ROLE OF INTERLEUKIN-1β AND NEUTROPHIL ACTIVATION DURING ACETAMINOPHEN OVERDOSE

BY

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Abstract

Acetaminophen (APAP) overdose is the leading cause of acute liver failure in the US. APAP is metabolized to a reactive metabolite that causes hepatotoxicity in a dose-dependent manner. A series of cascading intracellular events lead to cellular necrosis; this necrosis initiates a sterile inflammatory response which includes the production of multiple cytokines and chemokines that in turn recruit innate immune cells into the liver. Many of the inflammatory or corresponding anti-inflammatory mediators that are produced in this process have been linked to alterations in the subsequent injury as well as the injury resolution. This dissertation focuses on one particular inflammatory mediator, interleukin-1β (IL-1β), and its ability to modulate neutrophil priming, activation and hepatic recruitment. We show that mature IL-1β is produced during APAP overdose in a caspase dependent manner (Nalp3 inflammasome) which can be inhibited in vivo by a pan-caspase inhibitor, and that IL-1β is capable of activating neutrophils in vivo. However, the limited amount of IL-1β produced during APAP overdose is insufficient to activate or recruit neutrophils into the liver. In confirmation of these findings, genetic elimination of the components of the Nalp3 inflammasome or the IL-1 receptor does not alter APAP-induced injury or neutrophil recruitment. It has previously been shown that neutrophils do not participate in APAP-induced injury. This has been demonstrated in various ways, however, controversy arose when pretreatment of mice with neutropenia-inducing antibody resulted in protection from APAP toxicity. To further clarify this matter, CD18-deficient mice were subjected to APAP overdose, and in agreement with previous findings these mice were not protected from APAP overdose. Next we functionally characterized neutrophils during APAP overdose.
overdose to further confirm that neutrophils do not participate in exacerbation of injury. Interestingly, during injury resolution neutrophils become activated especially in peripheral blood but did not have enhanced reactive oxygen priming in the liver. These findings were confirmed with NADPH oxidase deficient mice which had no alteration in injury resolution. Interestingly, these data were very similar to neutrophil function in human APAP overdose patients. These data indicate that activation of neutrophils might be critical for maintaining host defense during hepatic impairment. As a whole, this dissertation shows IL-1β is a minor participant in the sterile inflammatory response following APAP overdose, but this response is critical for neutrophil activation and eventual injury resolution.
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1. Introduction

Portions adapted from:

1.1 History of APAP

The history of acetaminophen (APAP) dates back to the 1850’s when a French chemist, Charles Gerhardt, synthesized and described acetanilide, while APAP itself was first described in chemical literature in 1878. Around this time another drug, phenacetin, was also discovered. In 1886 acetanilide was inadvertently used in the clinic by the French doctors Arnold Cahn and Paul Hepp, who noticed that in addition to its antipyretic (fever reducing) properties it was also an analgesic (pain reducer). In 1899 it was demonstrated by the German chemist Karl Morner that acetanilide could be converted via hydroxylation to form APAP. For much of the first half of the 20th century these drugs were limited in usage due to fears of methemoglobinemia.

In the 1940s Julius Axelrod working in the laboratory of Bernard B. Brodie at the National Institutes of Health discovered that the active metabolite of both acetanilide and phenacetin is actually APAP. This discovery essentially ended the use of acetanilide and it was determined the methemoglobinemia induced by acetanilide is due to small percentage of the drug being converted to aniline. Phenacetin was still used clinically until the 1980s as a preparation of aspirin-phenacetin-caffeine (APC). A large percentage of phenacetin was converted to APAP through first pass metabolism however the parent drug also had pharmacologic actions. It was subsequently determined that phenacetin caused an increased risk of kidney injury and it was suspected as a carcinogen and therefore was subsequently removed from the market.

The findings of Axelrod and Brodie demonstrated that APAP is the dominant active pharmacologic compound and spurred its use clinically.
In 1951 the Institute for the Study of Analgesic and Sedative Drugs held a symposium in New York City regarding the efficacy of APAP. It was reported at this symposium that APAP was equally efficacious as aspirin for pain relief and fever reduction without the adverse side effects like gastrointestinal bleeding, stomach irritation and alterations in blood coagulation. This prompted McNeil Laboratories (now McNeil Consumer Health, a Johnson & Johnson company) to begin research and potential marketing of the drug. Two years later McNeil marketed the prescription elixir named Algoson which was a formulation of butabarbital and APAP.

Then in 1955 McNeil released a children’s elixir named Tylenol (N-aceTYL-p-aminophENOL) and in 1961 the adult formulation was marketed. These events gave rise to one of the most widely used and economically successful drugs ever produced. However, like with all drugs there is good and bad, and the story is not free from controversy.

1.2 Background of APAP

It is estimated that each week approximately 50 million Americans consume acetaminophen either alone in combination formulations. APAP can be used by itself in oral (tablet, capsule, caplet, liquid), rectal and intravenous formulations. It is found in numerous over the counter formulations like cold, flu and allergy combinations. Additionally, it is present in many prescription formulations that include controlled substances like Lortab®, Darvocet®, Vicodin®, Percocet® and many others. If taken at therapeutic doses APAP is very safe with few side effects, however overdose of APAP is currently the most frequent cause of acute liver failure in the US, the UK and many
other countries and accounts for nearly one half of all acute liver failure cases (Larson et al., 2005). The therapeutic dose of APAP for an adult is 4g per day (~60mg/kg/day); a serious risk of potentially fatal overdose can occur at approximately five times that dose (300mg/kg/day) and even 2.5-3 times a therapeutic dose can have toxic consequences. Cases of APAP overdose occur intentionally (suicide attempt) and unintentionally, with an increasing number of cases of unintentional overdosing due to the consumption of multiple drug preparations containing APAP.

Seminal work in the mechanism of APAP toxicity was performed in the early 1970's (Mitchell et al., 1973a,b; Jollow et al., 1973). These studies demonstrated that APAP hepatotoxicity involves the formation of a reactive metabolite, hepatic glutathione (GSH) depletion, and protein binding, all of which correlate with liver injury (Mitchell et al., 1973a,b; Jollow et al., 1973).

Although the focus of research in understanding the mechanisms of APAP-induced liver injury was always on intracellular events in hepatocytes, there is an increasing awareness that nonparenchymal cells of the liver and infiltrating inflammatory cells may be involved in the pathogenesis (Jaeschke 2005; Liu and Kaplowitz 2005). However, in contrast to the widely accepted contribution of inflammatory cells in the pathophysiology of hepatic ischemia-reperfusion injury, obstructive cholestasis and endotoxemia (Jaeschke 2003; Jaeschke 2006; Jaeschke and Hasegawa 2006), the contribution of inflammation in the mechanism of APAP-induced liver injury is highly controversial (Jaeschke 2008). The objective of these studies was to determine if the formation of IL-1β is a critical event in toxicity and if the production of IL-1β modulates neutrophil activity and function. Additionally we functionally characterized the role of neutrophils in
the pathophysiology of APAP overdose to determine their role in injury as well as injury resolution and tissue repair.

1.3 APAP compared to other drug induced liver toxicities

Adverse drug reactions can be divided into five categories. Type A (augmented) adverse reactions represent an exaggeration of pharmacologic effect; an example of this is excessive bleeding due to anticoagulant overdose. Type B (bizarre or idiosyncratic) reactions are not predictable based on dose, exposure and pharmacology; examples include troglitazone, flucloxacillin and other drugs that cause idiosyncratic drug-induced liver injury (IDILI). Type C (chemical) reactions have predictable toxicity based on the chemical structure of the parent drug or metabolite; APAP is the classical example of a chemical adverse reaction which was demonstrated by the four part series of Mitchell and coworkers in 1973. Type D (delayed) adverse reactions are normally a secondary effect of a previous drug treatment; a typical example would be a secondary cancer following chemotherapy. Type E (End-of-treatment) reactions occur upon removal of drug; an example would be the induction of seizure upon stoppage of phenytoin.

Drug disposition alone cannot explain the other causes of drug induced liver injury (DILI) because there is no association with the accumulation of excess drug or metabolite that would result in concentrations that are toxic to the liver (Kaplowitz, 2005). This is in contrast to what is seen in APAP overdose. Excessive concentration and hepatocytes-specific reactive metabolite generation result in this injury. The causes
of the other DILI are largely unknown but there are several theories to the mechanisms which include hapten formation, immune-mediated cell death, and mitochondrial stress. In most cases IDILI is regarded as dose-independent, however this is most likely an incorrect statement. IDILI is very rarely seen at doses of <10mg/day and more than three fourth of IDILI cases occur when the drug is given at >50mg/day (Zhang et al., 2011). The notion that IDILI is dose independent probably arises from the fact that the overwhelming majority of patients taking the drug are non-responders in regard to toxicity (Uetrecht, 2007).

Drugs with the highest IDILI concern are antineoplastic agents, NSAIDs, antivirals, antidepressants, and antimicrobials, and in the US, antimicrobials account for 46% of IDILI (Fontana et al., 2010). In a majority of cases (73%) a single prescription was implicated as the cause of IDILI (Chalasani et al., 2010). Clinically, the only effective treatment for DILI is to stop the administration of the drug and offer supportive care. The clear exception to this rule is the administration of N-acetylcysteine for APAP overdose (and potentially carnitine for valproic acid overdose).

There are several reasons to assume IDILI is immune mediated. The most striking is the time to onset; depending on the drug it could take months to over one year prior to initiation of IDILI which would indicate an adaptive immune response. Additionally elevated transaminase activities can be observed with some drugs up to one month following discontinuation of drug (Uetrecht, 2007). Some IDILI also involves the generation of antidrug antibodies or autoantibodies (Uetrecht, 2007). The very rapid onset to APAP-induced liver injury is in stark contrast to other drug-induced liver injury; additionally no biologically relevant clinical manifestations occur with APAP unless it is
taken at supra-therapeutic levels (occasionally transient ALT increases can be seen at therapeutic levels). Patients who overdosed on APAP have no additional increased probability of subsequent liver injury (autoantibodies or antidrug antibodies) and in fact the liver repairs completely without risk of fibrosis or cirrhosis.

1.4 Clinical implication and treatment of APAP overdose

APAP hepatotoxicity is initiated by the P450-mediated metabolic conversion to \( N \)-acetyl-\( \rho \)-benzoquinone imine (NAPQI) which is discussed in more detail (1.6.1 and 1.8). It has been recognized clinically since the 1960s (Thomas et al., 1966) as cause of severe liver injury which can ultimately lead to death. APAP overdose in the US results in ~50,000 emergency room visits, ~25,000 hospital admissions and ~500 deaths annually (Larson et al., 2005). After overdose, elevations in serum liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), can be seen within one day and generally peak around three days post overdose. This loss of hepatocyte mass prevents normal hepatic function like the production of serum proteins (coagulation factors, complement components, albumin and others) and the removal of endo- and xenobiotics. Impaired liver function can be monitored clinically by blood coagulopathies (increased international normalized ratio (INR)) and elevation in serum bilirubin. Upon liver recovery these laboratory results will begin to normalize, however if the liver mass is insufficient these lab results will continue to worsen.

The administration of \( N \)-acetylcysteine (NAC) was shown to be an effective antidote against APAP hepatotoxicity in clinical practice (Prescott et al., 1977) and still today is the only approved drug to treat APAP overdose patients (Polson et al., 2005).
Treatment involves the administration of NAC intravenously (i.v.) or orally. The i.v. regimen involves 150mg/kg/h over 1 h followed by 12.5mg/kg/h over 4 hours then 6.25mg/kg/h over 3 days. Oral dosing of NAC begins with a loading dose of 140mg/kg and 70mg/kg every four hours. Duration of treatment depends on clinically determined need but generally lasts a minimum of 3-5 days. The decision to treat patients with NAC generally occurs very quickly because timing is critical for patient outcome, so if very high serum transaminases are observed or the patient claims to have take large quantities of APAP NAC will started immediately. To aid in the decision making process, clinicians generally refer to the Rumack-Mathews nonogram. This is a plot of serum APAP concentration versus time post APAP-ingestion. High APAP concentration at a time many hours past ingestion is dangerous and will definitely initiate NAC treatment.

After overdose, in addition to NAC, patients receive supportive care like initial volume replacement therapy which reduces lactic acidosis and some patients may eventually need to be on renal supportive therapy. Renal dialysis can also help to reduce ammonia which is a very severe problem clinically. There are reports of renal toxicity due to APAP however many believe this toxicity may be mediated by the loss of liver function and altered metabolic state of the patient. Others feel that the expression of P450 enzymes in tubule cells are the cause of this injury as evidenced by renal GSH depletion (Mudge et al., 1978). Realistically the major clinical problem in these patients is the failing hepatic function and kidney injury is only a secondary problem. In addition patients that recover from the hepatic injury never sustain permanent renal damage. Excessive ammonia results in encephalopathy and coma. If these treatments fail then
the only remaining option is liver transplantation which is determined by using the King’s College Criteria. These criteria include INR, serum creatinine and encephalopathic grade; additionally, clinicians today use low arterial pH as another criterion. These patients are also at high risk of the development of infection or sepsis due to prolonged stay in the hospital and compromised immunity.

1.5 Epidemiology of APAP overdose
In the largest and most comprehensive retrospective study performed to date, which spanned from 1998 to 2007, it was reported that of all causes of acute liver failure (ALF), APAP was the predominant cause (46%) and liver injury from all other drugs accounted for 11% (Lee et al., 2008). Additionally, the spontaneous patient survival is quite different between etiologies; APAP showed a 65% spontaneous survival rate while the spontaneous survival rate from other drugs was only 29% (Lee et al., 2008). APAP overdose has a sex bias with approximately three-fourths of the patient being female (Lee et al., 2008); other forms of DILI also show a female predominance but not any other cause of ALF (Lee et al., 2008). Most emergency departments reporting APAP overdose show no difference in distribution of intentional versus unintentional overdose (Lee et al., 2008). Additionally, in these patients who overdosed there is no difference in quantity taken (average of 20-25 grams), maximum ALT, spontaneous survival, percentage of patients who underwent liver transplantation, or patients that died (Lee et al., 2008).
1.6 APAP pharmacokinetic (PK) and pharmacodynamics (PD)

The PD of APAP makes for a very interesting story. The mechanism of action of APAP for its pharmacologic activity *in vivo* is almost completely unknown. It is believed to act centrally for both its analgesic and antipyretic effects. It differs in mechanism from the commonly used NSAIDs and aspirin because it will not inhibit cyclooxygenases (COX) in the periphery, however it was reported that APAP inhibits COX-2 centrally by reducing the heme at the peroxidase domain rather than inhibiting the catalytic domain (Kis et al., 2005). Additionally, it is believed that APAP cannot act as an anti-inflammatory because of high peroxide levels in inflamed tissue (Kam and So, 2009). It was initially believed that APAP inhibited the previously undiscovered COX-3. In 2002, an alternative splicing variant of canine Cox-1 was discovered and named Cox-3 (Chandrasekharan et al., 2002); it was thought to answer the long sought after question of the APAP therapeutic target. The problem was a frameshift in rodents and humans prevented all activity of the COX-3 enzyme (Kis et al., 2005). Since this time, the COX-3 hypothesis has fallen out of favor. Most recently a group has reported that the mechanism of action might be the reactive metabolites acting centrally to reduce voltage-gated calcium and sodium currents in primary sensory neurons (Andersson et al., 2011). All mechanisms of therapeutic action to date are still speculative and highly scrutinized.

APAP has a relatively high pKa (9.5) therefore it does not ionize in compartments *in vivo*. Additionally being a small molecule (molecular weight: 151.2 Da) it is freely diffusible across cell membranes; this gives credence to its ability to act centrally. To be popular as an analgesic and antipyretic APAP should demonstrate an effect quickly and
generally it is regarded to have an onset of action within 30 minutes of oral dosing which should last between 4-6 hours (which also happens to be the dosing regimen). The half-life of APAP in humans ranges between 60 to 180 minutes with a normal half-life of approximately 90 minutes (Koch-Weser, 1976). In neonates and overdose patients the half-life is longer (2 to 5 hours) presumably due to poor glucuronidation or general liver impairment, respectively (Koch-Weser, 1976). Peak serum APAP concentrations in humans occurs as quickly as 10 minutes post ingestion but can occur in some people up to 1 hour post dosing and are generally around 30 μg/mL (~200 μmol/L). Since APAP is a small drug the excretion occurs mainly through the kidney, with 2-5% being eliminated as the parent drug, ~55% as the glucuronide metabolite (on the hydroxyl group), ~30% as the sulfate metabolite (on the hydroxyl group) and the remaining dose as mercapturic acid (GSH conjugated to APAP at the meta position). This is shown in 1.6.1.
Most of the ingested dose of APAP is conjugated by glucuronidation (~55-60%) or sulfation (~25-30%) and eliminated. The remaining APAP is converted to the quinoneimine (NAPQI). This electrophilic metabolite is detoxified by GSH and eliminated as mercapturic acid in the urine. If this detoxification pathway is overwhelmed adduction of cellular proteins can occur thereby initiating toxicity.
1.7 Animal models of APAP overdose

As with most drugs and toxicants, the most commonly used animal models are rodent models with rats and mice being the most common. Interestingly these two models are quite different in terms of toxicity and potentially mechanism of injury. Rats are resistant to APAP induced liver injury, but the reason for this insensitivity is unclear. Mice on the other hand present a liver injury similar to what is observed clinically in humans. The progression of injury is more rapid in mice than humans with liver enzymes peaking 12-24h post overdose rather than 1-2 days which is seen in patients. For this reason and the use of gene knockout animals, mice are the most commonly used model species for mechanistic studies. Other animal models have also been used but are not commonly studied. APAP in both dogs and pigs results in severe liver injury however many of these studies are highly variable in terms of liver injury and are not commonly used as models (Gazzard et al., 1975; Thompson et al., 1972). Cats are a poor model of APAP overdose because they are inefficient glucuronidators (Welch et al., 1966); in fact a very small amount of APAP is highly toxic in cats and can cause methemoglobinemia independent of liver injury (Welch et al., 1966). Many of these studies in larger animals (dogs and pigs) also report the development of methemoglobinemia which would make them poor models for human APAP overdose because this does not occur clinically (Gazzard et al., 1975; Thompson et al., 1972).

1.8 Intracellular mechanisms of APAP toxicity

Initiation Phase: APAP-induced liver injury depends on the metabolism of a small fraction of the overall dose of APAP (generally <10%) through the cytochrome P450
system (CYP) (Mitchell et al., 1973a). The most predominant isoform catalyzing this reaction is CYP2E1, however CYP1A2, CYP2D6 and potentially others form the reactive metabolite, \(N\)-acetyl-\(p\)-benzoquinone imine (NAPQI). Detoxification of NAPQI with GSH occurs either spontaneously or is catalyzed by glutathione-S-transferases (Dahlin et al., 1984). If the formation of NAPQI exceeds the capacity of GSH to eliminate this metabolite, NAPQI can react with protein sulphydryl groups, which leads to the formation of protein adducts (Mitchell et al., 1973b; Jollow et al., 1973). Initially, it was assumed that covalent binding of APAP to proteins was the cause of cell death (Mitchell et al., 1973a,b; Jallow et al., 1973). Although cell death correlates with early protein binding (Roberts et al., 1991), it was obvious that the total amount of adducts is limited and may be insufficient to cause cell death. Therefore it was hypothesized that NAPQI may selectively affect vital proteins within the cell. A substantial number of adducted proteins were identified (Cohen et al., 1997; Qiu et al., 1998), however none of the proteins adducted were so critical that a moderate loss of enzyme activity would account for the rapid cell death. Studies of the non-hepatotoxic regioisomer of APAP, 3'-hydroxyacetanilide (AMAP) suggested that binding of mitochondrial proteins was essential for toxicity (Qiu et al., 2001; Tirmenstein et al., 1989). The parallel recognition that APAP overdose can inhibit mitochondrial respiration (Meyers et al., 1988; Ramsay et al.; 1989) and causes a selective mitochondrial oxidant stress (Jaeschke 1990) led to the concept that early protein binding is the initiator of toxicity, which requires amplification and propagation in order to cause cell death (Jaeschke et al., 2003; Jaeschke and Bajt 2006).
Propagation Phase. Central to amplifying the initial stress of protein adduct formation are the mitochondria. Although some of the mechanistic details are still unknown, it is well established that the mitochondrial translocation of bax is a very early event (Jaeschke and Bajt 2006; Bajt et al., 2008; Bajt et al., 2006). Bax together with Bak form pores in the outer mitochondrial membrane that lead to the release of intermembrane proteins including cytochrome c, the second mitochondrial activator of caspases (smac), endonuclease G and apoptosis-inducing factor (AIF) (Bajt et al., 2008). Endonuclease G and AIF translocate to the nucleus (Bajt et al., 2006) and contribute to the characteristic nuclear DNA fragmentation and cell death (Bajt et al., 2008). Independent of bax, the mitochondrial protein binding causes inhibition of mitochondrial respiration resulting in oxidant stress and peroxynitrite formation in mitochondria (Knight et al., 2001; Cover et al., 2005). The oxidant stress is responsible for mitochondrial DNA damage (Cover et al., 2005) and the opening of the mitochondrial membrane permeability transition pore (MPT) (Masubuchi et al., 2005; Kon et al., 2004; Ramachandran et al., 2011), which triggers the collapse of the membrane potential and loss of ATP formation. The resulting mitochondrial swelling leads to the rupture of the outer membrane with release of intermembrane proteins and subsequent nuclear DNA fragmentation (Bajt et al., 2008). The selective scavenging of mitochondrial peroxynitrite by accelerating the recovery of mitochondrial GSH levels documented the critical role of peroxynitrite in the pathophysiology (Knight et al., 2002; Bajt et al., 2003). In addition, the supply of large doses of N-acetylcysteine and GSH supports mitochondrial function by providing substrates for ATP synthesis (Saito et al., 2010). Together the emerging evidence is
very strong that dysfunction of mitochondria and the resulting energy crisis and nuclear DNA damage are key events in causing oncotic necrotic cell death (Gujral et al., 2002).

