rpth
research & practice
in thrombosis & haemostasis

ORIGINAL ARTICLE

Neutrophil-dependent hepatic platelet accumulation and liver injury revealed by acetaminophen dose-response studies

Anthony Schulte[1] | Dafna J. Groeneveld[1] 🐦 | Zimu Wei[1] 🐦 | Bianca Hazel[2] |
Matthew P. Bernard[3] | Lauren G. Poole[2] 🐦 | James P. Luyendyk[1,3] 🐦

[1]Department of Pathobiology & Diagnostic Investigation, Michigan State University, East Lansing, Michigan, USA

[2]Department of Pharmacology, Robert Wood Johnson Medical School, Rutgers University, Piscataway, New Jersey, USA

[3]Department of Pharmacology & Toxicology, Michigan State University, East Lansing, Michigan, USA

Correspondence
James P. Luyendyk, Department of Pathobiology & Diagnostic Investigation, Michigan State University, 253 Food Safety and Toxicology Building, 1129 Farm Lane, East Lansing, MI 48824, USA.
Email: luyendyk@msu.edu

Handling Editor: Dr Michael Makris

Abstract

Background: Acetaminophen (APAP) overdose is a leading cause of drug-induced acute liver failure (ALF). Neutrophil activation has been associated with poor outcomes in patients with ALF and is proposed to amplify coagulation in this context. However, the precise role of neutrophils in APAP-induced liver injury is not known.

Methods: We used a dual antibody–mediated neutrophil depletion strategy to determine the role of neutrophils in mice challenged with different doses of APAP (300 or 600 mg/kg) that produce hepatotoxicity and ALF-like pathology.

Results: Flow cytometry confirmed depletion of neutrophils in whole blood prior to APAP challenge. Mice given isotype control and challenged with 300 mg/kg APAP developed marked hepatocellular necrosis and showed an increase in biomarkers of coagulation cascade activation. Neutrophil depletion (anti-Ly6G) did not affect either liver injury or coagulation activation in mice challenged with 300 mg/kg APAP. Mice given isotype control and challenged with 600 mg/kg APAP developed hepatic necrosis alongside marked hemorrhage and congestion indicative of vascular injury. Interestingly, hepatic neutrophil and platelet accumulation were increased in mice given 600 mg/kg APAP compared with those given the lower APAP dose. Neutrophil depletion significantly reduced the severity of liver necrosis in mice challenged with 600 mg/kg APAP, without significantly impacting biomarkers of coagulation activity. Notably, neutrophil depletion significantly reduced hepatic platelet accumulation in mice challenged with 600 mg/kg APAP.

Conclusion: The results indicate a role of neutrophils in APAP-induced liver injury that is dependent on the APAP dose and suggest involvement of neutrophil-platelet interactions in promoting hepatic injury in experimental APAP-induced ALF.

KEYWORDS
blood coagulation, chemical and drug induced liver injury, mice, neutrophils

Essentials

- Acetaminophen (APAP) leads to dose-dependent liver injury in mice.
- We defined the role of neutrophils in liver injury induced by different doses of APAP.
- Neutrophils promote liver injury at APAP doses that cause acute liver failure.
- Neutrophils promote hepatic platelet accumulation in mice with APAP-induced acute liver failure.

1 | INTRODUCTION

Acute liver failure (ALF) is associated with changes in plasma levels of both pro- and anticoagulant proteins in plasma [1]. Coagulation cascade activation is also evident in patients with ALF [2], a major cause of which is overdose of the common analgesic acetaminophen (APAP) [3]. Standard APAP overdose in mice (eg, 300 mg/kg) adequately models the clinical course of most APAP overdose in patients by producing acute hepatotoxicity, followed by robust liver repair [4], and this is associated with coagulation cascade activation [5,6]. Notably, recent studies found that doses of APAP (ie, 600 mg/kg) that produce indicators of ALF in mice [4] also trigger a marked coagulopathy not evident in simple APAP-induced hepatotoxicity [7].

It is increasingly appreciated that the mechanisms driving APAP-induced liver damage change with the intensity of the challenge (ie, the dose). For example, there is no conclusive evidence that neutrophils contribute to liver injury in mice challenged with 300 mg/kg APAP [8]. In contrast, hepatic neutrophil accumulation is exaggerated in mice challenged with a larger dose of APAP (600 mg/kg), and neutralization of the neutrophil chemokine macrophage inflammatory protein-2 reduced APAP-induced liver injury in mice challenged with 600 mg/kg APAP, but not 300 mg/kg APAP [9]. Interestingly, a recent study observed that myeloperoxidase (MPO)-DNA complexes, biomarkers of neutrophil extracellular traps (NETs), were associated with poor outcomes in patients with ALF, and their role in NET-dependent coagulation was suggested [10]. These studies suggest that a pathologic role of neutrophils emerges in the context of ALF. However, the connections between neutrophils and coagulation have not been explored in experimental APAP-induced liver injury.

Prior studies have routinely used neutrophil depletion as an approach to define the role of these cells in APAP-induced liver injury. The results obtained are mixed, explained in part by lack of antibody specificity for neutrophil epitopes, challenges in confirming neutrophil depletion, and indirect effects of neutrophil depletion that may impact APAP hepatotoxicity [8,11]. Here, we sought to define the contribution of neutrophils to liver injury and coagulation in mice with APAP hepatotoxicity (ie, 300 mg/kg) and APAP-induced ALF (ie, 600 mg/kg) using a recently described dual-antibody approach that induces robust and specific neutrophil depletion [12].

2 | METHODS

2.1 | Mice

Male C57Bl/6J wild-type mice between the ages of 8 and 10 weeks were purchased from The Jackson Laboratory. Mice were provided standard rodent diet (Teklad 2918) and drinking water *ab libitum* and were housed under a 12-hour light/dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University (MSU).

2.2 | Neutrophil depletion and detection of neutrophils and platelets in blood

A dual-antibody strategy was used to induce durable neutrophil depletion in blood, as described previously [12]. Twenty-four hours prior to APAP challenge, mice were treated with 50 μg of anti-Ly6G isotype antibody (clone 1A8; BioXCell, #BE0075-1) or 50 μg isotype control antibody (clone QA3; BioXCell, #BE0089) via intraperitoneal (i.p.) injection with saline as the vehicle. Two hours later, the mice were treated with 100 μg of antirat IgGk (clone MTA, #BE0122, i.p., saline as vehicle). Whole blood was collected from the retro-orbital venous plexus into a heparinized microcapillary tube and an EDTA-spray coated tube 22 hours after anti-Ly6G antibody administration to confirm neutrophil depletion by flow cytometry. Red blood cells in 50 μL of blood were lysed using RBC Lysis Buffer (BioLegend). Following Fc receptor blocking, blood was incubated with rat anti-CD11b, anti-F4/80, anti-Ly6C, anti-CD45, and anti-CD115 conjugated to unique fluorophores and 4',6-diamidino-2-phenylindole to assess viability (Supplementary Table S1). Cells were analyzed on a Cytek Aurora Spectral flow cytometer (Cytek Biosciences) in the MSU Flow Cytometry Core Facility. Neutrophil (SSC^{high} CD11b$^+$ F4/80$^-$ CD115$^-$ Ly6c^{mid}) percentage was calculated as a percentage of live CD45$^+$ leukocytes (gating strategy is outlined in Supplementary Figure S1). Flow cytometric analysis was performed using FCS Express (De Novo Software, version 7.18). In some studies, segmented neutrophil (manual) and platelet (automated) counts in EDTA-anticoagulated whole blood were determined by the MSU Veterinary Diagnostic Lab (https://cvm.msu.edu/vdl).

