Good cells go bad: immune dysregulation in the transition from acute liver injury to liver failure after acetaminophen overdose

James P. Luyendyk, Elena Morozova, Bryan L. Copple

JPL and EM: Department of Pathobiology & Diagnostic Investigation, Michigan State University, East Lansing, MI, USA.
BLC: Department of Pharmacology & Toxicology, Michigan State University, East Lansing, MI, USA.
Running Title: Immune Dysregulation in Acute Liver Failure

Co-corresponding Authors:
Dr. James P Luyendyk
Department of Pathobiology & Diagnostic Investigation
Michigan State University
253 Food Safety and Toxicology Building
East Lansing, MI 48824
E: luyendyk@msu.edu

Dr. Bryan L Copple
Department of Pharmacology and Toxicology
Michigan State University
221 Food Safety and Toxicology Building
East Lansing, MI 48824
E: copple@msu.edu

Number of text pages: 25
Number of Tables: 0
Number of Figures: 1
Number of References: 91
Abstract word count: 224
Introduction word count: 1252
Discussion word count: 3463

Nonstandard abbreviations:
APAP: acetaminophen
AALI: APAP-induced acute liver injury
AALF: APAP-induced acute liver failure
ALF: acute liver failure
ALI: acute liver injury
ALT: alanine aminotransferase
CARS: compensatory anti-inflammatory response syndrome
IL-10: interleukin-10
MDMs: monocyte-derived macrophages
MODS: multi-organ dysfunction syndrome
MDSCs: myeloid-derived suppressor cells
NAPQI: N-acetyl-p-benzoquinone imine
NETs: neutrophil extracellular traps
NK: natural killer cells
NKT: natural killed T cells
PD-L1: programmed death-ligand 1
SIRS: systemic inflammatory response syndrome
Abstract:

The role of inflammatory cells and other components of the immune system in acetaminophen (APAP)-induced liver injury and repair has been extensively investigated. Although this has resulted in a wealth of information regarding the function and regulation of immune cells in the liver after injury, apparent contradictions have fueled controversy around the central question of whether the immune system is beneficial or detrimental after APAP overdose. Ultimately, this may not be a simple assignment of “good” or “bad.” Clinical studies have clearly demonstrated an association between immune dysregulation and a poor outcome in patients with severe liver damage/liver failure induced by APAP overdose. To date, studies in mice have not uniformly replicated this connection. The apparent disconnect between clinical and experimental studies has perhaps stymied progress and further complicated investigation of the immune system in APAP-induced liver injury. Mouse models are often dismissed as not recapitulating the clinical scenario. Moreover, clinical investigation is most often focused on the most severe APAP overdose patients, those with liver failure. Notably, recent studies have made it apparent that the functional role of the immune system in the pathogenesis of APAP-induced liver injury is highly context dependent and greatly influenced by the experimental conditions. In this review, we highlight some of these recent findings, and suggest strategies seeking to resolve and build on existing disconnects in the literature.

Significance Statement:

Acetaminophen overdose is the most frequent cause of acute liver failure in the United States. Studies indicate that dysregulated innate immunity contributes to the transition from acute liver injury to acute liver failure. In this review, we discuss the evidence for this and the potential underlying causes.
In its simplest form, APAP overdose produces hepatocyte injury that triggers a rapid inflammatory response, culminating in the accumulation of neutrophils in the liver (Lawson et al., 2000). Soon after, monocytes are recruited and traffic into the regions of injury (Dambach et al., 2002) (Antoniades et al., 2012). Interplay between the neutrophils and monocytes orchestrates the clearance of dead cell debris and the conversion of the monocytes into pro-resolution macrophages that temper the inflammatory response (Graubardt et al., 2017b; Yang et al., 2019b). Coincident with the recruitment and activation of immune cells, hepatocytes and nonparenchymal cells proliferate, supporting restoration of the hepatic architecture and importantly, maintenance of liver function (Bhushan et al., 2014). The mechanisms underlying this well coordinated process have been obtained from studies in mice treated with doses of APAP at or near 300 mg/kg, which produces acute hepatotoxicity that is rapidly and predictably repaired (frequently referred to as APAP-induced liver injury; AILI). These experimental conditions most accurately reflect the pathogenesis of APAP overdose occurring in patients that spontaneously recover with standard care. Findings from these important studies have defined the pathways and mechanisms critical for hepatocyte injury and liver repair after APAP overdose. Moreover, these studies have demonstrated a central role for the immune system in these processes and have uncovered many of the critical regulatory mechanisms that coordinate immune cell function during liver injury and repair. This has laid the foundation for defining how disruption of these processes can facilitate the progression of acute liver injury to acute liver failure (ALF) producing a myriad of extrahepatic complications.

