

# A Novel Resolvin-Based Strategy for Limiting Acetaminophen Hepatotoxicity

Suraj J. Patel, MD, PhD<sup>1,2,5</sup>, Jay Luther, MD<sup>3,5</sup>, Stefan Bohr, MD<sup>1</sup>, Arvin Iracheta-Vellve, BS<sup>1</sup>, Matthew Li, PhD<sup>1</sup>, Kevin R. King, MD, PhD<sup>1</sup>, Raymond T. Chung, MD<sup>3</sup> and Martin L. Yarmush, MD, PhD<sup>1,2,4</sup>

**OBJECTIVES:** Acetaminophen (APAP)-induced hepatotoxicity is a major cause of morbidity and mortality. The current pharmacologic treatment for APAP hepatotoxicity, *N*-acetyl cysteine (NAC), targets the initial metabolite-driven injury but does not directly affect the host inflammatory response. Because of this, NAC is less effective if given at later stages in the disease course. Resolvins, a novel group of lipid mediators shown to attenuate host inflammation, may be a therapeutic intervention for APAP hepatotoxicity.

**METHODS:** The temporal patterns of liver injury and neutrophil activation were investigated in a murine model of APAP hepatotoxicity. In addition, the effect of neutrophil depletion and resolvin administration on the severity of liver injury induced by APAP was studied. *In vitro* studies to investigate the mechanism of resolvin effect on hepatocyte injury and neutrophil adhesion were performed.

**RESULTS:** We demonstrate that hepatic neutrophil activation occurs secondary to the initial liver injury induced directly by APAP. We also show that neutrophil depletion attenuates APAP-induced liver injury, and administration of resolvins hours after APAP challenge not only attenuates liver injury, but also extends the therapeutic window eightfold compared to NAC. Mechanistic *in vitro* analysis highlights resolvins' ability to inhibit neutrophil attachment to endothelial cells in the presence of the reactive metabolite of APAP.

**CONCLUSIONS:** This study highlights the ability of resolvins to protect against APAP-induced liver injury and extend the therapeutic window compared to NAC. Although the mechanism for resolvin-mediated hepatoprotection is likely multifactorial, inhibition of neutrophil infiltration and activation appears to play an important role.

*Clinical and Translational Gastroenterology* (2016) 7, e153; doi:10.1038/ctg.2016.13; published online 17 March 2016

Subject Category: Liver

## INTRODUCTION

Acetaminophen (APAP) and APAP-containing products are the most commonly used antipyretic-analgesic medications worldwide. Although APAP is safe when taken at therapeutic doses in the majority of patients, overdoses of APAP can lead to significant morbidity and mortality. In fact, APAP-induced hepatotoxicity accounts for 50% of acute liver failure cases, and is the leading reason for liver transplantation for acute liver failure in the United States.<sup>1–3</sup> In addition, the risk of developing APAP hepatotoxicity is further increased in the large cohort of patients with preexisting chronic liver disease.<sup>4</sup>

The pathogenesis of APAP-induced hepatotoxicity begins with its metabolism by perivenular hepatocytes, leading to the generation of reactive metabolites such as *N*-acetyl-*p*-benzoquinone-imine (NAPQI) that directly trigger oxidative stress, mitochondrial damage, and hepatocellular injury.<sup>5–7</sup> Growing evidence suggests that as this injury propagates throughout the hepatic lobule, an exuberant host

inflammatory response is activated, resulting in hepatic neutrophil infiltration and significant collateral damage.<sup>8–13</sup> Without timely treatment, many patients develop fulminant hepatic failure and multiorgan dysfunction.

*N*-acetyl cysteine (NAC) is the only FDA-approved pharmacologic therapy for APAP hepatotoxicity.<sup>1,14</sup> However, it suffers from a limited rescue window (the time between APAP ingestion and initiation of therapy), as it targets only the initial reactive metabolite-driven injury that occurs at the earliest stage of disease pathogenesis.<sup>14,15</sup> Unfortunately, many patients are asymptomatic during this stage and do not present for treatment. As such, there is a need to better understand the complete pathogenesis of APAP hepatotoxicity with a particular emphasis on the later stages, in order to develop novel therapies that extend the rescue window to beyond that of NAC.

Immune cells play a dynamic role in activating, maintaining, and resolving inflammation at the site of tissue injury. Kupffer cells, natural killer cells, and neutrophils have all been implicated in APAP hepatotoxicity by releasing various

<sup>1</sup>Center for Engineering in Medicine and the Department of Surgery, Massachusetts General Hospital and the Shriners Burns Hospital, Boston, MA, USA; <sup>2</sup>Harvard-MIT Division of Health Science and Technology, Harvard Medical School, Massachusetts Institute of Technology, Cambridge, MA, USA; <sup>3</sup>Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA and <sup>4</sup>Department of Biomedical Engineering, Rutgers University, Piscataway, NJ, USA

Correspondence: MLYarmush, MD, PhD, Center for Engineering in Medicine and the Department of Surgery, Massachusetts General Hospital and Shriners Burns Hospital, 51 Blossom Street, Boston, MA 02114, USA. E-mail: ireis@sbi.org

<sup>5</sup>These authors contributed equally to this work.

Received 30 June 2015; accepted 28 September 2015

inflammatory mediators including cytokines, chemokines, and reactive oxygen species.<sup>8,16–18</sup> Although neutrophil recruitment into the liver and peripheral activation has been demonstrated in APAP hepatotoxicity, neutrophil contribution to the progression and severity of injury is controversial. Whereas some data show that neutrophils establish a host inflammatory response that amplifies overall liver injury, other data suggest that their activation may be a critical event for injury resolution following APAP overdose.<sup>8,19–21</sup>

Recently endogenous lipid mediators derived from omega-3 polyunsaturated fatty acids have been shown to control important events during inflammation, such as neutrophil migration, adhesion, activation, and clearance.<sup>21–23</sup> In particular, docosahexaenoic acid-derived lipid mediators, known as resolvins, regulate critical cellular events in the resolution of inflammation.<sup>21–23</sup> Resolvins are synthesized by neutrophils during the resolution phase of inflammation, and serve to block the secretion of interleukin-1 beta and tumor necrosis factor- $\alpha$ , as well as stimulate nitric oxide production, thereby reducing neutrophil adhesion to the endothelium and inhibiting neutrophil infiltration into the tissue.<sup>24,25</sup> Resolvins have also been shown to potently and specifically inhibit neutrophil chemotaxis by directly acting on circulating neutrophils.<sup>26</sup> As such, resolvins have proven protective in murine models of inflammatory bowel disease, colitis, sepsis, asthmatic airway inflammation, conjunctivitis, myocardial ischemia-reperfusion injury, and burn injury.<sup>22,27–31</sup>

In this study, we tested whether inhibition of neutrophil recruitment attenuates APAP-induced hepatotoxicity. Specifically, we hypothesized that administration of a resolvin compound, 7S, 16R, 17S-trihydroxy-4Z, 8E, 10Z, 12E, 14E, 19Z-docosahexaenoic acid (RvD2),<sup>22</sup> after APAP overdose would lessen liver injury by reducing the secondary, neutrophil-predominant sterile inflammatory phase seen with APAP hepatic injury.

