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Role of the Inflammasome in Acetaminophen-induced Liver Injury and Acute Liver Failure

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Abstract

Drug-induced acute liver failure carries a high morbidity and mortality rate. Acetaminophen overdose is the number one cause of acute liver failure and remains a major problem in Western medicine. Administration of N-acetyl cysteine is an effective antidote when given before the initial rise in toxicity; however, many patients present to the hospital after this stage occurs. As such, treatments which can alleviate late-stage acetaminophen-induced acute liver failure are imperative. While the initial mechanisms of toxicity are well described, a debate has occurred recently in the literature over whether or not there exists a second phase of injury, mediated by inflammatory processes. Critical to this potential inflammatory process is the activation of caspase-1 and interleukin-1ß by a molecular complex known as the inflammasome. A number of different stimuli for formation of multiple different inflammasome complexes have been identified. Formation of the Nalp3 inflammasome in particular has directly been attributed to late-stage acetaminophen toxicity. In this review, we will discuss mechanisms of acetaminophen-induced liver injury in mice and man with a particular focus on the role of inflammation and the inflammasome.

Keywords

Acetaminophen; hepatotoxicity; sterile inflammation; inflammasome, neutrophil; monocyte; toll-like receptor

INTRODUCTION

Drug-induced liver injury and acute liver failure (ALF) remains a major problem in Western societies [1,2]. A majority of drug-induced liver injury and ALF occurs due to either accidental or intentional overdose of acetaminophen (APAP, paracetamol). Because its dose-

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dependent toxicity, APAP-induced liver injury can be studied in animal models and in isolated hepatocytes and most mechanisms are translatable to humans [3-5]. While significant progress has been made in the understanding of intracellular signaling mechanisms of APAP toxicity in hepatocytes, a considerable debate still occurs in the literature over the role of sterile inflammation in the pathophysiology. While the presence of an inflammatory infiltrate is obvious both histologically and biochemically, whether or not this infiltrate directly contributes to hepatocyte death remains controversial. At the core of many of these debates lies the role of many specific inflammatory processes associated with liver injury, including the activation of the inflammasome after APAP overdose. Breakthrough studies in the early-mid 2000's first identified the presence of a highly regulated signaling system in myeloid cells that responds rapidly to the presence of so-called damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs). This system, the inflammasome, has been extensively studied since then in the context of liver injury [6,7]. The purpose of this article is to review both recently discovered molecular mechanisms that control inflammasome activation and the role of the inflammasome in drug-induced liver injury, with a special emphasis on APAP overdose and APAP-induced ALF.

The Inflammasome – a Molecular Mechanism for Immune Cell Activation

Since the initial description of the activation and formation of the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome [8], there have been intensive studies on the molecular mechanisms that control the inflammasome. Ostensibly, the major purpose of the inflammasome is for immune cells to detect the presence of DAMPs and PAMPs in serum and respond with the activation of pro-inflammatory cytokines, interleukin-1ß (IL-1ß) and interleukin 18 (IL-18), through a proteolytic cleavage pathway mediated by the activation of caspase-1 [8]. IL-1ß is a potent activator of effector cells such as monocytes and neutrophils that express the interleukin-1 receptor (IL-1R). As such, the commonly measured primary outcomes of inflammasome activation are increased serum levels of IL-1ß and IL-18 [8] and subsequent recruitment of inflammatory cells (Figure 1). However, the mechanism of secretion of IL-1ß remains poorly defined [9]. The simplest explanation remains the idea that IL-1ß is produced in cells which undergo necrosis and then release IL-1ß passively [10]. This corroborates data that brefeldin A, a classical Golgi inhibitor, has no effective on IL-1ß secretion [11]. Other data have supported an unconventional secretion mechanism that bypasses the endoplasmic reticulum/Golgi apparatus. This may occur independent of cell death, and through mechanisms that involve autophagosomes typically associated with autophagy [12]. Vesicle and exosome release have also been implicated [13,14]. As such, it is probable that multiple mechanisms can contribute to IL-1ß secretion depending on the current microenvironment and relevant cell type. The degree to which each of these contribute versus cell death via necrosis or pyroptosis has yet to be determined.