When the hepatotoxic response to APAP overdose is not counterbalanced by appropriate activation of repair processes, this can lead to a continual deterioration of liver function (Schmidt and Dalhoff, 2005; Bhushan et al., 2014). This profoundly impacts multiple organ systems and can result in the development of life-threatening extrahepatic complications, such as hepatic encephalopathy (i.e., a disorder of the brain and a diagnostic criterion for ALF) and multiorgan dysfunction syndrome (MODS) (see these excellent reviews for a detailed discussion of the systemic impacts of ALF (Stravitz and Lee, ...
The underlying causes of dysfunctional liver repair and the mechanism(s) linking this to the pathogenesis of serious, extrahepatic complications remain unclear. Findings from clinical studies suggest that dysregulation of the immune system may be causally involved (Possamai et al., 2014). APAP-induced ALF patients with the poorest prognosis develop systemic inflammatory response syndrome (SIRS), a life-threatening condition characterized by the systemic release of high levels of proinflammatory cytokines (i.e., cytokine storm) (Rolando et al., 2000; Craig et al., 2011). This condition triggers a massive inflammatory response that can severely damage multiple organ systems leading to MODS and may be integral to the development of hepatic encephalopathy (Figure 1) (Rolando et al., 2000). To counter SIRS, immune cells often begin to express high levels of anti-inflammatory cytokines and immune suppressive ligands. This good faith effort can unfortunately produce severe immunosuppression and a condition referred to as compensatory anti-inflammatory response syndrome (CARS) (Antoniades et al., 2008). CARS can increase susceptibility to nosocomial infections and interfere with the ability of certain immune cells to facilitate liver repair (Figure 1) (Roth K, 2021; Triantafyllou et al., 2021).

These clinical findings illustrate the complexity of immune cell engagement in acute liver injury and underscore the importance of elucidating the mechanisms triggering dysregulation and counterbalancing of immune system components during the transition of AILI to AALF. A better understanding of these mechanisms could provide insight into approaches to “normalize” the immune response in ALF patients, allowing for repair processes to restore liver function while maintaining the capacity to kill pathogens. Because the transition from AILI to AALF (APAP-induced ALF) in mice occurs with increasing doses of APAP, it would be tempting to speculate that this is merely the result of increasing hepatocyte injury. Interestingly, though, Bhushan and colleagues demonstrated that liver injury, quantified histologically, was not different between mice treated with either 300 or 600 mg/kg APAP (i.e., 300 mg/kg APAP: mice with AALI or 600 mg/kg APAP: mice with AALF) (Bhushan et al., 2014). Similar findings have been reported in APAP overdose patients where clinical measures of liver
injury (e.g., alanine aminotransferase; ALT) are poorly predictive of outcome (Larson et al., 2005; Berry et al., 2010; Bernal et al., 2016). Notably, recent studies from our laboratories have demonstrated that mice treated with higher doses of APAP (i.e., 500 mg/kg APAP or greater; AALF) develop many features of SIRS and CARS similar to patients with AALF. These mice also exhibit clinical features of hepatic encephalopathy, including cerebral edema, and develop a coagulopathy and renal injury, a frequent component of multiorgan failure (Shah et al., 2013; Akakpo et al., 2020; Groeneveld et al., 2023). These findings suggest that the pathogenesis of AALF in mice is similar to that in APAP overdose patients. Importantly, these findings also lend further support for the hypothesis that immune dysregulation is causally involved in the transition from ALI to ALF. What remains unclear, however, is the underlying cause of immune dysregulation in ALF and its relationship to the development of systemic complications and ultimately outcome. In our opinion, the best strategy to investigate this is to compare findings from mice with AALF to those from mice with AALI, where many of the critical regulatory networks governing the immune response have been defined. With this premise in mind, in this review we cover the role of various immune cell types in the pathogenesis of APAP-induced liver injury and repair during conditions of AALI and further discuss how these processes are impacted under conditions of AALF.