## METHODS

**Animals.** C57BL/6 mice were purchased from Jackson Laboratory. Male mice between the ages of 6–8 weeks were used for all experiments. All animal protocols were approved by Massachusetts General Hospital Subcommittee on Research Animal Care. For survival experiments, animals were killed when they became moribund according to the criteria of lack of response to stimuli or lack of righting reflex. Ten animals per group were used for each experiment.

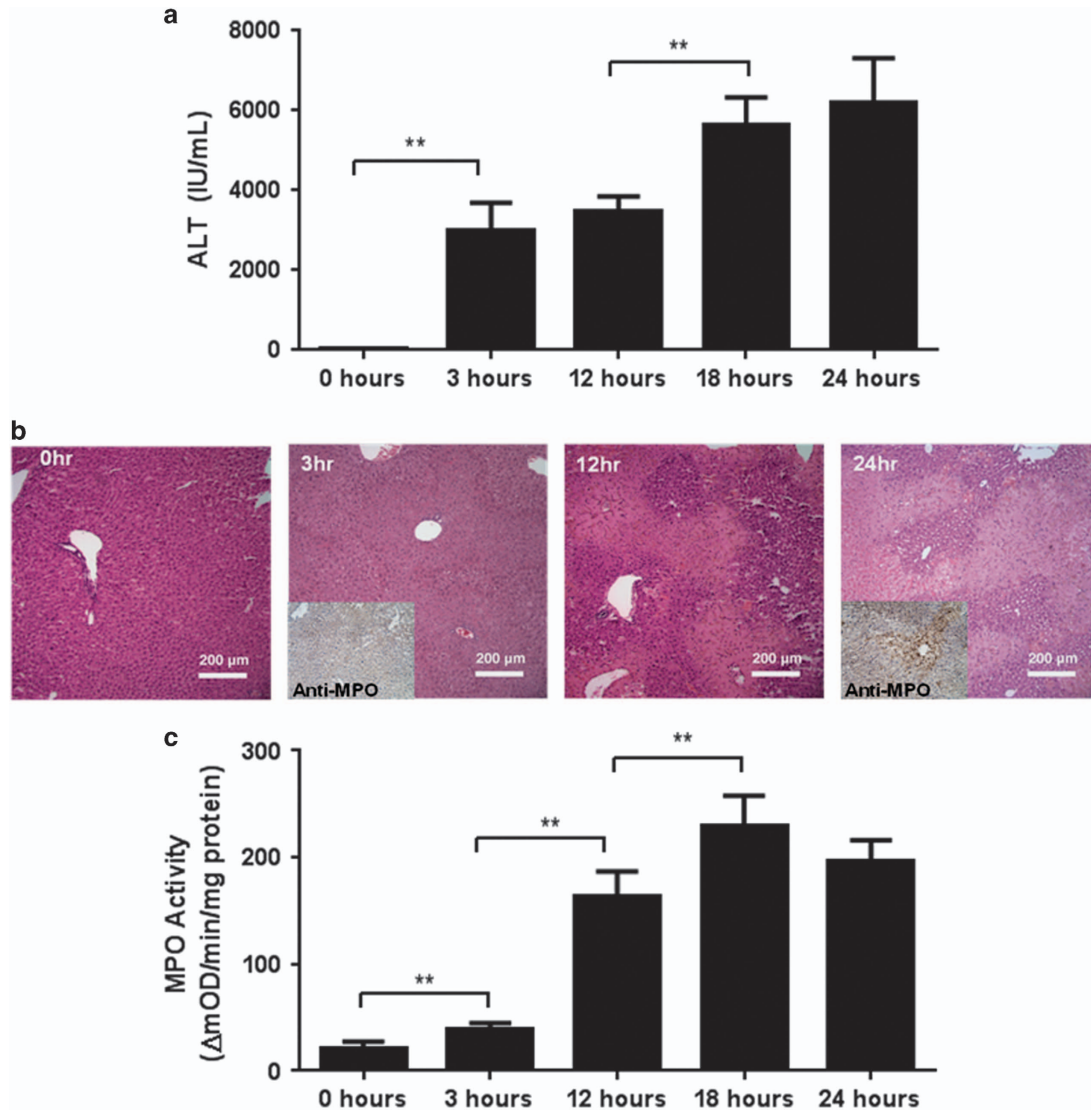
**Cell culture.** Primary rat hepatocytes were isolated and maintained as previously described.<sup>32</sup> Briefly, hepatocytes were isolated from female Lewis rats by a two-step collagenase perfusion technique. Isolation yields ranged from 200 to 300 million hepatocytes/rat with viabilities ranging from 85 to 95%, and purity was >95%. The culture medium for hepatocytes consisted of Williams E supplemented with 20 ng/ml epidermal growth factor (EGF), 14 ng/ml glucagon, 0.5 U/ml insulin, 7.5  $\mu$ g/ml hydrocortisone, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Isolated hepatocytes were stimulated in suspension (2 million cells/ml) with NAPQI (Sigma, St Louis, MO) at various concentrations (100  $\mu$ M,

250  $\mu$ M, 500  $\mu$ M, and 1,000  $\mu$ M) for 4 h, in the presence or absence of RvD2 (Cayman Chemical, Ann Arbor, MI) at 10  $\mu$ M and 100  $\mu$ M. Hepatocellular damage was assayed by centrifuging the cells and measuring alanine aminotransferase (ALT) in the supernatant using the Infinity ALT (GPT) Liquid Stable Reagent (Thermo Scientific, Middletown, VA). Cell cytotoxicity was assayed by measuring lactate dehydrogenase in the supernatant using an lactate dehydrogenase cytotoxicity Assay Kit (Cayman Chemical, Ann Arbor, MI). As a positive control, Triton X-100 was used to lyse hepatocytes (Sigma-Aldrich, St Louis, MO).

