	16 hour overnight incubation	4 hours of LPS and/ or MSO exposure	% reduction in TNF-α production caused by MSO treatment
1	+	+	+	8 – 37
2	+	+	_	7 – 15
3	+	_	_	6 -27
4	+	_	+	(-7.5) – 12.5

Table 4.1. The table describes the glutamine conditions to which cells were exposed. After peritoneal macrophage isolation cells were plated in presence of 2 mM glutamine-rich RPMI medium for 4 hours. Thereafter for 16 hours of overnight plating, cells were exposed to glutamine rich RPMI medium for the first two conditions (condition 1 and 2), whereas cells described in condition 3 and 4 were exposed to RPMI medium with no glutamine in it. During the 4 hours of the experiment (i.e. after the addition of 1 µg/ml LPS and/or 9 mM MSO) cells of condition 1 and 4 were exposed to glutamine rich cell culture medium and cells of condition 2 and 3 were incubated in RPMI with no glutamine. Thereafter, TNF-α was measured in the cell culture medium using an ELISA. Six separate experiments were carried out. Values are presented as % TNF-α production, comparing cytokine produced in the absence of MSO to that

produced in the presence of MSO in LPS stimulated cells. TNF-α was below detection limits for untreated and MSO alone treated cells. Under conditions of glutamine present throughout the experiment (condition 1) or glutamine absent throughout the experiment (condition 3) we see a statistically significant decrease in TNF-α production in cells treated with MSO and LPS as compared to LPS alone. This decrease is of 8- 37% and 6 – 27% respectively. Whereas, when glutamine is fluctuated during the experiment (conditions 2 or 4) the effect of MSO on TNF-α secretion by peritoneal macrophages stimulated with LPS is either reduced (condition 2) or lost altogether (condition 4). (The p values for all the experiments were calculated using paired t test).

As described in Chapter 3 (and published) TNF-α production by peritoneal macrophages is consistently reduced when cells are treated with MSO. Here we show that modulating exposure of cells to glutamine during the 16 hours overnight incubation and/or the 4 hours of LPS treatment affect TNF-α production. Thus, the presence or absence of glutamine in the cell culture medium can have an effect on TNF-α secretion by peritoneal macrophages. MSO has maximal and consistent effect on cytokine production when the cells are exposed to high levels of glutamine (condition 1) or no glutamine (condition 3) throughout the course of experiment. We did not pursue this question further, and have therefore not tested any possible explanations for these results. It may be that elimination of glutamine from the medium stimulates the production of GS which might then reduce or negate the "effective MSO" concentration in these experiments. But even though these results are difficult to interpret, it is clear that altering glutamine levels can influence the extent of the effect of MSO on TNF-α production, supporting the conclusion that glutamine presence/availability is involved in this effect.

Effect of MSO on protein production:

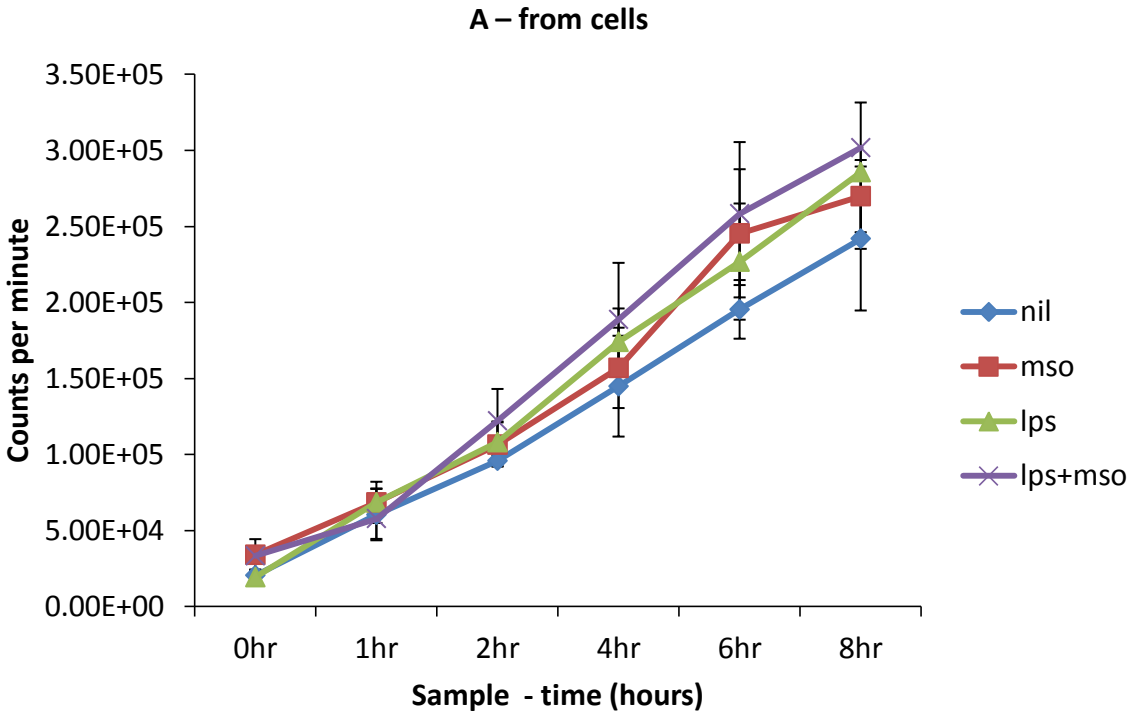
For these in vitro experiments, we used 9 mM MSO, which had been used in previously published studies on the effects of MSO on cultured cells. To assure ourselves that this