2.3 | Neutrophil and platelet immunohistochemistry

Neutrophil immunohistochemistry was performed on formalin-fixed paraffin-embedded liver sections using an anti-Ly6G antibody (clone 1A8; BioXCell, #BE0075-1) and rat-on-mouse HRP (Biocare Medical) by the MSU Investigative Histopathology Laboratory. Platelet immunohistochemistry was performed on formalin-fixed paraffin-embedded liver sections after heat retrieval at pH 9.0 using a recombinant rabbit monoclonal anti-CD41 antibody (Abcam, [EPR17876], ab181582) and rabbit on rodent HRP Polymer (Biocare Medical) incubated for 30

minutes. Reaction development used the Romulin AEC Kit (Biocare Medical). Quantification of CD41 and neutrophil (Ly6G) labeling was performed using QuPath (version 0.4.3) [13] by assigning and thresholding 3,3′-diaminobenzidine color and quantifying positive pixels occupied by 3,3′-diaminobenzidine labeling within the entire left lateral lobe (ie, approximately 120 mm^2).

2.4 | Acetaminophen challenge and quantification of liver injury

Male mice were fasted overnight (12-16 hours) prior to administration of 300 mg/kg or 600 mg/kg APAP (Sigma Aldrich, A7085, i.p., 10 mg/mL in warm sterile saline). This experimental setting and the use of male mice were selected following guidance from a prior study characterizing responses to each dose of APAP in male mice [4]. Twenty-four hours after the APAP challenge, liver and plasma were collected under deep surgical isoflurane anesthesia. Blood was collected from the vena cava into sodium citrate (final, 0.38%) and centrifuged at 4000 ×g for 10 minutes. Liver pieces were snap-frozen and stored at −80 °C, and the left lateral lobe was fixed in 10% formalin for 4 days. Formalin-fixed liver sections were stained with hematoxylin and eosin by MSU Investigative Histopathology Laboratory, and the area of hepatocellular necrosis was quantified as described previously [14]. Serum alanine-transaminase (ALT) activity was measured using commercial reagents (Pointe Scientific ALT, SGPT).

2.5 | RNA isolation and quantitative polymerase chain reaction

Frozen liver tissue (∼100 mg) was homogenized in TRI reagent (Molecular Research Center), and RNA was isolated using Direct-zol RNA MiniPrep Kit (Zymo Research). RNA (1 μg) was then converted to cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Ribolock RNase inhibitor from Thermo Fisher Scientific). Primers from Integrated DNA Technologies were designed to measure messenger RNA expression of metallothionein 1 (Mt1), metallothionein 2 (Mt2), GAPDH, and HPRT (Supplementary Table S2).

2.6 | Measurement of coagulation biomarkers

Plasma thrombin-antithrombin (TAT) concentration was measured using a commercial enzyme-linked immunosorbent assay (Enzygnost TAT Micro Kit, Siemens Healthcare Diagnostics). Plasma fibrinogen concentration was determined by enzyme-linked immunosorbent assay, as previously described [7] using 2 distinct polyclonal antifibrinogen antibodies. Hepatic fibrin(ogen) levels were determined in insoluble protein extracts, and the levels of α, β, and γ polypeptides were quantified using capillary Western blotting, as described previously [15] (Wes

platform, ProteinSimple) using antibodies selective for each fibrinogen chain (20645-1-AP, 16747-1-AP, 15841-1-AP, respectively) [16].

2.7 | Western blotting

Snap-frozen liver was homogenized in physiological lysis buffer with protease inhibitors, as described previously [17]. Soluble proteins were subjected to reducing conditions in 1× Laemmli Sample Buffer (Bio-Rad) containing 2.5% 2-mercaptoethanol and heat denatured at 95 °C for 10 minutes prior to separation on precast Criterion 4% to 12% Bis-Tris Protein Gels (Bio-Rad) in 1× XT MOPS Running Buffer (Bio-Rad) for CD41 detection. CD41 was detected with a recombinant rabbit monoclonal anti-CD41 antibody (Abcam, [EPR17876], ab181582). For detection of citrullinated histone H3 (H3-Cit) in citrated plasma samples, plasma was diluted 4× with Novex Tricine SDS Sample Buffer (1× final concentration) with NuPAGE Reducing Agent (Thermo Fisher Scientific). Samples were heat denatured at 85 °C for 2 minutes and then separated on a 10% Novex Tricine Gel (Thermo Fisher Scientific) and transferred to a polyvinylidene fluoride membrane. H3-Cit was detected using recombinant rabbit monoclonal anti-H3-Cit antibody (ERP20358-13) (Ab219406). HRP-conjugated goat antirabbit secondary antibody was used for detection, and Eco-Bright West Pico Reagent (Innovative Solutions) chemiluminescence was detected using an iBright instrument (Thermo Fisher Scientific) and no-stain total protein reagent (Thermo Fisher Scientific). Molecular weight was determined using PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Fisher Scientific).

2.8 | Statistical analysis

Comparison of 2 groups was performed using Student's t-test. Comparison of 3 or more groups was performed using two-way analysis of variance and Tukey post hoc test. Results were log-transformed if not normally distributed. Differences were considered significant when the P value was <.05. Analysis was performed using GraphPad Prism (version 9, GraphPad Software).

3 | RESULTS

3.1 | Effective depletion of neutrophils in mice using a dual-antibody approach

A prior study described an approach to deplete neutrophils specifically and efficiently in C57Bl/6J mice [12]. We treated mice with rat anti-mouse Ly6G IgG (clone 1A8) or isotype control (BioXCell, 50 μg, i.p.) and then 2 hours later with antirat kappa immunoglobulin light chain (clone MAR 18.5, BioXCell, 100 μg i.p.). Neutrophil depletion was confirmed by flow cytometry in EDTA-anticoagulated whole blood collected retro-orbitally 22 hours after administration of the anti-mouse Ly6G antibody. Neutrophils, defined as SSC^{high} CD11b$^+$ F4/