1.9 Sterile inflammation, cytokine formation and inflammasome activation

A fundamental principle of sterile inflammation is the requirement for necrotic cell death to trigger this inflammation (Scaffidi et al., 2002). Thus, some initial hepatocellular injury must occur to initiate the inflammatory response which may then modulate the injury. Following APAP-induced toxic insult the formation of cytokines such as TNF-\(\alpha\), IL-1\(\beta\) and others, has been well described (Blazka et al., 1995; Lawson et al., 2000; Gardner et al., 2003; James et al., 2005) but the initiating mechanisms emerged only recently. Damage associated molecular patterns (DAMPs) are molecules released from dying cells that are ligands for toll-like receptors (TLRs) on macrophages and other cell types (Schwabe et al., 2006; Jeannin et al., 2008). DAMPs identified to be released during APAP hepatotoxicity include high mobility group box 1 protein (HMGB1), heat shock proteins, mitochondrial and nuclear DNA fragments and others (Jaeschke 2008; Jahr et al., 2001; Martin-Murphy et al., 2010; Antoine et al., 2009). A hypoacetylated form of HMGB1 can be passively released by necrotic cells (Antoine et al., 2009) and a hyperacetylated form of HMGB1 is secreted by activated macrophages and indicates an inflammatory response (Bonaldi et al., 2003). Both hyper- and hypo-acetylated forms of HMGB1 are found in plasma after APAP overdose (Antoine et al., 2009). However, it appears that HMGB1 alone is less effective as a pro-inflammatory mediator; combinations of HMGB1 with other DAMPS such as DNA fragments are the most potent inflammasens (Bianchi 2009). Although these DAMPs are clearly released into
the plasma during APAP-induced liver injury, the impact on the injury mechanisms is controversial. Antibodies against HMGB1 have been shown to reduce hepatic neutrophil accumulation without effect on injury (Jaeschke 2008). However, other authors reported a minor reduction in liver injury (Antoine et al., 2010) or drastically reduced liver injury in the presence of HMGB1 antibodies (Chen et al., 2009). In addition, HMGB1 antibodies attenuated cytokine and chemokine (TNF-α, MCP-1, IL-6) formation (Antoine et al., 2009; Chen et al., 2009) supporting the hypothesis that HMGB1 is an important mediator of the inflammatory response after APAP overdose. Mice deficient in TLR4, a receptor for HMGB1, showed a moderate reduction in APAP-induced injury (Yohe et al., 2006) but mice deficient in TNF-α (Boess et al., 1998) were not protected. In contrast, TNF receptor 1 (TNFR1)-deficient mice had exaggerated liver injury, which correlated with accelerated iNOS induction and peroxynitrite formation (Chiu et al., 2003).

DNA fragmentation is a characteristic feature of APAP-induced cell death (Cover et al., 2005; Ray et al., 1990; Lawson et al., 1999). DNA fragments released during APAP-induced necrosis (Jahr et al., 2001; McGill et al., 2012) can be recognized by TLR9 (Imaeda et al., 2009). The pathophysiological relevance of TLR9 has been implicated by the protective effect of TLR9 antagonists and in TLR9-deficient mice (Imaeda et al., 2009). TLR9 stimulation can activate cytokine formation through activation of NF-κB (Imaeda et al., 2009). It was hypothesized that interleukin-1α and IL-1β are critical mediators of APAP hepatotoxicity based on the observation that IL-1 receptor-deficient (IL-1R1-/-) mice are protected (Chen et al., 2007) and the reports that mice deficient in components of the Nalp3 inflammasome show substantially reduced injury (Imaeda et al., 2009). This protein complex consists of Nalp3 (NACHT, LRR, and pyrin domain-
containing protein 3), ASC (apoptosis-associated speck-like protein containing a CARD), and caspase-1 (Lamkanfi et al., 2009). Based on the reduced injury in Nalp3-/-, ASC-/- and caspase-1-/- mice, it was concluded that processing of pro-IL-1β and pro-IL-18 to the active, soluble cytokines is critical for APAP-induced liver injury (Imaeda et al., 2009). The IL-1 receptor 1 (IL-1R1) and TLRs share a common signaling domain called the Toll-like/IL-1 Receptor (TIR) domain on the cytoplasmic side of the cell surface receptor as well as additional adapter molecules all of which contain additional TIR domains (O'Neill and Bowie, 2008; Kenny and O'Neill, 2008). The lack of a cytoplasmic death domain in the IL-1R makes it impossible for IL-1α or β to directly induce cell death (Sims et al., 2010) therefore this toxicity would be mediated through inflammatory cell activation and recruitment.

Taken together, there is established release of various DAMPs after APAP overdose triggering the formation of a number of cytokines and chemokines and initiating recruitment of neutrophils and monocytes into the liver. However, there is no convincing evidence to suggest that the pro-inflammatory mediator formation results in direct cell death (apoptosis) (Gujral et al., 2002) or a neutrophil- or macrophage-mediated injury (Jaeschke 2008). In contrast, inflammatory cytokines are able to modulate intracellular events within hepatocytes thereby altering toxicity.

1.10 Role of Kupffer cells in APAP hepatotoxicity

The resident macrophages of the liver (Kupffer cells) are activated within 1-2 h after APAP overdose in mice as indicated by the formation of cytokines (Blazka et al., 1995; Lawson et al., 2000; Gardner et al., 2003; James et al., 2005; Martin-Murphy et al.,
In this respect it is important to recognize that the first report on macrophage activation after APAP used a rat model (Laskin et al., 1986). The rat is a poor model for APAP overdose because the rat is highly resistant to toxicity, whereas the mouse model more closely mimics human APAP overdose (as described in 1.7). A similar investigation using GdCl₃ was later repeated in the mouse (Michael et al., 1999). In this report, GdCl₃ completely eliminated the oxidant stress and peroxynitrite formation and liver injury during the first 8 h after APAP (Michael et al., 1999). In this case, GdCl₃ clearly acted on Kupffer cells as infiltrating monocytes are not detectable before 12-24 h in the mouse (Holt et al., 2008). However, the main conclusion of this manuscript, i.e. that Kupffer cell-derived oxidants are responsible for the centrilobular injury (Michael et al., 1999), is highly questionable. First, the most active Kupffer cells in terms of reactive oxygen formation are located in the periportal area as part of the vital host defense function (Bautista et al., 1990; Jaeschke et al., 1991). Thus, it is unlikely that oxidants formed in the periportal area cause selectively damage to centrilobular hepatocytes. Second, mice with a genetic deficiency of a functional NADPH oxidase, the enzyme by which phagocytes such as Kupffer cells produce reactive oxygen, do not show reduced oxidant stress or peroxynitrite formation and liver injury after APAP overdose (James et al., 2003). Likewise, inhibitors of NADPH oxidase do not protect against APAP hepatotoxicity (Cover et al., 2006). Thus, it is highly unlikely that Kupffer cells are involved in the pathophysiology of APAP-induced liver injury by directly causing cell injury through reactive oxygen and peroxynitrite formation. In support of these findings, several subsequent studies using GdCl₃ found either very little or even no protection (Ito et al., 2003; Knight et al., 2004; Ju et al., 2002). Even the sinusoidal endothelial cell
injury and hemorrhage that occurs early after APAP overdose in certain mouse strains appears to be related to APAP toxicity in SEC rather than indirect cytotoxicity of Kupffer cells (Knight et al., 2001; Lawson et al., 2000; Ito et al., 2003; Ju et al., 2002; Yin et al., 2010). However, the most important finding related to Kupffer cells was reported by Ju et al (Ju et al., 2002). This study demonstrated that elimination of Kupffer cells by clodronate liposomes actually increased APAP toxicity. The most likely explanation is related to the elimination of the formation of IL-10, IL-6 and other cytokines and of cyclooxygenase products (Ju et al., 2002). IL-10 was shown to protect against APAP toxicity by downregulation of iNOS expression and peroxynitrite formation (Bourdi et al., 2002). In addition, cyclooxygenase products induce heat shock proteins (Reilly et al., 2001), which protect against APAP toxicity (Tolson et al., 2006). Together, these data demonstrate that the dominant effect of Kupffer cell activation after APAP is not to cause cytotoxicity by oxidant formation but to limit toxicity by preventing excessive iNOS induction, by promoting cytoprotective gene expression and by supporting regeneration.

1.11 Role of NK and NKT cells in APAP hepatotoxicity

It was reported that depletion of natural killer (NK) and NK T cells in the liver exerted a protective effect against APAP hepatotoxicity (Liu et al., 2004). The increase in a large number of cytokines and chemokines including interferon-γ (IFN-γ) after APAP treatment was substantially reduced in NK and NKT cell-depleted mice (Liu et al., 2004). In addition, down-regulation of the Fas receptor and reduced neutrophil accumulation in the NK/NKT cell-depleted mice was observed. Interestingly, the effect of APAP on cytokine and chemokine formation, Fas receptor expression and neutrophil
infiltration could also be reproduced in IFN-γ-deficient mice (Ishida et al., 2002). Because IFN-γ formation was mainly attributed to NK and NKT cells, it was concluded that these cell types are responsible for IFN-γ production and the more severe injury after APAP overdose (Liu et al., 2004). It was later determined that the presence of DMSO as a solvent in these studies activated NK and NKT cells which does not occur without DMSO (Masson et al., 2008). In all, it was found that DMSO used in these initial studies increased activation of these NK and NKT cells and in particular enhanced granzyme B and IFN-γ production. Depletion of NK and NKT cells does not alter APAP-induced injury unless these cell types are pre-activated with DMSO (Masson et al., 2008). Despite the limitations, the initial study did demonstrate that IFN-γ has the potential to modulate APAP-induced toxicity, even if it was induced under non-physiological conditions. Further confirming the potential of IFN-γ to alter APAP toxicity, IFN-γ-/- mice and wild-type mice treated with IFN-γ neutralizing antibody were used (Ishida et al., 2002). Depending on the time after APAP overdose IFN-γ-/- mice showed a 2- to 8-fold reduction in plasma ALT activities and the neutralizing antibody reduced ALT levels approximately 3-fold at 24 hours. The reduction in injury also correlated with reduced immune cell infiltration, cytokine and chemokine formation and liver Fas receptor expression (Ishida et al., 2002).

During APAP-induced sterile inflammation various immune cells are recruited into the liver many of which highly express Fas ligand (FasL), and it is well established that hepatocytes express Fas receptor. Increased circulating levels of Fas have been observed after APAP overdose in humans (Tagami et al., 2003). The Fas/FasL interaction has the potential to modulate APAP-induced injury by affecting intracellular
signaling mechanisms. Although the easiest interaction between FasL and the Fas receptor would be to trigger apoptosis, there is no evidence that apoptotic cell death makes a relevant contribution to the overall liver injury after APAP overdose (Williams et al., 2011). Nevertheless, several lines of evidence exist linking the Fas receptor and/or FasL to altered APAP toxicity. An in vivo study in mice showed that knocking-down the Fas receptor protected against 300 mg/kg APAP overdose but the protection was lost when the dose was increased to 700 mg/kg (Zhang et al., 2000). These experiments utilized antisense or scrambled oligonucleotides, which were injected once per day for four days prior to APAP overdose. In this study potential alterations in APAP metabolism and/or GSH levels were not evaluated (Zhang et al., 2000). This raises the possibility that the protection may have been an indirect effect. A different study demonstrated that subliminal activation of Fas by the Fas-activating antibody, Jo-2, enhanced APAP-induced injury (Tinel et al., 2004). In these experiments, the very low dose of Jo-2 itself caused no plasma ALT increase or any detectable caspase activation in the liver. If administered prior to APAP, however, Jo-2 caused a more than two-fold increase in APAP induced injury (Tinel et al., 2004). In this study it was shown that the subliminal Fas activation increased iNOS induction, which then enhanced APAP-induced injury (Tinel et al., 2004) linking Fas receptor activation to a critical intracellular signaling event. Thus, three independent studies demonstrated three unique ways that Fas receptor activation and cytokine formation can modulate APAP toxicity. Potential Fas activation mediated through infiltrating leukocytes (ie. NK and NKT cells) alters intracellular signaling within the hepatocyte to make them more vulnerable to APAP-
induced toxicity by a mechanism independent of the Fas/FasL apoptotic cell death pathway.

1.12 Role of neutrophils in APAP hepatotoxicity

The early release of DAMPs and formation of cytokines during the sterile inflammatory response after APAP overdose leads to recruitment of neutrophils into the hepatic vasculature (Scaffidi et al., 2002; Lawson et al., 2000). As has been demonstrated in a number of cases, given the appropriate chemotactic signal from the parenchyma, these leukocytes can extravasate and seriously increase liver injury (Jaeschke 2003; Jaeschke 2006; Jaeschke and Hasegawa 2006). Despite the initial results arguing against an active role of neutrophils in APAP hepatotoxicity (Scaffidi et al., 2002; Lawson et al., 2000), two studies using the identical experimental approach suggested that neutrophils are responsible in part for APAP-induced liver injury (Liu et al., 2006; Ishida et al., 2006). This conclusion was mainly based on the use of a neutropenia-inducing antibody, which was injected 24 h before APAP (Liu et al., 2006; Ishida et al., 2006). However, when animals were treated with the same antibody after APAP administration, but still before the onset of injury, neutropenia did not protect (Cover et al., 2006). The reason for these contradicting results using the same reagent was that the pretreatment regimen caused not only neutrophil depletion but also triggered a preconditioning effect due to the accumulation of the antibody-tagged neutrophils in the liver and the attempts of Kupffer cells to remove them (Bautista et al., 1994; Jaeschke and Liu 2007). Phagocytosis of inactivated neutrophils causes Kupffer cell activation (Bautista et al., 1994) and triggers a stress response in hepatocytes including induction
of inflammatory genes and a number of protective genes, e.g. metallothionein, heme oxygenase -1 and others (Jaeschke and Liu 2007). Consequently, hepatocytes from animals pretreated with this neutropenia antibody were more resistant to APAP toxicity independently of the lack of neutrophils in blood. This conclusion is further supported by the lack of protection of mice deficient in ICAM-1 (Cover et al., 2006) and phox91 (James et al., 2003) as well as the ineffectiveness of pharmacological inhibitors of NADPH oxidase (Cover et al., 2006).

1.13 Innate immunity and liver regeneration
In addition to the injury mechanisms, initiation of regeneration is critical for the repair of the damaged liver tissue and the recovery of the patient (Mehendale 2005). Vascular endothelial growth factor (VEGF), IL-6, TNF-α and other mediators have been implicated in promoting tissue regeneration after APAP overdose (Chiu et al., 2003; Croyle et al., 2009; Mehendale 2005; Donahower et al., 2006; Kato et al., 2010; James et al., 2003b). Dividing hepatocytes closest to the area of necrosis are replacing the dead cells (Bajt et al., 2003). However, a prerequisite of hepatocyte proliferation is the removal of necrotic cells by phagocytes. Both neutrophils and monocyte-derived macrophages are recruited into the area of necrosis. The infiltrating macrophages (M2) are distinct from activated resident macrophages of the liver (M1; Kupffer cells) in terms of their cytokine profile produced (Laskin et al., 2009; Adams et al., 2010). M2 macrophages generate IL-10 and other cytokines that down-regulate inflammation and promote tissue repair and have a high capacity for phagocytosis (Laskin et al., 2009; Adams et al., 2010). M2 macrophages are recruited into the liver within 12-24 h after
APAP overdose, i.e. after the peak of injury (Holt et al., 2008). The recruitment of M2 macrophages specifically into the area of necrosis occurs through formation of monocyte chemoattracant protein 1 (MCP-1) generated by injured hepatocytes and recruited macrophages (Dambach et al., 2002). Animals deficient in the C-C chemokine receptor 2 (CCR2), the receptor for MCP-1 on monocytes, experienced reduced M2 accumulation during APAP hepatotoxicity and consequently a substantial delay in tissue repair (Holt et al., 2008; Dambach et al., 2002). These data support the hypothesis that M2 macrophages are critical for the removal of necrotic cells and for tissue repair. Prevention of M2 infiltration in CCR2-/− mice led to an increase in hepatic neutrophil numbers and delayed clearance of liver neutrophils (Holt et al., 2008). Although the role of neutrophils in tissue regeneration has not been specifically investigated, these data are not consistent with a vital importance of neutrophils in this process. Clearly, M2 macrophages appear to be the most critical phagocytes for clearing necrotic cell debris and shutting down inflammation.

1.14 Purpose and Aims

It has been shown that sterile inflammation and the subsequent cytokine production can directly modulate the injury after APAP overdose. Additionally the initial inflammatory response also has an impact on liver repair and regeneration after APAP overdose. The mechanism of how this occurs is highly debated and there are two main theories. Some people believe this sterile inflammation activates immune cells and these immune cells come into the liver thereby increasing and amplifying the injury. The other belief is that these cytokines act on hepatocytes or other cells of the liver which alters how the
hepatocytes respond to the toxic insult. This could occur by increasing the expression and production of certain proteins that are inherently protective to the hepatocytes. Examples include metallothionein, heme oxygenase, heat shock proteins and others. Also these inflammatory mediators can act on other cells in the liver like resident macrophages. These cells can produce nitric oxide by the induction of inducible nitric oxide synthase (iNOS); this allows for the formation of peroxynitrite with enhances mitochondrial injury.

Numerous cytokines and cytokine signaling pathways have been demonstrated to modulate APAP induced injury in the past. Examples include: IL-10 is protective (Ju et al., 2002; Bourdi et al., 2002), IL-13 is protective (Yee et al., 2007), TNF-α is protective (Gardner et al., 2003), IP-10 is protective (Bone-Larson et al., 2001), CCR-chemokines are protective (Hogaboam et al., 2000), SOCS-3 is protective (Numata et al., 2007), IFN-γ increases injury (Liu et al., 2004), and the list continues.

Several papers (Chen et al., 2007; Imaeda et al., 2009) have implicated that the pro-inflammatory cytokine, interleukin-1β (IL-1β), is a critical mediator of APAP hepatotoxicity and this toxicity occurs predominantly through the recruitment of neutrophils. From what is known about the progression of injury and the subsequent inflammatory response following APAP overdose we wanted to evaluate the maturation of IL-1β and the role of IL-1β on the progression of liver injury. Additionally we wanted to further characterize the role neutrophils in this process.

Mature IL-1β is formed by post-translational modification which is dependent on the NACHT, LRR, and pyrin domain-containing protein 3 (Nalp3)-inflammasome (Franchi et al., 2009). The Nalp3-inflammasome is composed of three proteins including caspase-1
(formerly, interleukin-1 converting enzyme), Nalp3 and ASC (apoptosis-associated speck-like protein containing a caspase recruiting domain [CARD]) (Franchi et al., 2009). It has been shown that DAMPs can signal via toll-like receptors (TLRs) and this can then activate the inflammasome (Park et al., 2004; Lamkanfi and Dixit, 2009). Upon TLR stimulation the Nalp3-inflammasome is assembled and is then capable of processing pro-IL-1β to mature IL-1β; only the processed form of IL-1β can be released from cells which is then capable of signaling through the IL-1 receptor-1 (IL-1R1) (Sims and Smith, 2010). Both IL-1α and IL-1β signal through IL-1R1. The actions of these cytokines can be attenuated it two ways. The first is the presence of IL-1R2, which lacks the functional intracellular domain for signal transduction and therefore acts as a decoy receptor. The second is the soluble IL-1 receptor antagonist (IL-1ra); it acts as a competitive inhibitor for binding of IL-1α and IL-1β to IL-1R1 (Sims and Smith, 2010).

Previous studies implicating a central role of IL-1β in APAP hepatotoxicity (Chen et al., 2007; Imaeda et al., 2009) raise some concerns. The most important one is that IL-1 receptor signaling cannot directly induce cell death due to the absence of a death domain (Sims and Smith, 2010). Thus, IL-1β acts mainly as a pro-inflammatory mediator activating and recruiting leukocytes, especially neutrophils, into the liver (Bajt et al., 2001). However, there is evidence that neutrophils are not involved in the injury process after APAP overdose (Lawson et al., 2000; Cover et al., 2006; James et al., 2003). In addition, pan-caspase inhibitors did not protect during the early injury phase of APAP hepatotoxicity (Lawson et al., 1999; Jaeschke et al., 2006). However, it remained unclear if these pan-caspase inhibitors can actually affect IL-1β formation and are able to reduce liver injury at later time points when neutrophils are more likely to be involved.
Many inflammatory mediators are produced during APAP overdose, therefore it was our hypothesis that this mediator would have little impact on the progression of injury or hepatic neutrophil recruitment, thus, the objective of this investigation was to evaluate the potential role of IL-1β in hepatic neutrophil recruitment and progression of liver injury after APAP overdose.

**Specific Aim #1: Determine the role of IL-1β on APAP-induced injury**

- If IL-1β is responsible for enhanced injury during acetaminophen overdose then it must be produced after overdose, and be present in sufficient concentration for a sufficient amount of time to exert a biological effect and subsequently modulate injury. To determine this we evaluated the production and maturation of IL-1β during APAP overdose.
- Mature IL-1β is produced through a caspase-dependent mechanism. It was never evaluated if this occurs during APAP overdose. Therefore, mice were treated with pan-caspase inhibitor to block the maturation of IL-1β during APAP overdose.
- Efficacy of the caspase inhibitor was confirmed by inhibition of caspases in vivo by using the galactosamine/endotoxin model of liver injury.
- Mice with (vehicle) and without (caspase inhibited) mature IL-1β were compared after APAP overdose at several time points for liver injury (ALT, necrosis), oxidant stress (GSSG formation), neutrophil recruitment, and inflammatory mediator production (cytokine/chemokine protein and mRNA).
- Mice with (wild-type) and without (IL-1R1−/−) functional IL-1-signaling were compared after APAP overdose.
• Mice were treated with additional, very high doses of recombinant IL-1β after APAP to determine if it can enhance injury.

**Specific Aim #2: Determine the role of the Nalp3 Inflammasome on APAP-injury**

• Evaluate APAP induced injury in mice deficient for each component of the Nalp3 inflammasome versus control and compare liver injury (ALT, necrosis), oxidant stress (GSSG formation), neutrophil recruitment, DNA damage and inflammatory mediator production (cytokine/chemokine mRNA).

• Evaluate the release and role of DAMPs (nuclear DNA/histones, hyper- and hypo-acetylated HMGB-1, keratin-18) in the injury process in wild-type versus Nalp3-deficient mice.

• Confirm these findings using an inflammasome inhibitor (acetylsalicylic acid) to determine injury and inflammation after APAP-overdose.

**Specific Aim #3: Determine if CD18-/- is important in recruitment and activation status of neutrophils during the injury phase of APAP-overdose**

• Evaluate the role of neutrophils in the injury progression of APAP overdose in control and CD18-deficient mice (these mice are protected in other models of neutrophil-mediated liver injury).

• Compare the quantity and distribution of neutrophils after APAP to bile duct ligated mice (which is a known neutrophil mediated injury).

• Characterize the activation status of neutrophils in the blood and liver (surface marker expression and reactive oxygen priming) during the injury phase of APAP overdose.
- Enhance the activation of neutrophils (through the use of recombinant IL-1β or endotoxin) to see if they can exacerbate APAP-induced injury.

**Specific Aim #4: Determine if neutrophils are activated during liver repair**

- Evaluate a time course of APAP induced injury/injury resolution, DNA damage, hepatocyte proliferation and neutrophil activation and recruitment. Aim 3 evaluated early injury, this evaluates late injury and injury repair.
- If neutrophils are critical for repair of tissue then activation should be observed. To do this we measured liver and blood neutrophils throughout this time course.
- If NADPH oxidase is critical in these phagocytic cells then impaired recovery should be seen in NADPH oxidase deficient mice.
- Neutrophils may be critical for the recovery from APAP overdose in human overdose patients. To determine this we evaluated activation parameters of neutrophils in patients during the injury and injury resolution phases of APAP overdose.