concentration was not having a global effect on protein synthesis, we measured total protein synthesis in these cultures in the presence and absence of MSO. **Figure 4.5A** shows total cellular protein synthesis in peritoneal macrophages, and **figure 4.5B** shows proteins synthesized and secreted by cells into the cell culture medium, as measured by the incorporation of S^{35} labeled methionine and cysteine. TCA-precipitable radioactive proteins were measured for 1) control cells (untreated), 2) MSO (9 mM)-treated cells 3) LPS (1 µg/ml)-treated cells and 4) cells treated with both MSO (9 mM) and LPS (1 µg/ml).

In control untreated cells, 20500 ± 3700 cpm of radioactive labeled protein was detected at time zero. Incorporation of radioactivity increased linearly to 241900 ± 47300 cpm at 8 hours. With MSO treatment, incorporation of radioactivity increased from 34000 ± 10200 cpm at zero hours to 269800 ± 23700 cpm at 8 hours. In LPS-treated cells, the incorporation of radioactivity from 19300 ± 4200 cpm at zero hours to 285600 ± 45700 cpm at 8 hours.. Treatment with both LPS and MSO led to an increase in incorporation of radioactivity from 33300 ± 5300 cpm at zero hours to 301700 ± 66600 cpm at 8 hours. Each experiment was conducted 3 times and the results are presented as average ± SEM.

Similar results were observed for proteins secreted into cell culture medium. Here the incorporation of radioactivity radio labeled proteins was measured in 50 µl of cell culture medium and the results are shown in **figure 4.5B**. The control medium had 12100 ± 1900 cpm at time zero. This increased to 76900 ± 11200 cpm after 8 hours. Medium obtained from MSO alone and LPS alone conditioned cells had 14700 ± 2300 cpm and 15100 ± 5100 cpm at time zero. At 8 hours the incorporation of radioactivity increased to 58200 ± 6500 cpm and 57800 ± 5300 cpm respectively. MSO treated cells exposed to LPS showed an initial incorporation of radioactivity of 12600 ± 3900 cpm which increased to 41400 ± 12500 cpm at 8 hours. **Appendix**

2 shows values obtained in CPM for all the experiments. Values for all time points are presented for cellular protein as well as protein secreted in 50 µl of cell culture medium. Supernatant samples from cells treated with MSO alone and LPS alone exhibited a slight reduction in radio labeled protein being secreted into medium as compared to control cells. The cpm differences observed at various time points in MSO and LPS treated cells and medium were not statistically significant. These results indicated that 9 mM MSO does not affect protein production in cells and has a negligible effect on total secreted protein levels. It is important to remember that different genes are transcribed in resting and stressed cells. It is possible that MSO and LPS together as well as separately might activate different genes without affecting overall protein synthesis. Nonetheless, this experiment makes clear that 9 mM MSO does not have a global inhibitory effect on protein synthesis in the presence of glutamine.