A

B

C

D
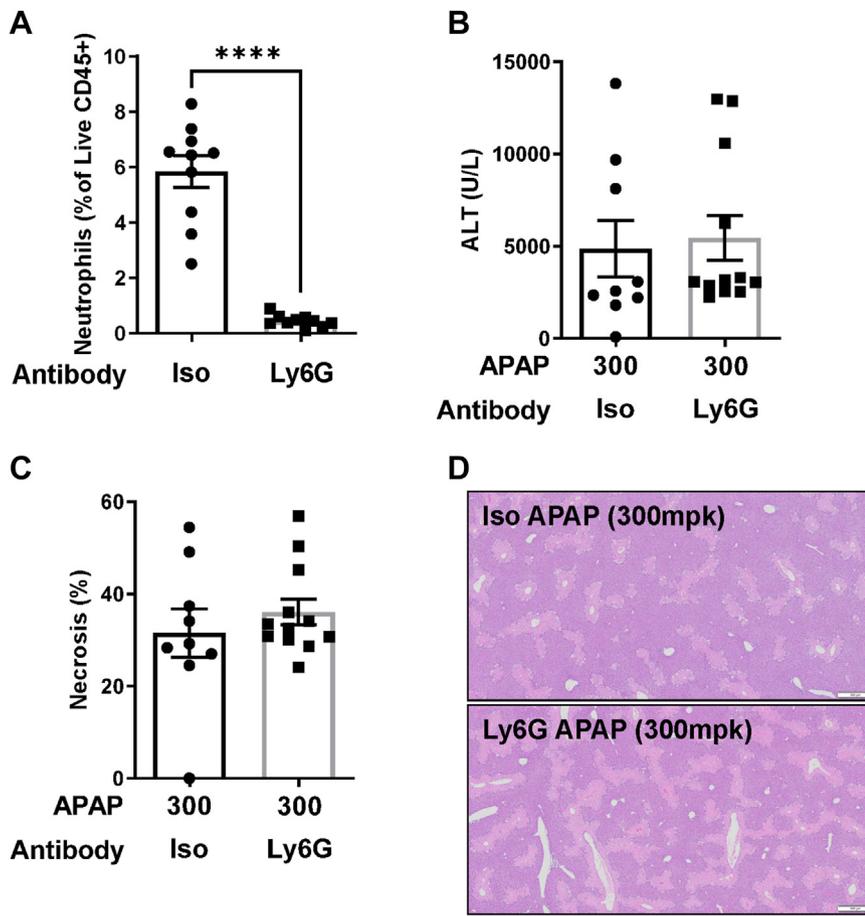
FIGURE 1 Confirmation of neutrophil depletion using flow cytometry and impact of neutrophil depletion on liver injury induced by 300 mg/kg acetaminophen (APAP) challenge. Male C57Bl/6J mice were treated with 50 μg of anti-Ly6G or isotype control antibody and then 2 hours later with 100 μg of anti-rat IgGk. (A) Anticoagulated whole blood was collected 22 hours after anti-Ly6G antibody administration to confirm neutrophil (SSC^{high} CD11b$^+$ F4/80$^-$ CD115$^-$ Ly6c^{mid}) depletion by flow cytometry. Neutrophils, determined as a percentage of singlet, live, CD45$^+$ cells, are shown (A). Mice were challenged with 300 mg/kg APAP 24 hours after administration of anti-Ly6G antibody. Twenty-four hours after APAP challenge, (B) plasma alanine-transaminase (ALT) activity and (C) hepatic necrosis were determined (see Methods). (D) Representative photomicrographs depicting hepatocellular necrosis in hematoxylin and eosin–stained liver sections. N = 9 to 12 mice per group. Data points for individual mice are shown, and the results are expressed as mean ± SEM. ****$P < .001$.

80$^-$ CD115$^-$ Ly6c^{mid} events, were calculated as a percentage of live CD45$^+$ leukocytes (Figure 1A). Similar results were obtained by manual quantification of segmented neutrophils in blood smears (Supplementary Figure S2A). These results confirm that sequential administration of antibodies in the previously published method [12] induces robust neutrophil depletion.

3.2 | Neutrophil depletion does not affect hepatotoxicity, coagulation activation, or hepatic platelet accumulation induced by 300 mg/kg APAP challenge

Hepatocellular injury induced by APAP challenge (300 mg/kg) was not affected by neutrophil depletion, indicated by similar plasma ALT activity (Figure 1B) and area of hepatocellular necrosis (Figure 1C, D) in mice after pretreatment with either isotype control or anti-Ly6G antibody. Next, the impact of neutrophil depletion on coagulation activation was determined after APAP challenge. Notably, plasma TAT complexes were elevated in APAP-challenged mice (normal concentration, ~5-10 ng/mL), but this was not affected by neutrophil depletion (Figure 2A). Plasma fibrinogen concentration after APAP challenge was unaffected by neutrophil depletion (Figure 2B). Hepatic deposition of fibrin(ogen) is a hallmark of APAP-induced liver injury [6]. Neutrophil depletion did not affect insoluble hepatic fibrin(ogen)

accumulation (Figure 2C) or accumulation of high-molecular-weight crosslinked fibrin(ogen) complexes (Figure 2D, E) in the APAP (300 mg/kg)-injured liver. Multiple studies report that hepatic platelet accumulation accompanies APAP-induced hepatotoxicity. Interactions between neutrophils and platelets can exacerbate tissue injury in some contexts [18,19], and hepatic platelet accumulation promotes APAP (300 mg/kg)-induced liver injury [20–22]. Notably, hepatic CD41 levels, reflecting hepatic platelet accumulation, were unaffected by neutrophil depletion in mice challenged with 300 mg/kg APAP (Figure 2F and Supplementary Figure S3). The results indicate that neutrophils do not contribute to acute liver injury and related changes in coagulation and platelet accumulation in mice challenged with a standard hepatotoxic dose of APAP.

3.3 | Neutrophils contribute to liver injury after challenge with 600 mg/kg APAP

Our laboratory recently uncovered dramatic hemostatic changes and coagulopathy associated with high-dose (600 mg/kg) APAP challenge, not recapitulated at lower doses [7]. Recent studies in patients with ALF linked neutrophil-derived mediators to poor outcomes [10]. Therefore, in a separate experiment, mice were challenged with 300 mg/kg or 600 mg/kg APAP following neutrophil depletion. Liver sections from APAP-challenged mice treated with