We hypothesized IL-1 signaling would have a very minor role in APAP induced injury. We did not expect to see neutrophil activation during the early injury but did expect modulation of function later as neutrophils could have an active role in hepatic injury resolution or maintenance of peripheral immune function. To better understand the role of neutrophils in the injury and injury resolution process we characterized activation and priming status of hepatic and peripheral neutrophils during APAP overdose.
Chapter 2:

Role of Caspase-1 inhibition and IL-1β formation during APAP overdose

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2.1 Abstract

Acetaminophen (APAP) overdose can result in serious liver injury and potentially death. Toxicity is dependent on metabolism of APAP to a reactive metabolite initiating a cascade of intracellular events resulting in hepatocellular necrosis. This early injury triggers a sterile inflammatory response with formation of cytokines and innate immune cell infiltration in the liver. Recently, IL-1β signaling has been implicated in the potentiating of APAP-induced liver injury. To test if IL-1β formation through caspase-1 is critical for the pathophysiology, C57Bl/6 mice were treated with the pan-caspase inhibitor Z-VD-fmk to block the inflammasome-mediated maturation of IL-1β during APAP overdose (300 mg/kg APAP). This intervention did not affect IL-1β gene transcription but prevented the increase in IL-1β plasma levels. However, APAP-induced liver injury and neutrophil infiltration was not affected. Similarly, liver injury and the hepatic neutrophilic inflammation were not attenuated in IL-1-receptor-1 deficient mice compared to wild type animals. To evaluate the potential of IL-1β to increase injury, mice were given pharmacological doses of IL-1β after APAP overdose. Despite increased systemic activation of neutrophils and recruitment into the liver, there was no alteration in injury. We conclude that endogenous IL-1β formation after APAP overdose is insufficient to activate and recruit neutrophils into the liver or cause liver injury. Even high pharmacological doses of IL-1β, which induce hepatic neutrophil accumulation and activation, do not enhance APAP-induced liver injury. Thus, IL-1 signaling is irrelevant for APAP hepatotoxicity. The inflammatory cascade is a less important therapeutic target than intracellular signaling pathways to attenuate APAP-induced liver injury.
2.2 Introduction

Acetaminophen (APAP) overdose is the most frequent cause of acute liver failure in the US and many European countries (Larson et al., 2005). Toxicity is dependent on the metabolic activation of APAP to the reactive metabolite \( N \)-acetyl-\( p \)-benzoquinone imine (NAPQI) within hepatocytes (Nelson, 1990) and sinusoidal endothelial cells (Holt et al., 2010). NAPQI initiates a series of intracellular events critical for hepatotoxicity including glutathione depletion and covalent protein modifications leading to mitochondrial dysfunction with formation of reactive oxygen species and peroxynitrite (Nelson, 1990; Jaeschke and Bajt, 2006; Jaeschke et al., 2003). The oxidant stress is ultimately responsible for the opening of the mitochondrial membrane permeability transition (MPT) pore (Kon et al., 2004) and necrotic cell death (Gujral et al., 2002). APAP-induced liver toxicity is accompanied by an inflammatory response involving activation of Kupffer cells and recruitment of neutrophils and mononuclear cells into the liver (Jaeschke, 2005; Liu and Kaplowitz, 2006; Laskin, 2009). The inflammatory response is initiated by release of cellular contents, some of which can function as damage associated molecular patterns (DAMPs) that can directly activate innate immune cells (Scaffidi et al., 2002; Martin-Murphy et al., 2010). During this sterile inflammation many cytokines and chemokines are up-regulated and innate immune cells begin to accumulate in the liver with neutrophils arriving early after the initial APAP-induced injury (Lawson et al., 2000). One of these pro-inflammatory cytokines, interleukin-1\( \beta \) (IL-1\( \beta \)), has been recently implicated as a critical mediator of APAP hepatotoxicity (Chen et al., 2007; Imaeda et al., 2009).
Mature IL-1β is formed by post-translational modification which is dependent on the NACHT, LRR, and pyrin domain-containing protein 3 (Nalp3)-inflammasome (Franchi et al., 2009). The Nalp3-inflammasome is composed of three proteins including caspase-1 (formerly, interleukin-1 converting enzyme), Nalp3 and ASC (apoptosis-associated speck-like protein containing a caspase recruiting domain [CARD]) (Franchi et al., 2009). It has been shown that DAMPs can signal via toll-like receptors (TLRs) and this can then activate the inflammasome (Park et al., 2004; Lamkanfi and Dixit, 2009). Upon TLR stimulation the Nalp3-inflammasome is assembled and is then capable of processing pro-IL-1β to mature IL-1β; only the processed form of IL-1β can be released from cells which is then capable of signaling through the IL-1 receptor-1 (IL-1R1) (Sims and Smith, 2010).

Previous studies implicating a central role of IL-1β in APAP hepatotoxicity (Chen et al., 2007; Imaeda et al., 2009) raise some concerns. The most important one is that IL-1 receptor signaling cannot directly induce cell death due to the absence of a death domain (Sims and Smith, 2010). Thus, IL-1β acts mainly as a pro-inflammatory mediator activating and recruiting leukocytes, especially neutrophils, into the liver (Bajt et al., 2001). However, there is extensive evidence that neutrophils are not involved in the injury process after APAP overdose (Lawson et al., 2000; Cover et al., 2006; James et al., 2003; Williams et al., 2010). In addition, pan-caspase inhibitors did not protect during the early injury phase of APAP hepatotoxicity (Lawson et al., 1999; Jaeschke et al., 2006). However, it remained unclear if these pan-caspase inhibitors can actually affect IL-1β formation and are able to reduce liver injury at later time points when neutrophils are more likely to be involved. Thus, the objective of this investigation was
to evaluate the potential role of IL-1β in hepatic neutrophil recruitment and progression of liver injury after APAP overdose in a well-established murine model.
2.3 Materials and Methods

2.3.1 Animals. Eight to twelve week old male C57BL/6J or IL-1 receptor 1 knock-out mice (B6.129S7-Il1r1tm1Imx/J), with an average weight of 18 to 22 g were purchased from Jackson Laboratory (Bar Harbor, Maine). All animals were housed in an environmentally controlled room with 12 h light/dark cycle and allowed free access to food (8604 Teklad Rodent, Harlan, Indianapolis, IN) and water. Experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Kansas Medical Center and followed the criteria of the National Research Council for the care and use of laboratory animals in research.

2.3.2 Experimental design. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. Mice were intraperitoneally injected with 300 mg/kg APAP (dissolved in warm saline) after overnight fasting, 700 mg/kg D-galactosamine (Gal) and 100 μg/kg endotoxin (ET), 20 μg/kg recombinant mouse IL-1β containing <0.1 ng endotoxin/μg protein (GenScript, Piscataway, NJ), or equivalent volumes of saline. Additionally, some mice were injected (i.p.) with 10 mg/kg Z-VD-fmk (EP1013; a generous gift from Dr. S. X. Cai, Epicept Corp., San Diego, CA) (dissolved in Tris-buffered saline) two hours after APAP treatment or three hours after Gal/ET. The animals were sacrificed 6 or 24 h after APAP or 6 h after Gal/ET. Blood was drawn into heparinized syringes for measurement of alanine aminotransferase (ALT) activity (Kinetic Test Kit 68-B, Biotron Diagnostics, Inc., Hernet, CA) and cytokine concentrations. The liver was removed and was rinsed in ice-cold saline; liver sections
were fixed in 10% phosphate buffered formalin for histological analyses. The remaining liver lobes were snap-frozen in liquid nitrogen and stored at -80 °C.

2.3.3

Histology. Formalin-fixed tissue samples were embedded in paraffin and 5 μm sections were cut. Sections were stained with hematoxylin and eosin (H&E) for blinded evaluation of the areas of necrosis by the pathologist. The percent of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the entire cross section. Additional sections were stained for neutrophils using the anti-mouse neutrophil allotypic marker antibody (AbD Serotec, Raleigh, NC) as described in detail (Williams et al., 2010). Positively stained cells consistent with cellular morphology were quantified in 15 high power fields (HPF). Some sections were also stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in situ cell death assay (Roche, Indianapolis, IN) as previously described (Lawson et al. 1999).

2.3.4

mRNA expression. Quantification of mRNA expression of several cytokines and chemokines was performed by real-time PCR (RT-PCR) analysis as described previously (Bajt et al., 2008). cDNA was generated by reverse transcription of total RNA by M-MLV reverse transcriptase in the presence of random primers (Invitrogen, Carlsbad, CA). Forward and reverse primers for the genes were designed using Primer Express software (Applied Biosystems, Foster City, CA). After normalization of cDNA concentration, SYBR green PCR Master Mix (Applied Biosystems) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values generated by the ABI 7900 instrument (Applied
Biosystems). All genes evaluated were first normalized to β-actin and then expressed as a fold increase relative to control which is arbitrarily set as 1.0. Calculations are made by assuming one cycle is equivalent to a two-fold difference in copy number which is the \( 2^{(-\text{ddCt})} \) formula.

2.3.5

*Plasma cytokine measurements.* Plasma cytokine concentrations were quantified by the Bio-Plex bead-based multiplex assay following the kit instructions (Bio-Rad Laboratories, Hercules, CA) and analyzed on the Bio-Plex 200 instrument (Bio-Rad).

2.3.6

*Tissue Caspase-3 Activity.* Quantification of hepatic caspase-3 activity was performed as previously described in detail (Lawson et al., 1999). Briefly, liver tissue was homogenized and protein concentration was normalized after BCA protein assay (Thermo Scientific, Rockford, IL). Homogenate was assayed with Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) (Enzo Life Sciences, Plymouth Meeting, PA) for change in fluorescent intensity over time by fluorometer.

2.3.7

*Flow Cytometric Analysis of CD11b Expression and Reactive Oxygen Formation: CD11b/Gr-1 staining.* Neutrophil priming as measured by CD11b expression was performed as previously described in detail (Williams et al., 2010). Briefly, heparinized whole blood was stained with PE-Cy5-labeled-anti-Gr-1 (BioLegend, San Diego, CA) and PE-labeled-anti-CD11b (BioLegend). After red cell lysis samples were analyzed on the FACSCalibur (BD, Franklin Lakes, NJ).
Reactive Oxygen Species (ROS) Production (Smith and Weideman, 1993; Daley et al., 2008) Neutrophil ROS priming was performed as previously described in detail (Williams et al., 2010). Briefly, whole blood was treated \textit{ex vivo} for 10 minutes with phorbol 12-myristate 13-acetate (PMA) or saline followed by the addition of dihydrorhodamine-123 for 10 minutes at 37ºC. Neutrophils were stained with PE-Cy5-labeled-anti-Gr-1 (BioLegend) then red blood cells were lysed. Samples were analyzed on the FACSCalibur and ROS production was evaluated in Gr-1$^\text{bright}$ neutrophils.

\textbf{2.3.8}

\textit{Statistics}. All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way ANOVA or, where appropriate, by two-way ANOVA, followed by a \textit{post hoc} Bonferroni test. If the data were not normally distributed, we used the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test. P < 0.05 was considered significant.
2.4 Results

2.4.1 Pharmacological inhibition of caspases in vivo during APAP toxicity.

Treatment of fasted C57Bl/6 mice with 300 mg/kg APAP resulted in substantial liver injury as indicated by the increase in plasma ALT activities at 6 h (2.4.1.1A). During the following 18 h, the injury was further increased (2.4.1.1A). Liver injury was also confirmed by histology (2.4.1.1B,C), which showed extensive centrilobular necrosis (2.4.1.1C). Quantitatively, about 55% of hepatocytes were necrotic at 24 h (2.4.1.1B). APAP-induced liver injury caused a significant accumulation of neutrophils in the liver, which was only moderate at 6 h but dramatically increased by 24 h (2.4.1.1C,D). In order to inhibit IL-1β processing, animals were treated with the potent pan-caspase inhibitor Z-VD-fmk (Jaeschke et al., 2000) 2 h after APAP. Z-VD-fmk had no significant effect on plasma ALT activities or the area of necrosis (2.4.1.1A-C). In addition, Z-VD-fmk had no significant effect on hepatic neutrophil accumulation at 6 or 24 h (2.4.1.1C,D). To evaluate the efficacy of the pan-caspase inhibitor, plasma IL-1β protein levels were measured at 6 h and 24 h after APAP. Six hours after APAP the circulating levels of IL-1β were increased three-fold over baseline (2.4.1.2A). This increase in IL-1β protein levels was completely prevented with the pan-caspase inhibitor demonstrating the efficacy of Z-VD-fmk to block caspase-1 activity (2.4.1.2A). Consistent with the function of caspase-1 to process pro-IL-1β, the caspase inhibitor did not affect the increase in IL-1β mRNA (2.4.1.2B). Furthermore, the pan-caspase inhibitor did neither modulate the mRNA or plasma protein levels of several other cytokines and chemokines including TNF-α, IL-18, IL-10, IL-6, KC and MIP-2 (data not shown).
Acetaminophen-induced liver injury in C57Bl/6 mice with or without caspase inhibitor. Animals were first treated with 300 mg/kg APAP and then two hours later with 10 mg/kg Z-VD-fmk or vehicle control. (A) Plasma ALT at 6 h and 24 h; (B) area of necrosis at 24 h. (C) Representative H&E-stained liver sections (x50 magnification) and immunohistochemistry for hepatic neutrophil accumulation (x200 magnification) are shown for controls and animals treated with APAP for 24 h. (D) Neutrophil numbers were quantified in 15 randomly selected high power fields (HPF; x400). Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to untreated controls).
Interleukin-1β (IL-1β) protein levels in plasma (A) and hepatic IL-1β mRNA levels (B) were measured 6 and 24 h after administration of 300 mg/kg acetaminophen (APAP) in C57Bl/6 mice with or without the pan-caspase inhibitor (Z-VD-fmk). mRNA levels are expressed as IL-1β mRNA-to-β-actin mRNA ratio. The values of untreated controls were set as 1 and the fold change of the treated animals was calculated. Data represent means ± SE of n = 4-5 animals per group. *P<0.05 (compared to untreated control). #P<0.05 (compared to APAP-only equivalent time point)
2.4.2 Efficacy of Z-VD-fmk in the galactosamine/endotoxin liver injury model

To further confirm the efficacy of the pan-caspase inhibitor, mice were treated with galactosamine/endotoxin (Gal/ET), which is a well established caspase-dependent model of hepatocellular apoptosis (Jaeschke et al., 1998). The 10 mg/kg dose and route of administration of Z-VD-fmk was effective in reducing 95% of the Gal/ET-induced increase in liver caspase-3 activity (2.4.2.1A). Blocking apoptosis also prevented secondary necrosis thereby reducing plasma ALT by nearly 90% (2.4.2.1B). To confirm the reduction in apoptotic cell death, liver sections were stained for DNA strand-breaks with the TUNEL assay. Gal/ET-induced caspase activation causes massive DNA fragmentation as indicated by the extensive staining of TUNEL-positive hepatocytes (2.4.2.1C). Again, the caspase-inhibitor almost completely prevented any TUNEL staining in these livers (2.4.2.1C). Together, these data confirm the high efficacy of this pan-caspase inhibitor against TNF-mediated apoptosis in the Gal/ET model of liver injury.
Effects of 10 mg/kg Z-VD-fmk on galactosamine/endotoxin (GalN/ET)-induced liver injury. Animals were treated with saline or 700 mg/kg GalN and 100 μg/kg ET and then three hours later with 10 mg/kg Z-VD-fmk or vehicle control. (A) Caspase-3 activity at 6 h; (B) Plasma ALT at 6 h. (C) Representative TUNEL staining of animals from each group. Data represent means ± SE of n = 3 animals per group. *P<0.05 (compared to saline-treated controls)
2.4.3 APAP toxicity in IL-1 receptor-1 knockout mice

The caspase inhibitor data indicate that IL-1β maturation does not appear to be a critical factor in APAP-induced toxicity in contrast to previous reports. To confirm these findings and to exclude a potential role of IL-1α, which is formed independent of the inflammasome, IL-1R1-/- mice were treated with APAP. Similar to the caspase inhibitor data, there was no significant effect of APAP on plasma ALT activities, area of necrosis and hepatic neutrophil accumulation in IL-1R1-/- mice compared to wild type animals at 6 or 24 h after APAP (2.4.3.1A-C). Despite similar neutrophil recruitment, both hepatic cytokine/chemokines mRNA production and plasma cytokine/chemokine levels were quantified to determine if the inflammatory response is altered or delayed in mice lacking IL-1 signaling. Transcription of inflammatory genes in the liver is unchanged between wild-type and knockout mice at 6 and 24 h (2.4.3.2), however at the six hour time-point IL-6 and KC plasma protein levels are reduced relative to the treated wild-type group (2.4.3.2), however these differences are not seen at 24 h. Despite this apparent delay in formation of some of the cytokine/chemokine, liver injury as well as the quantity and distribution of neutrophils were unchanged (2.4.3.1C,D).
Acetaminophen-induced liver injury in C57Bl/6 wild-type mice or IL-1R1-deficient mice. Animals were treated with 300 mg/kg APAP. (A) Plasma ALT at 6 h and 24 h; (B) area of necrosis at 24 h. (C) Representative H&E-stained liver sections (x50 magnification) and immunohistochemistry for hepatic neutrophil accumulation (x200 magnification) are shown for control and APAP-treated mice at 24 h; (D) Neutrophil numbers were quantified in 15 randomly selected high power fields (HPF; x400). Data represent means ± SE of n = 6 animals per group. *P<0.05 (compared to untreated controls).
2.4.3.2

Hepatic mRNA levels and peripheral blood cytokine/chemokine concentrations were measured 6 h and 24 h after administration of 300 mg/kg APAP in C57Bl/6 wild type animals or IL-1R1-deficient mice. mRNA levels are calculated as the cytokine mRNA-to-β-actin mRNA ratio. For mRNA calculations the values of untreated controls were set as 1 and the fold change of the APAP-treated animals is shown. Data represent means ± SE of n = 5-6 animals per group. *P<0.05 (compared to control). #P<0.05 (compared to wild type equivalent time point)

### Plasma Protein Concentration (pg/mL)

<table>
<thead>
<tr>
<th>Cytokine or Chemokine</th>
<th>Control C57Bl/6</th>
<th>APAP 6 h C57Bl/6</th>
<th>IL-1R1-/- C57Bl/6</th>
<th>APAP 6 h IL-1R1-/-</th>
<th>APAP 24 h C57Bl/6</th>
<th>APAP 24 h IL-1R1-/-</th>
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</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>3.3 ± 1.0</td>
<td>12.5 ± 3.2*</td>
<td>5.7 ± 0.7</td>
<td>2.4 ± 0.6</td>
<td>5.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>11.1 ± 3.0</td>
<td>41.3 ± 6.8*</td>
<td>26 ± 7.9</td>
<td>20.4 ± 3.7*</td>
<td>6.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>1.8 ± 0.7</td>
<td>52.8 ± 13.8*</td>
<td>10.2 ± 2.7*#</td>
<td>21.6 ± 5.6*</td>
<td>29.8 ± 12.6*</td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>4.9 ± 0.8</td>
<td>19.5 ± 3.4*</td>
<td>4.2 ± 0.8#</td>
<td>19.5 ± 2.5*</td>
<td>19.3 ± 1.7*</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>35.1 ± 5.2</td>
<td>79 ± 21.5*</td>
<td>16.9 ± 4.0*#</td>
<td>53.6 ± 29.9</td>
<td>77.9 ± 10.1*</td>
<td></td>
</tr>
</tbody>
</table>

### mRNA expression (fold increase)

<table>
<thead>
<tr>
<th>Cytokine or Chemokine</th>
<th>APAP 6 h C57Bl/6</th>
<th>APAP 6 h IL-1R1-/-</th>
<th>APAP 24 h C57Bl/6</th>
<th>APAP 24 h IL-1R1-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>3.1 ± 0.6*</td>
<td>2.4 ± 0.9*</td>
<td>3.0 ± 0.9*</td>
<td>2.8 ± 0.6*</td>
</tr>
<tr>
<td>IL-10</td>
<td>65.7 ± 15.6*</td>
<td>39.4 ± 10.8*</td>
<td>10.0 ±</td>
<td>8.9 ± 3.5*</td>
</tr>
<tr>
<td>IL-6</td>
<td>12.3 ± 6.1*</td>
<td>5.6 ± 0.5*</td>
<td>3.0 ± 0.6*</td>
<td>3.5 ± 1.1*</td>
</tr>
<tr>
<td>KC</td>
<td>6.1 ± 1.1*</td>
<td>6.1 ± 2.6*</td>
<td>10.8 ±</td>
<td>7.0 ± 1.7*</td>
</tr>
<tr>
<td>MIP-2</td>
<td>170 ± 40*</td>
<td>96 ± 52*</td>
<td>96 ± 56*</td>
<td>114 ± 23*</td>
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</tbody>
</table>
2.4.4 Effects of supra-physiological levels of IL-1β during APAP toxicity

Although our experiments so far were unable to support a role of IL-1β in the pathophysiology of APAP hepatotoxicity, we cannot rule out that the endogenous levels of IL-1β produced under our experimental conditions may have been too low to have a relevant impact on the injury mechanism. Therefore, we hypothesized that if IL-1β is critical for the injury via the recruitment of hepatic neutrophils or other leukocytes then an exacerbation of the injury should be observed when IL-1β is administered concurrently with APAP. Mice were injected i.p. with APAP and then given IL-1β or saline vehicle three hours later to mimic IL-1β, which would be produced during APAP-induced sterile inflammation. Administration of IL-1β alone caused recruitment of neutrophils into the liver within 90 minutes (2.4.4.1D) without causing injury (2.4.4.1A,B). When IL-1β was administered after APAP, the additional inflammatory mediators enhanced APAP-induced hepatic neutrophil accumulation by 35% (2.4.4.1D) but had no effect on plasma ALT values or the area of necrosis induced by APAP alone (2.4.4.1A-C). These data further support our findings that even excess IL-1β did not further increase APAP-induced liver injury.
Acetaminophen-induced liver injury in C57Bl/6 mice with or without IL-1β treatment. Mice were treated with 300 mg/kg APAP and/or 20 μg/kg IL-1β or vehicle. (A) Plasma ALT levels at 24 h; (B) area of necrosis at 24 h; (C) Representative H&E-stained liver sections (x50 magnification) and immunohistochemistry for hepatic neutrophil accumulation (x200 magnification) are shown for control and treated mice. (D) Neutrophil numbers were quantified in 15 randomly selected high power fields (HPF; x400). Data represent means ± SE of n = 6 animals per group. *P<0.05 (compared to untreated controls). #P<0.05 (compared to APAP only).
2.4.5 Neutrophil activation during IL-1β treatment

In order to verify that the dose of IL-1β was able to activate neutrophils, the enhanced expression of CD11b on neutrophils and priming for NADPH oxidase-derived reactive oxygen formation was evaluated on peripheral neutrophils. Previous data showed a close correlation between the activation status of peripheral and hepatic neutrophils (see Chapter 4). C57Bl/6 mice were treated in vivo with saline (6 h), 300 mg/kg APAP (6 h), or 20 μg/kg IL-1β (1.5 h). Neutrophils were gated as Gr-1bright cells because previous literature demonstrated that Gr-1bright cells being the same population as Ly6G+ cells, which are neutrophils (Daley et al., 2008). APAP treatment caused no increase in CD11b expression versus control, and the very high dose of IL-1β caused only a partial activation of peripheral neutrophils resulting in two distinct cell populations expressing CD11b (2.4.5.1A,B). Similarly, APAP did not cause increased ROS priming in peripheral neutrophils versus controls while supra-physiologic levels of IL-1β caused only a modest increase in ROS priming (2.4.5.1C,D).
2.4.5.1

Neutrophil priming in peripheral blood. C57Bl/6 mice (n=4 per group) were treated with 20 mL/kg saline (6 h), 300 mg/kg APAP (6 h), or 20 µg/kg IL-1β (1.5 h). Immediately after in vivo stimulation whole blood was stained for CD11b and Gr-1 surface expression and neutrophils (Gr-1 bright cells) were analyzed by flow cytometry. (A) The mean CD11b fluorescent intensities of each treatment group are shown and (B) representative CD11b surface-expression dot plots and histograms for saline, APAP and IL-1β. To determine reactive oxygen species (ROS) priming after in vivo treatment, whole blood was stimulated ex vivo with PMA or saline. Upon PMA-induced ROS production DHR-123 is converted to R-123 and quantified in neutrophils by flow cytometry. (C) The mean ROS fluorescent intensities of each treatment group are shown and (D) representative ROS dot plots and histograms for saline, APAP and IL-1β. Data represent means ± SE of n = 4 animals per group. *P<0.05 (compared to saline-treated controls).
2.5 Discussion

The primary objective of this study was to evaluate the formation and pathophysiological significance of IL-1β during APAP-induced hepatotoxicity. Although there were recent reports implicating the inflammasome (caspase-1) and IL-1β signaling in APAP overdose (Chen et al., 2007; Imaeda et al., 2009), there were some concerns whether a single pro-inflammatory mediator like IL-1β could be such a dominant factor in the mechanism of injury as suggested by these authors.