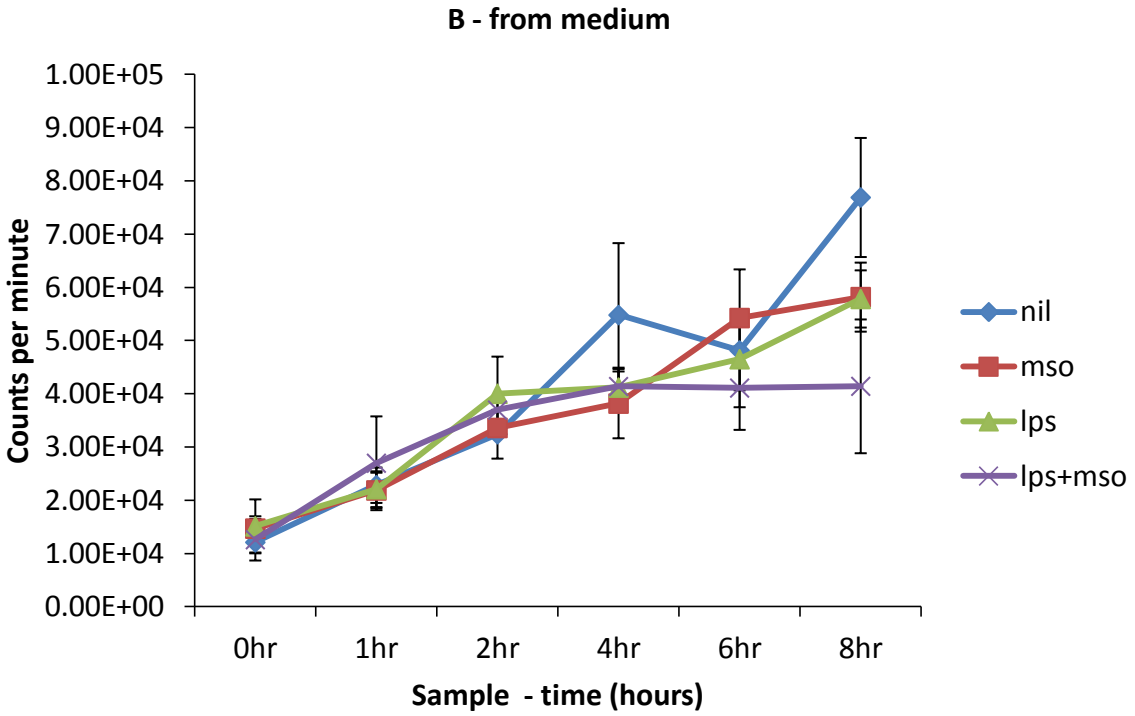


Figure 4.5. Protein synthesis measurements in peritoneal macrophages exposed to LPS and/ or MSO using radioactive S^{35}. The figures show amount of incorporated S^{35} in newly synthesized protein in untreated cells, cells treated with 9 mM MSO alone, cells treated with 1 µg/ml of LPS alone, and cells treated with 9 mM MSO and 1 µg/ml of LPS. Protein synthesis was measured at 0hrs, 1hr, 2hr, 4hr, 6hr, and 8hr after LPS and/ or MSO addition. The blue line shows S^{35} incorporation in untreated cells. The red shows S^{35} incorporation in MSO treated cells. The green line shows S^{35} incorporation in proteins of LPS–treated cells and the purple line shows protein synthesis in cells treated with both LPS and MSO. The results are the averages from three separate experiments conducted on three different days. Results are presented as counts per minute (CPM) of radioactive S^{35} incorporated in newly synthesized protein. All counts per minute (CPM) values are given as mean ± standard error of mean. **Figure 4.5.A** shows total protein synthesis in cells plus supernatant, **Figure 4.5.B** shows protein synthesis of cell secreted proteins (culture supernatant).

Conclusion

Acute liver failure is defined as the deterioration of liver function over a short period of time – typically a few weeks. Brain edema, a complication of ALF, first implicated ammonia and glutamine metabolism in disease pathology. ALF can be induced in mice by intra peritoneal administration of *E. coli* lipoplysaccharides (LPS) and the sugar D-Galactosamine (D-GalN).

The previous chapter focused on understanding the action of methionine sulfoximine (MSO) in preventing liver failure. MSO increases survival in this ALF mouse model. MSO treatment prevents caspase-3 and STAT-1 activation. MSO reduces the inflammatory immune response at some very early step, prior to TNF-α production. TNF-α is one of the first acute phase proteins to be secreted in ALF, and MSO reduces its production by more than 50% in the mouse model. TNF-α was secreted as early as 1 hour post LPS and D-GalN administration. Since MSO is a known irreversible inhibitor of glutamine synthetase (GS), GS and/or glutamine appear to be involved in ALF-associated immune response. Kupffer cells – the resident liver macrophages – have intrinsic GS activity and the GS transcripts are known to increase during stress ((Bode, Peters-Regehr et al. 2000)). Moreover, glutamine is required for the generation, propagation, and maintenance of the immune response (Crawford and Cohen 1985; Hörig, Spagnoli et al. 1993; Li, Yin et al. 2007).

In this chapter we explored the mechanistic action of MSO. We first used immunohistochemistry to determine the effects of MSO on glutamine synthetase and Kupffer cells. 2 hours after disease induction the MSO-treated and untreated tissue did not exhibit any differences in GS and Kupffer cell staining. GS staining was reduced in both LPS/D-GalN and MSO – LPS/D-GalN tissue as compared to tissue collected from a saline-treated control animal. This is indicative of a reduction in number of perivenous hepatocytes. Macrophages in both treated and untreated tissue were diffusely stained compared to control tissue. This is indicative of active macrophages. However, the staining patterns for MSO treatment and disease tissue were similar. Hence, MSO does not appear to exert its beneficial effects via GS protein levels or by directly acting upon macrophages.