FIGURE 2 Impact of neutrophil depletion on coagulation in mice challenged with 300 mg/kg acetaminophen (APAP). Male C57Bl/6J mice were treated with 50 µg of anti-Ly6G or isotype control antibody and then 2 hours later with 100 µg of anti-rat IgGk. Mice were challenged with 300 mg/kg APAP 24 hours after administration of anti-Ly6G antibody. Twenty-four hours after APAP challenge, (A) plasma thrombin-antithrombin (TAT) complexes, (B) plasma fibrinogen, (C) hepatic insoluble fibrinogen (Fib)β chain, and (D) high-molecular-weight (HMW) crosslinked insoluble Fibα (>250 kD) chain and (E) Fibγ chain (>100 kD) levels were determined (see Methods). (F) Hepatic CD41 levels were measured using Western blot (chemiluminescence, film), and total protein (Revert 700, Licor) was quantified and expressed as a ratio. N = 8 to 12 mice per group. Data points for individual mice are shown, and the results are expressed as mean ± SEM.
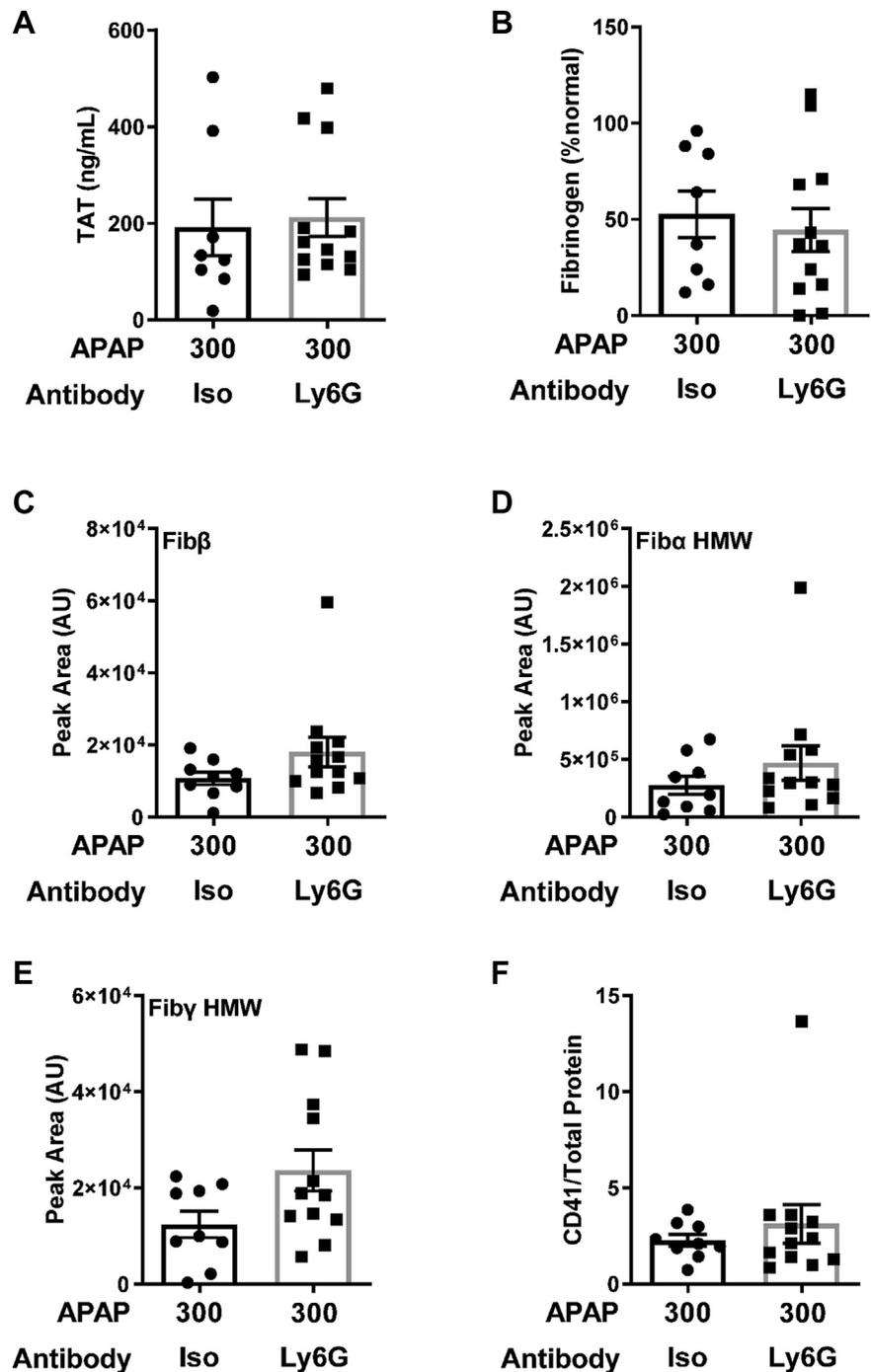

the isotype antibody were stained with the anti-Ly6G antibody, and neutrophil accumulation in the livers of mice challenged with 600 mg/kg APAP increased (P = .069) compared with mice challenged with 300 mg/kg APAP (Figure 3A, B), in agreement with recent studies [9]. Neutrophil depletion did not impact liver injury in mice challenged with 300 mg/kg APAP (Figure 3C, E), which is in agreement with the initial *in vivo* studies (Figure 1). Interestingly, at the 600 mg/kg APAP dose, plasma ALT activity was reduced (Figure 3C) in mice subjected to anti-Ly6G antibody neutrophil depletion compared with the isotype control treatment. Although not statistically significant (P = .06), these findings were further supported by a significant reduction in liver necrosis observed in

neutrophil-depleted mice challenged with 600 mg/kg APAP (Figure 3D, E). These results indicate that neutrophils contribute to liver injury in an experimental setting of high-dose APAP-induced ALF (ie, 600 mg/kg APAP challenge).

3.4 | Neutrophil depletion does not impact coagulation activation in mice challenged with 600 mg/kg APAP