2.5.1 Caspase-1 and IL-1β formation during APAP hepatotoxicity

The induction of IL-1α and IL-1β mRNA is well described after APAP overdose (Gardner et al., 2003; Cover et al., 2006; Ishibe et al., 2009; Imaeda et al., 2009; Martin-Murphy et al., 2010). Our data are in agreement with the generally moderate transcriptional activation of IL-1β in response to APAP reported by these studies. In addition, we could demonstrate that there are significant but modest increases in mature IL-1β protein levels. Most importantly, this increase in IL-1β protein could be eliminated by inhibition of caspase-1 without affecting the mRNA levels. These data support the hypothesis that the increased formation of the mature IL-1β protein during APAP hepatotoxicity is dependent on caspases, most likely on caspase-1. These findings indicate that there is activation of the inflammasome, which contributes to the formation of pro-inflammatory mediators after APAP. Previous studies indicated that the release of DAMPs from necrotic hepatocytes may contribute to the formation of cytokines by macrophages through activation of toll-like receptors (Imaeda et al., 2009; Martin-Murphy et al., 2010).
2.5.2 Effect of IL-1β on the inflammatory response during APAP hepatotoxicity.

In contrast to TNF receptor 1 or the Fas receptor, the IL-1 receptor does not have a death domain and therefore cannot directly trigger cell death. Thus, the only way IL-1β and the IL-1 receptor can be involved in cell injury is through the activation of cytotoxic leukocytes (Sims and Smith, 2010). During APAP-induced injury, the first leukocytes observed in the liver are neutrophils (Lawson et al., 2000), which have the capacity to severely enhance liver injury (Jaeschke, 2006). IL-1α is a potent activator of neutrophils that can trigger substantial hepatic neutrophil recruitment (Bajt et al., 2001). Our data with IL-1β confirm these findings and additionally show that adding high doses of endogenous IL-1β during APAP toxicity has an additive effect on hepatic neutrophil accumulation. However, eliminating the increase in IL-1β formation had no effect on the number of liver neutrophils. Thus, together these data suggest that the endogenous IL-1β formation during APAP hepatotoxicity is insufficient to have a relevant impact on hepatic neutrophil recruitment. Because IL-1R-deficient mice did not have less neutrophils in the liver compared to wildtype animals, it indicates that neither endogenous IL-1β nor IL-1α affects hepatic neutrophil accumulation. In addition, treatment with high doses of IL-1β activated and primed circulating neutrophils, which closely reflect the activation status of liver neutrophils (see Chapter 4). APAP alone did not activate circulating or liver neutrophils as indicated by absence of enhanced CD11b expression and no priming for ROS formation (2.4.5.1 and Chapter 4). This supports the conclusion that endogenously formed IL-1β is also insufficient to activate neutrophils. These findings, although opposite to what has been postulated (Chen et al., 2007; Imaeda et al., 2009), are not surprising given the substantial number of cytokines,
chemokines and other mediators formed during the extensive cell damage caused by APAP (Lawson et al., 2000; Gardner et al., 2003; Cover et al., 2006; Ishibe et al., 2009; Imaeda et al., 2009; Martin-Murphy et al., 2010).

Additional inflammatory cells activated during APAP hepatotoxicity include the resident macrophages of the liver (Kupffer cells) and newly recruited tissue macrophages derived from blood monocytes (Laskin, 2009). Emerging evidence indicates that Kupffer cells are mainly involved in the formation of pro- and anti-inflammatory cytokines but less in direct cytotoxicity (Ju et al., 2002; Martin-Murphy et al., 2010). The newly recruited tissue macrophages arrive mainly after the injury already peaked and appear to be involved in the resolution of inflammation and removal of necrotic cell debris (Dambach et al., 2002; Holt et al., 2008). Monocyte chemoattractant protein (MCP-1) rather than IL-1β is involved in the late monocyte recruitment (Dambach et al., 2002; Holt et al., 2008).

2.5.3 IL-1β and APAP-induced liver injury

The most critical question is whether IL-1β is involved in the injury process. In a previous paper it was reported that IL-1R-deficient mice are more than 90% protected against APAP hepatotoxicity based on plasma ALT activities (Chen et al., 2007). Using the same gene knockout mice, we did not find any evidence of protection at early or late time points after APAP overdose. When the authors used a combination of neutralizing antibodies against IL-1β, IL-1α, and IL-1R1, the protective effect was only 30% (Chen et al., 2007). Thus, even this study shows highly variable results on the role of IL-1. In addition, a second report claimed a critical role specifically for IL-1β due to the fact that gene knock-out mice of caspase-1 and different components of the inflammasome were
partially protected (Imaeda et al., 2009). In this study, neutralizing IL-1β or deficiency of IL-18, Nalp3, caspase-1, and ASC showed reduced mortality and lower plasma ALT levels by 50-60% after APAP overdose (Imaeda et al., 2009). The authors concluded that the transcriptional activation of IL-1β and IL-18 together with the processing to the mature cytokine through caspase-1 is critical for APAP hepatotoxicity (Imaeda et al., 2009). In sharp contrast, our data are consistently showing no relevant effect of IL-1β (or IL-1α) in the pathophysiology. The amount of IL-1β generated was minor and insufficient to trigger activation and recruitment of neutrophils into the liver. In addition, we did not find significant upregulation of IL-18 mRNA after APAP treatment. A caspase inhibitor, which completely prevented the APAP-induced mature IL-1β formation, did neither affect hepatic neutrophil recruitment nor liver injury. Furthermore, the IL-1R1-deficient mouse was not protected or showed reduced inflammation. Finally, even treating the animals with an extremely high dose of IL-1β, which by itself can activate neutrophils and cause their accumulation in the liver, did enhance the overall neutrophil count in the liver but had no effect on the injury. These findings are consistent with a large number of different experiments that all demonstrated that the inflammatory response with hepatic neutrophil infiltration does not increase APAP-induced liver injury (Lawson et al., 2000; James et al., 2003; Cover et al., 2006). In these experiments, blocking or eliminating adhesion molecules which have been shown to be required for neutrophil cytotoxicity in the liver, i.e. CD18 and intercellular adhesion molecule-1 (ICAM-1) (Jaeschke and Smith, 1997), had no effect against APAP hepatotoxicity (Lawson et al., 2000; Chapter 4). Furthermore, inhibiting or eliminating the capacity of neutrophils to generate reactive oxygen species, which is critical for neutrophil
cytotoxicity in vivo (Jaeschke et al., 1999; Gujral et al., 2004; Hasegawa et al., 2005), had no effect on acetaminophen toxicity (James, et al., 2003; Cover et al., 2006). Most importantly, even when large amounts of pro-inflammatory mediators are either injected or generated through endotoxin during APAP-induced liver injury, the additional activated neutrophils do not affect the overall injury. Thus, even if sufficient IL-1 or IL-18 would have been generated during APAP overdose, there is no evidence that these phagocytes contribute to the injury.

Why is there such a difference in the results and conclusions between studies despite the use of the same mouse strain and similar doses of APAP? There is no easy answer. However, in the previous studies where extensive protection was shown in a large number of different gene knock-out mice, it was never tested if there are differences in metabolic activation or other changes in the intracellular signal transduction pathways (Chen et al., 2007; Imaeda et al., 2009). Furthermore, the cells that actually cause the toxicity were never identified. Thus, the mechanisms of APAP toxicity in these knock-out mice remained unclear and it was not excluded that off-target effects could have been responsible for the protection. In support of this hypothesis, it was recently shown that IL-1 receptor antagonist-deficient (IL-1ra-/-) mice showed reduced liver injury to APAP (Ishibe et al., 2009). Since IL-1ra blocks the signaling of IL-1α and IL-1β through IL-1R1 and prevents the intracellular recruitment of IL-1RAP (Sims and Smith, 2010), this finding is in direct contrast to the hypothesis that the IL-1 axis increases injury. Most importantly, this study demonstrated that IL-1ra-/- mice have decreased metabolic activity and therefore generate less reactive metabolite, i.e. the protection was caused by modulation of intracellular events rather than inflammation (Ishibe et al., 2010).
However, consistent with our data, Imaeda et al. (2009) showed only a 6% increase of mature IL-1β in plasma after APAP. As we demonstrated, such a minute change in circulating IL-1β has no chance to cause neutrophil activation and recruitment into the liver and, therefore, it remained unclear how the activity of the inflammasome could have had any impact on APAP-induced liver injury.

2.5.4 Summary

Comprehensive analysis of the formation and potential role of IL-1β in the pathophysiology of APAP hepatotoxicity revealed no support that this cytokine is generated in sufficient quantities to activate neutrophils and neither inhibition of caspases and the inflammasome nor deficiency of the IL-1R had any impact on APAP-induced inflammation and liver injury. Although these data are opposite to several recent papers (Chen et al., 2007; Imaeda et al., 2009), they are fully consistent with our previous findings that neutrophils do not increase liver injury after APAP overdose. Thus, the inflammatory cascade is a less relevant therapeutic target than the intracellular signaling pathways to attenuate APAP-induced liver injury.
Chapter 3

Role of the Nalp3 inflammasome and sterile inflammation during APAP overdose

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3.1 Abstract

Acetaminophen (APAP) overdose is the leading cause of acute liver failure in the US and UK. Recent studies implied that APAP-induced injury is partially mediated by interleukin-1β (IL-1β), which can activate and recruit neutrophils, exacerbating injury. Mature IL-1β is formed by caspase-1, dependent on inflammasome activation. The objective of this investigation was to evaluate the role of the Nalp3 inflammasome on release of damage associated molecular patterns (DAMPs), hepatic neutrophil accumulation and liver injury (ALT, necrosis) after APAP overdose. Mice deficient for each component of the Nalp3 inflammasome (Caspase-1, ASC and NALP3) were treated with 300 mg/kg APAP for 24 h; these mice had similar neutrophil recruitment and liver injury as APAP-treated C57Bl/6 wildtype animals. In addition, plasma levels of DAMPs (DNA fragments, keratin-18, hypo- and hyper-acetylated forms of high mobility group box-1 protein) were similarly elevated with no significant difference between wildtype and gene knockout mice. In addition, aspirin treatment, which has been postulated to attenuate cytokine formation and the activation of the Nalp3 inflammasome after APAP, had no effect on release of DAMPs, hepatic neutrophil accumulation or liver injury. Together these data confirm the release of DAMPs and a sterile inflammatory response after APAP overdose. However, as previously reported minor endogenous formation of IL-1β and the activation of the Nalp3 inflammasome have little impact on APAP hepatotoxicity. It appears that the Nalp3 inflammasome is not a promising therapeutic target to treat APAP overdose.
3.2 Introduction

Acetaminophen (APAP) is a widely used safe analgesic and antipyretic drug. However, an overdose can cause hepatotoxicity and even liver failure in animals and humans (Larson et al., 2005). Early mechanistic studies identified formation of a reactive metabolite, glutathione depletion and covalent binding to cellular proteins as critical initiating events in the toxicity (Cohen et al., 1997; Nelson, 1990). More recent studies showed the central role of mitochondrial dysfunction, including oxidant stress and peroxynitrite formation, the mitochondrial membrane permeability transition pore opening (MPT) and nuclear DNA fragmentation as propagation events in APAP-induced cell death in the liver (Jaeschke et al., 2003; Jaeschke and Bajt, 2006).

In recent years the new concept emerged that APAP-induced cell death triggers a neutrophilic inflammatory response, which has the potential to further exacerbate the existing injury (Jaeschke, 2005; Liu and Kaplowitz, 2006). A neutrophil-mediated injury component has been identified in a variety of experimental conditions including hepatic ischemia-reperfusion injury, endotoxemia, alcoholic hepatitis, obstructive cholestasis and in several drug-induced liver injury models (Jaeschke, 2006; Jaeschke and Hasegawa, 2006). It has been recognized that the initial cell death triggers formation of inflammatory mediators including activated complement factors (Jaeschke et al., 1993), cytokines and chemokines (Okaya and Lentsch, 2003). A variety of molecules released from necrotic cells were identified that could induce cytokine formation through stimulating toll-like receptors (TLRs) (Schwabe et al., 2006). These molecules collectively termed damage-associated molecular patterns (DAMPs) include high mobility group box-1 (HMGB1) protein, heat shock proteins (HSPs), and DNA fragments...
(Bianchi, 2007). Similar DAMPs are also released and correlate with the degree of hepatic damage observed during APAP-induced liver injury (Antoine et al., 2009, 2010; Jahr et al., 2001; Martin-Murphy et al., 2010; Scaffidi et al., 2002) and are responsible for hepatic neutrophil accumulation (Scaffidi et al., 2002).

Despite extensive evidence against a direct involvement of neutrophils in APAP hepatotoxicity (Bauer et al., 2000; Cover et al., 2006; James et al., 2003; Lawson et al., 2000; Welty et al., 1993), several recent reports suggested a critical role of interleukin-1α (IL-1α) (Chen et al., 2007) and in particular IL-1β, in the pathophysiology (Imaeda et al., 2009). It was shown that stimulation of TLR9 by DNA fragments during early APAP-induced cell death can lead to the transcriptional activation of the IL-1β gene resulting in the formation of pro-IL-1β (Imaeda et al., 2009). The pro-form of IL-1β has to be proteolytically cleaved by activated caspase-1 (interleukin-1 converting enzyme) to yield the active cytokine (Sims and Smith, 2010). Caspase-1 activation is regulated by the assembly of the inflammasome, which consists of Nalp3 (NACHT, LRR, and pyrin domain-containing protein 3), ASC (apoptosis-associated speck-like protein containing a CARD), and caspase-1 (Kanneganti et al., 2007; Lamkanfi and Dixit, 2009). Imaeda et al. (2009) showed reduced APAP-mediated injury in gene knockout mice of each individual component of the inflammasome, suggesting an important role of inflammasome activation and IL-1β formation in the pathophysiology of experimental APAP hepatotoxicity. Moreover, inhibition of inflammasome activation by aspirin resulted in protection from APAP hepatotoxicity (Imaeda et al., 2009). These intriguing results open up new avenues of research, provide further opportunities for therapeutic strategies to treat APAP hepatotoxicity and can potentially lead to the identification of
human susceptibility factors for drug-induced liver injury. However, there are conflicting data associated with this concept that require reconciliation. One of the most critical observations arguing against a significant role played by the inflammasome is the fact that IL-1 signaling cannot directly induce cell death in vivo (Sims and Smith, 2010). The only way IL-1α or -1β can cause relevant liver injury is through activation of inflammatory cells, i.e. neutrophils (Bajt et al., 2001), and exacerbation of acetaminophen-induced hepatocyte damage. However, neutrophils are unlikely to cause liver injury after APAP overdose (Bauer et al., 2000; Cover et al., 2006; James et al., 2003; Lawson et al., 2000; Welty et al., 1993). In addition, we have shown that a potent pan-caspase inhibitor prevented mature IL-1β formation during APAP-induced liver injury (Williams et al., 2010b). However, various pan-caspase inhibitors, despite their high efficacy to inhibit caspases in vivo, had consistently no effect on APAP-mediated neutrophil accumulation or liver injury (Antoine et al., 2009; Lawson et al., 1999). Thus, evaluation of the role of the Nalp3 inflammasome and caspase-1 in APAP toxicity in vivo resulted in opposing findings when investigated through pharmacological interventions to inhibit caspase-1 activity compared to a genetic approach to eliminate this enzyme (Imaeda et al., 2009, Chapter 2). In order to reconcile these contradicting results, the objective of this investigation was to further re-evaluate the contribution of activation of the Nalp3 inflammasome in the pathogenesis of APAP hepatotoxicity by using gene knockout mice of all components and by using aspirin treatment.
3.3 Materials and Methods

3.3.1

Animals. Eight to twelve week old male ASC-/-, Caspase-1-/-, Nalp3-/- and C57BL/6 control mice with an average weight of 18 to 24 g were bred and maintained at St. Jude Children’s Research Hospital (Memphis, TN). Nalp3-/-, ASC-/- and Caspase-1-/- mice backcrossed to C57Bl/6 background for at least 10 generations have been described previously (Shaw et al., 2010). Additionally, C57BL/6 mice were bred and housed at the University of Liverpool (Liverpool, UK). All animals were housed in environmentally controlled rooms with 12 h light/dark cycle and allowed free access to food and water. Experiments followed the criteria of the National Research Council for the care and use of laboratory animals in research and guidelines set forth by the University of Liverpool Animal Ethics Committee, respectively.

3.3.2

Experimental design. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Mice were intraperitoneally (i.p.) injected with 300 mg/kg APAP (dissolved in warm saline) after overnight fasting or with an equivalent volume of saline. Additionally, some mice were given aspirin via drinking water (0.06 mg/mL or 1.0 mg/mL) for three days and fasted overnight prior to 530 mg/kg APAP (i.p.). All animals were sacrificed 24 h after APAP. Blood was drawn into heparinized syringes for measurement of alanine aminotransferase (ALT) activity (Pointe Scientific, Canton, MI) and then stored at -80°C. The liver was removed and was rinsed in cold saline; liver sections were fixed in 10% phosphate buffered formalin for histological
analyses. The remaining liver lobes were snap-frozen in liquid nitrogen and stored at -80°C.

3.3.3

*Histology.* Formalin-fixed tissue samples were embedded in paraffin and 5 μm sections were cut. Sections were stained with hematoxylin and eosin (H&E) for blinded evaluation of the areas of necrosis by the pathologist. The percent of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the cross sectional area. Additional sections were stained for neutrophils using the anti-mouse neutrophil allotypic marker antibody (AbD Serotec, Raleigh, NC) as previously described (Williams et al., 2010a). Positively stained neutrophils consistent with cellular morphology were quantified in 15 high power fields (HPF). Some sections were also stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) *in situ* cell death assay (Roche, Indianapolis, IN) as previously described (Lawson et al., 1999).

3.3.4

*mRNA expression.* Quantification of mRNA expression was performed by real-time PCR (RT-PCR) analysis as described previously (Bajt et al., 2008). cDNA was made by reverse transcription of total tissue RNA by M-MLV reverse transcriptase in the presence of random primers (Invitrogen, Carlsbad, CA). Forward and reverse primers for the genes were designed using Primer Express software (Applied Biosystems, Foster City, CA). After cDNA concentration normalization, SYBR green PCR Master Mix (Applied Biosystems) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values generated by
the ABI 7900 instrument (Applied Biosystems). All genes evaluated were first normalized to the β-actin gene and then expressed as a fold increase relative to control arbitrarily set as 1.0. Calculations are made by the $2^{\Delta\Delta Ct}$ formula.

3.3.5

*Glutathione quantification.* Glutathione (GSH) and glutathione disulfide (GSSG) were measured from liver homogenate using the Tietze method as previously described in detail (Jaeschke and Mitchell, 1990). Briefly, frozen tissue was homogenized in sulfosalicylic acid/EDTA. For total GSH determination samples were assayed using dithionitrobenzoic acid. Similarly, measurement of GSSG was performed using the same method after trapping and removal of GSH with N-ethylmaleimide.

3.3.6

*Plasma DNA fragments.* To quantify the release of nuclear DNA fragments into plasma the cell death detection ELISA (Roche, Indianapolis, IN) was used according to the manufacturer’s instructions.

3.3.7

*Plasma HMGB1 and Keratin-18.* Quantification of plasma HMGB1 and Keratin-18 were previously described in detail (Antoine et al. 2009, 2010). Briefly, serum proteins were immunoprecipitated and subsequently analyzed by LC-MS/MS.

3.3.8

*Statistics.* All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way ANOVA or, where appropriate, by two-way ANOVA, followed by a post hoc Bonferroni test. If the data were not
normally distributed, the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn’s Multiple Comparisons Test was used. P < 0.05 was considered significant.
3.4 Results

3.4.1 Liver injury and inflammation in Nalp3 inflammasome-deficient mice

To determine if the Nalp3 inflammasome is critical for APAP-induced toxicity, inflammasome-deficient mice (ASC-/-, Caspase-1-/- and Nalp3-/-) and C57BL/6 wild-type mice were given APAP and sacrificed 24 h later. Liver injury was determined by plasma ALT (3.4.1.1A) and histological quantification of liver necrosis (3.4.1.1B and 3.4.1.2). In contrast to previously published results (Imaeda et al., 2009), the injury in all genotypes was similar demonstrating the Nalp3 inflammasome does not appear to be a critical factor in murine APAP-induced liver injury. Additionally, it was hypothesized that APAP-induced sterile inflammation and IL-1β production mediates immune cell infiltration, potentiating injury. To evaluate if Nalp3 inflammasome activation is important for neutrophil recruitment during APAP overdose, hepatic neutrophils were quantified. Consistent with the injury, total hepatic neutrophil counts were not different between genotypes (3.4.1.1C and 3.4.1.2), which agrees with previously published results demonstrating IL-1β does not participate in neutrophil recruitment during APAP-overdose (Chapter 2). To further evaluate inflammation, hepatic mRNA levels for various cytokines, chemokines and adhesion molecules were compared (3.4.1.3). Interestingly, IL-18, a cytokine which also requires caspase-1 processing, was down-regulated after APAP overdose and IL-1β was only slightly increased similar to previously published results (Chapter 2). Despite the absence of each Nalp3 inflammasome component, there was no alteration in gene expression between genotypes of any cytokine (IL-1β, IL-18, IL-10, IL-6, TNF-α), chemokines (MIP-1α, MIP-1β, MIP-2α, MCP-1), or adhesion molecule (ICAM-1) measured (3.4.1.3). This
demonstrates a nearly identical inflammatory response, which appears to be independent of the Nalp3 inflammasome, during APAP overdose.
Liver injury and hepatic neutrophil recruitment after APAP-overdose in Nalp3 inflammasome-deficient mice. Liver injury in C57BL/6 (WT), ASC-/-, Caspase-1-/- and Nalp3-/- mice was measured by plasma ALT (A) and by histological scoring of liver necrosis (B) 24 hours after APAP overdose. Hepatic neutrophil recruitment was also quantified at 24 h by immunohistochemistry and expressed as total liver neutrophils counted in 15 high power fields (x400 magnification) (C). *P < 0.05 compared to untreated control.
3.4.1.2

TUNEL, H&E and neutrophil staining in Nalp3-inflammasome deficient mice. Liver tissue sections were stained with the TUNEL assay (x50 magnification) to demonstrate equivalent DNA damage in all genotypes [C57BL/6 (WT), ASC-/-, Caspase-1/- and Nalp3/- mice]. Area of necrosis was determined by H&E staining (x50 magnification). Neutrophil recruitment was identified by immunohistochemistry (x200 magnification). Control panels shown are C57BL/6 saline treated mice; knockout saline-treated mice look morphologically identical (not shown).
3.4.1.3

Hepatic mRNA levels 24 h after administration of 300 mg/kg APAP in C57BL/6 wild type animals or knockout mice. mRNA levels are calculated as the cytokine mRNA-to-β-actin mRNA ratio. For mRNA calculations the values of untreated controls were set as 1 and the fold change of the APAP-treated animals is shown. Data represent means ± SE of n = 5 animals per group. All data shown are significant as compared to untreated genotype-matched controls (P<0.05). No gene from APAP-treated knockout animals was significantly different as compared to APAP-treated C57BL/6 controls.