Human microvascular endothelial cells (HMVECs) (Lonza, United States) were cultured as previously described<sup>31</sup> using MCDB 131 media (Caisson Laboratories, North Logan, UT). Experiments were performed using standard phenol red free Dulbecco's modified Eagle's medium (Thermo Fisher, Waltham, MA, GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and L-glutamine (200 mM). Polymorphonuclear granulocytes (PMNs) were isolated from human peripheral blood by gradient separation using Lympholyte-poly (Cedarlane Labs, Burlington, NC) according to the manufacturer's protocol. To assay adhesion between PMNs and HMVECs *in vitro*, PMNs were co-cultured for 1 h with HMVECs in 12-well plates followed by three washing steps. Experimental pretreatment of HMVECs included NAPQI at 250  $\mu$ M for 4 h. Experimental pretreatment of PMNs included RvD2 at 1  $\mu$ M for 1 h. For image analysis, HMVEC nuclei were labeled with Hoechst 33342, PMNs with Calcein-AM stain (Life Technologies, Grand Island, NY). Images were acquired using an inverted microscope equipped with an incubation chamber (Axiovert, Zeiss, Oberkochen, Germany) and analyzed using ImageJ 1.43 u. To account for partial detachment of NAPQI-injured HMVECs, the number of adherent PMNs was normalized to the density of HMVECs. Washing steps effectively eliminated nonspecific PMN adherence to HMVEC-free areas.

**APAP-induced hepatotoxicity.** APAP (Sigma, St Louis, MO) solution was made fresh for each experiment in 0.9% saline at 20 mg/ml and heated until dissolved. APAP was dosed at 400 mg/kg, and injected intraperitoneally after 15 h of starvation. Animals were euthanized by ketamine/xylazine injection at various time points for collection of serum and liver tissue for modified colorimetric myeloperoxidase (MPO) activity assay, and histology. For survival studies, APAP was dosed at 750 mg/kg, and injected intraperitoneally after 15 h of starvation, as previously described.<sup>33</sup> These doses are higher than are seen in humans, as patients exceeding 200 mg/kg are at very high risk for APAP-induced liver injury. Serum ALT (GPT) levels were measured via chemical reaction using the Infinity ALT (GPT) Liquid Stable Reagent (Thermo Scientific, Middletown, VA).

**Myeloperoxidase assessment.** Freshly excised livers were snap-frozen and then crushed in liquid nitrogen. A MPO assay was performed on these samples. Briefly, pulverized liver tissue was added to a non-denaturing lysis buffer (TRIZMA, Sigma, 10% glycerol, and 0.2% Tween), and samples were exposed to three cycles of freeze-thawing, and then centrifuged to collect the lysate. The MPO reaction was



**Figure 1** Temporal evolution of acetaminophen hepatotoxicity. Characterization of (a) serum alanine aminotransferase (ALT) levels, (b) H&E liver histology (10× magnification) and liver tissue immunohistochemical staining for myeloperoxidase (MPO) liver tissue, and (c) MPO activity from wild-type (WT) mice ( $N=10$  mice) after acetaminophen treatment (APAP, 400 mg/kg).  $**P<0.005$ . H&E, haematoxylin and eosin.

carried out by first mixing 20  $\mu$ l of liver lysate with 20  $\mu$ l assay buffer (0.167 mg/ml *o*-dianisidine-HCL, 50 mM  $\text{Na}_2\text{HPO}_4$ , pH 5.4) in a 96-well plate, and then adding 200  $\mu$ l of development solution (0.1%  $\text{H}_2\text{O}_2$ , 50 mM  $\text{Na}_2\text{HPO}_4$ , pH 5.4). Absorbance was measured at 450 nm every 15 s. MPO activity is expressed as the change in absorbance per minute per milligram of liver lysate protein ( $\Delta\text{mOD}/\text{min}/\text{mg}$  protein). Immunohistochemical staining for MPO was also performed from excised liver tissue.

**In vivo neutrophil depletion.** Neutrophils were depleted using an antibody-mediated strategy using a method previously described.<sup>8</sup> Briefly, mice were injected intravenously with 2 mg/kg of Anti-Gr1 monoclonal antibody (Beckman Coulter, Brea, CA) followed by injection of 1 mg/kg 20 h later. Vehicle-control mice received rat IgG2b. APAP was

injected intraperitoneally 4 h after the second dose of anti-Gr1 neutrophil depleting antibody. Animals were killed by ketamine/xylazine injection at various time points for collection of serum and liver tissue for MPO activity assay and histology.

**Resolvin D2 treatment.** Resolvin D2 (Cayman Chemical, Ann Arbor, MI) was reconstituted in 0.9% saline. It was given intravenously via tail vein (25  $\mu$ g/kg) at various time points after APAP administration. This supraphysiologic dose of RVD2 was chosen to ensure optimal neutrophil interaction in this proof-of-concept study, and was based on previous investigations with RVD2.<sup>22</sup>

**N-acetylcysteine treatment.** NAC (Sigma) was made fresh for each experiment in 0.9% saline at 20 mg/ml. NAC

was dosed at 200 mg/kg, and injected intravenously in the tail vein of mice after 15 h of starvation, as previously described.<sup>33</sup>

## RESULTS

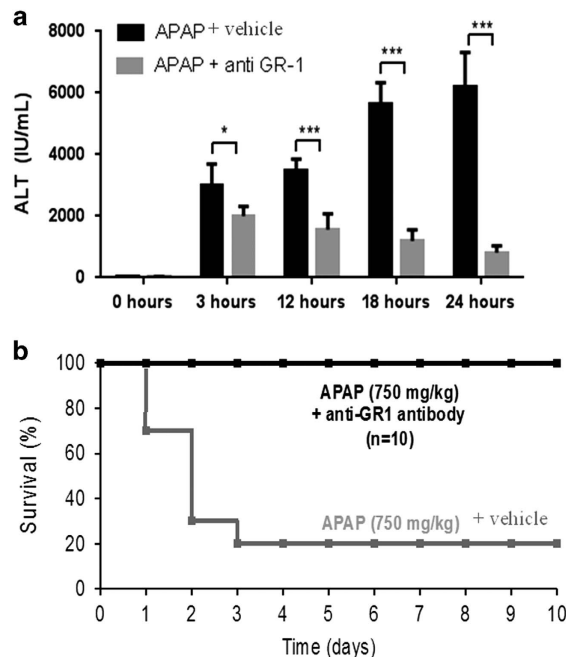
**Temporal evolution of APAP-induced hepatotoxicity.** In order to define the temporal pattern of APAP hepatotoxicity, mice were treated with a sub-lethal dose of APAP (400 mg/kg) and examined for liver injury at multiple time points following treatment. We found evidence of significant liver injury as early as 3 h after APAP administration. ALT, a serum marker for hepatocellular injury, was increased at hour 3 compared to hour 0 (3100 IU/l vs. 30 IU/l,  $***P < 0.001$ ). Evidence for ongoing liver injury in the form of rising ALT persisted until termination of the experiment at hour 24 (Figure 1a). In conjunction with ALT, histological evaluation of liver injury revealed progressively worsening inflammation, hemorrhage, and hepatocyte necrosis through the course of the experiment (Figure 1b). Taken together, these results show continuing liver injury up to 24 h after APAP administration.

