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Neutrophil Activation During Acetaminophen Hepatotoxicity and Repair in Mice and Humans

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Abstract

Following acetaminophen (APAP) overdose there is an inflammatory response triggered by release of cellular contents from necrotic hepatocytes into systemic circulation which initiates the recruitment of neutrophils into the liver. It has been demonstrated that neutrophils do not contribute to APAP-induced liver injury, but their role and the role of NADPH oxidase in injury resolution is controversial. C57BL/6 mice were subjected to APAP overdose and neutrophil activation status was determined during liver injury and liver regeneration. Additionally, human APAP overdose patients (ALT: >800U/L) had serial blood draws during the injury and recovery phases for determination of neutrophil activation. Neutrophils in the peripheral blood of 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, but no ROS priming was observed. Confirming that NADPH oxidase is not critical to injury resolution, gp91^{phox}^{-/-} mice following APAP overdose displayed no alteration in injury resolution. Peripheral blood from APAP overdose patients also showed increased neutrophil activation status after the peak of liver injury and remained elevated until discharge from the hospital. In mice and humans, markers of activation, like ROS priming, were increased and sustained well after active liver injury had subsided. The similar findings between surviving patients and mice indicate neutrophil activation may be a critical event for host defense or injury resolution following APAP overdose, but not a contributing factor to APAP-induced injury.

Keywords

acetaminophen; hepatotoxicity; neutrophils; inflammation; regeneration; innate immunity

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Introduction

Acetaminophen (APAP) overdose can result in severe liver injury, acute liver failure and potentially death (Larson, 2007; McGill et al., 2012). The hepatotoxicity of APAP begins with metabolic conversion of APAP to a reactive metabolite, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI). The reactive metabolite causes glutathione (GSH) depletion and binds to cellular proteins, which is the initiating event in toxicity (McGill and Jaeschke, 2013). Downstream of protein binding are mitochondrial dysfunction, increased oxidant stress, mitochondrial permeability transition pore (MPT) opening, DNA damage by mitochondrial endonucleases, and hepatocellular necrosis (Jaeschke et al., 2012a). 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 trigger a sterile inflammatory response resulting in increased cytokine/chemokine formation and recruitment of innate immune cells into the liver (Jaeschke et al., 2012b). In the mouse model, neutrophils are recruited to the site of injury first, followed by monocytes/macrophages (Lawson et al., 2000; Dambach et al., 2002; Holt et al., 2008). However, it is controversial whether or not neutrophils actually contribute to the injury (Jaeschke, 2008). A few studies that have used neutropenia-inducing antibodies have provided evidence for the involvement of neutrophils in the late-stage injury process (Ishida et al., 2006; Liu et al., 2006; Marques et al., 2012). However, this particular approach has been repeatedly criticized for potential off target effects (Jaeschke and Liu, 2007; Jaeschke et al., 2013). On the other hand, a substantial number of other studies using a wide variety of approaches to inhibit neutrophil function have not found any evidence for a neutrophil-induced injury phase (Connolly et al., 2012; Cover et al., 2006; Hou et al., 2012; James et al., 2003; Lawson et al., 2000; Williams et al., 2010a, 2010b). In addition, there is no support for activation of neutrophils in circulation or in the liver, a hallmark of any model where neutrophils are critical (Jaeschke et al., 1992; Gujral et al., 2003), during the early phase of APAP-induced liver injury at 6 h (Williams et al., 2010a).

Neutrophils are a first-line defense against pathogens and are essential for microbial phagocytosis and killing (Nathan, 2006). During APAP hepatotoxicity, this innate immune function is critical for host defense, especially if the patient progresses toward fulminant hepatic failure (FHF) (Antoniades et al., 2008; Taylor et al., 2013). 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 considerable interest. This loss of host defense could be the result of impaired synthesis of complement components, impaired phagocytosis of gut-derived bacteria by Kupffer cells, or multiple immune deficits which may include neutrophils (Wyke et al., 1980, 1982; Taylor et al., 2013).