<table>
<thead>
<tr>
<th>Gene</th>
<th>C57BL/6</th>
<th>ASC/-</th>
<th>Caspase1/-</th>
<th>Nalp3/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>2.5 ± 0.4</td>
<td>3.5 ± 0.8</td>
<td>2.1 ± 0.3</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>IL-18</td>
<td>-1.6 ± 0.2</td>
<td>-1.5 ± 0.1</td>
<td>-1.4 ± 0.3</td>
<td>-1.6 ± 0.1</td>
</tr>
<tr>
<td>IL-10</td>
<td>9.4 ± 3.3</td>
<td>7.9 ± 2.3</td>
<td>9.8 ± 2.0</td>
<td>11.9 ± 4.8</td>
</tr>
<tr>
<td>IL-6</td>
<td>3.9 ± 0.7</td>
<td>3.9 ± 0.6</td>
<td>4.1 ± 1.0</td>
<td>3.3 ± 1.0</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>4.4 ± 0.3</td>
<td>4.6 ± 0.4</td>
<td>4.5 ± 0.7</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>26.2 ± 8.5</td>
<td>27.8 ± 5.3</td>
<td>19.2 ± 2.8</td>
<td>13.3 ± 4.6</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>7.8 ± 0.5</td>
<td>10.3 ± 1.9</td>
<td>12.6 ± 2.9</td>
<td>11.3 ± 2.5</td>
</tr>
<tr>
<td>MCP-1</td>
<td>70.9 ± 16.9</td>
<td>68.8 ± 12.6</td>
<td>73.9 ± 19.1</td>
<td>58.5 ± 12.7</td>
</tr>
<tr>
<td>TNF-α</td>
<td>14.8 ± 3.1</td>
<td>14.9 ± 2.1</td>
<td>11.8 ± 3.0</td>
<td>9.7 ± 4.0</td>
</tr>
</tbody>
</table>
3.4.2 APAP-induced oxidant stress in Nalp3 inflammasome-deficient mice

To evaluate liver glutathione recovery and oxidant stress, hepatic GSH and GSSG levels were measured. All genotypes had equivalent GSH recovery at 24 h after APAP (3.4.2.1A) and untreated control mice had equivalent hepatic GSH levels between genotypes (data not shown). In addition, all genotypes had increased GSSG levels over controls with ASC-/− and Nalp3-/− groups having further elevated GSSG levels versus wildtype APAP-treated animals (3.4.2.1B). Despite significantly increased GSSG to GSH ratios in all Nalp3 inflammasome-deficient mice compared to wildtype controls (3.4.2.1C) there was no alteration in injury (3.4.1.1A,B). These data show increased and somewhat variable levels of oxidative stress between genotypes but this variability did not impact liver injury.
Liver glutathione and GSSG in Nalp3-inflammasome deficient mice. Total GSH was measured in liver tissue homogenate of saline treated C57BL/6 (control) and C57BL/6 (WT), ASC-/-, Caspase-1-/- and Nalp3-/- at 24 h after APAP (A). GSSG was measured at 24 h after APAP (B) and the ratio of GSSG to total GSH is shown (C). Knockout saline-treated control mice are not different from C57BL/6 saline-treated controls (not shown). *P < 0.05 compared to saline control. #P < 0.05 compared to APAP-treated C57BL/6 mice.
3.4.3 DAMP release is unaltered in Nalp3 inflammasome-deficient mice

Necrotic cells release damage associated molecular patterns (DAMPs) which can initiate an inflammatory response that was implicated in promoting further injury. DAMPs include high mobility group box-1 (HMGB1) proteins and DNA fragments (Bianchi, 2007). To eliminate the possibility that inflammasome-deficient mice have altered DAMP release, nuclear DNA fragments, keratin-18 and HMGB1 proteins were measured in plasma (3.4.3.1A-C). Consistent with cellular injury, the increase in plasma DNA fragments was similar in all genotypes (3.4.3.1A). Total keratin-18 (K-18) is another useful biomarker to demonstrate cellular injury (Antoine et al., 2009). Indeed, there was a significant increase in plasma keratin-18 compared to controls but no differences in plasma concentrations between genotypes (3.4.3.1B). Another DAMP released after APAP overdose is HMGB1. In addition to total plasma HMGB1 (3.4.3.1C), the hyper- and hypo-acetylated forms of the protein have been shown to originate from innate immune inflammation and necrotic tissue, respectively (Antoine et al., 2009). Correlating with equivalent inflammation and injury, there were no differences between genotypes of total, hyper- or hypo-acetylated HMGB1 (3.4.3.1C,D), and no differences in plasma DNA fragments, K-18 or HMBG1 were observed between genotypes of saline treated animals (data not shown). These data show equivalent DAMP release and a subsequent equivalent sterile inflammation with or without the presence of functional Nalp3 inflammasome.
3.4.3.1

Quantification of plasma DAMPs in Nalp3-inflammasome deficient mice. DNA fragments were measured in plasma of saline treated C57BL/6 (control) and C57BL/6 (WT), ASC-/-, Caspase-1/-/- and Nalp3/-/- at 24 h after APAP (A) and expressed as percent of control. Similarly, total plasma keratin-18 (B), total plasma HMGB1 (C), and hypo- and hyper-acetylated plasma HMGB1 (D) were measured. Knockout saline-treated mice are not different from C57BL/6 saline-treated controls (not shown). *P < 0.05 compared to saline control.
3.4.4 Aspirin does not alter APAP toxicity or DAMP release

It was hypothesized that aspirin (acetylsalicylic acid, ASA) is capable of inhibiting the Nalp3 inflammasome and it was reported that pretreatment with ASA in drinking water reduced APAP-induced injury (Imaeda et al., 2009). This study was repeated with the ASA dose previously published (0.06 mg/mL in drinking water) as well as a higher dose (1.0 mg/mL in drinking water). Data presented are for only the high dose of ASA because no effect on injury was observed for either dose. ASA in the drinking water of mice caused no liver injury nor was it capable of reducing the APAP-induced liver injury as measured by plasma ALT activities (3.4.4.1A) or the area of necrosis (3.4.4.1B,D). ASA pretreatment did not alter hepatic GSH levels nor did it cause differences in GSH recovery after overdose indicating it did not affect APAP metabolism (data not shown). ASA pretreatment caused a trend in decreasing hepatic neutrophil recruitment after APAP overdose, but the reduction was not statistically significant (3.4.4.1C,D). In addition, ASA pretreatment caused no alterations in DAMP release which is consistent with the results observed in Nalp3 inflammasome knockout mice. The APAP-induced increase in plasma levels of keratin-18 (3.4.4.2A), total HMGB1 (3.4.4.2B) and hyper- and hypo-acetylated HMGB1 (3.4.4.2C) were not significantly affected by ASA treatment. As a whole the genetic elimination of the Nalp3 inflammasome and pharmacologic inhibition of the Nalp3 inflammasome with ASA did nothing to alter APAP-induced injury.
Liver injury and hepatic neutrophil recruitment in aspirin-pretreated mice after APAP-overdose. ALT (A) was measured in the plasma of C57BL/6 mice pretreated with or without aspirin (ASA) in the drinking water and then subjected to APAP-overdose or saline injection. Similarly, histological area of necrosis (B), and neutrophil accumulation were quantified (C) were measured as previously described. Representative sections with H&E and neutrophil staining are shown (D). *P < 0.05 compared to the respective control with or without ASA.
Quantification of plasma DAMPs in aspirin-pretreated mice. Total keratin-18 (A) was measured in the plasma of C57BL/6 mice pretreated with or without aspirin (ASA) in the drinking water and then subjected to APAP-overdose or saline injection. Similarly, total plasma HMGB1 (B), and hypo- and hyper-acetylated HMGB1 (C) were measured. *P < 0.05 compared to the respective control with or without ASA.
3.5 Discussion

3.5.1 Role of the Nalp3 inflammasome in APAP-induced liver injury

The main objective of this investigation was to reconcile apparently contradictory results regarding the importance of the Nalp3 inflammasome in APAP hepatotoxicity obtained by different groups using a genetic versus a pharmacological approach. Imaeda et al. (2009) reported significantly reduced liver injury and improved survival in ASC-/-, Nalp3-/- and caspase1-/- mice. Although it was only shown for Nalp3-/- mice that the APAP-induced increase in IL-1β protein formation was prevented, the assumption was that all knockout mice were partially protected because processing of pro-IL-1β to the active, pro-inflammatory cytokine IL-1β should have been prevented when caspase-1 as part of the Nalp3 inflammasome was nonfunctional (Imaeda et al., 2009). These conclusions are consistent with a previous report showing that IL-1 receptor-deficient mice were almost completely (95%) protected against APAP-induced liver injury (Chen et al., 2007). It was assumed that inflammatory cells played a deleterious role in the pathogenesis (Imaeda et al., 2009; Chen et al., 2007). In contrast to these reports, our current findings indicate very clearly that ASC-/-, Nalp3-/- and caspase1-/- knock-out mice are neither protected (ALT, area of necrosis, oxidant stress, DNA damage) nor do these animals show any evidence of a reduced inflammatory response (cytokine and chemokine formation, hepatic neutrophil accumulation). These data are fully consistent with our previous report where we found not only increases of IL-1β mRNA after APAP overdose but also moderate increases in caspase-dependent mature IL-1β protein formation (Chapter 2). However, the caspase inhibitor had no protective effect on liver injury or hepatic neutrophil accumulation, a concept consistent with other published
findings (Antoine et al., 2009). It is notable that the 300% increase of IL-1β formation after APAP as reported in our study (Chapter 2) is actually higher than the 13% increase measured by Imaeda et al. (2009). The mechanistic basis of this still requires further investigation. Independent of these differences in pathological outcome, both studies clearly show that the absolute plasma levels of IL-1β protein are very low even after APAP overdose (Imaeda et al., 2009; Chapter 2). Thus, in order to assess if levels of IL-1β that have been shown to activate and recruit neutrophils into the liver (Bajt et al., 2001), can impact APAP toxicity, animals were treated with pharmacological levels of murine recombinant IL-1β (about 3-4 orders of magnitude above endogenous concentrations). Although this indeed caused more recruitment of activated neutrophils into the liver, it still had no effect on liver injury (Chapter 2). In agreement with these findings, IL-1R-/- mice were not protected against APAP hepatotoxicity (Chapter 2). These data together indicate that despite the fact that some IL-1β is generated in a caspase-dependent manner after APAP overdose, it is highly unlikely that this minor, endogenously formed cytokine has any major impact on the inflammatory response induced during APAP hepatotoxicity. In addition, neither our previous study (Chapter 2) nor our present experiments showed any evidence for increased formation of IL-18, another cytokine that is processed by caspase-1, after APAP overdose. Moreover, theoretically, any pathophysiological effect of both cytokines would have to involve cytotoxicity mediated by inflammatory cells. However, there is extensive experimental evidence to suggest that neutrophils are not involved in APAP-induced liver injury (Bauer et al., 2000; Cover et al., 2006; James et al., 2003; Lawson et al., 2000; Welty et al., 1993). Thus, based on the currently reported data from our study activation of the
Nalp3 inflammasome does not play a critical role in the exacerbation of APAP hepatotoxicity.

In addition to playing a critical role in the formation of the Nalp3 inflammasome, recent reports have suggested that ASC is involved in inflammasome-independent cellular functions such as lymphocyte survival (Shaw et al., 2010), antigen-induced T cell activation by dendritic cells (Ippagunta et al., 2010) and antigen-specific humoral immunity (Ellebedy et al., 2011). The mechanism(s) underlying the critical role of ASC in these functions is still unknown, but it is possible ASC is involved in other inflammasome-independent functions in various cell types. However, ASC-/- mice developed APAP-induced liver injury to the same degree as wildtype animals suggesting that all inflammasome-dependent and inflammasome-independent functions of ASC do not play a relevant role in APAP hepatotoxicity.

3.5.2 The role of aspirin in APAP hepatotoxicity

Imaeda et al. (2009) also reported that a low dose of aspirin (N-acetylsalicylic acid) can reduce APAP hepatotoxicity independent of its effect on platelet aggregation or cyclooxygenase-1 or -2. It was concluded that aspirin protected due to inhibition of the transcriptional activation of cytokine formation (IL-1β, IL-18, TNF-α and interferon-γ) and also inhibition of the Nalp3 inflammasome pathway, which processes IL-1β and IL-18 (Imaeda et al., 2009). Again, the assumption was that the reduced cytokine formation attenuates liver injury through reduced inflammatory cell activation and recruitment. However, using the same pretreatment regimen as described by Imaeda et al. (2009) with the same or higher doses of aspirin, no significant effect on APAP-induced liver injury was observed when compared to APAP-treated mice in our study. Aspirin
treatment showed a trend to attenuate the number of neutrophils in the liver but the overall effect was statistically not significant and indeed, the quantification of serum biomarkers of toxicity (ALT, HMGB1, K-18) was not different between groups. These data further support the concept that aspirin pretreatment has no significant effect on the overall extent of experimental APAP hepatotoxicity. APAP metabolism was shown to be unaffected by aspirin pretreatment in our investigation, but this was not assessed in the previous study (Imaeda et al., 2009). Thus, in our hands aspirin was not effective in preventing APAP hepatotoxicity. Even if aspirin would reduce hepatic neutrophil recruitment, the fact that many other interventions against neutrophils were ineffective in this model (Bauer et al., 2000; Cover et al., 2006; James et al., 2003; Lawson et al., 2000; Welty et al., 1993), are consistent with the lack of protection by aspirin.

3.5.3 DAMPs and APAP hepatotoxicity

The sterile inflammatory response after APAP overdose requires the release of DAMPs. Previous studies reported measurement of DAMPs including HMGB1, heat shock proteins, and DNA fragments in plasma of APAP-treated animals (Antoine et al., 2009, 2010; Jahr et al., 2001; Martin-Murphy et al., 2010; Scaffidi et al., 2002). In the case of HMGB1, it was shown that a hypo-acetylated form is passively released by necrotic cells and can be used similar to keratin-18 as a biomarker of necrosis (Antoine et al., 2009). In addition, a hyper-acetylated form of HMGB1 can be actively secreted by macrophages or monocytes (Bonaldi et al., 2003) and thus can represent a potential biomarker of inflammation (Antoine et al., 2009). Consistent with these previous observations, we found a substantial increase in nuclear DNA fragments, in plasma keratin-18 and in total HMGB1 levels after APAP treatment. However, the increase in
these plasma biomarkers of hepatic necrosis was neither affected by aspirin treatment nor by deficiency of inflammasome genes. Furthermore, the levels of hypo-acetylated HMGB1 (necrosis) and hyper-acetylated HMGB1 (inflammation) when determined by mass spectrometry were not modulated in the knockout mice or affected by aspirin treatment. These data are consistent with both the histology data and other markers (ALT) and demonstrate that the overall APAP-induced cell necrosis in these livers is the same in all groups. Moreover, the similar release of DAMPs in all groups is consistent with the similar overall induction of pro-inflammatory cytokine and chemokine genes and recruitment of neutrophils into the livers. Thus, despite the minor effect of IL-1β processing caused by the activation of the Nalp3 inflammasome (Imaeda et al., 2009; Chapter 2), the overall inflammatory response after APAP overdose is not dependent on the Nalp3 inflammasome and does not impact on the overall extent of observed toxicity.

The data presented in this manuscript are fully consistent with a previous study (Chapter 2) showing that IL-1β formation and processing by the inflammasome has no relevant impact on the degree of APAP hepatotoxicity in mice. However, this is not consistent with the results and conclusions presented by Imaeda et al. (2009) and in part by Chen et al. (2007). The conclusions presented herein are based on data from three independent research groups, that the activation of the Nalp3 inflammasome does not play a significant role in the pathogenesis of experimental APAP hepatotoxicity. We have extended the original investigations reporting a critical role for the Nalp3 inflammasome in APAP toxicity by inclusion of the characterization of the inflammatory response, a more extended quantification of cell death and associated biomarker release. Each of these factors was not altered between the genetic or pharmacological
manipulation of the inflammasome and the overall degree of toxicity. It is important to
point out only low levels of IL-1β produced in the APAP-treated mice could be detected
in all studies, but the evidence for a functional consequence of such cytokine release
was inconsistent. There is no obvious explanation for these discrepancies. However,
there are minor differences in the experimental design that need to be considered
between our present study and the experiments reported by Imaeda et al. (2009). For
the experiments with the knock-out mice, we used our standard dose of 300 mg/kg
compared to 500 mg/kg by Imaeda et al., (2009). However, the aspirin study was
performed administering the same dose of APAP as used by Imaeda et al. (2009) but
still resulted in no protection. In addition, we terminated our experiments at 24 h
compared to 12 h in the Imaeda study. It appears unlikely that there could be protection
at earlier time points that is lost at later times given the fact that inflammatory liver injury
generally occurs at later time points. Lastly, the sources of the mice used in these
studies were different. Although it cannot be completely ruled out that the different
sources of mice may have affected the results, the fact that our results with the various
knock-out mice are fully consistent with pharmacological interventions (caspase
inhibitors, aspirin) and additional manipulations (injection of pharmacological doses of
IL-1β) (Chapter 2) as well as a battery of studies on the role of neutrophils in the
pathogenesis of APAP hepatotoxicity in rats and various mouse strains (Bauer et al.,
2000; Cover et al., 2006; James et al., 2003; Lawson et al., 2000; Welty et al., 1993),
gives us confidence in the general applicability of our data and conclusions. Although,
an important aspect of the present study was to demonstrate that the pharmacological
manipulations used for investigation of the inflammasome did not alter the metabolic
activation of APAP to its toxic metabolite. In addition to unrecognized effects on drug metabolism, pharmacological interventions and genetic manipulations can trigger stress responses with expression of protective genes or cause compensatory responses that may affect APAP toxicity (Jaeschke et al., 2011).

It is well recognized that an inflammatory response after an acute tissue injury can have multiple opposing effects (Bulkley and Roberts, 1974). Although inflammation may enhance tissue injury under certain circumstances, the recruited neutrophils and especially macrophages are critical for removal of necrotic cell debris and promotion of tissue repair. Our data provide further support that in the APAP model, inflammatory cells do not directly cause liver damage. However, there is evidence to indicate that suppressing the late inflammatory response delays liver regeneration (Dambach et al., 2002; Holt et al., 2008).

3.5.4 Summary

Our data clearly demonstrated that APAP overdose caused substantial liver injury, release of DAMPs and a sterile inflammatory response. However, mice deficient in components of the Nalp3 inflammasome (ASC-/-, Nalp3-/- and caspase1-/-) or wild type mice treated with aspirin showed very similar responses to APAP as wild type animals treated with APAP alone. These data are consistent with previous pharmacological approaches to inhibit caspase-1, with data obtained in IL-1 receptor/-/- mice, and with the lack of effects when pharmacological doses of IL-1β were administered (Chapter 2). All these data together strongly support the conclusion that the minor endogenous formation of IL-1β and the activation of the Nalp3 inflammasome has no relevant impact on APAP hepatotoxicity and therefore the Nalp3 inflammasome may not be a viable
therapeutic target to treat APAP overdose. Caution must therefore be exercised in translating this concept to man with respect to aspirin acting as a hepatoprotective agent against APAP-induced liver injury.
Chapter 4

Role of CD18 and neutrophil priming during APAP overdose

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Liver International, 30(9):1280-1292, Acetaminophen-induced hepatic neutrophil accumulation and inflammatory liver injury in CD18-deficient mice, 2010, with permission from Wiley.
4.1 Abstract

Acetaminophen (APAP) hepatotoxicity is currently the most frequent cause of acute liver failure in the US and many European countries. Although intracellular signaling mechanisms are critical for hepatocellular injury, a contribution of inflammatory cells, especially neutrophils, has been suggested. However, conflicting results were obtained when using immunological intervention strategies. The role of neutrophils was investigated using a CD18-deficient mouse model. Treatment of C57Bl/6 wild type mice with 300 mg/kg APAP resulted in severe liver cell necrosis at 12 and 24 h. This injury was accompanied by formation of cytokines and chemokines and accumulation of neutrophils in the liver. However, there was no difference in the inflammatory response or liver injury in CD18-deficient mice compared to wild type animals. In contrast to treatment with endotoxin, no upregulation of CD11b or priming for reactive oxygen was observed on neutrophils isolated from the peripheral blood or the liver after APAP administration. Furthermore, animals treated with endotoxin 3 h after APAP experienced an exaggerated inflammatory response as indicated by substantially higher cytokine and chemokine formation and twice the number of neutrophils in the liver. However, liver injury in the two-hit model was the same as with APAP alone. Our data do not support the hypothesis that neutrophils contribute to APAP hepatotoxicity or that a neutrophil-mediated injury phase could be provoked by a second, pro-inflammatory hit. Thus, APAP-induced liver injury in mice is dominated by intracellular mechanisms of cell death rather than by neutrophilic inflammation.
4.2 Introduction

Acetaminophen (APAP) overdose is currently the most frequent cause of acute liver failure in the US and many other countries (Lee, 2004; Larson et al., 2005). The mechanisms of cell injury induced by APAP have been extensively studied in vivo and in primary cultured hepatocytes during the last several decades. The main intracellular events critical for hepatotoxicity include the formation of a reactive metabolite, which causes glutathione depletion and covalent protein modifications, mitochondrial dysfunction and oxidant stress, peroxynitrite formation, and nuclear DNA fragmentation by mitochondrial-derived endonuclease G and apoptosis-inducing factor (Nelson, 1990; Jaeschke et al., 2003; Jaeschke and Bajt, 2006). Ultimately these events, especially the oxidant stress and peroxynitrite formation in the mitochondria (Cover et al., 2005), trigger the opening of the membrane permeability transition pore and the collapse of the mitochondrial membrane potential leading to necrotic cell death (Kon et al., 2004; Gujral et al., 2002).