Multiple different inflammasome complexes exist **(Supplementary Table 1)** [6,8,15], and generally, these different pathways converge at the activation of caspase-1 and the subsequent activation of IL-1ß [8,16-19]. The intracellular priming mechanisms for inflammasome activation have been studied intensely; however, no consensus activation signal has been detected [6]. Instead, a number of different extracellular signals have been

defined that activate the inflammasome, which are detected by a family of proteins called nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing protein (NLR) proteins. These include, among others, NLRP1, NLRP3, and NLRC4 (reviewed in [6]). Each of these family members are capable of assembling a complex with a caspase recruitment domain (CARD) via the adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), that forms the protein complex responsible for the binding and cleavage of caspase-1 [20,21]. The two most commonly discussed inflammasomes in the liver are NLRP1 and NLRP3, with NLRP3 being identified as a potential mediator in the mouse model of APAP overdose [22]. Additional representative pathways of inflammasome activation include the activation of the NLRP1 pathway by the *B. anthrax* toxin, wherein the toxin enters the cell through an enzymatic reaction and then releases the lethal factor subunit which activates the NLRP1 inflammasome formation [23,24]. NLRP3 activation is characterized by a diverse array of signals, but can be activated in the liver by local release of ATP, which binds the purinergic P2X receptor 7 (P2X7R) and activates the inflammasome through a decrease in intracellular potassium [17,25] or potentially through reactive oxygen species generation [26]. In addition to canonical inflammasome activation, there is a non-canonical pathway that is mediated by caspase-11 in humans, which corresponds to caspase-4/5 in mice [27,28]. Currently, this non-canonical form of inflammasome activation is poorly understood in the context of drug-induced liver injury, but given the liver's high exposure to LPS via the portal tract, activation of this pathway is a possibility in some disease states. Activation of these caspases by LPS results in formation of the NLRP3 inflammasome independent of receptor binding, and activates caspase-1, but without the subsequent activation of pro-IL-1ß [29]. This results in pyroptosis, a form of necrotic cell death featuring the activation of caspase-1 that is implicated in other liver injury models [30,31]. Of note, pyroptosis also occurs internally in hepatocytes, indicating activation of the inflammasome also occurs outside of just innate or recruited inflammatory cells in the liver [30]. While the mechanisms are not fully delineated, initial work indicates similar mechanisms of Nalp3 formation and caspase-1 cleavage are occurring. Chronic activation of Nalp3 in hepatocytes results in inflammation-mediated cell death that is partially dependent on IL-1R receptor activation, indicating that both secreted IL-1ß and internal activation of the inflammasome can generate toxicity in this model [30]. Recent data indicate that the protein gasdermin D might be the critical mediator of pyroptosis [32-35]. N-terminal cleavage of gasdermin D by caspase-1, and other pro-inflammatory caspases such as caspase-4/5 and caspase-11, results in unfolding of the protein, and translocation to the plasma membrane where it binds cardiolipin, phosphatidylinositol phosphates, and phosphatidylserine and initiates pore formation resulting in the characteristic loss of membrane integrity, cytosolic swelling and release of cellular contents [32,35].

Regardless of the intracellular signaling mechanisms, the primary outcome of inflammasome activation is the activation of caspase-1 and cleavage of pro-IL-1ß to IL-1ß, which binds to the IL-1R, and stimulates hepatic recruitment of IL-1R expressing cells, resulting in inflammation.

Sterile Inflammation and Inflammasome Activation

The current hypothesis on the initiation of sterile inflammation involving inflammasome activation in the liver requires a two-step process [22,36-38]: First, the transcriptional activation of pro-IL-1ß, and second, the cleavage of pro-IL-1β by the inflammasome-activated caspase-1 and release of the active cytokine [36,38] (Figure 1). Pattern recognition receptors (PRR) or DAMP receptors are thought to increase expression of pro-IL-1ß transcripts when activated by their respective ligand [22,37]. One of the commonly studied PRR families is the Toll-Like receptor (TLR) family, ubiquitously expressed on macrophages and other immune cells present in the liver. Two of the most well studied TLRs in the liver are TLR4 [39-41] and TLR9 [22]. TLR4 is ligated by a number of different compounds, but primarily is thought to be activated by the PAMP lipopolysaccharide (LPS) [42], and by the DAMP high mobility group box-1 protein (HMGB1) [43], which also binds to additional receptors including the receptor for advanced glycation end products (RAGE) [44]. TLR9 is activated by unmethylated cytosine deoxynucleotide-guanine deoxynucleotide oligo sequences [45]. These sequences are common in the microbial genome, but are relatively uncommon in the vertebrate genome, and thus, the mammalian innate immune system has evolved to detect and respond to these sequences [45]. Ligation of either TLR by its ligand results in increased pro-IL-1ß gene formation [22,36,46]. In addition, IL-18 is thought to be released through a similar process, although its role in liver pathophysiology is, up to this point, relatively less studied. Given the diverse array of signals that can activate DAMP receptors, it seems probable that during APAP-induced necrosis a number of different cellular components can be released that will both activate cytokine transcription through TLR binding, and inflammasome activation through purinergic receptor stimulation.