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 (Dambach et al., 2002; Holt et al. 2008; You et al., 2013). No characterization of neutrophils following the injury phase of APAP has been performed, however, and for this reason the activation status of peripheral blood and liver-sequestered neutrophils beyond the early injury phase of APAP remains unclear. In this study, we determined the progression of neutrophil activation and hepatic accumulation in the mouse model and utilized mice deficient for NADPH oxidase activity to investigate the importance of phagocytic oxidative burst. Additionally, we evaluated the activation status of human granulocytes in several APAP overdose patients during the course of their hospital stay and

compared the activation status to what we observed in the mouse model, in which the mechanisms of APAP toxicity are better understood.

Methods

Animals

Male gp91^{phox}-deficient (aka: Nox-2^{-/-}, Cybb^{-/-}, Phox^{-/-}) mice (B6.129S-Cybb^{tm1Din}/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 the University of Kansas Medical Center and followed the criteria of the National Research Council for the care and use of laboratory animals in research.

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 and then sacrificed (approximately at 10 am).

Patient selection and consent

Blood samples were obtained from APAP overdose patients (n=14; 9 female and 5 male) admitted to the University of Kansas Hospital in Kansas City, Kansas. 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) (Table 1). 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 *N*-acetylcysteine (NAC) treatment.

Histology

Formalin-fixed mouse 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). Quantification was performed by staining tissue sections, randomly selecting 10 high power fields (HPF, x400) and counting positively stained cells consistent with neutrophil morphology. The sum of these 10 HPFs for each animal are then used to determine the

group mean and standard error. 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 (careful determination was made to not count binucleated cells twice) were quantified in ten randomly selected high power fields (HPF, x400) in a manner consistent with the neutrophil quantification. 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).

Isolation of hepatic non-parenchymal cells

The procedure was adapted from a method described by 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 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 50 mL conical tube. The cell suspension was centrifuged at $50 \times g$ for 2 minutes to remove hepatocytes and large debris. The supernatant containing non-parenchymal cells was then centrifuged at $350 \times g$ for 5 minutes and cells were passed through a 40 μ m 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.

Flow Cytometric Analysis of Neutrophil Function

Neutrophil CD11b staining—(Bajt et al., 2001; Daley et al., 2008). 5 μ g Fc receptor (FcR) blocking antibody (BioLegend, San Diego, CA) diluted in 100 μ L 0.1% BSA in PBS was added to 100 μ L non-parenchymal cell suspension for 20 minutes on ice (mouse only). To 50 μ L heparinized whole blood (mouse or human) or 200 μ L 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 NH_4Cl , 10 mM KHCO_3 , 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^{high} cells, which are the Ly6G-positive population and identified as neutrophils by cell morphology (Daley et al., 2008). Other leukocytes are either Gr-1^{intermediate} (mainly monocytes and eosinophils) or Gr-1^{negative} (monocytes and lymphocytes) (Daley et al., 2008). Human granulocytes (primarily comprised of neutrophils) were gated to exclude monocytes and lymphocytes by forward- and side-scatter characteristics. The data were analyzed using the BD FACSDiva 6.0 software.

Reactive Oxygen Production—(Emmendorffer et al., 1990; Smith and Weidemann, 1993). To 50 μ L heparinized whole blood (mouse or human) or 100 μ L hepatic non-parenchymal cells (mouse only), 2 μ M phorbol 12-myristate 13-acetate (PMA) or saline was added and incubated for 10 minutes at 37°C. Subsequently, 4 μ M 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 measured on the FACSCalibur (BD) and the data were analyzed using the BD FACSDiva 6.0 software.

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 μ L aliquots of heparinized whole blood, 20 μ L of diluted opsonized-FITC-*E. coli* were added and incubated for 5, 10, 15 and 20 min at 37°C. 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.

Statistics

All results were expressed as mean $\pm$ 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.