In addition to the intracellular signaling events, a potential contribution of inflammatory cells to the overall injury after APAP overdose came into focus during the last decade (Jaeschke, 2005; Liu and Kaplowitz, 2006). During an inflammatory response, neutrophils can accumulate in sinusoids, extravasate and kill stressed hepatocytes mainly through reactive oxygen formation (Jaeschke 2006). It is well documented that liver injury can be exacerbated by neutrophils during ischemia-reperfusion injury (Jaeschke et al., 1990), endotoxemia (Jaeschke et al., 1991), alcoholic hepatitis (Bautista 1997; Ramaiah and Jaeschke 2007), and obstructive cholestasis (Gujral et al., 2003) as well as during drug- and chemical-induced hepatotoxicity by α-
naphthylisothiocyanate (Dahm et al., 1991; Kodali et al., 2006), halothane (You et al., 2006) and concanavalin A (Bonder et al., 2004). However, the role of neutrophils in acetaminophen hepatotoxicity is controversially discussed (Jaeschke 2008). Evidence for (Smith et al., 1998; Liu et al., 2006; Ishida et al., 2006) and against (Lawson et al., 2000; Bauer et al., 2000; Cover et al., 2006) a contribution of neutrophils to the overall injury has been presented.

More recent reports show that danger-associated molecular patterns (DAMPs) released from necrotic hepatocytes after APAP overdose can activate toll-like receptors on Kupffer cells and induce the formation of inflammatory mediators through the inflammasome (Chen et al., 2007; Imaeda et al., 2009; Chen et al., 2009). The implication is again that inflammatory cytokines such as interleukin-1β promote a neutrophil-mediated aggravation of the initial injury (Chen et al., 2007; Imaeda et al., 2009; Chen et al., 2009). A problem with the previous studies evaluating a potential effect of neutrophils on APAP hepatotoxicity was the use of immunological agents, i.e. neutropenia-inducing antibodies, which resulted in conflicting results (Smith et al., 1998; Liu et al., 2006; Bauer et al., 2000; Cover et al., 2006). To avoid these issues, we applied a genetic approach by using mice deficient in the β-chain (CD18) of β2 integrins (CD11/CD18). β2 integrins on neutrophils are critical for their adhesion to receptors on endothelial cells and hepatocytes as well as the adherence-dependent generation of reactive oxygen species (Smith, 1993). Consequently, neutrophil accumulation during peritonitis was drastically reduced in CD18-deficient mice (Wilson et al., 1993). In addition, we showed previously in these mice impaired extravasation of neutrophils and reduced reactive oxygen formation in the liver resulting in less inflammatory cell injury
during obstructive cholestasis (Gujral et al., 2003). Based on these data, we tested the hypothesis that if neutrophils play a relevant role in the progression of APAP hepatotoxicity, CD18-deficient mice should be at least partially protected.
4.3 Materials and Methods

4.3.1

**Animals.** Male CD18-deficient mice (B6.129S7-Itgb2tm1Bay/J; Stock number: 002128), which are backcrossed to a C57BL/6J background and their age-matched control littermates (C57BL/6J) with an average weight of 18 to 20 g were purchased from Jackson Laboratory (Bar Harbor, Maine). All animals were housed in an environmentally controlled room with 12 h light/dark cycle and allowed free access to food (# 8604 Teklad Rodent, Harlan, Indianapolis, IN) and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Kansas Medical Center and followed the criteria of the National Research Council for the care and use of laboratory animals in research.

4.3.2

**Experimental design.** All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. Mice were intraperitoneally injected with 300 mg/kg APAP (dissolved in warm saline) after overnight fasting. The animals were killed 12 or 24 h after APAP treatment, blood was withdrawn from the vena cava into a heparinized syringe for measurement of alanine aminotransferase (ALT) activities (Kinetic Test Kit 68-B, (Biotron Diagnostics, Inc., Hernet, CA) and cytokine levels. The liver was removed and was rinsed in saline; liver sections were fixed in 10% phosphate buffered formalin for histological analyses. The remaining liver was snap-frozen in liquid nitrogen and stored at -80 °C. In additional experiments, groups of animals were first treated with 300 mg/kg APAP and 3 h later either with saline (10 ml/kg) or 100 μg/kg Salmonella abortus equi endotoxin (ET) (i.p.). As positive controls for neutrophil-mediated liver injury, a few
animals were subjected to common bile duct ligation (BDL) for 2 days as described in
detail (Gujral et al., 2003).

4.3.3

Histology. Formalin-fixed tissue samples were embedded in paraffin and 5 μm sections
were cut. Sections were stained with hematoxylin and eosin (H&E) for blinded
evaluation of the areas of necrosis by the pathologist. The percent of necrosis was
estimated by evaluating the number of microscopic fields with necrosis compared to the
entire cross section. Additional sections were stained for neutrophils. Endogenous
peroxidase was suppressed with peroxidase suppressor (Thermo, Waltham, MA).
Tissue was blocked with serum free blocker (Dako, Carpinteria, CA). Trypsin antigen
retrieval was performed (Abcam, Cambridge, MA). Anti-mouse neutrophil allotypic
marker antibody (AbD Serotec, Raleigh, NC) was incubated for 1 hour at room
temperature. Secondary antibody coupled with biotin was then added (Vector,
Burlingame, CA) followed by streptavidin-HRP conjugate (Vector). DAB substrate was
used (Dako) and then counterstained with hematoxylin (Sigma). Positively stained cells
consistent with neutrophil morphology were quantified in randomly selected high power
fields (HPF, x400).

4.3.4

mRNA expression. Quantification of mRNA expression of several cytokines and
chemokines was performed by real-time PCR (RT-PCR) analysis as previously
described (Bajt et al., 2008). cDNA was generated by reverse transcription of total RNA
by M-MLV reverse transcriptase in the presence of random primers (Invitrogen,
Carlsbad, CA). Forward and reverse primers for the genes were designed using Primer
Express software (Applied Biosystems, Foster City, CA). After normalization of cDNA concentrations, SYBR green PCR Master Mix (Applied Biosystems) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values generated by the ABI 7900 instrument (Applied Biosystems). All genes evaluated were first normalized to β-actin and then expressed as a fold increase relative to control which was arbitrarily set as 1.0. Calculations are made by assuming one cycle is equivalent to a two-fold difference in copy number which is the $2^{\Delta\Delta Ct}$ formula.

4.3.5

*Plasma cytokine measurements.* Quantification of cytokines was performed in plasma by the Bio-Plex bead-based multiplex assay following the kit instructions (Bio-Rad Laboratories, Hercules, CA) and analyzed on the Bio-Plex 200 instrument (Bio-Rad).

4.3.6

*Isolation of hepatic non-parenchymal cells.* The procedure was adapted from a method described by Watanabe et al. (Watanabe et al., 1992). Under isoflurane anesthesia mice were exsanguinated from the caudal vena cava into heparinized tubes and the blood was placed on ice; the liver was then perfused through the portal vein with 1.0 mL ice-cold Dulbecco’s PBS. The liver was immediately excised, placed in ice-cold PBS and minced with scissors. The tissue was then pressed through a 200-gauge stainless steel mesh into a 50mL conical tube. The cell suspension was centrifuged at 50 x g for 2 minutes to remove hepatocytes and large debris. The supernatant containing non-parenchymal cells was then centrifuged at 350 x g for 5 minutes and cells were washed
twice in a 15 mL conical bottom tube. Viable, nucleated cells were counted by trypan blue exclusion and brought to a uniform cell density.

4.3.7

Flow Cytometric Analysis of CD11b Expression and Reactive Oxygen Formation.

CD11b/Gr-1 staining (Bajt et al., 2001; Daley et al., 2008). 5µg Fc receptor (FcR) blocking antibody (BioLegend, San Diego, CA) diluted in 100µL 0.1% BSA in PBS was added to 100µL non-parenchymal cell suspension for 20 minutes on ice. To 50µL whole blood or 200µL FcR-blocked hepatic non-parenchymal cells, saturating concentrations of PE-Cy5-labeled-anti-Gr-1 (BioLegend) and PE-labeled-anti-CD11b (BioLegend) diluted in 0.1% BSA in PBS were added. Tubes were incubated in the dark, on ice for 30 minutes. After washing with 0.1% BSA, red blood cells were lysed using RBC lysing solution (0.155 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Pellets were washed three times with 0.1% BSA then fixed with 2.5% buffered formalin. Samples were measured on the FACSCalibur (BD, Franklin Lakes, NJ). We gated on the Gr-1<sup>high</sup> cells, which is also the only Ly6G-positive population (Daley et al., 2008). Cells, which are Gr-1<sup>high</sup> were identified as neutrophils by cell morphology. Other leukocytes are either Gr-1<sup>intermediate</sup> (mainly monocytes and eosinophils) or Gr-1<sup>negative</sup> (monocytes and lymphocytes) (Daley et al., 2008). The data were analyzed using the BD FACSDiva 6.0 software.

Reactive Oxygen Production (Emmendorffer et al., 1990; Smith and Weidemann, 1993). To 50µL whole blood or 100µL hepatic non-parenchymal cells, 10 µM diphenyleneiodonium or vehicle (0.1% DMSO) was added to each tube for 20 minutes. 2 µM phorbol 12-myristate 13-acetate (PMA) or saline was added and incubated for 10
minutes at 37°C. 4 µM dihydrorhodamine-123 was added and incubated for 10 minutes at 37°C. Ice-cold 0.1% BSA in PBS was added and pellets were washed. The non-parenchymal cell suspension was blocked with FcR blocking antibody (BioLegend) as previously described and cells were stained with saturating concentrations of PE-Cy5-labeled-anti-Gr-1 (BioLegend) diluted in 0.1% BSA. Tubes were incubated in the dark, on ice for 30 minutes. After washing with 0.1% BSA, red blood cells were lysed using RBC lysing solution. Pellets were washed three times with 0.1% BSA then fixed with 2.5% buffered formalin. Samples were measured on the FACSCalibur (BD) and the data were analyzed using the BD FACSDiva 6.0 software.

4.3.8

Statistics. All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way ANOVA or, where appropriate, by two-way ANOVA, followed by a post hoc Bonferroni test. If the data were not normally distributed, we used the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn’s Multiple Comparisons Test. P < 0.05 was considered significant.
4.4 Results

4.4.1 Liver injury and oxidant stress in C57Bl/6 and CD18-deficient mice

Administration of 300 mg/kg APAP to C57Bl/6 and CD18-deficient mice resulted in severe liver injury at 12 and 24 h as indicated by the highly elevated plasma ALT activities (4.4.1.1A) and the extensive centrilobular necrosis (4.4.1.1B,C). Blinded quantification of cell death revealed that approximately 50% of all hepatocytes were necrotic in wild type and CD18-deficient animals at 24 h (4.4.1.1B). Hepatic glutathione (GSH+GSSG), which is depleted by >90% during the first hour after APAP, was only partially recovered at 12 h but reached baseline values at 24 h (4.4.1.2A). APAP induced a significant oxidant stress as indicated by the increase of hepatic GSSG values and the increase of the GSSG-to-GSH ratio (4.4.1.2B,C).
Acetaminophen-induced liver injury in C57Bl/6 wild type and CD18-deficient mice. Animals were treated with 300 mg/kg APAP and plasma ALT was measured at 12 h and at 24 h (A) and the area of necrosis was quantified at 24 h (B). Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to untreated controls). C. Representative H&E-stained liver sections of animals treated for 12 h and 24 h with 300 mg/kg APAP (x50 for all panels).
Hepatic content of total GSH (GSH + GSSG) (Panel A) and GSSG (Panel B) were measured in untreated controls (C) and in C57Bl/6 wild type and CD18-deficient mice treated with 300 mg/kg APAP for 12 h or 24 h. In addition, the GSSG-to-GSH ratio (Panel C) was calculated from each animal. Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to respective control)
4.4.2 Liver neutrophil recruitment in C57Bl/6 and CD18-deficient mice following APAP or bile duct ligation

Immunohistochemical staining for neutrophils showed extensive accumulation of these leukocytes in the liver at 12 h and a substantial further increase at 24 h (4.4.2.1A). However, despite the fairly high numbers of neutrophils around the area of necrosis after APAP (4.4.2.1B), animals subjected to bile duct ligation, where neutrophils play a critical role in liver injury (Gujral et al., 2003; Gujral et al., 2004), showed a substantially higher accumulation of neutrophils around the bile infarcts (necrotic foci) (4.4.2.1B). Overall, the APAP data are consistent with previous findings regarding the degree of injury, oxidant stress and inflammation in this model (Knight et al., 2001). Treatment of CD18-deficient mice with APAP resulted in similar liver injury as compared to wild type animals as judged by both plasma ALT activities and histology (4.4.1.1) and showed similar oxidant stress (4.4.1.2) and hepatic neutrophil accumulation (4.4.2.1).
4.4.2.1

Hepatic neutrophil accumulation after APAP treatment in C57Bl/6 wild type and CD18-deficient mice. A. Neutrophil numbers were quantified in 15 randomly selected high power fields (HPF; x400). Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to untreated controls). B. Comparison of neutrophil accumulation in an area of necrosis 12 h after 300 mg/kg APAP or 2 days after common bile duct ligation (BDL). (x200). Red arrows highlight selected neutrophils in sinusoids and on the periphery of necrosis in the APAP-treated liver.
4.4.3 Inflammatory response in C57Bl/6 and CD18-deficient mice

To evaluate pro-inflammatory mediator formation, the hepatic mRNA of selected cytokines and chemokines were measured (4.4.3.1). A variable but significant increase above untreated controls was found for all cytokines/chemokines at 12 h and at 24 h after APAP. Consistent with these findings, plasma protein levels of most of these cytokines and chemokines were significantly increased at both time points (4.4.3.2). However, the increase was generally moderate and limited to levels between 2-fold to less than 10-fold above baselines. Most importantly, no significant difference in mRNA or serum protein levels of any cytokine or chemokine between wild type and CD18-deficient animals was observed (except the 12 h plasma TNF-α levels). These data support the conclusion that a neutrophilic inflammatory response occurs early after the APAP-induced liver injury. However, animals with a deficiency in CD18 develop the identical degree of injury after APAP overdose suggesting that neutrophils do not play a critical role in the pathophysiology.
Hepatic cytokine/chemokine mRNA levels were measured 12 and 24 h after administration of 300 mg/kg acetaminophen (APAP) in C56Bl/6 wild type animals and in CD18-deficient mice. mRNA levels are expressed as the cytokine mRNA-to-β-actin mRNA ratio. The values of untreated controls were set as 1 and the fold change of the APAP-treated animals was calculated. Data represent means ± SE of n = 6-7 animals per group. All cytokines and chemokines were significantly elevated over control (P<0.05) however differences between genotypes could be observed.
Plasma cytokine and chemokine levels after acetaminophen overdose in C57Bl/6 wild type and CD18-deficient mice. Animals were treated with 300 mg/kg APAP and plasma cytokine/chemokine levels were measured at 12 h and at 24 h after APAP using the Bioplex system. Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to untreated controls). #P<0.05 (compared to wild type animals).
4.4.4 Characterization of Neutrophils after APAP overdose

In order to further characterize a potential neutrophil priming or activation after APAP, the surface expression of Mac-1 (CD11b/CD18) and the capacity for the formation of reactive oxygen species (ROS) was determined in peripheral neutrophils (4.4.4.1) and in liver neutrophils (4.4.4.2) at 6 h after 300 mg/kg APAP. Upregulation of Mac-1 and priming for reactive oxygen formation of neutrophils is a hallmark of all models where neutrophils actively contribute to liver injury (Gujral et al., 2003; Bajt et al., 2001; Jaeschke et al., 1993). Despite significant injury at 6 h (ALT: 2840 ± 300 U/L; n=5), ROS formation and the expression of Mac-1 on peripheral neutrophils (4.4.4.1) and from hepatic neutrophils (4.4.4.2) from APAP-treated animals were similar compared to cells obtained from untreated controls. As a positive control experiment, animals were treated with 100 µg/kg of endotoxin. Peripheral blood and hepatic neutrophils were evaluated at 1.5 h. The well-known cytokine response after endotoxin caused a 2.6-fold (2.0-fold) increase in ROS formation and a 25-fold (7.5-fold) increase in Mac-1 expression on peripheral (hepatic) neutrophils (4.4.4.1; 4.4.4.2). The quantitatively slightly lower activation of the hepatic neutrophils compared to the peripheral neutrophils may be due to the increased stress of isolating the cells from the liver. However, most importantly, the data obtained from peripheral neutrophils closely reflects the activation status of liver neutrophils. Measurements of blood and liver neutrophils in CD18-deficient mice showed similar ROS formation as obtained in wild type animals (data not shown). The reason for this is the use of PMA as stimulant. PMA directly activates protein kinase C intracellularly and does not depend on surface receptors. As
expected (Gujral et al., 2003), CD11b expression was substantially lower in CD18-deficient samples compared to wild type (data not shown).
Continued to following page.
Reactive oxygen species (ROS) priming and CD11b expression on peripheral neutrophils. C57Bl/6 mice (n=4 per group) were treated with 20 ml saline/kg for 6 hours, 100 µg endotoxin/kg for 90 minutes or 300 mg APAP/kg for six hours. A. To determine reactive oxygen species (ROS) priming, mice were treated with APAP or endotoxin \textit{in vivo} then whole blood was stimulated \textit{ex vivo} with PMA. Upon PMA-induced ROS production DHR-123 is converted to R-123 and quantified in neutrophils by flow cytometry. In addition, immediately after \textit{in vivo} stimulation with APAP or endotoxin whole blood was stained for CD11b surface expression and neutrophils were analyzed by flow cytometry. The Gr-1\textsuperscript{high} cell population consists of neutrophils. Representative ROS or CD11b histograms or mean fluorescence intensities (B) for saline, the positive control endotoxin, and APAP are shown. *P<0.05 (compared to saline controls)
4.4.4.2

Continued to following page.
Reactive oxygen species (ROS) priming and CD11b expression on liver accumulated neutrophils. C57Bl/6 mice (n=4 per group) were treated with 20 ml saline/kg for 6 hours, 100 µg endotoxin/kg for 90 minutes or 300 mg APAP/kg for six hours. A. To determine reactive oxygen species (ROS) priming, mice were treated with APAP or endotoxin in vivo, non-parenchyma cells were isolated and then stimulated ex vivo with PMA. Upon PMA-induced ROS production DHR-123 is converted to R-123 and quantified in neutrophils by flow cytometry. In addition, immediately after in vivo stimulation with APAP or endotoxin, the isolated non-parenchymal cells were stained for CD11b surface expression and neutrophils were analyzed by flow cytometry. The Gr-1<sup>high</sup> cell population consists of neutrophils. Representative ROS or CD11b histograms or mean fluorescence intensities (B) for saline, the positive control endotoxin, and APAP are shown. *P<0.05 (compared to saline control)
4.4.5 Enhanced neutrophil recruitment with endotoxin after APAP overdose

Because endotoxin caused a substantial activation of neutrophils, it was tested if treatment with endotoxin 3 h after APAP enhances neutrophil recruitment into the liver and potentially increases APAP-induced liver injury during the 24 h observation period. However, liver injury after the sequential administration of APAP and ET was the same as with APAP alone (4.4.5.1A,C) despite the doubling of the number of neutrophils in the liver (4.4.5.1B). Although the peaks of cytokine/chemokine formation after endotoxin occur well before the 24 h time point (Dorman et al., 2005), there was still a more than 5-fold elevation of all mRNA levels and a 2- to 80-fold increase of plasma protein levels detectable at this late time (4.4.5.2). These data demonstrate that the additional treatment with endotoxin drastically enhanced the pro-inflammatory mediator formation and further promoted hepatic neutrophil accumulation. However, despite this substantial boost of the inflammatory response, liver injury was not different from injury with APAP alone (4.4.5.1).
Effect of endotoxin treatment on acetaminophen-induced liver injury. Animals were first treated with 300 mg/kg APAP and 3 h later either with saline (10 ml/kg) or 100 μg/kg endotoxin (ET) (i.p.). Plasma ALT activities (A) and the number of liver accumulating neutrophils (B) were measured at 24 h. Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to untreated controls). #P<0.05 (compared to APAP alone).

C. Representative H&E-stained liver sections (x50) and neutrophil staining (x100) of animals treated for 24 h with 300 mg/kg APAP alone or in combination with endotoxin.
Plasma cytokine/chemokine concentrations and hepatic mRNA levels were measured 24 h after administration of 300 mg/kg acetaminophen (APAP). Some animals were additionally treated with 100 µg/kg endotoxin (ET) at 3 h after APAP. mRNA levels are expressed as the cytokine mRNA-to-β-actin mRNA ratio. The values of untreated controls were set as 1 and the fold change of the APAP-treated animals was calculated. Data represent means ± SE of n = 4-5 animals per group. *P<0.05 (compared to APAP alone)

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<th>Cytokine</th>
<th>Plasma protein levels (pg/ml)</th>
<th>Real-time PCR (fold change)</th>
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<tr>
<td></td>
<td>APAP 24 h</td>
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<tr>
<td>TNF-α</td>
<td>10 ± 1</td>
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<td>IL-1β</td>
<td>17 ± 3</td>
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<td>IL-6</td>
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<td>KC</td>
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4.5 Discussion

The main objective of this investigation was to test in a model with genetic deficiency of neutrophil function if these phagocytes are sufficiently activated and receive the appropriate signals during APAP overdose to further increase liver injury. This approach was necessary because previous attempts to evaluate the role of neutrophils in APAP hepatotoxicity yielded conflicting results due to the use of immunological intervention strategies, i.e. neutropenia-inducing antibodies (Liu et al., 2006; Ishida et al., 2006; Cover et al., 2006). The problem with this approach is that treatment with such an antibody results in the accumulation of antibody-tagged neutrophils in sinusoids. During the 24 h pretreatment time used in experiments that showed a protection against APAP toxicity (Liu et al., 2006; Ishida et al., 2006), Kupffer cells remove these inactivated neutrophils by phagocytosis, which causes the activation of Kupffer cells (Bautista et al., 1994). Cytokines produced by these phagocytes act on hepatocytes and induce a number of genes, e.g., metallothionein, heme oxygenase-1 and others, some of which have been shown to protect against APAP overdose (Jaeschke and Liu, 2007). Although these findings cannot rule out that the protective effect with pretreatment of a neutropenia-inducing antibody was caused by the elimination of neutrophils, there are at least serious concerns that the induction of hepatoprotective genes (preconditioning effect) was at least in part if not completely responsible for the reduced injury after APAP. Our current experiments with CD18-deficient mice, which showed no protection against APAP hepatotoxicity, strongly support the hypothesis that neutrophils are not involved in the injury process in mice. In addition, the fact that endotoxin, which
dramatically exaggerated the inflammatory response, did not affect the overall injury further supports this conclusion.