Impact of the Immune System on Xenobiotic Metabolism—A Cautionary Note.

Before discussing the potential for immune cells to modulate APAP-induced liver injury, it is important to review the metabolism of APAP and discuss the potential for immune mediators, among other factors, to impact this process. At therapeutic doses, APAP is largely metabolized by glucuronidation and sulfation pathways with a minor fraction being oxidized by cytochrome P450s to the hepatotoxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI) (for a detailed review see Mazaleuskaya et al., 2015). At these lower doses, NAPQI is rapidly detoxified through reaction with glutathione. On the other hand, at hepatotoxic doses of APAP, glucuronidation and sulfation pathways become saturated shifting the metabolism of APAP towards NAPQI formation. As levels of NAPQI increase, glutathione stores become depleted, resulting in the persistence of NAPQI. This highly reactive...
metabolite binds to sulphydryl groups on cellular macromolecules ultimately triggering cell death by mechanisms reviewed in detail elsewhere (Jaeschke and Ramachandran, 2023; Ramachandran and Jaeschke, 2023).

The enzymes that metabolize xenobiotics, including drugs, are frequently induced or repressed in response to shifts in the chemical composition of the environment. This can occur though the action of nuclear receptors acting as xenobiotic sensors or through signals released by other cells (Omiecinski et al., 2011). Of relevance to this review, studies have identified several immunomodulatory cytokines that impact the expression of enzymes involved in the biotransformation of xenobiotics (Renton, 2005; Gramignoli et al., 2022; Wang et al., 2022). For example, studies have shown that a loss of IL-4 decreases the expression of γ-glutamylcysteine ligase, an enzyme important for the synthesis of glutathione (Ryan et al., 2012). As a result, hepatic glutathione levels are reduced making IL-4 knockout mice more sensitive to the toxic effects of NAPQI (Ryan et al., 2012). Therefore, throughout this review, we will highlight instances where manipulations, intended to modulate the immune system, impact the metabolism or detoxification of APAP.

MYELOID CELLS

Neutrophils

Activated neutrophils produce an array of mediators capable of exacerbating hepatic injury including reactive oxygen species, proteases, and neutrophil extracellular traps (NETs) (Jaeschke et al., 1996; Papayannopoulous, 2018). This has led to the omnipresent hypothesis that neutrophil accumulation in the APAP-injured liver functions solely to amplify the injury. Although there is clear literature to support a pathologic role of neutrophils in liver injury induced by ischemia-reperfusion and bile duct ligation (Gujral et al., 2003; Jaeschke, 2006), decades of experimentation have revealed that the precise role of neutrophils in APAP-induced liver injury is context dependent. Moreover, the idiosyncrasies of the tools used to evaluate neutrophil involvement are equally critical to consider. Induction of neutropenia using
the monoclonal antibody Gr-1 significantly reduced APAP-induced liver injury (Liu et al., 2006). Although these initial studies entrenched the concept that neutrophils contributed to acetaminophen hepatotoxicity, several challenges are noted. The Gr-1 antibody is not specific for a neutrophil epitope, binding both Ly6G (neutrophils) and Ly6C (inflammatory monocytes) (Fleming et al., 1993). Moreover, the same antibody used to deplete neutrophils has often been used to confirm neutrophil depletion, invoking the possibility of epitope masking masquerading as depletion efficacy. Finally, antibody-mediated cell depletion may alter APAP hepatotoxicity by mechanisms independent of the target cell deficiency. For example, depletion of neutrophils with Gr-1 increases hepatic expression of metallothionein (Jaeschke and Liu, 2007), a protein shown to reduce APAP hepatotoxicity (Saito et al., 2010). These challenges have been addressed in part by studies using anti-Ly6G antibodies and complementary approaches using Cxcl2-deficient mice. The collective impression offered by these studies is that neutrophils do not exacerbate hepatotoxicity in mice with AALI but may in fact play an important role in liver repair (Williams et al., 2014; Yang et al., 2019b). Specifically, neutrophils shape the monocyte/macrophage response to liver injury which is essential for the clearance of dead cell debris and for triggering the resolution of inflammation (Graubardt et al., 2017b; Yang et al., 2019b; Chauhan et al., 2020). Moreover, these same studies demonstrated that hepatocyte proliferation was reduced in neutropenic mice treated with APAP. Collectively, these studies in AALI mice indicate a protective role for neutrophils.