Results

Time course of APAP-induced hepatic injury

In C57BL/6 mice, following an i.p. dose of APAP (300mg/kg), there was substantial hepatic injury at 6 h, as indicated by the significant increase of plasma ALT activities (Figure 1A) and extensive areas of necrosis (Figure 1B,C). The injury further increased up to 24 h. At or around 24 h, the injury resolution phase begins (Figure 1A-C). This was confirmed by TUNEL staining from representative mice (Figure 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 are much less, and by 72 h fragmented DNA is almost undetectable in the liver despite the presence of necrotic lesions. Administration of 100 μ g/kg endotoxin did not cause any increase in plasma ALT activities or liver cell necrosis (Figure 1 A,B), or DNA fragmentation (data not shown) at 90 min.

Hepatic neutrophil recruitment and hepatocyte regeneration

It has been shown that neutrophils are recruited into the liver after APAP overdose in mice (Lawson et al., 2000; Cover et al., 2006). Consistent with these findings, we observed progressive neutrophil accumulation in livers of APAP-treated animals, with peak levels at 24 h and declining values afterwards (Figure 2A). Histological analysis indicated that at early time points (6, 12 h) most neutrophils were located at the periphery of injury and in sinusoids of healthy tissue (Figure 2B). By 24 h, the neutrophil count peaked, with most of the neutrophils located within the necrotic lesions. Forty-eight hours post-overdose, the number of neutrophils decreased dramatically and continued to decline to 72 h. By this time, the neutrophils were located exclusively within the injured areas. Recovery from injury requires regeneration and replacement of necrotic hepatocytes. Hepatocytes surrounding the area of necrosis enter the cell cycle (as indicated by PCNA expression), divide and ultimately replace the damaged cells (Bajt et al., 2003). PCNA-positive hepatocytes began to appear around 24 h post-overdose in mice (Figure 2C and 2D). The peak of PCNA-positive hepatocytes was observed at 48 h, with many proliferating hepatocytes still present at 72 h. This pattern was confirmed by western blotting for PCNA in total liver homogenates (data not shown). Most of the PCNA positive cells were located at the edge of necrosis, and the recruited immune cells were within this area. Endotoxin also triggered neutrophil recruitment into the liver (Figure 2A), but most of the neutrophils were located in sinusoids (Figure 2B), and due to the lack of injury no PCNA-positive cells were detected (Figure 2C).

Hepatic and peripheral blood neutrophil priming and activation

Neutrophils recruited into the liver can either further aggravate the injury or participate in removing cell debris and promote regeneration. Both functions require activation of the neutrophils. In determining the activation status, we compared neutrophil CD11b surface expression (Figure 3A) and reactive oxygen (ROS) priming (Figure 3B) 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 (Figure 3A,B). Following APAP overdose, peripheral blood neutrophils had increased CD11b expression at 12 h and 24 h (Figure 3C), which correlated with the highest levels of most circulating cytokines, chemokines and DAMPs following APAP overdose (Antoine et al., 2009; James et al., 2005; Lawson et al., 2000; Williams et al. 2010a; 2011). Correlating with increased CD11b on peripheral neutrophils, hepatic neutrophils also demonstrated increased CD11b expression, but this expression persisted much longer (Figure 3D). No priming for ROS formation was observed in blood neutrophils during the main injury phase (12 h); in contrast, priming was detected at 24-48 h (Figure 3E). 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 (Figure 3F). In contrast, blood and hepatic neutrophils were primed for ROS formation after exposure to endotoxin (Figure 3E,F).

Liver regeneration in NADPH oxidase-deficient (gp91^{-/-}) mice following APAP overdose

Since C57BL/6 mice showed no hepatic neutrophil ROS priming throughout the time course, we used mice deficient in the subunit gp91(phox) of the ROS-generating enzyme NOX2 (nicotinamide adenine dinucleotide phosphate oxidase 2) to confirm that phagocyte ROS was not critical for liver repair following APAP overdose. In addition to neutrophils, monocytes and macrophages also lack the capacity to generate ROS in gp91^{-/-} mice. James et al. (2003) demonstrated that gp91^{-/-} mice are not protected against APAP hepatotoxicity (24 h) but did not evaluate recovery from APAP-induced injury in these mice. To investigate the role of neutrophil-derived ROS, gp91^{-/-} and C57BL/6 control mice were treated with APAP and sacrificed at 24 h, 48 h and 72 h. Consistent with the previous study (James et al., 2003), there was no difference in the peak of injury at 24 h between gp91^{-/-} and C57BL/6 mice as measured by plasma ALT (Figure 4A) and necrosis scoring (Figure 4B) of H&E stained liver sections (Figure 4C). 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 (NOX2) the progression and resolution of injury is unaltered, indicating removal of cellular debris by phagocytes does not require ROS formation.