Since TNF-α levels reach maximal amount after one hour in plasma, we also examined GS transcript levels and TNF-α transcript levels in tissue collected one hour after LPS/D-GalN administration. Negligible TNF-α transcripts were detected in both saline treated and MSO treated control animals (no LPS/D-GalN). In LPS/D-GalN-treated animals, MSO-treated and untreated tissue exhibited 100 fold and 115 fold increases respectively. On other hand, GS transcript levels increased by 1.5 fold in liver tissue from LPS/D-GalN tissue as compared to liver tissue from saline treated mice. Control tissue from MSO treated mice also showed a 1.5 fold increase in transcript levels, and tissue from MSO treated LPS/D-GalN mice showed a 0.7 fold decrease. Since the fold changes in transcript amounts are quite small, much smaller than the effect of MSO on the enzymatic activity of GS, MSO's mechanistic effects – reduction in TNF-α secretion in plasma of LPS/D-GalN treated mouse – probably does not involve alterations in GS transcript levels at one hour after disease induction. MSO treatment clearly does not affect TNF-α transcript but might have a small effect on the GS transcription.

At this point, we can summarize the in vivo experiments as follows:

1) MSO treatment reduces TNF-α in plasma (chapter 3)
2) MSO does not affect GS protein levels in liver sections
3) MSO does not affect macrophages – Kupffer cells – in liver sections
4) MSO does not affect TNF-α transcript levels and might have a very small effect on GS transcript levels in LPS-treated mice.

MSO's inhibition of GS had no effect on protein levels or transcription in vivo. MSO or glutamine must therefore affect either translation or secretion of TNF-α and perhaps other proteins. To investigate this question we conducted in vitro experiments using a cell culture model of primary peritoneal macrophages. Protein production in macrophages stimulated with

LPS in the presence or absence of MSO was measured by radioactive incorporation of S^{35}. Protein synthesis within cells as well as secreted proteins in medium was monitored for up to 8 hours after LPS stimulation. MSO and LPS did not affect total protein production in cells. However, different proteins might have been produced in resting untreated cells and LPS and/or MSO treated cells.

TNF-α secretion by peritoneal macrophages was then measured in cell culture medium in an experiment where cells were incubated with or without glutamine either during the 16 hour overnight incubation phase before treatment or during the 4 hour LPS or LPS+MSO treatment phase. Maximal inhibitory effect of MSO was observed in either presence or absence of glutamine. When glutamine was either removed before the experiment or added during LPS stimulation of cells or vice – a – versa MSO's inhibitory effect was either lost or diminished. There is no clear explanation for these results besides the observation that the effect of MSO could be altered by altering culture medium glutamine.

At this point our results do not fully explain the role of glutamine and glutamine synthetase in cytokine production by immune cells. Glutamine is involved in formation of other amino acids and energy producing intermediates. Our model shows glutamine levels are depleted in the plasma of MSO treated LPS/D-GalN injected mice. Work done by Komano et al, shows that supplementing rat diet with 10% glutamine prevented serum transaminase elevation in D-GalN induced liver injury. Serum transaminases are indicators of "liver health". However, this 10% glutamine supplementation was unable to prevent TNF-α and caspase-3 activation (Komano, Yokoyama Funakoshi et al. 2009). Thus, it seems that glutamine is acting via some other pathway to reduce cytokines and prevent caspase-3 activation.

Glutamine is also required by various liver cell types for regeneration. Regeneration is activated in stressed liver, and the ability of glutamine to form glutamate, glucosamine-6-phosphate, GMP, NAD etc is critical (Gebhardt, Baldysiak-Figiel et al. 2007; Chang and Xu 2010). 90% of the energy required by liver parenchymal cells is obtained from glycolysis. However, for Kupffer cells glutamine is the main source of energy (Spolarics, Lang et al. 1991). Since glutamine is primary source of energy for immune cells, during stress it can become a rate-limiting factor for processes following proliferation – such as phagocytosis or antibody production (Karinch, Pan et al. 2001), these and other processes involved in regeneration might explain the mechanism of action of MSO. The reduction of glutamine might have no effect on transcription within or proliferation of Kupffer cells, but might be having an effect on antibody secretion and immune cell function triggered by liver damage. Such an explanation, however, still does not tell us the exact mechanism by which MSO prevents the production of TNF-□.

Another contradictory fact is that cytokine release is generally controlled at the level of gene expression. Hence, intracellular signaling mechanisms which induce transcription of late and early response inflammatory genes might be more important in this model. As mentioned in the discussion of the previous chapter, β – catenin and mTOR pathways are involved with glutamine metabolism. β-catenin mutated hepatic carcinoma cell lines are "addicted" to glutamine. β-catenin acts to inhibit apoptosis and glutamine synthetase is under its control (Bioulac-Sage, Cubel et al. 2011). Depletion of glutamine pools by MSO in these cells initiates autophagy and apoptosis. Whereas, in some cell lines MSO causes accumulation of glutamine within the cells leading to mTOR activation and cell survival (Tardito, Chiu et al. 2011). Direct activation of mTOR in cells by MSO has also been observed. Moreover this effect is more pronounced in glutamine depleted cells (Tardito, Chiu et al. 2011). mTOR is a serine/ theronine protein kinase.