Although the mechanisms that control the activation of the inflammasome are still under intensive study, the above mechanisms are widely accepted (Figure 1). Despite this, the role of the inflammasome in APAP-induced liver injury remains under considerable debate.

Drug-Induced Liver Injury in Mice and Man

APAP-induced liver injury remains the number one cause of drug-induced liver injury and acute liver failure in the West [1]. While the antidote N-acetyl cysteine (NAC) is highly effective in early presenting patients, patients that do not receive NAC in time undergo severe liver injury, which can progress to acute liver failure (ALF). ALF after APAP overdose carries a high mortality rate and features severe liver dysfunction, immune paresis, major bleeding disorders, hepatic encephalopathy and predisposes the patients to sepsis and systemic inflammatory response syndrome (SIRS) [47]. Liver transplantation is the only definitive cure at this stage, but carries a life-long need for anti-rejection medication and continued medical follow up. As such, therapeutic options that target the later stages of the injury have become the most probable for improving patient survival and reducing the number of transplants needed. While the initial stage of toxicity is mediated by reactive metabolite formation and mitochondrial dysfunction (reviewed in [48,49], a number of studies during the last decade have suggested a later stage of injury that is potentially mediated, at least in part, by the recruitment of inflammatory leukocytes such as neutrophils and monocytes [22,50-53]. One common explanation for the recruitment of these inflammatory cells is activation of the inflammasome through release of local DAMPs and

other cellular constituents [22,54]. Unquestionably, both mice and humans undergo a sterile inflammatory response after APAP overdose. The current critical question is whether modulation of this response could be beneficial to human patients.

Drug Induced Liver Injury – Oncotic Necrosis and the Initiation of Sterile Inflammation

The murine model of APAP overdose is a high fidelity model with numerous consistencies between mouse and man. As such, a number of the mechanisms that control APAP toxicity in mice are reasonably well understood [48] (Figure 2), and have been validated in the metabolically competent human hepatoma cell line HepaRG [55], in primary human hepatocytes [56] and in APAP overdose patients [57]. Therapeutic doses of APAP are mainly glucuronidated or sulfated, and only a small amount is metabolized by cytochrome P450 enzymes causing formation of the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which results in limited protein adduct formation [58,59]. After an overdose, sulfation is saturated and glucuronidation, despite its dramatic stimulation, is not able to prevent a substantial increase in NAPQI formation [60]. As a consequence, there is extensive glutathione depletion and protein adduct formation, especially in mitochondria, which results in a mitochondrial oxidant stress and activation of mitogen-activated protein kinases including c-jun *N*-terminal kinase (JNK) to amplify the oxidant stress [48,49] (Figure 2). Ultimately, the mitochondrial oxidant stress triggers the mitochondrial permeability transition (MPT) pore opening, which results in mitochondrial matrix swelling, rupture of the outer membrane and release of intermembrane proteins such as endonuclease G and apoptosis-inducing factor (AIF), which translocate to the nucleus and cause DNA fragmentation [48] (Figure 2). These events trigger cell necrosis with release of cellular contents including the potent DAMPs HMGB1 (nuclear protein) [61-63], nuclear DNA fragments [54,57], mitochondrial DNA [57], uric acid [64], ATP [65,66] and many others. These DAMPs cause the transcriptional activation of pro-inflammatory cytokines in macrophages through TLRs and inflammasome activation. In support of this hypothesis, cytokines (e.g., TNF-α, IL-1β, IL-6, IL-10) and chemokines (e.g., MCP-1, MIP-2, IL-8) are detectable in plasma of animals and patients after APAP overdose [67-74]. These pro-inflammatory mediators can activate and recruit first neutrophils [50,67,75] and later monocytes [53,68,76] to the liver (Figure 3). Despite these effects, the pathophysiological role of this sterile inflammatory response after APAP remains a topic of considerable debate.