The temporal pattern of APAP-induced hepatic neutrophil infiltration was then evaluated using hepatic MPO activity as a surrogate for neutrophil activation. We observed an increase in neutrophil activation at 3 h following APAP administration. However, we observed a much sharper rise in hepatic neutrophil infiltration 12 h following APAP administration (Figure 1b, c).

**Neutrophil depletion attenuates APAP-induced liver injury.** Mice were pretreated with an antibody directed against granulocyte-differentiation antigen-1 (anti-GR1) at 20 and 4 h before APAP treatment. Anti-GR1 monoclonal antibodies have previously been shown to specifically deplete both peripheral and liver neutrophils in mice, with efficiency rates of  $>95\%$ .<sup>34,35</sup>

Mice pretreated with anti-GR1 antibody before APAP displayed attenuated hepatocellular injury, based on ALT values, compared to vehicle-treated mice at all time points throughout the study. These results are consistent with previously published data.<sup>8,36</sup> Notably, the difference in liver injury between anti-GR1-treated and vehicle-treated mice was most significant at later time points (12 and 24 h) after APAP administration (Figure 2a), suggesting that neutrophils play a more prominent role in ongoing liver injury and inflammation as opposed to the initial injury. Furthermore, mice treated with anti-GR1 before a lethal dose of APAP exhibited no mortality when followed for 10 days after APAP administration, in contrast to vehicle-treated mice receiving APAP, which experienced significant mortality (Figure 2b).

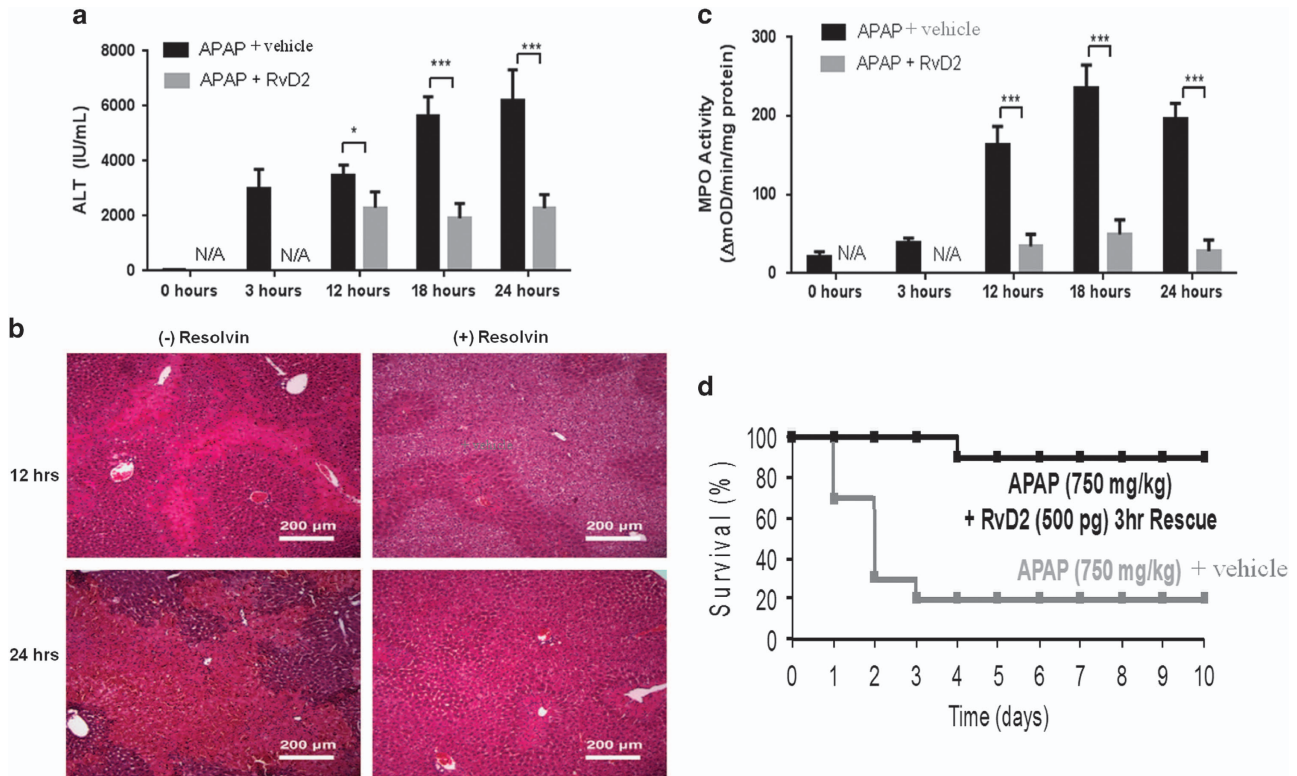
**Resolvin therapy attenuates APAP-induced hepatotoxicity.** Mice were treated with resolvin D2 (RvD2) at various time points following APAP administration and examined for markers of liver injury. RvD2, an endogenous lipid mediator with anti-inflammatory properties, has been previously shown to inhibit neutrophil trafficking to sites of inflammation.<sup>22</sup>



**Figure 2** Neutrophil depletion attenuates acetaminophen (APAP)-induced hepatotoxicity. Neutrophils were depleted using an antibody-mediated strategy.<sup>33,34</sup> Wild-type (WT) mice were intravenously injected with 2 mg/kg of Anti-GR1 antibody and then with 1 mg/kg 20 h later. Vehicle-treated mice were injected with control rat IgG2b. APAP treatment began 4 h after the second dose of anti-GR1 neutrophil depleting antibody. (a) Time course of serum alanine aminotransferase (ALT) levels from neutrophil-depleted WT mice or vehicle-treated WT mice ( $N = 10$  mice/group) after APAP treatment (intraperitoneally (IP), 400 mg/kg). (b) Kaplan–Meier survival curve for neutrophil-depleted WT mice or vehicle-treated WT mice ( $N = 10$  mice/group) after a single lethal dose of APAP (750 mg/kg).  $**P < 0.005$ ;  $***P < 0.001$ .