Administration of a direct thrombin inhibitor reduced liver damage in mice challenged with 600 mg/kg APAP [7]. Neutrophils are linked to
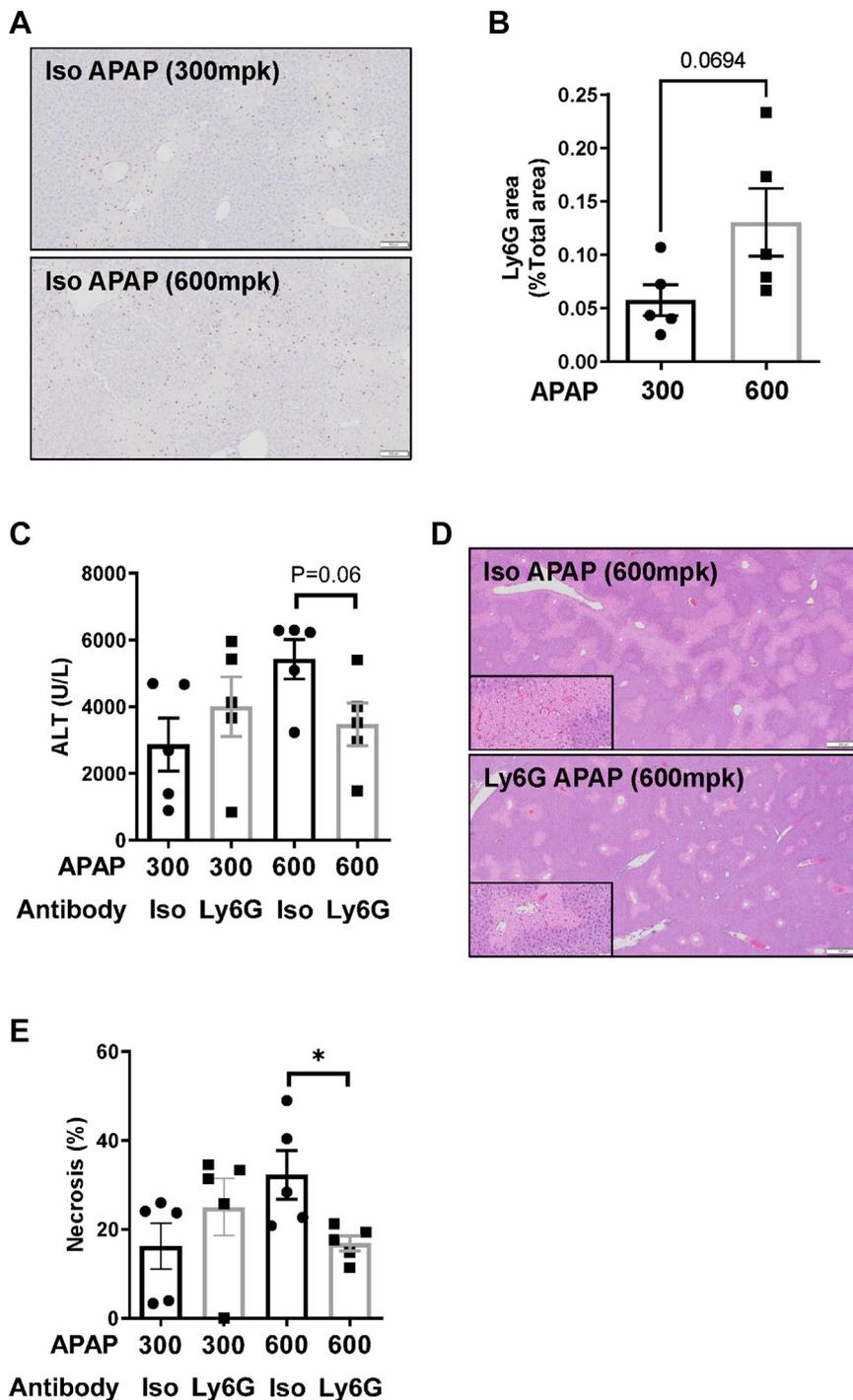
FIGURE 3 Impact of neutrophil depletion on liver injury in mice challenged with different doses of acetaminophen (APAP). Male C57Bl/6J mice were treated with 50 μg of anti-Ly6G or isotype control antibody and then 2 hours later with 100 μg of anti-rat IgGk. Mice were challenged with 300 or 600 mg/kg APAP 24 hours after administration of anti-Ly6G antibody. Liver and plasma samples were collected 24 hours after APAP challenge. (A) Representative photomicrographs showing liver sections stained for neutrophils (Ly6G) and (B) quantification of Ly6G area, expressed as a percentage of positive area to total area. (C) Plasma alanine aminotransferase (ALT) and (E) area of hepatic necrosis were determined as described (see Methods). (D) Representative photomicrographs depicting hepatocellular necrosis in hematoxylin and eosin–stained liver sections. N = 5 mice per group. Data points for individual mice are shown, and the results are expressed as mean ± SEM.

coagulation cascade activation and are proposed to contribute to pathology in ALF by driving coagulation activation [10,23]. Thus, we determined if neutrophils contribute to exaggerated coagulation in mice challenged with 600 mg/kg APAP. Neutrophil depletion did not have a statistically significant effect on plasma TAT complex concentration (ie, biomarker of thrombin generation) or fibrinogen concentration in mice challenged with either dose of APAP (Figure 4A, B). Moreover, hepatic insoluble fibrin(ogen) accumulation and formation of crosslinked high-molecular-weight fibrin(ogen) were not impacted by neutrophil depletion (Figure 4C–E). The results indicate that neutrophils do not drive exaggerated coagulation in mice challenged with 600 mg/kg APAP.

3.5 | Impact of neutrophil depletion on hepatic platelet accumulation in mice challenged with 600 mg/kg APAP