Members of \( \beta_2 \) integrin family, especially CD11a/CD18 and CD11b/CD18, are essential for firm adhesion of neutrophils to endothelial cells via binding to intercellular adhesion molecule-1 (ICAM-1), for their extravasation and for the adhesion to potential target cells, where a long-lasting, adhesion-dependent oxidant stress can be triggered (Smith, 1993). The neutrophil-dependent oxidant stress is responsible for target cell killing (Entman et al., 1992). In contrast to this general mechanism of neutrophil adhesion, which applies mainly to postcapillary venules in a variety of vascular beds, in the liver, the pathophysiologically most relevant accumulation of neutrophils occurs in sinusoids (Chosay et al., 1997). Because of the low shear stress in sinusoids, selectins, CD18 or ICAM-1 are not necessary for hepatic neutrophil recruitment (Wong et al., 1997; Jaeschke et al., 1996). If the sinusoidal endothelium is intact, blocking these adhesion molecules with an antibody or deficiency of CD18 or ICAM-1 does not prevent neutrophil accumulation in sinusoids (Bautista, 1997; Jaeschke et al., 1996; Essani et al., 1995). An effect on neutrophil numbers in the liver can only be seen after longer time period as documented in CD18- and ICAM-1-deficient animals 3 days after bile duct ligation (Gujral et al., 2003; Gujral et al., 2004). This reflects the reduced inflammatory response due to the reduced tissue injury rather than a direct inhibition of neutrophil adhesion (Gujral et al., 2003; Gujral et al., 2004). However, blocking CD18 inhibits the extravasation of neutrophils and prevents the neutrophil-induced oxidant stress and injury (Jaeschke et al., 1991; Gujral et al., 2003; Gujral et al., 2004; Essani et al., 1995). Even if the endothelium is severely damaged and does not provide a relevant
barrier for neutrophils, e.g. during hepatic ischemia-reperfusion injury, CD18 antibodies are still highly effective due to the attenuation of the adhesion-dependent oxidant stress (Jaeschke et al., 1991; Jaeschke et al., 1993). In all models where neutrophils increase liver injury it was consistently shown that a neutrophil component was evident during the first 24 h (Jaeschke et al., 1990; Jaeschke et al., 1991; Dahm et al., 1991; Dold et al., 2009). Taken together, if neutrophils contributed to the APAP hepatotoxicity, a beneficial effect should have been observed in CD18-deficient mice during the first 24 h after APAP administration. However, despite the mild inflammatory response as judged by cytokine and chemokine formation and the accumulation of neutrophils in the liver, there was no evidence that any of these effects are reduced in CD18-deficient animals. Because neutrophil-induced cell killing in the liver also requires reactive oxygen (Jaeschke, 2006), these data are consistent with previous reports showing that inhibitors of NADPH oxidase (Cover et al., 2006) or genetic deficiency in the function of this enzyme (James et al., 2003) do not protect against APAP-mediated liver injury. The lack of priming for reactive oxygen formation in peripheral and in liver accumulated neutrophils is also in agreement with this finding. Nevertheless, the fact that neutrophil accumulation is moderate, occurs mainly after the onset of severe cell injury (Lawson et al., 2000; Cover et al., 2006) and can be reduced by antibodies against DAMPs, e.g. high mobility group box proteins (Chen et al., 2009; Scaffidi et al., 2002), suggest this is a classical scenario of a sterile inflammatory response.

Because it appears that the modest activation of neutrophils during APAP hepatotoxicity is insufficient to trigger an active attack of these phagocytes, a more aggressive inflammatory response was induced by treatment with endotoxin several hours after
APAP. This schedule was chosen to mimic a realistic scenario of massive cytokine formation and neutrophil activation at the time of APAP-induced injury. Previously, we could show that administration of a low dose of endotoxin after hepatic ischemia-reperfusion drastically increased the inflammatory response including neutrophil activation and the neutrophil-mediated injury (Liu et al., 1994; Liu et al., 1995). In this combination model of two insults, even the delayed treatment with an antibody against CD18 was highly effective in reducing reperfusion injury (Liu et al., 1995). Although the enhanced cytokine and chemokine formation after endotoxin recruited substantially more activated neutrophils into the liver after APAP overdose, no increased injury was detected. These results may be surprising, however, they are consistent with our mechanistic understanding of neutrophil-mediated liver injury (Jaeschke, 2006; Ramaiah and Jaeschke, 2007; Jaeschke and Hasegawa, 2006). Various cytokines (TNF-α, IL-1), chemokines (IL-8, MIP-2, KC), activated complement factors (C5a) and other proinflammatory mediators can individually and in combination activate neutrophils to various degrees and induce their accumulation in sinusoids (Bajt et al., 2001; Chosay et al., 1997; Essani et al., 1995). This process does not require CD18 (Jaeschke et al., 1996), hence CD18-deficient mice did not show less sinusoidal neutrophil accumulation. In addition, primed neutrophils in sinusoids do not randomly cause liver injury (Chosay et al., 1997). For neutrophils to actually participate in killing of larger cells such as hepatocytes, neutrophils need to extravasate and adhere to the target. This process requires CD18 and ICAM-1 (Jaeschke et al., 1991; Gujral et al., 2003; Gujral et al., 2004; Essani et al., 1995). In addition, it requires a distress signal from the parenchyma that indicates to neutrophils located in the sinusoid a cell might be
dying and needs to be removed (Ramaiah and Jaeschke, 2007; Gujral et al., 2004b). 
The attack on the target involves an adherence-dependent oxidant stress (CD18-
dependent) and killing by reactive oxygen including hypochlorous acid (Jaeschke et al.,
1991, Gujral et al., 2003; Gujral et al., 2004a; Gujral et al., 2004b; Jaeschke et al.,
1993). Whether or not such an attack actually enhances liver injury depends on the 
initial insult. If the stress is mild and non-lethal, the stressed cell can trigger a neutrophil 
attack, which will kill the cell and therefore increase the injury. If the stress is already 
lethal by itself, e.g. APAP toxicity in hepatocytes, the accumulation and attack of 
neutrophils will have no impact on the overall injury. Thus, levels of cytokines and other 
pro-inflammatory mediators determine the degree of neutrophil activation and 
accumulation in sinusoids; the distress signal from injured cells determines the degree 
of extravasation and attack on hepatocytes. However, whether this makes a difference 
in the extent of liver damage is determined by the original stress or insult. Based on our 
data we can conclude that the intracellular signaling mechanisms induced by APAP in 
hepatocytes determine whether or not a cell will die and the neutrophilic inflammatory 
response has no impact on the injury. However, this does not exclude a critical role of 
neutrophils and macrophages in the removal of cell debris and preparation for 
regeneration.

In summary, our data documented severe liver injury after APAP overdose, which was 
accompanied by pro-inflammatory mediator formation and moderate hepatic neutrophil 
accumulation. However, animals with a deficiency of CD18, which has been shown to 
be critical for neutrophil-induced liver injury in a number of liver disease models, were 
not protected against APAP hepatotoxicity. In addition, stimulated cytokine formation
and enhanced hepatic recruitment of activated neutrophils did not enhance APAP-induced liver injury. Together these data do not support the hypothesis that neutrophils contribute to APAP hepatotoxicity or that a neutrophil-mediated injury phase could be provoked by a second, pro-inflammatory hit. Thus, APAP-induced liver injury in mice is dominated by intracellular mechanisms of cell death rather than by neutrophilic inflammation.
Chapter 5

Role of neutrophils and NADPH oxidase during APAP injury resolution
5.1 Abstract

Acetaminophen (APAP) is a widely used antipyretic and analgesic drug (Tylenol®); overdose is the most frequent cause of acute liver failure in the US. Following APAP-overdose there is a robust inflammatory response triggered by the release of cellular contents from necrotic hepatocytes into systemic circulation. This inflammatory response initiates the recruitment of neutrophils into the liver, followed by monocyte infiltration. The primary mechanism by which phagocytes can exacerbate tissue injury is by the production and release of reactive oxygen species (ROS) generated by NADPH oxidase. It has been demonstrated that neutrophils and resident liver macrophages do not contribute to APAP-induced liver injury but their role, and the role of NADPH oxidase, in injury resolution is unclear. To investigate this, C57BL/6 mice were subjected to APAP-overdose and sacrificed at various times (24-72 h) during liver regeneration. Neutrophils in the peripheral blood of these mice showed increasing activation status (CD11b expression and ROS priming) during and after the peak of injury but returned to baseline levels prior to complete injury resolution. Hepatic sequestered neutrophils showed an increased and sustained CD11b expression, however at no time had enhanced ROS priming. To confirm these findings, gp91phox-/- (NADPH oxidase defective) mice were subjected to APAP-overdose and displayed no alteration in injury resolution indicating phagocytic ROS is not essential for injury resolution. Peripheral blood from human APAP-overdose patients also showed increased neutrophil activation status after the peak of liver injury. These findings show similar neutrophil activation in peripheral blood of humans and mice which may be a critical event for host defense or injury resolution following APAP-overdose.
5.2 Introduction

Acetaminophen (APAP) overdose is the leading cause of acute liver failure in the US, and can result in severe liver injury and potentially death. The hepatotoxicity of APAP is initiated by metabolic conversion of APAP to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). This reactive metabolite results in glutathione (GSH) depletion and eventually causes adduction of cellular proteins which is the initiating event in toxicity (Jaeschke and Bajt, 2006). Downstream of protein binding is mitochondrial dysfunction, increased oxidant stress, mitochondrial permeability transition pore (MPT) opening, DNA damage by endonucleases, and hepatocellular necrosis. The massive cellular necrosis results in the release of damage associated molecular patterns (DAMPs) into systemic circulation; these DAMPs (including HMGB-1, nuclear DNA, and numerous others) then triggers a sterile inflammatory response resulting in increase cytokine/chemokine formation and recruitment of innate immune cells into the liver. In the mouse model neutrophils are initially recruited to the site of injury followed by monocytes/macrophages. Previously it was believed that that this inflammatory response and neutrophil influx exacerbated the injury (Liu et al., 2006), but extensive evidence has been presented to the contrary (Cover et al., 2006; Lawson et al., 2000; Jaeschke and Liu, 2007; James et al. 2003; Ju et al., 2002; Chapters 2-4).

Neutrophils are a first-line defense from pathogens and are essential for microbial phagocytosis and killing. During APAP overdose this innate immune function is critical for host defense especially if the patient progresses toward fulminant hepatic failure (FHF). It was reported that bacteremia occurs in up to one third of FHF patients and bacterial infection may be the cause of death for up to 20% of FHF patients (Wyke,
1987). This link between progression of liver injury toward liver failure and innate immune function is of great interest; potentially this loss of host defense could be mediated by impaired synthesis of complement components, impaired phagocytosis of gut-derived bacteria by Kupffer cells, or multiple immune deficits which may or may not include neutrophils.

Characterization of resident and infiltrating macrophages/monocytes has been performed in the mouse model of APAP overdose following the peak of injury, and it was demonstrated that the infiltrating monocyte/macrophage population is inherently anti-inflammatory and needed for injury resolution (Holt et al. 2008). No characterization of neutrophils following the injury phase of APAP has been performed however, and for this reason the role of peripheral blood and hepatic-sequestered neutrophils beyond the injury phase of APAP has remained unclear. In this study we determined the progression of neutrophil activation and hepatic accumulation in the mouse model. In conjunction, we investigate if NADPH oxidase (phagocyte ROS) is critical for injury resolution following APAP overdose; it has already been shown that NADPH oxidase does not participate in injury (James et al., 2003). Additionally we evaluated the activation status of human granulocytes in several APAP overdose patients and compared the activation status to what we observed in the better mechanistically understood mouse model.
5.3 Materials and Methods

5.3.1

Animals. Male gp91phox-deficient (aka: Nox-2-/-, Cybb-/-, Phox-/-) mice (B6.129S-Cybbtm1Din/J; Stock number: 002365) and C57BL/6J control mice (Stock number: 000664) with an average weight of 18 to 20 g were purchased from Jackson Laboratory (Bar Harbor, Maine). All animals were housed in an environmentally controlled room with 12 h light/dark cycle and allowed free access to food (# 8604 Teklad Rodent, Harlan, Indianapolis, IN) and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Kansas Medical Center and followed the criteria of the National Research Council for the care and use of laboratory animals in research.

5.3.2

Experimental design. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. Mice were intraperitoneally (i.p.) injected with 300 mg/kg APAP (dissolved in warm saline) or saline vehicle after overnight fasting. The animals were sacrificed 6, 12, 24, 48 or 72 h after APAP treatment, blood was withdrawn from the vena cava into a heparinized syringe for measurement of alanine aminotransferase (ALT) activities (Pointe Scientific, Canton, MI) and flow cytometric analysis. The liver was removed and rinsed in saline; liver sections were fixed in 10% phosphate buffered formalin for histological analyses. The remaining liver was used for isolation of non-parenchymal cells (as described below). Additionally, as a positive control, some mice were treated (i.p.) with 100 μg/kg Salmonella abortus equi endotoxin (ET) for 90 min.
5.3.3

Patient selection and consent. Patients admitted to the University of Kansas Hospital in Kansas City, Kansas following APAP overdose. The study protocol and design were approved by the institutional review board (IRB). Acute and chronic APAP overdose patients were from the emergency department or admitted to the intensive care unit with evidence of overdose. The diagnosis was made by a physician on site and all study subjects were required to sign a consent form. The inclusion criteria were two or more of the following: (1) patient-reported APAP overdose, (2) high serum APAP levels, and (3) abnormal liver function tests (based on ALT, AST, PT, bilirubin). Patients were excluded if there was reasonable evidence for liver injury due to another cause (viral hepatitis, ischemic liver, etc.). All overdose patients received standard of care NAC treatment.

5.3.4

Histology. Formalin-fixed tissue samples were embedded in paraffin and 5 µm sections were cut. Sections were stained with hematoxylin and eosin (H&E) for blinded evaluation of the areas of necrosis by the pathologist. The percent of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the entire cross sectional area. Additional liver sections were stained for neutrophils with anti-mouse neutrophil allotypic marker antibody (AbD Serotec, Raleigh, NC) as described in detail (Williams et al., 2010a). Positively stained cells consistent with neutrophil morphology were quantified in randomly selected high power fields (HPF, x400). Additional sections were stained for proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology, Santa Cruz, CA) as described in detail (Chosay et al., 1998).
Positively stained hepatocytes were quantified in randomly selected high power fields (HPF, x400). Additionally, some sections were stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Roche, Indianapolis, IN) as described in detail (Lawson et al., 1999).

5.3.5

Isolation of hepatic non-parenchymal cells. The procedure was adapted from a method described by Watanabe et al. (34). Under isoflurane anesthesia mice were exsanguinated from the caudal vena cava into heparinized tubes and the blood was placed on ice. The liver was immediately excised, placed in ice-cold PBS and minced with scissors. The tissue was then pressed through a 200-gauge stainless steel mesh into a 50mL conical tube. The cell suspension was centrifuged at 50 x g for 2 minutes to remove hepatocytes and large debris. The supernatant containing non-parenchymal cells was then centrifuged at 350 x g for 5 minutes and cells were passed through a 40 μm nylon screen and washed twice in a 15 mL conical bottom tube. Viable, nucleated cells were counted by trypan blue exclusion and brought to a uniform cell density.

5.3.6

Flow Cytometric Analysis of Neutrophil Function.

5.3.6.1

Neutrophil CD11b staining (Bajt et al., 2001; Daley et al., 2008). 5μg Fc receptor (FcR) blocking antibody (BioLegend, San Diego, CA) diluted in 100μL 0.1% BSA in PBS was added to 100μL non-parenchymal cell suspension for 20 minutes on ice (mouse only). To 50μL heparinized whole blood (mouse or human) or 200μL FcR-blocked hepatic non-parenchymal cells (mouse only), saturating concentrations of PE-Cy5-labeled-anti-
Gr-1 [mouse only (BioLegend)] and PE-labeled-anti-CD11b [human or mouse (BioLegend)] diluted in 0.1% BSA in PBS were added. Tubes were incubated in the dark, on ice for 30 minutes. After washing with 0.1% BSA, red blood cells were lysed using RBC lysing solution (0.155 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Pellets were washed three times with 0.1% BSA then fixed with 2.5% buffered formalin. Samples were measured on the FACSCalibur (BD, Franklin Lakes, NJ). Mouse neutrophils were gated on the Gr-1<sup>high</sup> cells, which are the Ly6G-positive population and identified as neutrophils by cell morphology (Daley et al., 2008). Other leukocytes are either Gr-1<sup>intermediate</sup> (mainly monocytes and eosinophils) or Gr-1<sup>negative</sup> (monocytes and lymphocytes) (Daley et al., 2008). Human granulocytes were gated to exclude monocytes and lymphocytes by forward- and side-scatter characteristics. The data were analyzed using the BD FACSDiva 6.0 software.

5.3.6.2

**Reactive Oxygen Production** ( Emmendorffer et al., 1990; Smith and Weidemann, 1993).

To 50 µL heparinized whole blood (mouse or human) or 100µL hepatic non-parenchymal cells (mouse only), 2 µM phorbol 12-myristate 13-acetate (PMA) or saline was added and incubated for 10 minutes at 37°C. 4 µM dihydrorhodamine-123 was added and incubated for 10 minutes at 37°C. Ice-cold 0.1% BSA in PBS was added and pellets were washed. The non-parenchymal cell suspension was blocked with FcR blocking antibody (BioLegend) as previously described and cells were stained with saturating concentrations of PE-Cy5-labeled-anti-Gr-1 (BioLegend) diluted in 0.1% BSA (mouse only). Tubes were incubated in the dark, on ice for 30 minutes. After washing with 0.1% BSA, red blood cells were lysed using RBC lysing solution. Pellets were
washed three times with 0.1% BSA then fixed with 2.5% buffered formalin. Samples were measured on the FACSCalibur (BD) and the data were analyzed using the BD FACSDiva 6.0 software.

5.3.6.3

*Neutrophil Phagocytosis* (Sahlin et al., 1983; Bassoe et al., 1983) FITC-labeled *E. coli* particles (Life Technologies, Grand Island, NY) were opsonized in heat-inactivated pooled-normal human serum per manufacturer’s recommendation. To 50 µL aliquots of heparinized whole blood, 20 µL of diluted opsonized-FITC- *E. coli* were added and incubated for 5, 10, 15 and 20 min at 37ºC. Aliquots were then placed on ice and 0.4% trypan blue added to quench soluble and surface bound FITC-*E. coli*. Samples were measured on the FACSCalibur (BD) and the data were analyzed using the BD FACSDiva 6.0 software.

5.3.7

*Statistics.* All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way ANOVA or, where appropriate, by two-way ANOVA, followed by a *post hoc* Bonferroni test. If the data were not normally distributed, we used the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn’s Multiple Comparisons Test. *P* < 0.05 was considered significant.
5.4 Results

5.4.1 Time course of APAP-induced hepatic injury

It has been demonstrated that neutrophils do not contribute to APAP-induced liver injury with a multitude of genetic and pharmacologic interventions (reviewed in detail: Jaeschke et al., 2011). We have also shown that 6h post APAP neutrophils in the peripheral blood and liver are not primed or activated (Chapter 4); additionally, increasing the hepatic accumulation of PMNs (with recombinant IL-1β or LPS) does not enhance injury (Chapter 2 and 4, respectively). It has been demonstrated in many studies that neutrophils are recruited into the liver following APAP overdose, however the activation status of these leukocytes has never been evaluated; therefore a time course of hepatic injury and resolution was performed. In C57BL/6 mice, following an i.p. dose of APAP (300mg/kg), there is substantial hepatic injury at 6 h and continues to increase until 24 h; at or around 24 h the injury resolution phase begins. This is demonstrated by increasing plasma ALT until 24 h and rapidly decreasing plasma ALT beyond this time (5.4.1.1A). Confirming the ALT data is hepatic necrosis scoring (5.4.1.1B) determined from H&E stained liver sections (5.4.1.1C). The differences in plasma ALT and the total area of necrosis at 48 h and 72 h show no active injury but rather the resolution of injury. Confirming this is TUNEL staining from representative mice (5.4.1.1D). At 6 h, 12 h and 24 h increasing DNA damage can be seen. The area and intensity of TUNEL staining at 48 h is much less, and by 72 h fragmented DNA is almost undetectable in the liver despite the presence of necrotic lesions.
**5.4.1.1**

*Hepatic injury and resolution following APAP overdose: C57BL/6 mice were treated with 300 mg/kg APAP (i.p.) for 6, 12, 24, 48 or 72 h or with 100 μg/kg LPS for 90 min. Heparinized blood was used for the determination of plasma ALT (A). Area of necrosis was quantified in blinded fashion by the pathologist from H&E stained liver sections (B). Representative liver sections (50x magnification) are shown (C). TUNEL staining (100x magnification) shows increasing DNA damage within necrotic areas until 24 h and resolution of fragmented DNA at 48 and 72 h (D). *P<0.05 compared to 0 h control. (n = 4-7 mice per group)*
5.4.2 Hepatic neutrophil recruitment and hepatocyte regeneration

It has been shown that the recruitment of neutrophils into the liver trails behind the
progressing liver injury (Lawson et al., 2000). If neutrophils were causing injury it would
be logical to see the maximal number of neutrophils prior to the peak of injury, however
the peak of neutrophil accumulation occurs at the peak of injury as resolution of injury
begins (5.4.2.1A). The hepatic accumulation of neutrophils can be seen at 6 h with
most neutrophils located at the periphery of injury and in sinusoids of healthy tissue
(5.4.2.1B). At 12 h the total number of hepatic neutrophils continues to increase
however the neutrophil distribution remains similar. By 24 h the neutrophil count peaks
with most neutrophils located within the necrotic lesions. Forty-eight hours post
overdose, the number of neutrophils decrease dramatically and continues to decline by
72 h, however these neutrophils are located exclusively within the injured tissue. As
stated previously, the injury resolution (as determined by PCNA positive hepatocytes
which identifies cells in S phase) begins around 24 h post-overdose in the mice
(5.4.2.1C,D). The peak of PCNA positive hepatocytes was observed at 48 h with many
proliferating hepatocytes still observed at 72 h. Additionally, this pattern was confirmed
by western blotting for PCNA of total liver homogenate (data not shown). The location
of PCNA positive cells occurs mostly at the edge of necrosis and the recruited immune
cells reside within this area.
5.4.2.1

Hepatic neutrophil accumulation and PCNA-positive hepatocytes following APAP overdose: Following APAP overdose hepatic neutrophils were identified by immunohistochemistry (IHC) and morphological features. Neutrophil numbers increased during the injury phase until peaking at 24 h and decreased as injury is resolved (A). Distribution of hepatic neutrophils changed throughout the time course (200x magnification). Initially neutrophils could be observed in healthy and injured areas at 6 h and 12 h. At 24 h, 48 h and 72 h neutrophils could be seen exclusively within the necrotic areas (B). Hepatocyte proliferation was quantified by counting...
PCNA-positive cells and could not be observed prior to 24 h and peaked at 48 h (C). The distribution of PCNA-positive hepatocytes remained similar throughout the time course as shown by IHC (200x magnification) (D). *P<0.05 compared to 0 h control. (n = 4-7 mice per group)
5.4.3 Hepatic and peripheral blood neutrophil priming and activation

Phagocytic cells recruited into injured tissue can function in several roles: (1) phagocytose and remove cellular debris (2) produce factors that stimulate cell proliferation and (3) provide host defense from pathogens. We have shown that the hepatic neutrophil counts correlate with injury resolution, so it was logical to determine if these neutrophils are activated. In determining activation status we compared neutrophil reactive oxygen (ROS) priming and CD11b surface expression in both peripheral blood and hepatic neutrophils. As an in vivo positive control, neutrophil activation from APAP-treated mice was compared to LPS-treated mice. Following APAP overdose, peripheral blood neutrophils had increased CD11b expression at 12 h and 24 h (5.4.3.1A), which correlates with the highest levels of most circulating cytokines, chemokines and DAMPs following APAP overdose (Antoine et al., 2009; Chapters 3 & 4). Correlating with increased CD11b on peripheral neutrophils, hepatic neutrophils also demonstrated increased CD11b expression, but this expression persisted much longer (5.4.3.1B). This sustained hepatic neutrophil CD11b expression could have been caused by the local environment enhancing CD11b expression.