These findings contrast with studies from the ALF Study Group, however, which demonstrated that biomarkers of NETs, an indicator of neutrophil activation, were predictive of poor outcome in ALF patients (von Meijenfeldt et al., 2022). Moreover, it has been reported that blood neutrophils from patients with ALF exhibit features of “exhaustion” suggesting wide-spread, systemic activation (Manakkat Vijay et al., 2016). Although the importance of this to the mechanism of liver injury can only be inferred, it suggests a potential pathological role for neutrophils in the pathogenesis of APAP-induced ALF in patients. These findings have spurred investigations into the role of neutrophils in mice with
AALF. In these studies, the hepatic expression of the neutrophil chemokine Cxcl2 was dramatically increased resulting in a greater accumulation of neutrophils in the liver as compared to mice with AILI (Nguyen et al., 2023). Importantly, Cxcl2 deficiency reduced hepatic neutrophils and liver injury in mice with AALF but had no effect on liver injury in mice with AILI (Nguyen et al., 2023). These findings suggest a pathological role for neutrophils under conditions of APAP overdose that produce ALF.

Overall, while it would be simpler to assign a singular role for neutrophils in APAP-induced liver injury, this is likely context dependent, with multiple facets of the experimental setting impacting interpretation. Increasing the severity of the APAP challenge uncovers a pathologic role for neutrophils and recent observations in ALF patients suggest a potential role for NETs in the pathogenesis. To date, however, there are no published studies documenting a role for NET formation/involvement in experimental APAP overdose (Li et al., 2022).

**Monocytes/Macrophages**

In addition to neutrophils, other myeloid cell populations have been investigated for their role in APAP-induced liver injury and repair, and over the years, there has been considerable debate regarding the precise function of these cells. Among the myeloid cells studied, Kupffer cells have received particular attention. Kupffer cells are the resident macrophages in the liver, originating from yolk sac progenitors and residing within the sinusoids, strategically positioned to clear pathogens entering the portal circulation (Schulz et al., 2012; Gomez Perdiguero et al., 2015). In addition to acting as a barrier between the gut and systemic circulation, these cells also serve various homeostatic functions which have been extensively reviewed elsewhere (Klein et al., 2007; Dixon et al., 2013). Kupffer cells are replenished through local proliferation, except in cases of significant loss, where circulating monocytes originating from bone marrow progenitors take their place (Zigmond et al., 2014; Lee et al., 2018b). Once these cells are positioned in the appropriate hepatic niche, they begin to take on characteristics of Kupffer cells (Scott et al., 2016; Bonnardel et al., 2019). The liver is also home to a population of dendritic cells which reside in close proximity to periportal vessels (reviewed in detail elsewhere (Lian et al., 2003; Soysa et
al., 2017). Unlike splenic dendritic cells, hepatic dendritic cells exhibit an immune suppressive phenotype, playing a crucial role in inducing immune tolerance (Pillarisetty et al., 2004; Dou et al., 2018). This unique phenotype of hepatic dendritic cells may function to prevent immune reactions against substances absorbed from the gut or protein-chemical haptens that may arise from the metabolism of xenobiotics in the liver.

In addition to resident myeloid populations, Ly6C^{hi} monocytes are recruited from the blood to the liver after APAP overdose and after injury to the liver by other hepatotoxicants (Zigmond et al., 2014). These cells produce proinflammatory cytokines and ultimately mature into monocyte-derived macrophages (MDMs), contributing to the clearance of dead cell debris (Holt et al., 2008; Zigmond et al., 2014; Graubardt et al., 2017b; Yang et al., 2019b). All of these myeloid cell populations have been implicated in the pathogenesis of APAP-induced liver injury or in the mechanisms driving liver repair. In the subsequent section, we will briefly discuss the current knowledge on the involvement of these cell types in this condition.