We observed that mice treated with RvD2 9 h following APAP administration exhibited less severe hepatic injury. Specifically, ALT levels in RvD2-treated mice were significantly reduced at all time points compared to vehicle-treated mice (Figure 3a). Furthermore, the severity of hepatocyte necrosis, inflammation, and hemorrhage was reduced in RvD2-treated mice both at 12 and 24 h post APAP overdose (Figure 3b). In concordance with the serologic and histological data, we also found that treatment with RvD2 offered significant survival benefit as compared to vehicle-treated mice (Figure 3c). To confirm that RvD2 treatment resulted in diminished hepatic neutrophil infiltration, MPO activity from livers of RvD2 and vehicle-treated mice was measured. We observed a significant reduction in hepatic MPO activity in RvD2-treated mice as compared to vehicle-treated animals (Figure 3d).

**RvD2 extends the APAP-treatment rescue window compared to NAC.** We next sought to compare the ability of RvD2 to rescue from APAP hepatotoxicity with that of NAC, the “gold-standard” therapy for APAP overdose. We found that NAC therapy rescued mice from APAP-induced liver injury if given within 90 min of APAP administration. However, NAC therapy given 3 h following APAP had no effect on liver injury (Figure 4a). This finding likely relates to the fact that



**Figure 3** Resolvin treatment attenuates acetaminophen (APAP) hepatotoxicity. Time course of (a) serum alanine aminotransferase (ALT) levels, (b) H&E liver histology (10 × magnification), and (c) liver myeloperoxidase (MPO) activity from wild-type (WT) mice after acetaminophen treatment (intraperitoneally (IP), 400 mg/kg) followed by IV RvD2 or saline vehicle treatment 9 h later (N = 10 mice/group). (d) Kaplan–Meier survival curve for WT mice after a single lethal dose of APAP with IV RvD2 or saline vehicle treatment 3 h later (N = 10 mice/group). \*P < 0.05; \*\*\*P < 0.001.

NAC primarily targets reactive metabolite-induced injury that occurs within the first hour or so of APAP overdose. These results are also consistent with clinical data showing that the efficacy of NAC decreases in humans when treatment is delayed following APAP ingestion.<sup>37–40</sup> Conversely, treatment with RvD2 up to 12 h following APAP led to significant attenuation of liver injury (Figure 4b).

**RvD2 does not protect against NAPQI-induced hepatotoxicity but does inhibit neutrophil activation *in vitro*.** To investigate the mechanistic aspects of our *in vivo* observations, we studied the effect of RvD2 on NAPQI-induced hepatocyte injury *in vitro*. Specifically, rat primary hepatocytes were cultured with NAPQI in the presence or absence of RvD2 and examined for hepatocyte injury. Our *in vivo* data suggest neutrophils likely do not contribute to initial injury induced by APAP, supporting the notion that direct toxicity from NAPQI drives initial injury. Therefore, we hypothesized that RvD2 would not affect NAPQI-related hepatocyte injury. Accordingly, we found that the treatment with escalating doses of RvD2 offered no protection against hepatocyte injury and death induced by NAPQI (Figure 5a).

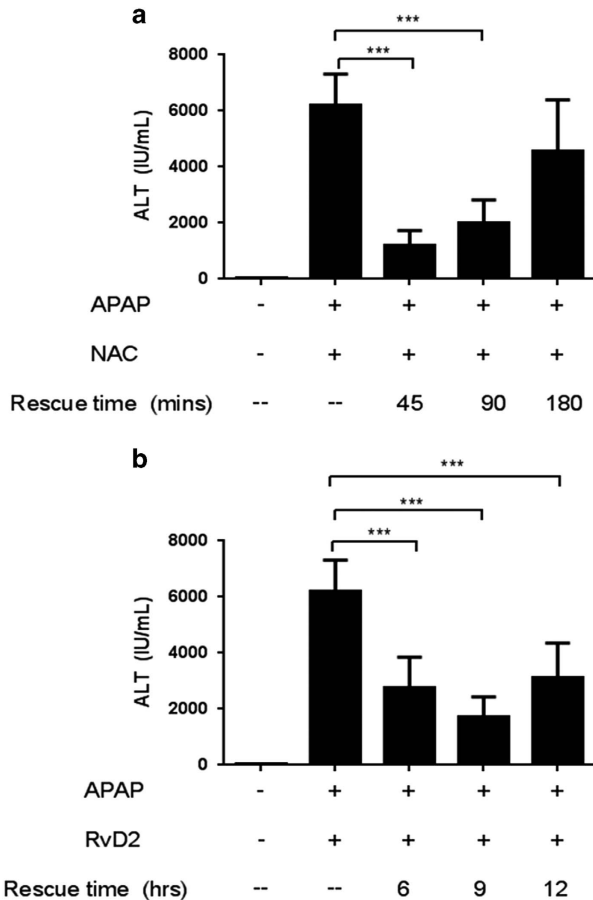
Multiple inflammatory mediators activate neutrophils and enhance their interaction with endothelial cells, thereby promoting neutrophil infiltration into active sites of tissue

injury.<sup>40,41</sup> Previous work has demonstrated the ability of RvD2 to inhibit neutrophil migration to sites of injury.<sup>22</sup> We showed that co-culturing of human neutrophils and endothelial cells in the absence of NAPQI leads to minimal neutrophil adhesion. Conversely, the addition of NAPQI strongly increases neutrophil adhesion to endothelial cells. Notably, NAPQI-induced neutrophil adhesion to endothelial cells is significantly attenuated by RvD2 treatment (Figure 5b).

## DISCUSSION

In this study we demonstrate that hepatic neutrophil infiltration occurs secondary to APAP-induced liver injury, and that neutrophil depletion attenuates liver injury. Furthermore, we show that administration of RvD2 after APAP overdose attenuates liver injury and extends the therapeutic rescue window as compared to the gold standard of treatment, NAC. Mechanistic *in vitro* analysis highlights RvD2's ability to inhibit neutrophil attachment to endothelial cells in the presence of NAPQI, the reactive metabolite of APAP, suggesting RvD2 may prevent neutrophil migration into the liver following APAP injury.