It regulates cell proliferation, cell survival, transcription and translation. It has capacity to integrate the nutrient status of cell with energy and redox status. It is especially sensitive to glutamine. These two pathways might be influenced by MSO in our model of liver failure, and by their action we see the protective effects of MSO being exercised.

Clearly these studies are just the beginning of understanding the therapeutic effects of MSO in this mouse model of ALF. Further studies need to address the importance of signaling pathways such as how the β-catenin and mTOR pathways respond in the presence MSO. At the same time, glutamine's actions need to be scrutinized at the levels of transcription, translation and secretion. Glutamine compartmentalization within various liver cell types – specifically periportal hepatocytes, pericentral hepatocytes and Kupffer cells – needs to be explored. The current studies need to be elaborated to include tissue collected at more time intervals after LPS/D-GalN administration. MSO's effects on translation and protein secretion have to be scrutinized to determine which step in TNF- production is most strongly affected. We need to look at TNF-α secretion rather than total secreted proteins to determine MSO's effect. Lastly, while continuing the experiments with peritoneal macrophages it would be essential to remember that the peritoneal macrophages are not Kupffer cells, and the specialized liver macrophages might be involved in the disease progression by novel mechanisms.

CHAPTER-5

Conclusion and future directions

The liver is an important organ, responsible for detoxification, protein synthesis, glycogen storage, gluconeogensis, cholesterol synthesis, drug metabolism, production of digestive juices, and ammonia detoxification. Acute liver failure (ALF) is a condition characterized by development of coagulopathy and hepatic encephalopathy in a previously healthy individual. ALF is caused by acetaminophen overdose, viral hepatitis (A, B and E), autoimmune hepatitis, severe alcohol intake and idiosyncratic reaction to drugs such as tetracycline. Severe systemic inflammation is mainly responsible for disease pathogenesis. Resident immune cells generate multiple cytokines at the site of injury and inflammation. Primarily macrophages i.e. Kupffer cells secrete cytokines which activate other immune cells in the vicinity (i.e. PMNs, NK and NKT cells) and thereafter attract and activate systemic immune cells, resulting in the generation of multiple cytokine and chemokines, hence the term cytokine storm is often used to explain ALF associated inflammation. Multiple cytokines are capable of initiating apoptotic death pathways in hepatocytes.

Methionine sulfoximine (MSO) is an irreversible inhibitor of glutamine synthetase (GS). GS plays an important role in ammonia detoxification. It generates glutamine from glutamate and ammonia. Glutamine is an energy source for immune cells. It is a conditionally essential amino acid. During stress conditions glutamine becomes rate limiting for immune cells. Our preliminary results show that inhibiting GS by treatment with MSO dramatically improved survival in a mouse model of acute liver failure.

The aims of my project were to 1) characterize the mechanism of action of MSO on the mouse model for acute liver failure (ALF) by screening for changes in plasma metabolites and

signaling molecules associated with the steps involved in different death pathways, 2) identify which step in the inflammatory response was inhibited by MSO treatment, and 3) to characterize the mechanism of action of MSO on the inflammatory immune response. The previous chapters have described the studies that answered aims 1 and 2, and have also described our first studies toward addressing aim 3.

For this project we used a model of lipopolysaccharides (LPS) and D- galactosamine (D-GalN) administration in mice. Our initial experiments were conducted on mice, but it was necessary to study macrophages *in vitro* to address mechanistic questions, so we included experiments on primary peritoneal macrophage cell culture to address how MSO might be affecting the inflammatory immune response. The major conclusion from both *in vivo* and *in vitro* studies is that the non essential amino acid glutamine and enzyme glutamine synthetase (GS) appear to influence the disease process, and that inhibition of GS by MSO prevents ALF associated inflammatory responses.

The inflammatory immune response that occurs during ALF involves the production of many cytokines, the most prominent of which are TNF-α, IFN-γ and IL-6. These cytokines activate death pathways by activation of caspase 3 and STAT1, both of which are capable of causing cellular death by apoptosis. Inhibition of glutamine synthetase by pre-treating animals with MSO both increased survival and prevented activation of caspase 3 and STAT1, in addition to reducing the levels of most plasma cytokines tested, especially TNF-α and IFN-γ, both of which were reduced by more than 50%.

In LPS/D-GalN treated animals, plasma glutamate levels were doubled but plasma glutamine levels were unaffected one hour after disease induction. With MSO pre-treatment, glutamate levels were same as control and were double at one hour after LPS/D-GalN injection.

However, plasma glutamine levels were reduced by 80% in MSO treated animals for both control and LPS/D-GalN treated group of animals. GS activity, observed from 1 hour to 5 hours after disease induction, in MSO pre-treated livers was reduced by more than 90% compared to livers from untreated animals, where activity did not fluctuate over the same 5 hours (result shown in chapter 3). MSO pre-treatment did not affect TNF-α transcript levels which increased 100 fold one hour after LPS/D-GalN administration in both saline and MSO treated animals. At the same time, a 1.5 fold increase was seen in GS transcripts of diseased and MSO control animals. MSO treated disease animals exhibited a 0.7 fold reduction in GS transcript levels. Therefore, MSO did not significantly affect the transcription of either TNF-α, which was substantially elevated in LPS-treated animals, or GS, which was substantially unchanged in LPS-treated animals.