Investigations into the role of macrophage populations in the development of liver injury following APAP overdose have employed diverse methods to ablate or inhibit the function of these cell populations. Unfortunately, the varied mechanisms and unintended impacts of these methods have generated contradictory findings across different studies. The first studies to investigate a role for macrophages utilized gadolinium chloride, which depletes Kupffer cells and other resident macrophage populations in other organs (Laskin et al., 1995). In these investigations, gadolinium chloride reduced liver injury after APAP overdose, suggesting that Kupffer cells may be directly involved in the mechanism of liver damage. The underlying mechanism was proposed to involve a decrease in reactive nitrogen species levels, which had previously been linked to APAP-induced liver injury (Laskin et al., 1995). Subsequent reports revealed, however, that gadolinium chloride impacts multiple processes beyond macrophage depletion, including the inhibition of glutathione-S-transferases and microsomal epoxide hydrolase (Kim and Choi, 1997; Abdel-Zaher et al., 2007). Consequently, due to these unintended effects,
it becomes challenging to definitively attribute the liver-protective effect of gadolinium chloride against APAP overdose to macrophage depletion.

Subsequent to studies with gadolinium chloride, another macrophage depleting agent, liposomal clodronate, was used to investigate the role of macrophages in APAP-induced liver injury. Contrary to findings with gadolinium chloride, liposomal clodronate worsened liver injury after APAP overdose (Ju et al., 2002). The underlying mechanism was attributed to a reduction in the levels of the anti-inflammatory cytokine, interleukin-10, which had previously been reported to protect the liver from injury after APAP overdose. In addition to enhanced parenchymal cell injury, liposomal clodronate also markedly increased red blood cell pooling in the liver (i.e., congestion and hemorrhage), suggesting that Kupffer cells protect the sinusoidal endothelium from injury after APAP overdose (Holt et al., 2010). Interestingly, the necrotic lesions in these mice resemble those produced in mice treated with a higher dose of APAP that progresses to ALF (AALF). Clinical endpoints of ALF, including evidence of hepatic encephalopathy, however, were not specifically investigated. Evidence of ALF could indicate a link between the presence of hepatic vascular damage and the progression to ALF. Further studies would be needed, however, to establish a mechanistic link. Similar to gadolinium chloride, liposomal clodronate has the capacity to deplete cells with phagocytic activity in multiple tissue compartments (Buiting and Van Rooijen, 1994; van Rooijen and Hendrikx, 2010). Therefore, specifically linking an effect of this compound to Kupffer cells becomes complicated. Moreover, it was recently reported that treating mice with multiple doses of liposomal clodronate protects mice from APAP-induced liver injury by a mechanism that accelerates the re-synthesis of hepatic glutathione.

A frequently overlooked factor that could complicate the interpretation of findings from macrophage depletion studies is the vital role these cells play in clearing alanine aminotransferase (ALT) from the bloodstream (Horiuchi et al., 1985). When hepatocytes die, ALT is released passively into the blood, forming the basis for measuring this enzyme as a biomarker of hepatocyte injury. It was recently reported, however, that macrophages are required for the clearance of circulating ALT from the blood and...
that liposomal clodronate increases blood ALT levels independently of hepatocyte injury (Horiuchi et al., 1985; Radi et al., 2011). Therefore, cautious interpretation of this and related biomarkers should be considered when interpreting results from macrophage depletion studies.