Although inflammation is host protective against a variety of insults and pathogens, excessive inflammation can be damaging. For example, sepsis results from an overly exuberant and persistent inflammatory response to a



**Figure 4** Resolvin extends the temporal window for rescue from acetaminophen (APAP) hepatotoxicity compared to *N*-acetyl cysteine (NAC). (a) Serum alanine aminotransferase (ALT) levels 24 h after APAP treatment (400 mg/kg), with an IV rescue injection of NAC (300 mg/kg) or saline vehicle 45, 90, or 180 min after APAP challenge ( $N=5$  mice/group). (b) Serum ALT levels 24 h after APAP treatment (400 mg/kg), with an IV rescue injection of RvD2 (500 µg/20 g) or saline vehicle 6, 9, or 12 h after APAP challenge ( $N=5$  mice/group). \*\*\* $P < 0.001$ .

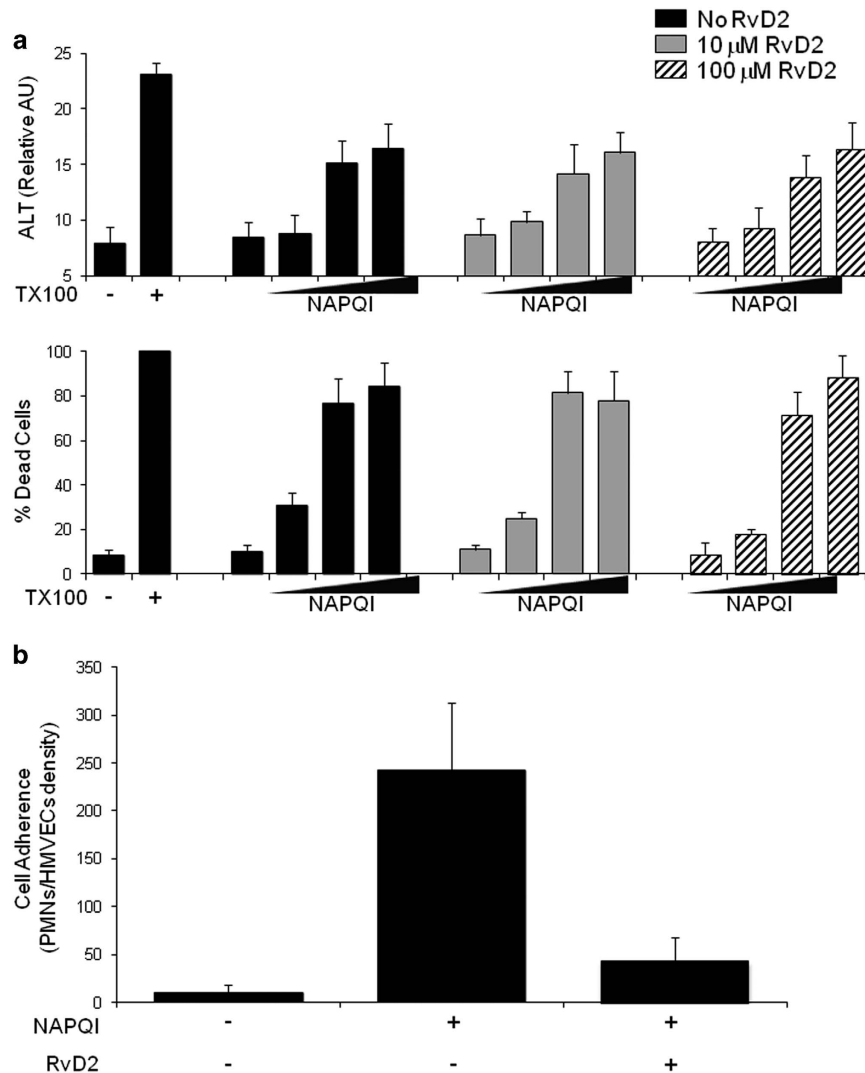
pathogen, resulting in multisystem organ dysfunction. Furthermore, the inability to dampen inflammation may contribute to the development of certain chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis. Accordingly, the ability to modulate the resolution phase of the inflammatory response may prove critical in the treatment of these disorders. Endogenous lipid mediators, termed resolvins, have been shown to actively contribute to the inflammation resolution phase.<sup>23</sup> In fact, exogenous administration of resolvins has decreased the severity of inflammation in multiple preclinical models of disease, including sepsis and inflammatory bowel disease. These encouraging preclinical animal data have led to the development of clinical programs investigating the use of pro-resolving mediators for human disease, in particular ocular and neurodegenerative diseases.<sup>42</sup> Although the mechanism for the observed protection is not fully understood, it has been demonstrated that resolvins regulate trafficking of immune cells to active sites of inflammation, downregulate immune cell production of

pro-inflammatory cytokines, and preserve the vascular network surrounding sites of inflammation.<sup>25,26,31</sup> It is increasingly appreciated that APAP-induced hepatotoxicity results not only from reactive metabolite-driven injury but also as a result of an exuberant host inflammatory response. Supporting this theory are the data that show APAP-related hepatocellular injury occurs long after hepatotoxic metabolites such as NAPQI have been removed from systemic circulation, suggesting an inflammation-driven secondary component to APAP hepatotoxicity.<sup>43</sup> With these data in mind, we tested the hypothesis that exogenously administered RvD2 would dampen hepatic inflammation and injury induced by APAP. Indeed, RvD2 given as late as 12 h after APAP attenuated liver injury. Our data suggest that this protective effect is driven, at least in part, by inhibition of neutrophil migration into the liver.

It is well-recognized that although NAC offers benefit to patients with APAP overdose, its efficacy is largely based on the timing of administration. NAC offers best protection against hepatotoxicity if given within 10 h of APAP ingestion.<sup>37-40</sup> Unfortunately, only a minority of patients present in this narrow time frame. Accordingly, additional therapies that offer benefit at later time points following APAP ingestion are critically needed. An intriguing finding in our study was the ability of RvD2 to extend the therapeutic rescue window following APAP overdose. In particular, RvD2 was able to rescue from APAP-induced liver injury when given up to eight times later than NAC. We believe this extended protection stems from the fact that RvD2 targets the resolution phase of the inflammatory pathways induced by APAP, whereas NAC targets the earliest phases of injury in the pathogenesis of APAP-induced liver injury. This exciting finding deserves further investigation as a potential treatment strategy, either as an alternative to or in combination with NAC, for the treatment of APAP overdose in patients.