Drug-Induced Liver Injury – Evidence for a Pathophysiological Role of Sterile Inflammation

After early reports could not find evidence for a pathophysiological contribution of neutrophil recruitment into the liver after APAP overdose [67,77], a paper indicated that NK/NKT cells modulate the immune response in the mouse liver, leading to increased neutrophil recruitment [78]. A follow-up paper confirmed these data by demonstrating that depletion of neutrophils also protected against APAP toxicity when given as a 24 hour pretreatment before the administration of APAP [50]. This has led to a considerable number of papers presenting, or assuming, the hypothesis that the effector cell for inflammation-mediated toxicity after APAP overdose is the neutrophil. In addition, it was reported that IL-1R might have a substantial role in APAP-induced liver injury [79]. IL-1R-deficient mice were shown to be almost completely protected against APAP-induced liver injury, although this same study demonstrated that antibodies against IL-1α, but IL-1ß, attenuated APAP

hepatotoxicity [79]. A subsequent study has indicated that administration of a recombinant human IL-1R antagonist might also be protective against APAP-induced liver injury [80]. These data were partially corroborated by a study demonstrating that mice deficient in TLR9, Nalp3, caspase-1, or ASC, as well as pharmacological antagonism of TLR9 attenuated APAP-induced liver injury and increased survival 72 hours post treatment in mice given an overdose [22]. The authors proposed that this protection occurred through the Nalp3 inflammasome, via a reduction in the expression of pro-IL-1ß, a subsequent reduction in serum IL-1ß and a reduction in $Gr1^+$ neutrophils that likely mediated the effects [22]. This concept was largely corroborated by a number of recent papers indicating that DAMP release can guide neutrophils to the site of hepatic injury, wherein it was assumed that neutrophils exacerbated the injury. Administration of benzyl alcohol blocked APAP-induced liver injury, as well as blocking plasma IL-1ß and IL-18 release through a TLR4 dependent mechanism, as either global knockout of TLR4 or myeloid specific knockout of TLR4 resulted in a loss of the protective effect of benzyl alcohol [46]. In contrast, knockout of TLR4 had no effect in a separate paper wherein neutrophil recruitment was suggested to occur through release of HMGB1 and its ligation to the RAGE receptor [52]. Furthermore, formyl peptides released during necrosis were demonstrated to guide neutrophils to the area of hepatic necrosis via a formyl peptide receptor-1 (FPR1)/CXC chemokine receptor-2 interaction and both neutrophil recruitment and hepatic injury were ameliorated by blockade of TLR9 or by blockage of FPR1/CXCR2 [51]. A similar interaction was noted wherein DNA released from dying hepatocytes guided neutrophils to the site of injury [54]. Injection of DNAse I to lyse plasma DNA eliminated the localized increase in DNA, preventing inflammation and reducing hepatic injury [54]. This interaction is also potentially enhanced by release of intracellular ATP during necrosis as both antagonism of P2XR7 with a pharmacological agent and knockout of P2XR7 [65] as well as treatment with apyrase to disrupt ATP [65,66] reduced APAP-induced liver injury, presumably through a reduction of inflammation. However, the importance of ATP and its receptor P2XR7 could not be confirmed as critical mediators for hepatic neutrophil infiltration or injury during APAP hepatotoxicity in a different study [81]. Another, inflammasome-independent pathway of neutrophil activation has been suggested [82]. The release of HMGB1 from necrotic hepatocytes activates Kupffer cells through TLR4, which generate IL-23. This cytokine stimulates γδT cells to produce IL-17A, which then recruits neutrophils in the liver [82]. The assumption is that these neutrophils aggravate the injury (Figure 3).