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), lung inflammation (Morgenstern et al., 1997) and arthritis (van de Loo et al., 2003) mouse models, there is increased and sustained innate immune cell infiltration in these animals. Following APAP overdose in gp91^{-/-} mice a marked further increase in hepatic neutrophil recruitment could be observed versus C57BL/6 mice (Figure 5A). This increase was most dramatic 24 h post-overdose but neutrophil counts were still significantly elevated at 48 h; by 72 h, the difference in hepatic neutrophil counts between groups was not significantly different (Figure 5A). The distribution of neutrophils was not altered between groups. In C57BL/6 mice at 24 h most neutrophils were located within the necrotic lesions and the same was observed in gp91^{-/-} mice (Figure 5B). By 48 h and 72 h most neutrophils were 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 (Figure 5C) and the pattern of positive cells was not different, with most PCNA-positive cells being observed adjacent to the injury (data not shown).

Granulocyte activation following APAP overdose in patients

Neutrophil function in human APAP overdose patients can only be evaluated in peripheral blood because of the risk of liver biopsies due to frequent coagulopathies 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. Unfortunately, limitations in sample collection and analysis did not permit us to evaluate these parameters daily.

Similar to the previously described mouse neutrophil flow cytometric analysis, human granulocytes were evaluated by flow cytometry (n=14 patients; 9 female and 5 male). Human granulocytes were gated by characteristic forward and side scatter properties (Figure 6A). These granulocytes are predominantly neutrophils (accounting for 40-70% of blood leukocytes) with a minor population of eosinophils (1-3% of blood leukocytes) and will subsequently be referred to as 'neutrophils.' Activation status was determined by neutrophil 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. Ex vivo, the whole blood was incubated with fluorescently labeled bacteria to determine the neutrophil phagocytic capacity. A representative patient showed increased phagocytic capacity from day one to day four by the characteristic right shift in neutrophil fluorescence (Figure 6B). Additionally, the blood was used to determine NADPH oxidase-mediated ROS production after stimulation ex vivo. Similar to the phagocytic capacity, PMA-mediated ROS production increased from day one to day four in this patient as seen by increased mean fluorescence (Figure 6C), which also demonstrates enhanced neutrophil function during patient recovery.

A detailed time course of the neutrophil activation status in three representative patients that fully recovered from APAP overdose are presented in Figure 7. Overlaying these activation parameters are plasma ALT levels, indicating the progression and resolution of liver injury in these patients. Two patients (Figure 7A and 7B) were admitted to the hospital prior to the peak of liver injury and one patient arrived at or after the peak of liver injury (Figure 7C). The activation status of neutrophils was determined by measuring the mean fluorescent intensity of all neutrophils and normalized to the mean fluorescent intensity of the first day sampled; therefore the data is shown as a fold-increase over the first day measured. The patients represented in Figure 7A and 7C had low neutrophil function parameters at or near the peak of injury that increased as the injury was resolving. The patient represented in Figure 7B had increasing peripheral blood neutrophil activation as the injury was progressing, however this activation status continued to rise and remained elevated during resolution until the patient was discharged from the hospital nine days post-admission.