First, we determined if hepatic platelet accumulation increases in a dose-dependent manner in APAP-challenged mice. Hepatic CD41 levels were significantly increased in mice challenged with 600 mg/kg APAP compared with those challenged with 300 mg/kg APAP (Figure 5A, B). Neutrophil depletion did not affect blood platelet count in unchallenged mice (Supplementary Figure S2B). We also used immunohistochemistry to stain liver sections for CD41 (ie, platelets).
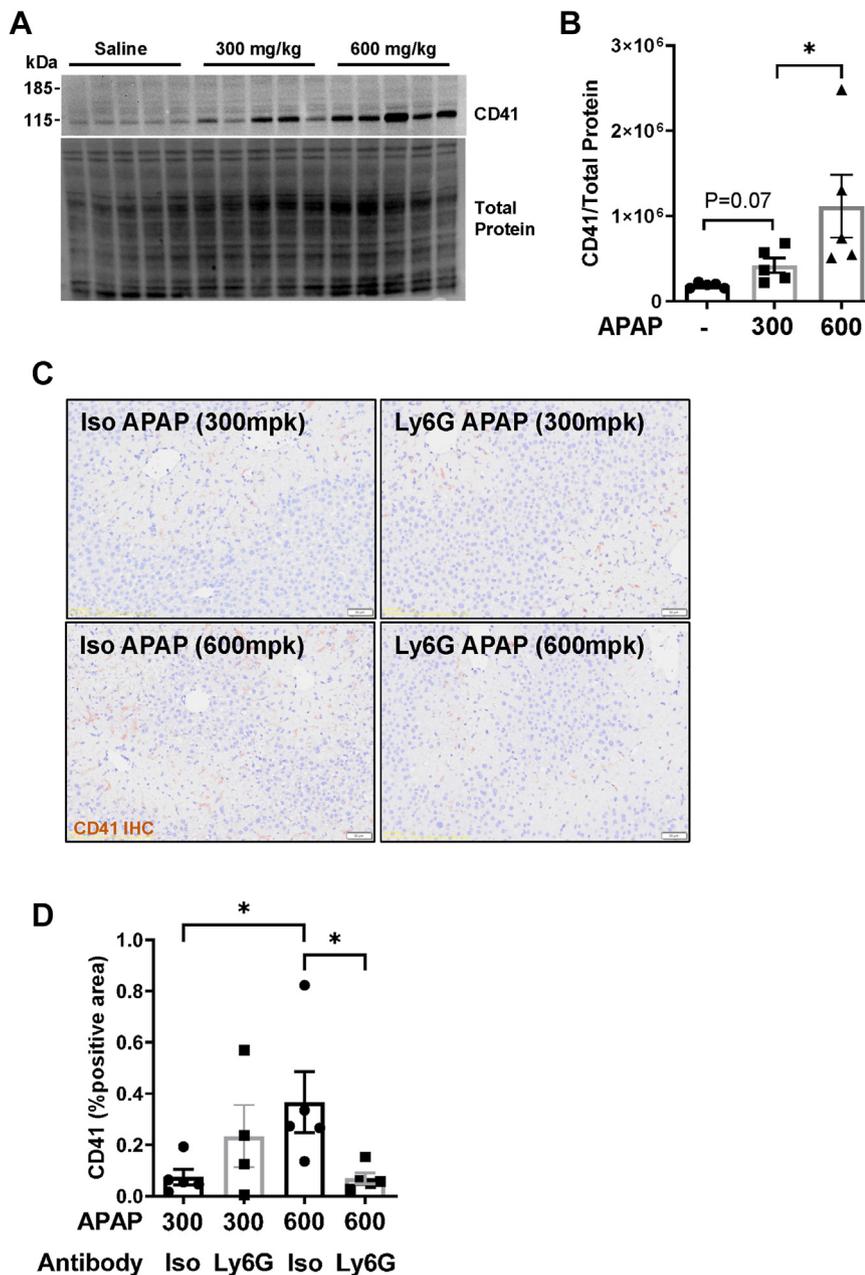
FIGURE 4 Impact of neutrophil depletion on coagulation in mice challenged with different doses of acetaminophen (APAP). Male C57Bl/6J mice were treated with 50 µg of anti-Ly6G or isotype control antibody and then 2 hours later with 100 µg of anti-rat IgGk. Mice were challenged with 300 or 600 mg/kg APAP 24 hours after administration of anti-Ly6G antibody. Twenty-four hours after APAP challenge, (A) plasma thrombin-antithrombin (TAT) complexes, (B) plasma fibrinogen, (C) hepatic insoluble fibrinogen (Fib)β chain, and (D) high-molecular-weight (HMW) crosslinked insoluble Fibα chain (>250 kD) and (E) Fibγ chain (>100 kD) levels were determined (see Methods). N = 5 mice per group. Data points for individual mice are shown, and the results are expressed as mean ± SEM. *P < .05.
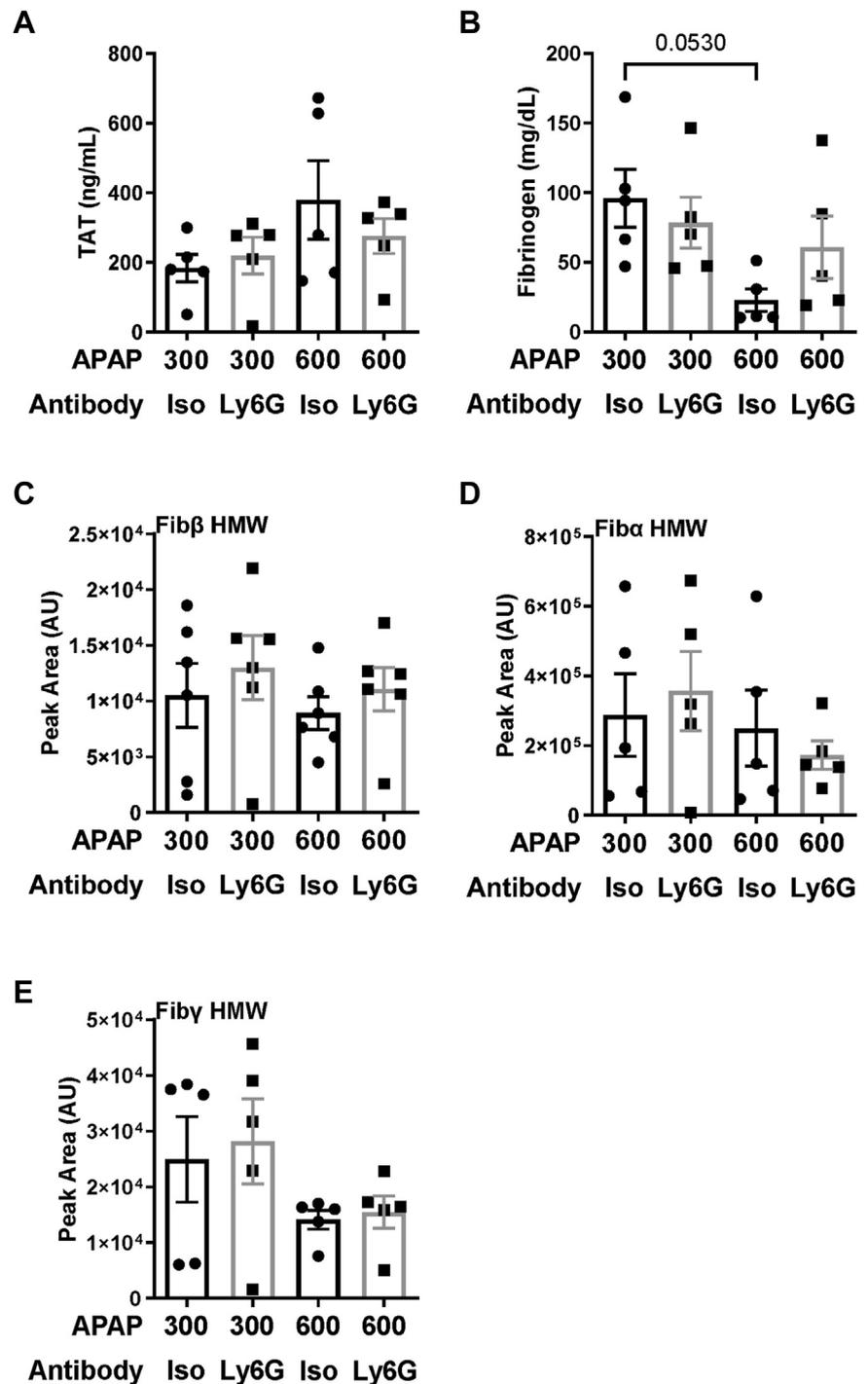
This revealed a marked increase in platelet accumulation in livers of isotype-treated mice challenged with 600 mg/kg APAP compared with isotype-treated mice given 300 mg/kg APAP. Platelets were particularly evident in the sinusoids in and around necrotic lesions (Figure 5E). Notably, hepatic platelet accumulation tended to increase in neutrophil-depleted mice challenged with 300 mg/kg APAP, whereas hepatic platelet accumulation was significantly reduced in neutrophil-depleted mice challenged with 600 mg/kg APAP (Figure 5C, D). Collectively, the results indicate that neutrophils enhance hepatic platelet accumulation at a dose of APAP that causes ALF.

4 | DISCUSSION

Although multiple prior studies have examined the role of neutrophils in APAP-induced liver injury in mice, a unified conclusion on the precise role of neutrophils is only now emerging. Indeed, the role of neutrophils in APAP-induced liver injury appears to depend on the magnitude of the challenge (ie, the dose of APAP). Neutrophils are posited to be bystanders or even to play a proresolution role at lower APAP doses [9,24]. In contrast, our findings in this study suggest that neutrophils contribute to experimental APAP-induced ALF, an

FIGURE 5 Role of neutrophils in hepatic platelet accumulation after challenge of mice with different doses of acetaminophen (APAP). Male C57Bl/6J mice were challenged with 300 or 600 mg/kg APAP or vehicle (saline), and hepatic CD41 levels were measured by Western blotting 24 hours after APAP challenge. (A) CD41 Western blot (chemiluminescence) and (B) total protein (no-stain total protein) were visualized and quantified using iBright software. $N = 5$ mice per group. (C and D) Male wild-type mice were treated with 50 µg of anti-Ly6G or isotype control antibody and then 2 hours later with 100 µg of anti-rat IgGk. Mice were challenged with 300 or 600 mg/kg APAP 24 hours after administration of anti-Ly6G antibody. (C) Representative photomicrographs showing immunohistochemical staining for CD41 (brown) that was quantified (D) using QuPath (see Methods). $N = 4$ to 5 mice per group. Data points for individual mice are shown, and the results are expressed as mean ± SEM.

observation aligned with that of other recent studies [9]. Using a recently described dual-antibody approach to magnify neutrophil depletion with an anti-Ly6G antibody [12], we observed that neutrophil depletion significantly reduced liver injury induced by 600 mg/kg APAP without impacting liver damage induced by a lower dose of APAP (ie, 300 mg/kg). This result fully aligns with observations from published studies using both male and female mice deficient in the neutrophil chemokine macrophage inflammatory protein-2 [9]. The results corroborate prior studies [9] and strongly suggest that neutrophils play a pathologic role in experimental liver injury in doses of APAP that produce ALF-like features in mice [4]. The precise mechanisms driving neutrophils to acquire a proinjury role at larger doses of APAP require additional experimentation but may relate simply to increased chemokine induction at larger APAP doses.