A more recent study suggested that recruitment of bone marrow-derived monocytes may contribute to the aggravation of APAP hepatotoxicity [53]. These cells, which express CCR2 receptors, are being recruited into the liver and into areas of necrosis by MCP-1 (CCL2) [53]. The conclusion of the importance of monocyte-induced liver injury during APAP overdose is based on the reduced liver injury in CCR2-deficient mice and pharmacological inhibitors of MCP1 and CCR2, which reduced hepatic monocyte recruitment [53]. However, since neutrophil accumulation was not affected, this study does not allow room for an effect of neutrophils, which makes it somewhat contradictory to the previously discussed studies.

A study using clodronate liposomes to eliminate the resident macrophages of the liver (Kupffer cells) reduced IL-10 formation and showed enhanced APAP-induced liver injury [83]. Subsequent experiments with IL-10-deficient mice demonstrated an aggravation of

APAP-induced liver injury, which correlated with induction of inducible nitric oxide synthetase (iNOS) [84]. Since IL-10 mainly attenuates pro-inflammatory cytokine formation in macrophages, the authors concluded that IL-10 acts to suppress cytokine formation, which is responsible for iNOS induction and thus aggravation of liver injury by enhanced NO and consequently peroxynitrite formation [84]. Since peroxynitrite is a critical mediator of APAP toxicity through mitochondrial dysfunction [85,86], this hypothesis links the DAMP-mediated cytokine formation to intracellular signaling mechanisms of injury rather than inflammatory cell activation.

Currently, the most widely accepted hypothesis behind inflammation after APAP overdose is that the process occurs through a sterile inflammatory response. A largely unexplored hypothesis is that part of the inflammation is driven by LPS translocation due to gut barrier dysfunction. Especially in human patients, the role of LPS and other PAMPs should be assessed and verified. The only current information on the topic of note is the recent report that germ-free mice experience the same degree of hepatic necrosis as conventionally housed animals or endotoxin-resistant mice after APAP overdose [87]. Regardless, the potential for LPS or other PAMPs mediating some portion of the inflammation and contribute to the development of acute liver failure still exists.

Given the totality of this information, it is apparent that there is massive DAMP release following APAP-induced necrosis and that multiple DAMPs can lead to the characteristic neutrophil and monocyte recruitment seen after APAP overdose [67,68,75,76] and affect intracellular signaling mechanisms [84]. Moreover, some increases in pro-IL-1ß gene expression occur, suggesting that DAMP release after APAP can activate the inflammasome, and there is a small, but routinely detectable difference in plasma IL-1ß after APAP overdose, which can be attenuated with a pan-caspase inhibitor [72]. As such, the idea that a sterile inflammatory response potentially involving inflammasome activation could contribute to APAP-induced liver injury in the mouse is realistic and is apparently supported by a number of compelling pieces of evidence. Whether this inflammatory injury component requires leukocytes recruited into the liver is less clear as discussed later.

Drug-Induced Liver Injury – Evidence against a Pathophysiological Role of Sterile Inflammation

Despite this mounting evidence in favor of a second phase of injury, mediated at least in part by sterile inflammation with or without inflammasome participation, substantial experimental evidence also exists that raises concerns about this hypothesis. Foremost are the minimal increases found in caspase-1 activation and plasma IL-1ß or IL-18 levels in the murine model of APAP overdose [72]. Interestingly, even studies that advocate a role for the inflammasome and IL-1β agree with the very low formation of this cytokine during APAP toxicity [22,46]. The fact that administration of even very high doses of murine recombinant IL-1β only moderately enhance hepatic neutrophil accumulation, but do not affect APAP-induced liver injury, argues against a critical role of IL-1β in the pathophysiology [72]. This conclusion is supported by the observation that IL-1R-deficient mice are not protected, and that pan-caspase inhibitors do not reduce APAP-induced liver injury [72,88,89], despite the fact that the inhibitor prevented the formation of the active cytokine [72]. In addition, uricase

transgenic animals, which have very low levels of the DAMP uric acid, showed 80% less neutrophil accumulation after APAP but no reduction in liver injury [64]. Furthermore, studies using mice deficient in inflammasome components Nalp3, caspase-1 or ASC could not confirm previous reports [22], and showed instead that these KO mice were not protected [90]. The reason for these opposite results remains unclear. In contrast, the effective protection by the purinergic receptor antagonist A4380797 as reported by Hoque et al. [65] was reproducible, however, the mechanism of protection proved to be inhibition of P450 enzymes rather than attenuation of inflammasome activation [91]. This issue is a common problem with using pharmacological interventions as many chemicals and the solvents needed to get them into solution can have off-target effects. In particular, the potential effect on the metabolic activation of APAP needs to be meticulously investigated to avoid misinterpretation of experimental results [92]. This issue has been raised for a number of recently used drugs and their solvents [89,91,93-95].