The contribution of neutrophils to the pathogenesis of APAP-induced hepatotoxicity has been the subject of debate. Consistent with our data, previous reports have documented attenuation of APAP liver injury in neutrophil-depleted mice, suggesting an essential role for neutrophils in the injury process.<sup>8,36</sup> In addition, recent data show neutrophils contribute to the sterile inflammatory response seen in APAP-induced liver injury in a TLR-9-dependent manner.<sup>44</sup> However, other reports suggest that neutrophils are not critical to the inflammatory response induced by APAP, and, in fact, may contribute to liver regeneration and recovery following the APAP insult.<sup>19,45-48</sup> Although our study does not provide definitive evidence for the role of neutrophils in APAP liver injury, it provides experimental support for their importance.

Despite these exciting findings, certain limitations need to be noted. As this was a proof-of-concept study, we did not test various doses of RvD2 to ascertain the lowest dose needed to obtain protection. It is unclear if the supraphysiological dose used in this study would be tolerated clinically; therefore, the use of RvD2 as a therapeutic target in humans with APAP overdose remains uncertain. Furthermore, it must be noted that in this study we did not investigate the effects of RvD2 administration systemically and on immune cells other than neutrophils, such as macrophages and natural killer cells.



**Figure 5** RvD2 does not protect against *N*-acetyl-*p*-benzoquinone-imine (NAPQI)-induced hepatotoxicity but does inhibit neutrophil activation *in vitro*. (a) Rat primary hepatocytes were cultured in various concentrations of NAPQI, the reactive metabolite of acetaminophen (APAP), in the presence or absence of RvD2. Supernatant alanine aminotransferase (ALT) levels and percentage cell death were measured 1 hr later. Detergent Triton X-100 (TX100) was used as a positive control to induce cell necrosis/lysis. (b) Human peripheral neutrophils were isolated from healthy donors and added to a monolayer of human microvascular endothelial cells (HMVECs) treated with NAPQI, in the presence or absence of RvD2. Neutrophils were stained with Calcein-AM and HMVECs were stained with Hoechst dye. The number of adherent neutrophils relative to HMVECs was quantified 3 h later.

Further work is needed to comprehensively define the effect of resolvins on the multiple inflammatory pathways involved in the APAP liver injury.

In summary, we highlight the ability of RvD2 to protect against APAP-induced liver injury, and compared to NAC therapy, significantly extend the therapeutic rescue window by eightfold following APAP overdose. Although the mechanism for RvD2-induced hepatoprotection is likely multifactorial, inhibition of neutrophil migration and activation plays an important role. Further study into the potential use of RvD2 in the treatment of APAP-induced hepatotoxicity is thus warranted.

#### CONFLICT OF INTEREST

**Guarantor of the article:** Martin L. Yarmush, MD, PhD.

**Specific author contributions:** Suraj J. Patel and Jay Luther designed the concept, performed experiments, and wrote manuscript. Stefan Bohr, Arvin Iracheta-Vellve, Matthew Li, and Kevin R. King performed experiments. Raymond T. Chung and Martin L. Yarmush provided final approval.

**Financial support:** Shriner Hospital for Children Postdoctoral Fellowship Award to Suraj J. Patel; AASLD Clinical and Translational Research Grant to Jay Luther.

**Potential competing interests:** None.

## Study Highlights

### WHAT IS CURRENT KNOWLEDGE

- ✓ Acetaminophen (APAP) overdose can induce severe liver injury and failure.
- ✓ APAP-induced hepatotoxicity accounts for 50% of acute liver failure cases, and is the leading reason for liver transplantation for acute liver failure (ALF) in the United States.
- ✓ N-acetyl cysteine (NAC), the only FDA-approved therapy for APAP overdose, has a limited therapeutic window, limiting its clinical efficacy.

### WHAT IS NEW HERE

- ✓ Resolvins can rescue from APAP-induced hepatotoxicity and improve morbidity and mortality in an animal model as compared to current gold standard therapy with NAC.
- ✓ Resolvins also extend the therapeutic window eightfold compared to NAC, which is of critical importance given the clinical need for therapies that provide protection when given long after APAP ingestion.
- ✓ Resolvins act by dampening the secondary host inflammatory response generated by APAP instead of acting on the toxic metabolite.
- ✓ Resolvins block neutrophil entry and activation in hepatic tissue, thereby limiting inflammation.