I conducted immunohistochemistry experiments to see what effect MSO might be having on total amounts of observable glutamine synthetase in normal and diseased livers, and also to see if the total numbers of liver macrophages (Kupffer cells) were obviously altered by MSO treatment. Both experiments showed no effect of MSO, so it appears that this drug does not affect the amount of GS or the numbers of macrophages.

We conducted preliminary tests of the glutamine requirement for the TNF-α production in cell cultures of peritoneal macrophages. Although MSO-treatment consistently reduced TNF-α production in these cultures treated with LPS, that effect could be modified by the presence or absence of glutamine in the culture. Detailed experiments with varying conditions of glutamine during overnight cell incubation and treatment with LPS and/ or MSO produced conflicting effects of MSO on TNF-α production. Keeping glutamine constant during the experiments i.e. either conducting the experiments in presence of 2 mM glutamine and in complete absence of

glutamine showed maximal MSO inhibition of TNF-α production in presence of LPS. However, if glutamine in the medium was varied during the experiments, MSO's effects were either diminished or lost. Especially if glutamine was removed from cells until LPS and/ or MSO addition, TNF-α production was unaffected or even increased in the presence of MSO for LPS treated cells. These results are difficult to interpret, but they do show that glutamine levels in the culture medium can affect the action of MSO on TNF-α production.

One control experiment that was easy to interpret was my investigation on the effects of MSO on overall levels of protein synthesis, using S^{35}-labelled methionine and cysteine to quantitate the synthesis of both cellular and secreted proteins. In both untreated cells and cells treated with LPS to induce a cytokine response, the presence of 9 mM MSO had no effect on TCA-precipitable counts from either cells or the culture supernatant. Therefore, the effect of MSO was not a global effect on cellular protein synthesis.

In summary, MSO does not affect transcription of GS or TNF-α. It does not affect observable levels of GS in liver, and it does not affect the numbers of macrophages in liver. In cell culture, 9 mM MSO does not affect protein synthesis, but its inhibitory effect on cytokine production can be modulated somewhat by the presence or absence of culture glutamine – a not unexpected result.

My studies have shown that some connection exists between glutamine synthetase and the very early steps of the inflammatory immune response. That connection probably involves glutamine levels, although these studies do not address the specific involvement of plasma glutamine or some localized concentration of intracellular glutamine. As alluded to above, we found that starving peritoneal macrophages for glutamine overnight made them resistant to the cytokine-inhibitory effect of MSO when glutamine was added during the immune response, but

cells fed glutamine overnight and then starved of glutamine during the immune response were as sensitive to MSO as cells that had never been starved. Therefore, as we expected, glutamine levels matter, but we still don't know how.

The question of how GS is involved in the inflammatory immune response will have to be addressed in the future. The initial experiments will confirm the involvement of GS in this process by either 1) knocking down the expression of GS in peritoneal macrophages and repeating the experiments in cells with no GS, or by using another macrophage cell line which is capable of secreting TNF-α but does not have GS, or 2) using the liver specific GS knockout mouse model (liver specific β – catenin knockout mouse model). We would predict that the absence of GS in any of these systems would make these cells unable, or less able, to produce cytokines when stimulated by LPS. If cytokines were produced in the absence (or reduction) of GS, it would suggest that MSO might have one or more additional targets that are involved in the cytokine response.

But if, as we expect, GS really is the functional target for MSO in this system, future studies will involve a detailed analysis of cytokine synthesis and secretion, with the major question being what effect does MSO treatment have on the translation and secretion/release of TNF-α, arguably the most important inflammatory cytokine in this system as well as in many others? We have shown that MSO does not affect transcription. We will address this question in two ways – immunohistochemical and immunoprecipitate. We will try to use immunohistochemistry of liver slices to determine if the amounts of TNF-α produced in the presence of MSO are similar to the amounts produced in the absence of MSO. If so, then the results would support the hypothesis that MSO prevents release of TNF-α from the cells or cell surfaces. If not, and we see a significant reduction in the anti- TNF-α fluorescence in slices from

MSO-treated animals, then MSO is preventing synthesis of the cytokine, specifically by inhibiting translation. However, these will be difficult experiments because it may be difficult or impossible to visualize TNF-α in tissue slices. Or the amounts of intracellular TNF-α and membrane-associated TNF-α might be too low to be analyzed by this approach. It may therefore be necessary to use an immunoprecipitation approach using either mouse liver tissue or peritoneal macrophages, or both.