Interestingly, significant numbers of Kupffer cells are lost from necrotic regions of liver after APAP overdose (Dambach et al., 2002). Similar effects on resident macrophage populations have been observed in other organs, especially after exposure to certain pathogens. For instance, intraperitoneal bacterial infection triggers a rapid loss of resident macrophages known colloquially as the "macrophage disappearance reaction" (Barth et al., 1995). This phenomenon was recently linked to specific cell death mechanisms, including pyroptosis and necroptosis and it is proposed that this is important for triggering a rapid inflammatory response to clear the invading pathogens (Degterev et al., 2005; Miao et al., 2010). The involvement of these processes in regulating hepatic macrophage function after sterile liver injury, however, has only recently been investigated. After ischemia/reperfusion in the liver, Kupffer cells die by pyroptosis, and macrophage-specific deletion of gasdermin D, a protein essential for triggering pyroptosis, reduces proinflammatory cytokine levels and measures of liver injury (Li et al., 2020). The mechanisms driving Kupffer cell loss after APAP overdose, however, are less well understood. Recently, it was reported that whole body gasdermin D knockout mice are protected from APAP-induced liver injury (Yang et al., 2019a). Whether this specifically involved changes to Kupffer cells, however, was not specifically explored. Others have reported that caspase 3 is activated in Kupffer cells after APAP overdose in mice, implying that these cells might die through apoptosis (Lopes et al., 2022). The precise trigger for Kupffer cell apoptosis and its relationship to the mechanism of liver injury, however, remain to be defined. Remarkably, despite years of investigation, the precise role of Kupffer cells in the mechanism of liver injury after APAP overdose remains unresolved. The use of more selective tools to modulate Kupffer cell numbers, however, should begin to uncover the role of these cells in APAP-induced liver injury.

Monocytes:
Recently, significant attention has been directed towards monocytes due to their pivotal role in the mechanisms governing the resolution of injury and inflammation following an overdose of APAP. In mice with AILI, Ly6C\textsuperscript{hi} monocytes expressing high levels of proinflammatory cytokines begins accumulating in the liver shortly after neutrophils, with their numbers peaking at 24 hours (Zigmond et al., 2014). The recruitment of these cells depends upon the release of the chemokine Ccl2 by cells in the liver, triggering monocyte chemotaxis through a mechanism dependent on the receptor Ccr2 (Dambach et al., 2002; Holt et al., 2008). Within the liver, these monocytes contribute to the production of proinflammatory cytokines and navigate towards the damaged regions by a process facilitated by components of fibrinolysis (Zigmond et al., 2014; Roth et al., 2019). Notably, these monocytes release a factor or factors that activate neutrophils, prompting them to generate reactive oxygen species (Yang et al., 2019b). This activation results in the transformation of the Ly6C\textsuperscript{hi} monocytes into MDMs with a pro-resolution phenotype (Graubardt et al., 2017a; Yang et al., 2019b). Concurrently, neutrophils undergo apoptosis and are subsequently engulfed by MDMs, along with cellular debris. This reciprocal communication between monocytes and neutrophils ultimately leads to the resolution of both the injury and the ensuing inflammation. The intricate aspects of this well-coordinated sequence remain undefined, including the precise mechanisms governing the shift from Ly6C\textsuperscript{hi} monocytes to pro-resolving macrophages, as well as the specific signals connecting neutrophils to this process.

These mechanisms were largely defined in mice with AILI. Under conditions of ALF, however, this finely orchestrated process becomes disrupted, giving rise to immune dysregulation. Bhusan and colleagues presented evidence suggesting that the mechanisms responsible for the clearance of dead cells from the liver were perturbed in mice with AALF (Bhushan et al., 2014). To ascertain the underlying cause of this impairment, our laboratories determined whether the recruitment and/or function of Ly6C\textsuperscript{hi} monocytes, which clear the dead cell debris, was affected in AALF mice (Roth K, 2021). Our studies demonstrated that monocyte recruitment to the liver was markedly reduced in mice with AALF, indicating a defect in the mechanisms controlling recruitment of these cells (Roth K, 2021). As discussed
earlier, clinical studies have revealed that levels of interleukin-10 (IL-10), a component of CARS, are
greatest in ALF patients with the poorest prognosis (Berry et al., 2010; Woolbright et al., 2022). Building
upon this clinical foundation, we proposed the hypothesis that high levels of IL-10 restrict monocyte
recruitment to the liver during ALF. In mice with AALI, IL-10 mRNA levels in the liver did not
significantly increase until 72 hours after APAP treatment, coinciding with the resolution of inflammation
(Roth K, 2021). In contrast, mRNA levels of IL-10, in a hepatic population of F4/80+ macrophages, was
markedly elevated by 24 hours in AALF mice (Roth K, 2021). Although F4/80 is typically linked to
Kupffer cells, these cells co-expressed Cx3Cr1, suggesting that they may have arisen from recruited
monocytes (Roth K, 2021). Moreover, these cells expressed the immune suppressive ligand, programmed
death-ligand 1 (PD-L1), which has been associated with an elevated infection risk in ALF patients (Roth
K, 2021; Triantafyllou et al., 2021). While the precise source of the IL-10-secreting cells remains to be
determined, blocking IL-10 activity beginning at 24 hours after APAP treatment augmented hepatic
monocyte recruitment and restored dead cell clearance in AALF mice (Roth K, 2021). Conversely,
administration of recombinant IL-10 to AALI mice, beginning at 24 hours after APAP treatment, reduced
hepatic monocyte recruitment, resulting in the persistence of necrotic cells (Roth K, 2021). Based upon
these findings, we propose a model in ALF, whereby early induction of IL-10 prevents hepatic monocyte
recruitment which contributes in part to the failure of liver repair. Although an area of active
investigation, the cellular source of IL-10 expressed features of myeloid-derived suppressor cells
suggesting these cells as a potential source (Roth K, 2021). Consistent with this possibility, studies have
reported higher numbers of MDSCs in patients with severe ALF (Bernsmeier et al., 2018).