1. Larson AM, Polson J, Fontana RJ *et al.* Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* 2005; **42**: 1364–1372.
2. Mitchell JR. Acetaminophen toxicity. *N Engl J Med* 1998; **319**: 1601–1602.
3. Lee WM. Drug-induced hepatotoxicity. *N Engl J Med* 2003; **349**: 474–485.
4. Lewis JH. The rationale use of potentially hepatotoxic medications in patients with underlying liver disease. *Expert Opin Drug Saf* 2002; **1**: 159–172.
5. Lindros KO. Zonation of cytochrome P450 expression, drug metabolism and toxicity in liver. *Gen Pharmacol* 1997; **28**: 191–196.
6. Tujjos S, Fontana RJ. Mechanisms of drug-induced liver injury: from bedside to bench. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 202–211.
7. Bartolone JB, Cohen SD, Khairallah EA. Immunohistochemical localization of acetaminophen bound liver proteins. *Fundam Appl Toxicol* 1989; **13**: 859–862.
8. Liu ZX, Han D, Gunawan B *et al.* Neutrophil depletion protects against murine acetaminophen hepatotoxicity. *Hepatology* 2006; **43**: 1220–1230.
9. Chen CJ, Kono H, Golenbock D *et al.* Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* 2007; **13**: 851–856.
10. Imaeda AB, Watanabe A, Sohail MA *et al.* Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. *J Clin Invest* 2009; **119**: 305–314.
11. Cavassani KA, Moreira AP, Habel D *et al.* Toll like receptor 3 plays a critical role in the progression and severity of acetaminophen-induced hepatotoxicity. *PLoS One* 2013; **8**: e65899.
12. Marques PE, Amaral SS, Pires DA *et al.* Chemokines and mitochondrial products activate neutrophils to amplify organ injury during mouse acute liver failure. *Hepatology* 2012; **56**: 1971–1982.
13. Patel SJ, Milwid JM, King KR *et al.* Gap junction inhibition prevents drug-induced liver toxicity and fulminant hepatic failure. *Nat Biotechnol* 2012; **30**: 179–183.
14. Whyte IM, Francis B, Dawson AH. Safety and efficacy of intravenous N-acetylcysteine for acetaminophen overdose: analysis of the Hunter Area Toxicology Service (HATS) database. *Curr Med Res Opin* 2007; **23**: 2359–2368.
15. Kerr F, Dawson A, Whyte IM *et al.* The Australian Clinical Toxicology Investigators Collaboration randomized trial of different loading infusion rates of N-acetylcysteine. *Ann Emerg Med* 2005; **45**: 402–408.
16. Dambach DM, Watson LM, Gray KR *et al.* Role of CCR2 in macrophage migration into the liver during acetaminophen-induced hepatotoxicity in the mouse. *Hepatology* 2002; **35**: 1093–1103.
17. Laskin DL, Gardner CR, Price VF *et al.* Modulation of macrophage functioning abrogates the acute hepatotoxicity of acetaminophen. *Hepatology* 1995; **21**: 1045–1050.
18. Liu ZX, Govindarajan S, Kaplowitz N. Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. *Gastroenterology* 2004; **127**: 1760–1774.
19. Williams CD, Bajt ML, Sharpe MR *et al.* Neutrophil activation during acetaminophen hepatotoxicity and repair in mice and humans. *Toxicol Appl Pharmacol* 2014; **275**: 122–133.
20. Jaeschke H, McGill MR, Williams CD. Pathophysiological relevance of neutrophils in acetaminophen hepatotoxicity. *Hepatology* 2013; **57**: 419.
21. Serhan CN, Hong S, Gronert K *et al.* Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med* 2002; **196**: 1025–1037.
22. Spite M, Norling LV, Summers L *et al.* Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature* 2009; **461**: 1287–1291.
23. Schwab JM, Chiang N, Arita M *et al.* Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* 2007; **447**: 869–874.
24. Arita M, Yoshida M, Hong S *et al.* Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Proc Natl Acad Sci USA* 2005; **102**: 7671–7676.
25. Serhan CN, Gotlinger K, Hong S *et al.* Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis. *Prostaglandins Other Lipid Mediat* 2004; **73**: 155–172.
26. Jones CN, Dalli J, Dimio L *et al.* Microfluidic chambers for monitoring leukocyte trafficking and humanized nano-proresolving medicines interactions. *Proc Natl Acad Sci USA* 2012; **109**: 20560–20565.
27. Krishnamoorthy S, Recchiuti A, Chiang N *et al.* Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proc Natl Acad Sci USA* 2010; **107**: 1660–1665.
28. Duffield JS, Hong S, Vaidya VS *et al.* Resolvin D series and protectin D1 mitigate acute kidney injury. *J Immunol* 2006; **177**: 5902–5911.
29. Aoki H, Hisada T, Ishizuka T *et al.* Protective effect of resolvin E1 on the development of asthmatic airway inflammation. *Biochem Biophys Res Commun* 2010; **400**: 128–133.
30. Dartt Da, Hodges RR, Li D *et al.* Conjunctival goblet cell secretion stimulated by leukotrienes is reduced by resolvins D1 and E1 to promote resolution of inflammation. *J Immunol* 2011; **186**: 4455–4466.
31. Bohr S, Patel SJ, Sarin D *et al.* Resolvin D2 prevents secondary thrombosis and necrosis in a mouse burn wound model. *Wound Repair Regen* 2013; **21**: 35–43.
32. Jindal R, Patel SJ, Yarmush ML. Tissue-engineered model for real-time monitoring of liver inflammation. *Tissue Eng Part C Methods* 2011; **17**: 113–122.
33. Patel SJ, Milwid JM, King KR *et al.* Gap junction inhibition prevents drug-induced liver toxicity and fulminant hepatic failure. *Nat Biotechnol* 2012; **30**: 179–183.
34. Conlan JW, North RJ. Neutrophils are essential for early anti-Listeria defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. *J Exp Med* 1994; **179**: 259–268.
35. Emoto M, Miyamoto M, Emoto Y *et al.* Highly biased type I immune responses in mice deficient in LEA-1 in *Listeria monocytogenes* infection are caused by elevated IL-12 production by granulocytes. *J Immunol* 2003; **171**: 3970–3976.
36. Ishida Y, Kondo T, Kimura A *et al.* Opposite roles of neutrophils and macrophages in the pathogenesis of acetaminophen-induced acute liver injury. *Eur J Immunol* 2006; **36**: 1028–1038.
37. Prescott LF, Illingworth RN, Critchley JA *et al.* Intravenous N-Acetylcysteine: the treatment of choice for paracetamol poisoning. *BMJ* 1979; **2**: 1097–1100.
38. Rumack BH, Peterson RG. Acetaminophen overdose: incidence, diagnosis, and management in 416 patients. *Pediatrics* 1978; **62**: 898–903.
39. Rumack BH, Peterson RC, Koch GG *et al.* Acetaminophen overdose. 662 cases with evaluation of oral acetylcysteine treatment. *Arch Intern Med* 1981; **141**: 380–385.
40. Smilkstein MJ, Knapp GL, Kulig KW *et al.* Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. Analysis of the national multicenter study (1976–1985). *N Engl J Med* 1988; **319**: 1557–1562.
41. Tonnesen MG. Neutrophil-endothelial cell interactions: mechanisms of neutrophil adherence to vascular endothelium. *J Invest Dermatol* 1989; **92**: 53S–58S.
42. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 2014; **510**: 92–101.
43. Skoglund LA, Ingebrigtsen K, Lausund P *et al.* Plasma concentration of paracetamol and its major metabolites after p.o. dosing with paracetamol or concurrent administration of paracetamol and its N-acetyl-DL-methionine ester in mice. *Gen Pharmacol* 1992; **23**: 155–158.
44. Marques PE, Oliveria AG, Pereira RV *et al.* Hepatic DNA deposition drives drug-induced liver injury and inflammation in mice. *Hepatology* 2014; **61**: 348–360.
45. Cover C, Liu J, Farhood A *et al.* Pathophysiological role of the acute inflammatory response during acetaminophen hepatotoxicity. *Toxicol Appl Pharmacol* 2006; **216**: 98–107.
46. Jaeschke H, Liu J. Neutrophil depletion protects against murine acetaminophen hepatotoxicity: another perspective. *Hepatology* 2007; **45**: 1588–1589.
47. Jaeschke H. Innate immunity and acetaminophen-induced liver injury: why so many controversies? *Hepatology* 2008; **48**: 699–701.
48. Jaeschke H, Williams CD, Ramachandran A *et al.* Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. *Liver Int* 2012; **32**: 8–20.



**Clinical and Translational Gastroenterology is an open-access journal published by Nature Publishing Group.**

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/4.0/>