From the representative patients shown in Figure 7 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, 14 patients with liver injury (ALT: >800 U/L) (Table 1) that ultimately fully recovered after APAP overdose have been evaluated and are presented in Figure 8. At or near the peak of liver injury (highest measured plasma ALT levels), plasma ALT and neutrophil function tests 72 to 96 hours after the peak of injury (as determined by sample availability) were compared. The declining ALT values indicating the end of active cell death and the beginning of repair are shown in Figure 8A. Consistent with the representative patients, no correlation between declining injury and CD11b expression was

observed (Figure 8B). Reactive oxygen priming induced by PMA (Figure 8C) and opsonized *E. coli* (Figure 8D) was significantly increased following the peak of injury. Likewise, the increased phagocytosis capacity of neutrophils further supports the conclusion that neutrophils are more activated several days after the peak of injury than during the injury phase itself. These preliminary data demonstrate an increased neutrophil activation status in patients that recover from APAP overdose. In patients admitted with APAP overdose that never presented with increased liver enzymes the neutrophil function remained unaltered compared to healthy volunteers (data not shown), indicating injury and potentially suggesting that the release of DAMPs drives neutrophil activation. In addition, the unaltered PMN functions in patients with or without NAC treatment suggest that NAC had no direct effect on the results.

Discussion

The objective of the current investigation was to comprehensively assess the neutrophil activation status in mice during APAP hepatotoxicity and in patients admitted to KU Hospital with liver injury due to APAP overdose. Our results in mice and in patients indicate that neutrophil activation occurs mainly during the very late injury phase and during recovery, suggesting that neutrophils are more likely recruited to clean up cell debris than to actively participate in the injury process during APAP hepatotoxicity in mice and humans.

Neutrophil activation during APAP hepatotoxicity in mice

A role of neutrophils in liver injury models such as ischemia-reperfusion (Jaeschke et al., 1990), endotoxemia (Jaeschke et al., 1991; Hewett et al., 1992), obstructive cholestasis (Gujral et al., 2003), alcoholic hepatitis (Bautista, 1997; Bertola et al., 2013) and sepsis (Molnar et al., 1997) has been clearly demonstrated. However, the large number of studies that have been published investigating a potential involvement of neutrophils in APAP hepatotoxicity have reported conflicting results (reviewed: Jaeschke et al., 2012b). Although there is little doubt that extensive APAP-induced hepatocellular necrosis causes the release of many DAMPs including HMGB1, mtDNA, nuclear DNA fragments, and heat shock proteins (Antoine et al., 2009; Ju, 2012; Martin-Murphy et al., 2010; McGill et al., 2012), and triggers a sterile inflammatory response which includes the recruitment of neutrophils into the liver, the pathophysiological relevance of these cells remains controversial. Many experimental interventions are prone to off-target effects. In order to get closer to the potential mechanisms of neutrophil-induced liver injury in APAP hepatotoxicity, the activation status of neutrophils was determined in blood and the liver. In models with liver injury, there is upregulation of the adhesion molecule CD11b on circulating neutrophils (Jaeschke et al., 1993; Witthaut et al., 1994; Bajt et al., 2001; Gujral et al., 2003) and increased priming and ROS formation in liver-sequestered neutrophils (Jaeschke et al., 1991, 1992). Interestingly, after APAP in mice, no increased CD11b expression was observed at 6 h on blood or liver neutrophils (Lawson et al., 2000; Williams et al., 2010a). Our current results confirmed the lack of CD11b expression during the early injury phase and showed a minor increase of CD11b 12-24 h after APAP (compared to endotoxin) on both circulating and liver neutrophils. Liver neutrophils maintained CD11b expression throughout the injury resolution process which may have been caused by the local environment enhancing CD11b expression. During inflammation, hepatocytes can generate CXC chemokines (Allen et al., 2011; Maher et al., 1997), which can induce neutrophil activation as indicated by L-selectin shedding and CD11b upregulation (Bajt et al., 2001). Compared to the positive control, APAP-induced maximal CD11b expression never exceeded 30% and ROS never exceeded 70% of values induced by endotoxin in the absence of any liver injury. These data suggest that the level of neutrophil activation as measured by upregulation of CD11b or ROS priming is not an indicator of liver injury.