In vivo, we could solublize liver homogenates from control animals, LPS-treated animals, or LPS-treated animals pretreated with MSO, then attempt to immunoprecipitate those extracts with anti-TNF-α, followed by immunostain analysis to determine if the presence of MSO significantly reduced the total amount of TNF-α in the tissue. In vitro, we could label LPS-treated cells with S^{35}, then immunoprecipitate whole cells (solublized) and cell supernatant with anti- TNF-α to determine if MSO treatment reduces the total amount of TNF-α produced in cells treated with LPS. We hope these approaches will provide a more definitive answer to the question of which step in the early immune response is affected by MSO treatment.

The subsequent studies to define the mechanism more exactly will depend on the results of those experiments. We will either be studying the factors involved in TNF-α translation more carefully to determine how they are affected by MSO, or we will be studying the processing and release of TNF-α from the cell surface to see how that process is affected by MSO. As mentioned above, it is possible – although we feel unlikely – that these studies will show that MSO has another target involved in the inflammatory immune response besides (or in addition to) glutamine synthetase, in which case we would try to identify that target.

The involvement in the response to LPS of neutrophils and natural killer T cells has also been identified in this thesis. Their infiltration pattern in the presence and absence of MSO pre-

treatment will be investigated by immunohistochemistry. It is possible that MSO prevents tissue infiltration by other immune cells and thus prevents the cytokine storm. Another method of studying the mechanism is to modify the disease model. The use of acetaminophen, thioacetamide or chronic alcoholic liver failure model will provide diversity in terms of immune response. Each model is perpetuated by a type of immune cell and the effect of MSO on a specific type of immune cell within a whole animal can thus be easily studied.

Finally, there is a vast amount of research being carried out to understand the role of glutamine in inflammation. In the previous chapters we described the involvement of the mTOR and □-catenin pathways in glutamine metabolism. MSO treatment might be acting, in part, by influencing one or both of those signaling pathways. Contradictory literature of glutamine's effect on cytokine production and immune cell proliferation is indicative of the complexity of its role. Dietary glutamine is not necessarily beneficial in all conditions of stress. At the same time compartmentalization of GS within the liver shows that it plays a bigger role than just ammonia detoxification and glutamine homeostasis. Understanding these complex questions will involve experiments building on the results of my studies to provide new directions in this research.

APPENDIX A

Condition	Sample	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6
1 Gln=+/+/+	Nil	BDL	BDL	BDL	BDL	BDL	BDL
	MSO	BDL	BDL	BDL	BDL	BDL	BDL
	LPS	495	548	491	361	654	610
	LPS + MSO	421	442	394	228	604	564
2 Gln=+/+/-	Nil	BDL	BDL	BDL	BDL	BDL	BDL
	MSO	BDL	BDL	BDL	BDL	BDL	BDL
	LPS	742	117	1120	759	118	1142
	LPS + MSO	682	102	1046	701	110	971
3 Gln=+/-/-	Nil	BDL	BDL	BDL	BDL	BDL	BDL
	MSO	BDL	BDL	BDL	BDL	BDL	BDL
	LPS	556	557	420	523	569	567
	LPS + MSO	522	479	307	454	510	BDL
4 Gln= +/-/+	Nil	BDL	BDL	BDL	BDL	BDL	BDL
	MSO	BDL	BDL	BDL	BDL	BDL	BDL
	LPS	592	673	645	559	407	508
	LPS + MSO	616	587	661	517	389	546

Appendix A. TNF-α values obtained from cultured primary peritoneal macrophages are presented above. Cells were cultured in RPMI medium containing 5% FCS and in presence or absence of 2 mM glutamine. 50 µl of cell culture medium was used for ELISA measurement of the cytokine. Sensitivity of ELISA was 8 – 1000 pg/ml. Cells were stimulated with 1 µg/ml of LPS for 4 hours and then the culture medium was collected for TNF-α production. Experiment consisted of controls such as untreated cells and cells treated with 9 mM MSO alone. Both these conditions produced undetectable amounts of TNF-α (BDL = below detectable limits). For cells treated with LPS alone and LPS and MSO TNF-α values obtained are presented as pg/ml in the above table. %decrease presented in table 4.1 of the thesis has been calculated from the above shown values. Also, these values were used to run a paired t test and obtain the p values which have been described in the chapter 4. This experiment shows that varying glutamine in cell culture medium has an effect on TNF-α secretion.

APPENDIX B

Experiment 1

Sample	0hr	1hr	2hr	4hr	6hr	8hr
Nil	13360	56509	88840	111052	206269	236167
MSO	51449	69374	78617	132651	188186	290040
LPS	11641	50506	89645	188478	228665	247254
LPS + MSO	24824	46553	107817	190337	229813	310109

Experiment 2

Sample	0hr	1hr	2hr	4hr	6hr	8hr
Nil	22068	91301	99267	211055	221853	326559
MSO	16055	91393	127629	209576	327435	296849
LPS	20004	80160	134466	202575	291853	376599
LPS + MSO	43125	84553	163202	252229	350060	412424