Despite enhanced neutrophil CD11b expression in the blood and liver the magnitude of CD11b expression was much less than that observed following LPS treatment. Blood neutrophil ROS priming was elevated at 24 h and remained elevated at 48 h, but was not increased over control during the injury phase at 6 h and 12 h (5.4.3.1C). This ROS priming in the blood could be critical for host defense during liver function impairment. Interestingly, despite transient ROS priming in the blood no increase over control could
be observed in hepatic neutrophil ROS priming at any time during injury or liver repair (5.4.3.1D).
Activation of peripheral blood and hepatic neutrophils following APAP overdose

The activation status of neutrophils (Gr-1<sup>high</sup> population) was determined by surface CD11b expression and PMA-induced reactive oxygen production. Overlaying the neutrophil activation status is plasma ALT to show the correlation with injury. Peripheral blood neutrophils showed increased CD11b expression at only 12 h and 24 h (A). A similar time course of activation could also be observed from liver neutrophils, however the neutrophils in the liver past the peak of injury also showed enhanced CD11b expression (B). In peripheral blood neutrophils demonstrate ROS priming at 24 h and 48 h (C). Interestingly, no ROS priming was ever seen in liver neutrophils following APAP overdose (D). *P<0.05 compared to 0 h control (for activation status only, ALT shown in Fig. 1) (n = 4-7 mice per group)
5.4.4 Liver regeneration in NADPH oxidase-deficient mice following APAP overdose

Since C57BL/6 mice show no hepatic neutrophil ROS priming throughout the time course we used NADPH oxidase-deficient (gp91-/-) mice to confirm that phagocyte ROS was not critical for liver repair following APAP overdose. In addition to neutrophils, monocytes and macrophages also lack respiratory burst in gp91-/- mice. James et al. (2003) demonstrated that gp91-/- mice have no modulation of injury following APAP overdose but did not evaluate recovery from APAP-induced injury. To evaluate this gp91-/- and C57BL/6 control mice were treated with APAP and sacrificed at 24 h, 48 h and 72 h. Not surprisingly there was no difference in the peak of injury at 24 h between gp91-/- and C57BL/6 mice as measured by plasma ALT (5.4.4.1A) and necrosis scoring (5.4.4.1B) as determined by H&E staining (5.4.4.1C). Additionally, at 48 h and 72 h the decrease in plasma ALT and area of necrosis was not different between genotypes. These findings demonstrate that despite the lack of functional NADPH oxidase the progression and resolution of injury is unaltered indicating removal of cellular debris by phagocytes does not require oxidative burst.
5.4.4.1

Injury in gp91-/- (Phox-/-) and C57BL/6 control mice following APAP: Mice were treated with 300 mg/kg APAP (i.p.) for 24, 48 or 72 h. Heparinized blood was used for the determination of plasma ALT (A). Area of necrosis was quantified in blinded fashion by the pathologist from H&E stained liver sections (B). Representative liver sections (50x magnification) are shown (C). *P<0.05 compared to 0 h genotype control. (n = 3-4 mice per group)
5.4.5 Hepatic neutrophil recruitment and hepatocyte regeneration in gp91-/- mice

It has been shown that inflammation and innate cell recruitment is altered in gp91-/- mice. In peritonitis (Pollock et al., 1995; Rajakariar et al., 2009), bronchial inflammation (Morgenstern et al., 1997) and arthritis (van de Loo et al., 2003) gp91-/- mouse models there is increased and sustained innate immune cell infiltration. Following APAP overdose in gp91-/- mice a marked increase in hepatic neutrophil recruitment could be observed versus C57BL/6 mice (5.4.5.1A). This increase was most dramatic 24 h post-overdose but still significantly elevated at 48 h; by 72 h, the difference in hepatic neutrophil counts between groups was not significantly different (5.4.5.1A). The distribution of neutrophils was not altered between groups however. In C57BL/6 mice at 24 h most neutrophils are located within the necrotic lesions and the same was observed in gp91-/- mice (5.4.5.1C). By 48 h and 72 h most neutrophils are even more centrally located within the necrotic tissue in both genotypes despite a substantial reduction in total counts. Interestingly, despite this difference in inflammation the proliferation of hepatocytes was unaffected. PCNA positive hepatocytes at each time were not different between genotypes (5.4.5.1B) and the pattern of positive cells was not different, with most PCNA-positive cells being observed adjacent to the injury (data not shown).
Neutrophil recruitment and hepatocytes proliferation in gp91-/- mice: As shown previously, neutrophil quantification was determined by IHC. Neutrophil recruitment in gp91-/- mice was markedly elevated over C57BL/6 controls at 24 h and 48 h (A). The distribution of neutrophils was not different between genotypes, with most neutrophils accumulating with the areas of necrosis (B). Quantification of PCNA-positive hepatocytes by IHC was not different between genotypes with the peak of PCNA-positive hepatocytes being observed at 48 h post APAP (C). *P<0.05 compared to 0 h genotype control. #P<0.05 compared to time matched control. (n = 3-4 mice per group)
5.4.6 Human granulocyte activation following APAP overdose

The neutrophil function in human overdose patients can only be evaluated in peripheral blood because there is no therapeutic benefit of liver biopsy especially in light of frequent coagulopathy in these patients. Therefore the deleterious or beneficial role of neutrophils following APAP overdose must be determined from circulating neutrophils. To determine the activation status of neutrophils several priming or activation parameters were evaluated serially from freshly drawn heparinized whole blood, however limitations in sample collection and analysis did not permit us to evaluate these parameters daily.

Three representative patients that fully recovered from APAP-overdose are presented in Figure 6. Activation status was determined by granulocyte CD11b surface expression, phagocytic efficiency of FITC-labeled opsonized *E. coli* particles, and PMA- and opsonized *E. coli*-induced ROS production as determined by DHR123 to R123 conversion. Overlaying these activation parameters are plasma ALT levels indicating the progression and resolution of liver injury in these patients. Two patients (5.4.6.1A,B) were admitted to the hospital prior to the peak of liver injury and one patient arrived at or after the peak of liver injury (5.4.6.1C). The activation status of neutrophils (gating on the forward and side scatter properties of granulocytes) was determined by measuring the mean fluorescent intensity (MFI) of all neutrophils and normalized to the MFI of the first day sampled; therefore the data is shown as a fold-increase over the first day measured. The patients represented in 5.4.6.1A and 5.4.6.1C had low neutrophil function parameters at or near the peak of injury that increased as the injury was resolving. The patient represented in 5.4.6.1B had increasing peripheral blood
neutrophil activation as the injury was increasing, however this activation status continued to rise and remained elevated during resolution until he was discharged from the hospital nine days post-admission.

From the representative patients shown (5.4.6.1) the trend is clear that the activation of neutrophils continues well after active liver injury has subsided and in most cases begins after the injury phase. In total, ten patients with liver injury (ALT: >800 U/L) that ultimately fully recovered after APAP-overdose have been evaluated and are presented (5.4.6.2). At or near the peak of liver injury (highest measured plasma ALT), plasma ALT and neutrophil function tests 72 to 96 hours after the peak of injury (as determined by sample availability) were compared. These declining ALT values are shown (5.4.6.2A). Consistent with the representative patients no correlation between declining injury and CD11b expression was observed (5.4.6.2B). Reactive oxygen priming that was induced by PMA (5.4.6.2C) and opsonized E. coli (5.4.6.2D) was significantly increased following the peak of injury. The phagocytic rate of neutrophils showed a trend toward increased activation but with this sample size was not significantly increased.

These preliminary data demonstrate an increasing neutrophil function in patients that recover from APAP overdose. Some patients show increasing PMN activation prior to injury however most patients evaluated thus far show activation following the peak of injury. These findings show the possibility that neutrophils could aid in injury resolution via the removal of cellular debris or enhanced host defense functions.
Neutrophil function in representative human APAP overdose patients: Three representative patients are shown. Progression and resolution of liver injury is presented as plasma ALT. Gating on the granulocyte population (predominantly neutrophils) the activation status was determined by CD11b expression, rate of phagocytosis of FITC-labeled \textit{E. coli}, and PMA- and \textit{E. coli}-induced ROS production. (A) Patient 1 was admitted to the hospital prior to the peak of ALT and neutrophil function tests were performed on days 2, 3, 4 and 8. The patient was discharged on day 8. (B) Patient 2 was admitted prior the peak of injury and neutrophil function tests were measured on days 1, 2, 4 and 9. The patient was discharged on day 9. (C) Patient 3 was admitted at or shortly after the peak of liver injury and neutrophil function tests were measured on days 2, 6, 7 and 12. The patient was discharged on day 12.
Comparison of neutrophil function in human APAP overdose patients at the peak of injury and during liver recovery: Ten patients with moderate to severe liver injury that fully recovered from APAP-overdose were evaluated for neutrophil activation at or near the peak of liver injury and then again 3-4 days later (depending on sample availability). All flow cytometric data are presented as mean fluorescent intensity (MFI) of the total granulocyte population. (A) Three to four days post overdose all patient plasma ALT values declined. (B) Peripheral blood neutrophil CD11b expression did not change.
during this time. (C) PMA-induced reactive oxygen priming was elevated post-injury.

(D) Similarly, *E. coli*-induced reactive oxygen priming was elevated post-injury as well.

(E) A trend toward enhanced phagocytosis of FITC-labeled opsonized *E. coli* was seen post-injury. Post-injury data shown were measured at 72 h (n = 6) and 96 h (n = 4).

*P*<0.05 as determined by paired-Students T-test.
5.5 Discussion

It is well established in the mouse model of APAP-overdose that innate immune cells, in particular neutrophils, do not contribute to the progression of injury. Due to the many functions of neutrophils, including host defense from invading pathogens and removal of cellular debris, it was important to characterize the neutrophil activation status following APAP overdose. NADPH oxidase-mediated ROS production is the primary mechanism used by neutrophils to cause cellular injury during endotoxemia (Gujral et al., 2004) and ischemia-reperfusion injury (Hasegawa et al., 2005). Despite numerous studies investigating the role of NADPH oxidase during injury, it has not been previously demonstrated if NADPH oxidase is or is not critical for resolution of acute liver injury.

5.5.1 Role of gp91 during inflammation

NADPH oxidase-deficient mice in various models of injury show prolonged inflammation and impaired injury resolution. It has been shown that sterilized fungal preparations administered to gp91-/- mice were capable of causing a chronic bronchopneumonia with profound neutrophil infiltrates into the lung and prolonged elevations in inflammatory cytokines (Morgenstern et al., 1997). In other models of sterile inflammation, peritonitis was induced with thioglycollate (Pollack et al., 1995) or zymosan A (Rajakariar et al., 2009); in these models gp91-/- mice had increased numbers of neutrophils (two- to three-fold increase) and these neutrophils remained in the peritoneal exudate longer than control mice. Zymosan was also able to induce a more robust and prolonged inflammation marked with neutrophil infiltrates in a phox-deficient mouse arthritis model (van de Loo et al., 2003). These increases in neutrophils infiltration was not due to
enhanced peripheral blood counts or altered chemotaxis (Pollock et al., 1995). It was hypothesized that chronic inflammatory response in gp91-/- mice could be due to inefficient debris clearance or the inability to inactivate chemotactic signals (Pollock et al., 1995). Clearly from the neutrophil counts following APAP overdose there is an enhanced inflammatory response in gp91-/- mice but no effect on injury or injury resolution could be observed. Additionally the disappearance of neutrophils occurred quickly with no difference from C57BL/6 mice by 72 h post-APAP.

5.5.2 Innate immune function during APAP overdose

It is well known that during acetaminophen overdose liver function is greatly impaired resulting in decreased production of complement components including C3, C4 and C5 (Wyke et al. 1980; 1982) and an increased susceptibility to infection (Wyke, 1987). This decrease in innate immune defense could prompt a compensatory response of enhanced neutrophil activation. It was reported that neutrophil ROS production was reduced in APAP overdose patients that have progressed to acute liver failure (Clapperton et al., 1997) however the neutrophil activation was only evaluated at one time point and did not evaluate if patients that recover from severe liver injury have modulation of neutrophil function. Subsequent studies evaluated the efficacy of recombinant G-CSF administration to APAP-induced ALF patients (Rolando et al., 2000a; 2000b). In these studies neutrophil function was restored as determined by enhanced phagocytosis and bacterial killing both in vivo and in vitro. Following standard of care, all APAP-overdose patients were receiving N-acetylcysteine (NAC). It has been reported that NAC alters respiratory burst and phagocytosis in neutrophils,
however these studies were performed in critically ill patients with sepsis or systemic inflammatory response syndrome (Heller et al., 2001). As NAC is the standard of care for APAP overdose patients this not a variable that can be eliminated in the human studies.

APAP overdose patients that progress to fulminant hepatic failure have very severe (<20% normal activity) deficiencies of serum complement factors resulting in severe defects of in vitro test with phagocytosis as an endpoint (Wyke et al. 1980). Additionally, it has been established that during acute liver failure the ability of neutrophils to produce ROS was reduced and partially attributable to impaired opsonization (Clapperton et al. 1997). Our current study did not evaluate opsonization in the study design. Opsinization of *E. coli* and FITC-labeled *E. coli* was done with pooled normal human serum to only evaluate neutrophil priming and not complicate results with potential impairment of patient complement components.

5.5.3 NADPH oxidase is not needed for injury resolution

From the data generated it is clear that NADPH oxidase mediated ROS production is not a critical function in removal of cellular debris. This was demonstrated by a lack of ROS priming observed in hepatic-sequestered neutrophils and confirmed in NADPH oxidase-defective mice. Despite this lack of ROS priming in the liver, priming was observed in peripheral blood; it is not clear if this priming was compensatory due to impaired host defense or a secondary effect from circulating cytokines or DAMPs. In human overdose patients this enhanced neutrophil function could be critical to prevent infection or simply function as a biomarker of positive patient outcome. Clearly there
appears to be link between peripheral neutrophil activation and favorable outcome, however more patients need to be evaluated to draw more substantial conclusions.
6 Conclusions and future directions

Acetaminophen overdose can be divided into various stages. The injury phase is composed of two parts, the initiation and propagation phases (discussed in detail in Chapter 1). As the injury begins to peak, the regenerative processes begin to repair the damaged tissue. This involves replication of lost hepatocytes, removal of damaged cells and extracellular debris and the reformation of normal liver architecture. From our data and the data of multiple other groups, the lost hepatocytes mass is replenished by surviving adjacent hepatocytes. Ultimately, these processes repair the liver completely without any adverse long term effects. The mechanisms and orchestration of all these processes (especially tissue repair) during and after APAP overdose have yet to be fully explained.

This dissertation shows that IL-1β is produced during APAP overdose in a caspase dependent manner which can be inhibited *in vivo* by the use of pan-caspase inhibitor. Preventing the maturation of IL-1β protein by inhibiting the Nalp3 inflammasome and caspase-1 does not alter the APAP-induced injury nor does it alter hepatic neutrophil recruitment. Additionally, IL-1 receptor deficient mice are not protected from APAP induced injury. The use of supra-physiological doses of recombinant IL-1β or large doses of endotoxin can greatly enhance the number of neutrophils recruited into the liver however the injury is not altered by this influx. This further confirms the benign role of neutrophils in the APAP injury process. Quantitative assessment of neutrophil activation was also performed during APAP overdose and during endotoxemia as a positive control. During the early injury phase neutrophils are not primed or activated in the blood or in the liver. As the injury phase ends and the regeneration phase is more
apparent neutrophils show enhanced activation and priming. This activation is then sustained for several days as the injury is repaired. Similar activation and priming can be seen in human APAP overdose patients. It is not clear if the neutrophils participate in the injury resolution or if they provide enhanced host defense during the period of impaired hepatic function.

The mechanism of APAP hepatotoxicity is dominated by intracellular events including the formation of a reactive metabolite, GSH depletion, and protein adduct formation, which initiates a mitochondrial oxidant stress and peroxynitrite formation. Ultimately, this oxidant stress and peroxynitrite are responsible for the MPT, nuclear DNA fragmentation and necrotic cell death. The subsequent release of DAMPs results in activation of Kupffer cells with cytokine and chemokine formation and recruitment of neutrophils and monocytes into the liver. Despite the substantial sterile inflammatory response after the initial cell death, there is no convincing experimental evidence to support the hypothesis that Kupffer cells, neutrophils or monocytes directly cause cell injury by producing cytotoxic mediators. In contrast, pro-and anti-inflammatory cytokines produced mainly by activated Kupffer cells (M1) modulate intracellular mechanisms of cell death by regulating gene expression of iNOS, heat shock proteins, heme oxygenase and others. In addition, monocyte-derived macrophages (M2) and potentially neutrophils are instrumental in removing necrotic cell debris and promoting hepatocyte proliferation ultimately resulting in tissue repair and resolution of the inflammatory response. Thus, in contrast to other acute injury models the innate immune response after APAP-induced liver cell injury is mainly beneficial and is well orchestrated to assist in the repair of the tissue damage.
Future additional work needs to be done to more completely understand the liver repair mechanisms after APAP overdose. If treated early enough the survival rate is exceptionally high and it is possible to completely prevent injury even after a massive overdose. It seems unlikely that a better, more cost effective antidote will be discovered to replace NAC. NAC is very efficient at scavenging the reactive metabolite and subsequent oxidative stress. Most likely improvement of patient outcome will occur by promoting liver growth and repair.

The outcome after liver injury cannot be efficiently predicted by the measurement of serum transaminases. Patients with very high AST/ALT will recover completely while a patient with a similar overdose will have much lower transaminases but ultimately die. This points to two possible reasons: 1) the patient in the first case, despite high ALT, still has a larger proportion of functioning liver mass and is therefore able to recover, or 2) the second patient is unable to regenerate functional hepatic mass and ultimate dies because of it. If most functional hepatocytes are lost the only hope for these patients is to replace dead hepatocytes.

Currently there are multiple liver assist devices (LAD) being used clinically. There are two main types of these devices. The first and most commonly used are artificial liver support systems that utilize dialysis membranes and albumin or activated charcoal to remove toxins. The second type of LAD uses human hepatoblastoma cells or porcine hepatocytes in a matrix exposed to patient blood or plasma which attempts to simulate and replace liver function lost during ALF. In theory this will allow for the not only the removal of toxins but also replace the liver’s synthetic and metabolic functions. These bioartifical liver systems are still in their infancy and time will tell if these can improve
patient outcome. In theory these devices could decrease the burden of the remaining liver mass to help promote liver repair.

Currently in the clinic the search for better predictors of patient outcome is a primary goal. Recently the use of serum alpha-fetoprotein (Schmidt and Dalhoff, 2005) showed promise as a good predictor of patient outcome. Our preliminary data in patients shows enhanced neutrophil activation in surviving patients. Potentially this could serve as a functional biomarker of patient survival, but more data needs to be collected to confirm this finding. It has been known for decades that during fulminant hepatic failure innate immune function, and neutrophil function in particular, is impaired (Wyke et al., 1980; Clapperton et al., 1997). The use of G-CSF in patients showed a restoration of neutrophil function however these studies were of insufficient size to determine patient outcome (Rolando et al., 2000a,b). It is still possible that this treatment could have beneficial outcomes in larger studies.

Repair is the most important factor in ultimate survival from APAP-induced ALF. During acute injury hepatocyte mass is replaced by the replication of differentiated hepatocytes and not from liver progenitor cells (Malato et al., 2011). Determination of how to stimulate the proliferation of remaining hepatocytes could be very useful clinically. It has been shown in animal models that the cytokines produced during APAP overdose can modulate injury, it is not hard to believe that cytokines could also modulate tissue repair following APAP overdose. Orchestration of these processes will be complex, so the use of animal models is essential to determine how these cytokines can modulate various pro-survival/pro-proliferative pathways within the hepatocytes.
It is still not clear how neutrophils could be participating in the injury resolution after APAP-overdose. This could be occurring through various mechanisms: 1) the most straightforward mechanism would involve phagocytosis and cleanup of damaged tissue, 2) enhanced immune surveillance and prevention of infection in the periphery, or 3) production of pro-regenerative factors that could act directly on hepatocytes or other hepatic cells (6.1).

To better understand these mechanisms more experiments need to be performed in the mouse model. This could be accomplished by depleting mice of neutrophils and observing hepatocytes proliferation and liver regeneration. This depletion could be achieved by neutropenia-inducing antibody or genetic elimination of G-CSF or the G-CSF receptor. If neutrophils are essential for removal of damaged tissue these mice should have delayed or prevented liver recovery. If defective regeneration was observed it would also be useful to compare the cytokine milieu produced in these mice.

We have shown that CD18 is not essential for neutrophil recruitment into the liver, however hepatic sequestered neutrophils show enhanced CD11b expression. Using CD18-deficient mice we could determine if this is essential for cleanup after APAP overdose. Without CD18 leukocytes lose functionality of Mac-1 (CD18/CD11b); it is unknown if this plays an essential role in injury resolution. It is possible Mac-1 prolongs neutrophil half-life in the tissue or enhances phagocytic rate of these cells.

Early events in pathophysiology generally impact the later events. The primary reason for sterile inflammation is to limit injury and promote tissue repair. Occasionally, unchecked inflammation is deleterious; however this is not the case with APAP overdose. The inflammation does not make the injury worse; in fact it most likely helps
to resolve injury by creating an environment to promote repair and regeneration. We have shown that IL-1β on its own is produced in insufficient quantity to modulate injury during APAP overdose; however acting in concert with other inflammatory mediators it helps recruit leukocytes into the liver. These cells become activated in the blood and the liver and have functions which still need to be determined. Better understanding of immune activation during late stage liver injury will be critical to predict patient outcome (need for transplantation) and improve survival rates. Advances in this field will hopefully decrease the morbidity and mortality of the leading cause of acute liver failure in the US.
Sterile inflammation is initiated by the release of DAMPs from injury hepatocytes. This results in cytokine/chemokine-induced leukocyte recruitment. We have shown these innate immune cells do not contribute to the injury but may be essential in tissue repair.
7 References


Bianchi, M.E., 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. J. Leukoc. Biol. 81, 1-5.