Neutrophil cytotoxicity is generally dependent on ROS (Entman et al., 1992; Jaeschke, 2006). ROS formation starts with formation of superoxide by NADPH oxidase (NOX2) and hydrogen peroxide, which is converted by myeloperoxidase to hypochlorous acid (Nathan, 2006). Both neutrophil-derived hydrogen peroxide as well as hypochlorite has been shown to diffuse into hepatocytes during neutrophil-mediated injury mechanisms (Gujral et al., 2003, 2004; Hasegawa et al., 2005; Jaeschke et al., 1999). However, gp91^{-/-} mice, which lack a functional NADPH oxidase so that neither Kupffer cells nor neutrophils are able to generate ROS, did not have altered APAP-induced liver injury or recovery. These data indicate that neither injury nor clean-up function are dependent on NADPH oxidase-derived ROS in this model.

Neutrophil activation in APAP overdose patients

During APAP toxicity in humans, which is characterized by extensive necrosis, substantial release of DAMPs (e.g., mtDNA, DNA fragments, and HMGB1) has been demonstrated (Antoniades et al., 2012; McGill et al., 2012). Antoniades et al. (2012) showed that both neutrophils and monocytes accumulate in livers of patients with APAP-induced acute liver failure as a result. A caveat of that study, however, is that the tissue was from explanted livers, i.e. the tissue was obtained well after the injury phase from patients which did not recover (Antoniades et al., 2012). In fact, these patients actually show significantly impaired neutrophil function (Clapperton et al., 1997; Rolando et al., 2000; Taylor et al., 2013). In contrast, our study focused on the early injury phase and the subsequent recovery. Similar to mice, patients have very limited activation (CD11b, ROS, phagocytosis) of circulating neutrophils during the peak of injury after APAP overdose. Because of the close correlation between the activation status of circulating and liver-sequestered neutrophils, these data suggest that neutrophils do not contribute to liver injury in patients. In striking contrast, the neutrophil activation status substantially increased in most patients during the recovery phase, suggesting that neutrophils, in addition to recruited monocytes (Antoniades et al., 2012), may participate in the removal of necrotic cell debris. This conclusion is supported by the finding that neutrophil activation was observed only in those patients who experienced severe liver injury and recovered but not in overdose patients who never developed any liver injury.

Our study only focused on patients who spontaneously recovered within 6-10 days after taking the overdose. This is in contrast to previous studies, which investigated the activation status of circulating neutrophils in severe acute liver failure patients without recovery. 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* tests 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 the opsonization capacity of patient serum; rather opsonization 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. Overall, these observations in humans indicate that the early necrosis with its massive release of DAMPs contributes to neutrophil activation and hepatic recruitment during the later injury and during the regeneration phase. Thus, the presence of activated and functioning PMNs during the later phase of APAP injury suggest a role of neutrophils in recovery and not liver injury.

Differences in neutrophil activation between mice and humans

We have demonstrated that both mice and humans have increased neutrophil activation status during injury resolution following APAP overdose. Additionally, mice and humans

have comparatively low neutrophil activation status during the injury phase. This is a temporal relationship between activation status and injury/repair. Because of this relationship we conclude that neutrophils are not participating in the injury process however from our study this is not definitive.

Also, differences exist between the activation of neutrophils between mice and humans. Peripheral blood neutrophils showed a transient ROS priming and CD11b expression. Human neutrophils had enhanced ROS priming throughout the injury resolution process and never displayed increased CD11b expression. Therefore, both species showed increased neutrophil activation, however the activation parameters were different. Why this difference exists is not entirely clear. Reasons could include the time and progression of injury, supportive care administered to clinical patients or simply species variation.

Summary and Conclusions

Our data suggest that NADPH oxidase-mediated ROS production is not a critical function in the recovery from APAP-induced liver injury. This was demonstrated by a lack of ROS priming observed in hepatic-sequestered neutrophils and confirmed in NADPH oxidase-defective mice. Overall, the data from mice indicate, at best, a very limited activation of neutrophils at late time points of injury and during regeneration, suggesting that neutrophils do not contribute to the injury but may be involved in removal of cell debris during regeneration. Consistent with these findings, there was only very limited activation of neutrophils in APAP overdose patients during the injury phase. In contrast, neutrophils show increased phagocytosis capacity and priming for ROS during the regeneration phase in patients who spontaneously recover. 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 a link between peripheral neutrophil activation and favorable outcome. More patients need to be evaluated to further support this conclusion.