Multiple studies have used antibody-mediated depletion strategies to define the role of neutrophils in APAP-induced liver injury [24]. There are unique caveats and challenges to each approach, and our approach sought to address these challenges. Initial studies used an anti-Ly6C/Ly6G antibody (Gr-1) [25] that lacks specificity for neutrophils, as monocytes are also targeted with this strategy [26]. Administration of the Gr-1 antibody also increases hepatic expression of Mt [11], which reduces APAP hepatotoxicity [27]. Use of an anti-Ly6G antibody confers neutrophil selectivity and, paired with an antirat IgGk antibody, induces durable and more specific neutrophil depletion [12]. Using this approach, we observed robust neutrophil depletion in the absence of hepatic induction of Mt1 or Mt2 expression (Supplementary Figure S2). It is possible that the anti-Ly6G antibody elicits protective effects independent of neutrophil

depletion, such as baseline effects on coagulation, complement consumption, etc. These changes would be likely to reduce liver injury at both doses of APAP, but we did not observe an effect of neutrophil depletion on liver injury in mice challenged with 300 mg/kg APAP. Additionally, the same anti-Ly6G antibody clone used to deplete neutrophils is often used in assays to confirm efficacy of neutrophil depletion, with epitope masking potentially being confused for complete neutrophil depletion [12]. To circumvent this potential issue, we used established flow cytometric analysis gating strategies (SSC^{high} granulocyte population gated as CD11b$^+$ F4/80$^-$ CD115$^-$ Ly6c^{mid} surface expression phenotype) that avoid the use of anti-Ly6G antibodies for neutrophil detection [12] to confirm neutrophil depletion prior to APAP challenge. Our results highlight the utility of a novel dual antibody–mediated depletion approach to evaluate mechanisms of liver injury.

Mice challenged with 600 mg/kg APAP exhibit hepatic necrosis alongside evidence of hepatic dysfunction, failed repair, profound cytokine elevation, and indicators of hepatic encephalopathy [4,7,28,29]. Moreover, challenging mice with 600 mg/kg APAP induces more robust hepatic neutrophil accumulation and coagulation cascade activation [7,9]. Notably, recent studies suggesting a link between neutrophils (ie, NETs) and poor outcomes in ALF hypothesized that one connection may be exaggerated activation of coagulation activity [10]. We were not able to detect an obvious neutrophil-dependent increase in H3-Cit in plasma of APAP-challenged mice (Supplementary Figure S4), in agreement with prior studies, which also did not readily detect markers of NETs in the APAP-injured liver [30,31]. Routinely used biomarkers of NET formation, including cell-free DNA and MPO-DNA complexes, may also be complicated to interpret in the context of neutrophil-dependent hepatocellular necrosis, where DNA liberated from dying hepatocytes is thought to drive hepatic injury [30]. Given the connection between MPO-DNA complexes and outcomes in patients with ALF, a potential role of NETs should not be excluded. Notably, neutrophil depletion did not significantly alter plasma concentrations of TAT or plasma fibrinogen, suggesting that neutrophils do not play a major role in driving coagulation activity after APAP challenge. However, one limitation of this experiment is that analyzing these biomarkers near peak of liver injury may miss a contribution of neutrophils to coagulation activation during development of hepatotoxicity.

Platelets accumulate in the APAP-injured liver. Several studies suggest a pathologic role of platelets in the APAP-injured liver, and thrombocytopenia predicts poor outcomes in patients with ALF [20–22,32]. Notably, there are multiple mechanisms driving platelet-neutrophil interaction, and the formation of these cellular complexes has been shown to be an important driver of thromboinflammation [18]. We found that hepatic platelet accumulation was significantly increased in livers of mice challenged with 600 mg/kg APAP compared with those of mice challenged with lower dose of APAP and that hepatic platelet accumulation was reduced by neutrophil depletion. This suggests that neutrophils play a key role in promoting hepatic platelet accumulation in experimental APAP-induced ALF. Extensive interaction between neutrophils and platelets has been identified [33], and it is conceivable that each cell type reciprocally recruits the other to the APAP-injured liver. At present, it is unclear whether neutrophils contribute to the thrombocytopenia observed in mice after APAP challenge. This could be particularly intriguing and extend to observations in patients with ALF, where both MPO-DNA complexes and thrombocytopenia predict poor outcomes.

In summary, we found that antibody-mediated neutrophil depletion significantly attenuated liver damage in an experimental setting that resembles features of APAP-induced ALF in patients. Neutrophil depletion did not reduce liver injury induced by a standard hepatotoxic dose of APAP. These results agree with recent studies showing that neutrophil chemokine deficiency confers protection from APAP-induced liver injury only at large APAP doses. Notably, neutrophil depletion had minimal effect on coagulation cascade activation but substantially reduced hepatic platelet accumulation after APAP challenge. Defining the precise link between platelets and neutrophils in experimental APAP-induced ALF may uncover a mechanistic observation for clinical observations connecting these 2 cell types to outcomes in patients with ALF.

AUTHOR CONTRIBUTIONS
A.S., D.G., Z.W., B.H., M.B., L.P., and J.L. performed experiments and analyzed results. J.L. and A.S. drafted the manuscript, and all authors provided comments/edits and were aware of plans for submission.

RELATIONSHIP DISCLOSURE
A.S., D.G., Z.W., B.H., M.B., L.P., and J.L. have no relevant conflicts of interest to disclose.

TWITTER
Dafna J. Groeneveld @dafnagroeneveld
Zimu Wei @wei_zimu
Lauren G. Poole @PoolePAR_ty
James P. Luyendyk @TFFVIIa

REFERENCES

[1] Lisman T, Stravitz RT. Rebalanced hemostasis in patients with acute liver failure. *Semin Thromb Hemost.* 2015;41:468–73.

[2] Elvers FL, Stamouli M, Adelmeijer J, Jeyanesan D, Bernal W, Maas C, et al. In vivo generation of thrombin in patients with liver disease without apparent evidence of activation of the intrinsic or extrinsic pathway of coagulation. *J Thromb Haemost.* 2023;8:2078–88.