**Dendritic Cells:**

The role of dendritic cells in APAP-induced liver injury has not been extensively investigated. However,
depletion of dendritic cells increased APAP hepatotoxicity, whereas expansion of dendritic cells protected
from liver toxicity (Connolly et al., 2011). Remarkably, the mechanism by which dendritic cells modulate
liver toxicity after APAP overdose remains unknown. Interestingly, though, these same studies
demonstrated that dendritic cell numbers markedly decrease early after APAP treatment of mice. In a subsequent study, it was reported that macrophage migration inhibitory factor mediates this decrease through the induction of dendritic cell apoptosis. Following APAP (600 mg/kg) challenge increased hepatic MIF expression was accompanied by a reduction in hepatic dendritic cells. Conversely, administration of anti-MIF antibodies increased the number of dendritic cells in the liver (Wu et al., 2023). These studies suggest DCs exert protective effects in the APAP-injured liver injury.

**Eosinophils:**

Prior studies have shown that eosinophils traffic to the APAP-injured liver in humans and in mice (Pham et al., 2001; Xu et al., 2022). The accumulation of eosinophils in the liver of mice after APAP treatment is reported to occur secondary to IL-33 activation of eosinophils and amplification of eosinophil recruitment by macrophage production of the chemokine Ccl24 (Xu et al., 2022). Notably, eosinophil deficiency was found to exaggerate APAP-induced liver injury and the protective effects of eosinophils appeared to be mediated by the production of cytokines including IL-4 and IL-13 (Xu et al., 2022). Notably, the case of IL-4 in APAP-induced liver injury seems equally complex, with multiple studies concluding that IL-4 deficiency reduces APAP-induced hepatotoxicity (Pires et al., 2014) or increases APAP hepatotoxicity (Ryan et al., 2012).