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Highlights

- Neutrophil (PMN) function increases during liver repair after acetaminophen overdose
- Liver repair after acetaminophen (APAP)-overdose is not dependent on NADPH oxidase
- Human PMNs do not appear to contribute to acetaminophen (APAP)-induced injury
- Human PMNs have enhanced activation during the resolution of liver injury after APAP

Figure 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 μ g/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 (50 $\times$ magnification) are shown (C). TUNEL staining (100 $\times$ 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)

Figure 2.

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 (200× magnification). Initially neutrophils could be observed in healthy and injured areas at 6 h and 12h. 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 (200× magnification) (D). *P<0.05 compared to 0 h control. (n = 4-7 mice per group)

Figure 3.

Activation of peripheral blood and hepatic neutrophils following APAP overdose. The activation status of neutrophils (Gr-1^{high} population) was determined by surface CD11b expression and PMA-induced reactive oxygen production. Gr-1^{high} (upper quadrants) represent neutrophils and a shift to the right indicates enhanced CD11b expression as shown in peripheral blood neutrophils from representative control-, 24h APAP- and LPS-treated mice (A). Similarly, for neutrophil ROS production Gr-1^{high} (upper quadrants) were gated and a shift to the right indicates enhanced ROS production as determined by DHR123 to R123 conversion in peripheral blood neutrophils from representative control-, 24h APAP- and LPS-treated mice (B). 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 (C). 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 (D). Peripheral blood neutrophils show ROS priming at 24 h and 48 h (E). Interestingly, no ROS priming was ever seen in liver neutrophils following APAP overdose (F). *P<0.05 compared to 0 h control (for activation status only, ALT shown in Fig. 1) (n = 4-7 mice per group)

Figure 4.

Injury in gp91^{-/-} 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 (50× magnification) are shown (C). *P<0.05 compared to 0 h genotype control. (n = 3-4 mice per group)

Figure 5.

Neutrophil recruitment and hepatocyte 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 within 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)

Figure 6.

Gating and activation of human granulocytes (neutrophils) in APAP overdose patients. APAP overdose patients had serial blood draws while admitted to the hospital for determination of neutrophil activation status. Peripheral blood was used for determination of multiple neutrophil activation markers. Neutrophils were gated by forward and side scatter properties (A). Changes in the ability of neutrophils to phagocytize FITC-labeled bacteria were determined. Quantification of increased phagocytosis (right shift) was shown in a representative surviving patient from day one to day four (B). Similarly, an increase in PMA-induced ROS production in neutrophils could be observed in the same representative surviving patient (C).

Figure 7.

Neutrophil function in representative human APAP overdose patients.

Full time courses for three representative patients are shown. Progression and resolution of liver injury is presented as plasma ALT. Gating on the neutrophils, the activation status was determined by CD11b expression, rate of phagocytosis of FITC-labeled *E. coli*, and PMA- and *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 to 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.

Figure 8.

Comparison of neutrophil function in human APAP overdose patients at the peak of injury and during liver recovery.

Fourteen 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 neutrophil 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) Enhanced phagocytosis of FITC-labeled opsonized E. coli was seen post-injury. Post-injury data shown were measured at 72 h (n = 9) and 96 h (n = 5). *P<0.05 as determined by paired-Student's t-test.

Patient Information

Table 1

	<i>n</i> = 14		
	Median (range)	Sex (% Female)	Survival (%)
Age	44 (19 – 65)	64	100
Peak ALT (U/L)	2,851 (842 – 5,365)		
Peak PT (s)	41.3 (14.0 – 121.8)		
Peak Bilirubin (mg/dL) [†]	5.3 (1.6 – 9.1)		

ALT = Alanine aminotransferase. PT = Prothrombin time.

[†]When available.