[3] Stravitz RT, Lee WM. Acute liver failure. *Lancet.* 2019;394:869–81.

[4] Bhushan B, Walesky C, Manley M, Gallagher T, Borude P, Edwards G, et al. Pro-regenerative signaling after acetaminophen-induced acute liver injury in mice identified using a novel incremental dose model. *Am J Pathol.* 2014;184:3013–25.

[5] Sullivan BP, Kopec AK, Joshi N, Cline H, Brown JA, Bishop SC, et al. Hepatocyte tissue factor activates the coagulation cascade in mice. *Blood.* 2013;121:1868–74.

[6] Ganey PE, Luyendyk JP, Newport SW, Eagle TM, Maddox JF, Mackman N, et al. Role of the coagulation system in acetaminophen-induced hepatotoxicity in mice. *Hepatology.* 2007;46:1177–86.

[7] Groeneveld DJ, Poole LG, Bouck EG, Schulte A, Wei Z, Williams KJ, et al. Robust coagulation activation and coagulopathy in mice with experimental acetaminophen-induced liver failure. *J Thromb Haemost.* 2023;21:2430–40.

[8] Jaeschke H, Ramachandran A. Mechanisms and pathophysiological significance of sterile inflammation during acetaminophen hepatotoxicity. *Food Chem Toxicol.* 2020;138:111240.

[9] Nguyen NT, Umbaugh DS, Smith S, Adelusi OB, Sanchez-Guerrero G, Ramachandran A, et al. Dose-dependent pleiotropic role of neutrophils during acetaminophen-induced liver injury in male and female mice. *Arch Toxicol.* 2023;97:1397–412.

[10] von Meijenfeldt FA, Stravitz RT, Zhang J, Adelmeijer J, Zen Y, Durkalski V, et al. Generation of neutrophil extracellular traps in patients with acute liver failure is associated with poor outcome. *Hepatology.* 2022;75:623–33.

[11] Jaeschke H, Liu J. Neutrophil depletion protects against murine acetaminophen hepatotoxicity: another perspective. *Hepatology.* 2007;45:1588–9; author reply 1589.

[12] Boivin G, Faget J, Ancey PB, Gkasti A, Mussard J, Engblom C, et al. Durable and controlled depletion of neutrophils in mice. *Nat Commun.* 2020;11:2762.

[13] Bankhead P, Loughrey MB, Fernandez JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: open source software for digital pathology image analysis. *Sci Rep.* 2017;7:16878.

[14] Pant A, Kopec AK, Baker KS, Cline-Fedewa H, Lawrence DA, Luyendyk JP. Plasminogen activator inhibitor-1 reduces tissue-type plasminogen activator-dependent fibrinolysis and intrahepatic hemorrhage in experimental acetaminophen overdose. *Am J Pathol.* 2018;188:1204–12.

[15] Poole LG, Kopec AK, Groeneveld DJ, Pant A, Baker KS, Cline-Fedewa HM, et al. Factor XIII cross-links fibrin(ogen) independent of fibrin polymerization in experimental acute liver injury. *Blood.* 2021;137:2520–31.

[16] Poole LG, Pant A, Baker KS, Kopec AK, Cline-Fedewa HM, Iismaa SE, et al. Chronic liver injury drives non-traditional intrahepatic fibrin(ogen) crosslinking via tissue transglutaminase. *J Thromb Haemost.* 2019;17:113–25.

[17] Aleman MM, Byrnes JR, Wang JG, Tran R, Lam WA, Di Paola J, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest.* 2014;124:3590–600.

[18] Mandel J, Casari M, Stepanyan M, Martyanov A, Deppermann C. Beyond hemostasis: platelet innate immune interactions and thromboinflammation. *Int J Mol Sci.* 2022;23:3868.

[19] Denorme F, Rustad JL, Campbell RA. Brothers in arms: platelets and neutrophils in ischemic stroke. *Curr Opin Hematol.* 2021;28:301–7.

[20] Shan Z, Li L, Atkins CL, Wang M, Wen Y, Jeong J, et al. Chitinase 3-like-1 contributes to acetaminophen-induced liver injury by promoting hepatic platelet recruitment. *Elife.* 2021;10:e68571. https://doi.org/10.7554/eLife.68571

[21] Miyakawa K, Joshi N, Sullivan BP, Albee R, Brandenberger C, Jaeschke H, et al. Platelets and protease-activated receptor-4 contribute to acetaminophen-induced liver injury in mice. *Blood.* 2015;126:1835–43.

[22] Chauhan A, Sheriff L, Hussain MT, Webb GJ, Patten DA, Shepherd EL, et al. The platelet receptor CLEC-2 blocks neutrophil mediated hepatic recovery in acetaminophen induced acute liver failure. *Nat Commun.* 2020;11:1939.

[23] de Bont CM, Boelens WC, Pruijn GJM. NETosis, complement, and coagulation: a triangular relationship. *Cell Mol Immunol.* 2019;16:19–27.

[24] Jaeschke H, Williams CD, Ramachandran A, Bajt ML. Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. *Liver Int.* 2012;32:8–20.

[25] Liu ZX, Han D, Gunawan B, Kaplowitz N. Neutrophil depletion protects against murine acetaminophen hepatotoxicity. *Hepatology.* 2006;43:1220–30.

[26] Fleming TJ, Fleming ML, Malek TR. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol.* 1993;151:2399–408.

[27] Saito C, Yan HM, Artigues A, Villar MT, Farhood A, Jaeschke H. Mechanism of protection by metallothionein against acetaminophen hepatotoxicity. *Toxicol Appl Pharmacol.* 2010;242:182–90.

[28] Roth K, Strickland J, Gonzalez-Pons R, Pant A, Yen TC, Freeborn R, et al. Interleukin-6 drives key pathologic outcomes in experimental acetaminophen-induced liver failure. Preprint. Posted online November 16, 2021 bioRxiv 468664. https://doi.org/10.1101/2021.11.15.468664

[29] Borude P, Bhushan B, Apte U. DNA damage response regulates initiation of liver regeneration following acetaminophen overdose. *Gene Expr.* 2018;18:115–23.

[30] Marques PE, Oliveira AG, Pereira RV, David BA, Gomides LF, Saraiva AM, et al. Hepatic DNA deposition drives drug-induced liver injury and inflammation in mice. *Hepatology.* 2015;61:348–60.

[31] Li X, Gao Q, Wu W, Hai S, Hu J, You J, et al. FGL2-MCOLN3-autophagy axis-triggered neutrophil extracellular traps exacerbate liver injury in fulminant viral hepatitis. *Cell Mol Gastroenterol Hepatol.* 2022;l14:1077–101.

[32] Stravitz RT, Ellerbe C, Durkalski V, Reuben A, Lisman T, Lee WM, et al. Thrombocytopenia is associated with multi-organ system failure in patients with acute liver failure. *Clin Gastroenterol Hepatol.* 2016;14:613–20.e4.

[33] Lisman T. Platelet-neutrophil interactions as drivers of inflammatory and thrombotic disease. *Cell Tissue Res.* 2018;371:567–76.

SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.rpth.2024.102323.