**Immune cell potpourri**

Without question, the largest consensus supporting involvement of immune/inflammatory cells in APAP-induced liver injury exists for monocytes/macrophages and neutrophils. With that said, the complexities that remain despite the depth of investigation are substantial. Multiple other immune and inflammatory cell populations have been implicated as either damaging or protective after APAP treatment. Although perhaps equally important, these cell types are less investigated and clarity on their precise involvement has not been achieved. Natural killer (NK) and natural killer T (NKT) cells have been implicated in APAP-induced liver injury. However, a unified role for these cells in the pathogenesis has not been
identified. Depletion of NK/NKT cells was reported to attenuate IFN-gamma expression and hepatocellular injury (Liu et al., 2004), but discovery of this mechanism was ultimately suspected to originate from the use of dimethylsulfoxide as the vehicle for APAP (Masson et al., 2008). Notably, another example where seemingly subtle changes in the experimental context have the potential to alter mechanistic interpretation. Parallel studies using genetic approaches have also been performed. NKT cell-deficient mice (Vα14iNKT cell-deficient; Jα18−/− mice) treated with 600 mg/kg APAP displayed reduced liver injury attributed to altered APAP metabolism (Downs et al., 2012). Interestingly, hepatic injury was increased in Jα18−/− and CD1d−/− mice (i.e., NKT deficient mice) treated with lower doses of APAP (~200-350 mg/kg) (Martin-Murphy et al., 2013), a dichotomy consistent with emerging studies on neutrophils in APAP overdose. Hepatic accumulation of NK/NKT cells as well as other lymphocyte subsets are seemingly controlled by the chemokine receptor CCR6. CXCR6-deficient mice displayed a reduction in specific immune cell populations, particularly CD4+ T cells, natural killer (NK) cells, and most significantly, NKT cells (Heymann et al., 2023). The ultimate effect of these changes was exaggerated liver injury ascribed to proinflammatory activation of myeloid cells, supporting the concept that interaction between various lymphocyte and monocyte cell populations may determine the extent of liver injury. Additional lymphocyte populations including γδ T cells have been implicated in APAP-induced liver injury through production of cytokines including IL-17 (Wang et al., 2013; Zhu and Uetrecht, 2013; Lee et al., 2018a). The complexity of lymphocyte involvement requires further study as specific T cell populations have been shown to play both damaging and protective roles in APAP-induced liver injury (Numata et al., 2007; Getachew et al., 2014; Wang et al., 2015).

Applicability of findings in APAP-induced ALF to ALF produced by other drugs:

Much of our understanding of the pathogenesis of drug-induced ALF is from clinical studies or from studies in APAP-treated mice. ALF caused by other drugs is frequently the result of idiosyncratic drug-induced liver injury which is exceedingly rare in patients and is often difficult to reproduce in mice. As a result, the underlying mechanisms are not well understood. While it would be tempting to extrapolate
findings from APAP-induced ALF to ALF caused by other drugs, it is unlikely that the mechanisms are fully congruent. Clinically, compared to patients with APAP-induced ALF, patients with ALF produced by other drugs present with lower aminotransferase activity, higher bilirubin levels, and these patients often have a worse outcome (Stravitz and Lee, 2019). While the reasons for this are not known, it suggests that the pathogenesis of APAP-induced ALF may be quite different from that produced by other drugs. Clearly, though, this requires further investigation.

Summary and conclusions:

Cellular and soluble components of the innate and adaptive immune systems clearly play a role in both liver injury and liver repair after APAP overdose. Apparent controversy fueled by dichotomous findings may ultimately be unraveled by a deeper understanding of the mechanisms that drive transition of simple acute liver injury to liver failure, and engagement of immune cells in this process. Pursuit of relevant differences in experimental context, even retrospectively in established literature, has strong potential to inform on next steps. Moreover, continued movement toward standardization of experimental approaches and careful partnerships between hepatologists, immunologists, and toxicologists are most likely to unravel what is clearly a complex interplay driving immune cell effector functions and contributions in the context of acute liver injury.

Data Availability Statement: N/A, there are no datasets in this manuscript.

Author Contribution

Wrote or contributed to the writing of the manuscript: Luyendyk, J., Morozova, E., and Copple, B.
References:


Funding

Supported by National Institutes of Health grant DK135649 to James Luyendyk and Bryan Copple.

Conflict of Interest Statement

No author has an actual or perceived conflict of interest with the contents of this article.

Figure Legends

Figure 1: Pathogenesis of APAP-induced ALI and ALF. APAP-induced liver injury in mice and patients triggers a rapid inflammatory response. A tightly regulated balance between inflammatory and repair pathways facilitates the resolution of injury and the restoration of liver function resulting in a full recovery. In a subset of patients and in mice treated with a high dose of APAP, acute liver injury produces an exaggerated inflammatory response leading to SIRS. This condition contributes to the development of multiorgan dysfunction syndrome (MODS) and hepatic encephalopathy, a feature of ALF. To counter the hyperactive immune response, high levels of anti-inflammatory mediators are produced leading to CARS. This immunosuppressive condition increases susceptibility to infections and disrupts immune mediated repair pathways. Created with Biorender.com.
Figure 1