Experiment 3

Sample	0hr	1hr	2hr	4hr	6hr	8hr
Nil	26155	33288	99393	112384	157873	163059
MSO	34532	44529	113086	128350	220429	222511
LPS	26226	74708	100061	131006	159692	233035
LPS + MSO	31991	42390	95352	123756	195092	182560

Appendix B.1. CPM values obtained by TCA precipitation of radio labeled proteins from cultured primary peritoneal macrophage cells. Three separate experiments are listed above. 20,000 cells were cultured in wells of a 6 well plate. Radioactive S^{35}, LPS and MSO were added at the start of the experiment (0hr). At various time points (0hr, 1hr, 2hr, 4hr, 6hr and 8hr) after S^{35}, LPS and MSO addition medium was removed and cells were washed once. The cells were then scraped in 500 µl of PBS and proteins were TCA precipitated with 10 ml of ice cold 10% TCA. The precipitates were collected on glass fiber filter paper and washed twice with 10% TCA. The filters were air dried and thereafter the radio labeled protein formed was measured in cpm (counts per minute) in a scintillation counter.

Experiment 1

Sample	0hr	1hr	2hr	4hr	6hr	8hr
Nil	15104	23803	29241	32621	34350	99174
MSO	14786	24733	31218	33867	37230	50428
LPS	6313	22905	28315	35830	28532	47131
LPS + MSO	7005	43854	35292	40658	26428	16627

Experiment 2

Sample	0hr	1hr	2hr	4hr	6hr	8hr
Nil	12695	28083	41462	52622	60008	67434
MSO	10649	25658	35876	50986	68514	53096
LPS	15236	27489	52434	46280	56241	63535
LPS + MSO	10740	22885	42159	47873	53232	57143

Experiment 3

Sample	0hr	1hr	2hr	4hr	6hr	8hr
Nil	8585	16686	26610	79236	50145	64140
MSO	18678	15240	33754	29724	57038	71057
LPS	23845	15948	39382	41509	54832	62896
LPS + MSO	20110	14214	33474	35675	43666	50543

Appendix B.2. CPM values obtained by TCA precipitation of radio labeled proteins from cell culture medium of cultured primary peritoneal macrophage cells. Three separate experiments are listed above. 20,000 cells were cultured in wells of a 6 well plate. Radioactive S^{35}, LPS and MSO were added at the start of the experiment (0hr). At various time points (0hr, 1hr, 2hr, 4hr, 6hr and 8hr) after S^{35}, LPS and MSO addition 50 µl of medium was removed and 10 ml of ice cold TCA was added to it for protein precipitation. The precipitates were collected on glass fiber filter paper and washed twice with 10% TCA. The filters were air dried and thereafter the radio labeled protein formed was measured in cpm (counts per minute) in a scintillation counter.

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ABSTRACT

USE OF METHIONINE SULFOXIMINE TO DISSECT THE ROLE OF GLUTAMINE SYNTHETASE AND GLUTAMINE IN PROGRESSION OF ACUTE LIVER FAILURE

by

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Methionine sulfoximine (MSO) is a modified amino acid and a well characterized irreversible inhibitor of glutamine synthetase (GS) enzyme. Glutamine is synthesized by GS enzyme and it is the most abundant amino acid in the body. Glutamine is required by immune cells for generation, propagation and maintenance of an immune response.

To induce acute liver failure (ALF) in mice, animals were given intraperitoneal injections of *E. coli* lipopolysaccharides (LPS) and sugar D-galactosamine (D-GalN). When these animals were pretreated with MSO, 80% of the animals were completely rescued from liver failure. Moreover, when we characterized the immune response generated during ALF using cytokine antibody microarrays, we observed 1) that the macrophages and neutrophils appeared to be responsible for the cytokine storm generated during ALF, and 2) MSO pretreatment reduced the entire measured cytokine response.

Using ELISA assays we observed that MSO pretreatment reduced plasma cytokine values of TNF-α, IFN-γ and IL-6 by more than 50%. MSO inhibited GS by more than 90% in liver extracts and reduced plasma glutamine by around 75%. MSO pretreatment had no effect on GS protein levels and on kupffer cell (liver macrophages) infiltration in liver. GS and TNF-α transcript levels were unaffected by MSO pretreatment. Also, preliminary results show that MSO does not inhibit cellular and secreted protein synthesis globally in LPS stimulated peritoneal macrophage cell culture. However, MSO treatment reduces TNF-α production in the LPS stimulated peritoneal macrophages.

Thus, it seems that inhibiting GS during stress conditions such that low levels of plasma glutamine are maintained can lead to a reduction in inflammatory cytokines. Also, MSO might have targets other than glutamine synthetase. These results show a novel use for MSO in attenuating the overall immune inflammatory response in liver.

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Jambekar, A. A., E. Palma, et al. (2011). **"A glutamine synthetase inhibitor increases survival and decreases cytokine response in a mouse model of acute liver failure."** Liver international: official journal of the International Association for the Study of the Liver **31** (8): 1209 - 1221.

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