In addition to IL-1β, other cytokines are formed during APAP toxicity including mKC, MIP-2, TNF-α, IL-6 and more in mice [67,70,71] and IL-6, IL-8 and IL-10 in humans [69,74,96]. TNF-α has been implicated based on neutralizing antibodies [97]. However, neither TNF-α knockout mice [98] nor TNF receptor-1-deficient animals [71,99] showed reduced liver injury after APAP treatment suggesting that TNF-α is unlikely to be a critical pro-injury mediator. The monocyte chemoattractant protein 1 (MCP-1, CCL2), which acts mainly on monocytes through the CCR2 receptor, has been suggested to be important for the inflammatory injury after APAP based on CCR2-deficient mice and pharmacological intervention studies [53]. However, these results contradict a number of previous reports using both CCR2-deficient mice [68,76,100,101] and MCP-1-deficient animals [68], which showed no protection. Again, it remains unclear why the same KO mice respond differently in the hands of different investigators. However, additional support against a cytotoxic role of monocytes turned macrophages comes from the fact that mice deficient in a functional NADPH oxidase (NOX2), which generates reactive oxygen species in macrophages and other phagocytes, are not protected and show the same oxidant stress as wild type animals [102,103].

Some of the potential experimental design problems as the authors see them and the relevant solutions are listed in Table 1.

Drug-Induced Liver Injury – What is the Effector Cell if Inflammation Exacerbates Acetaminophen Toxicity?

Despite the common citation of inflammation being responsible for a second phase of APAP toxicity, there has yet to be a definitive demonstration of a specific inflammatory cell that is directly responsible for cell death after APAP overdose. The most commonly associated cell type with cytotoxicity is the neutrophil, which is known to cause liver injury in other models including hepatic ischemia-reperfusion [104], obstructive cholestasis [105], endotoxin shock [106] and alcohol-induced liver injury [107]. What these studies, and many others, unequivocally showed was that in order to get a neutrophil to attack and cause liver injury, it requires activation and priming of the neutrophil, adhesion in sinusoids and transmigration, the adherence to the target cell and an adherence-dependent oxidant stress, which will kill

the target [108,109]. The adhesion molecules involved include β_2 integrins (CD11b/CD18) on the neutrophil and intercellular adhesion molecule-1 (ICAM-1) on sinusoidal endothelial cells and hepatocytes [108,109]. In addition, the enzymes NADPH oxidase (NOX2) and myeloperoxidase of the neutrophil are needed to generate superoxide and hypochlorous acid, respectively [110-112]. The inhibition or knock-out of any of these genes effectively prevents neutrophil-mediated liver injury in models of ischemia-reperfusion injury, obstructive cholestasis and endotoxemia [108,109,113,114]. However, in striking contrast, antibodies against CD18, which functionally inactivate neutrophils, or inhibitors of NADPH oxidase, which prevent reactive oxygen formation and cytotoxicity of neutrophils, do not protect against the APAP-induced liver injury [67,75]. Furthermore, mice deficient in CD18, ICAM-1, or gp91phox, a component of NADPH oxidase, are not protected [75,102,103,115]. The ineffectiveness of specific interventions that functionally inactive neutrophils makes it almost impossible for the neutrophil to be part of the injury process.

Is there any direct evidence to suggest that neutrophils are involved in APAP toxicity? The most cited approach is the use of neutropenia-inducing antibodies being administered 24h before APAP [50,51,101,116]. Interestingly, these antibodies do not seem to be beneficial when given shortly after APAP [75] despite the fact that such an antibody can remove 90% of neutrophils from the blood within less than 1h [104]. Furthermore, these antibodies are protective when given at the same time as the toxin in the case of alpha-naphthylisothiocyanate [117]. Given these observations, what is special about the 24h pretreatment requirement in the APAP model? The most likely answer is that the pretreatment regimen causes off-target effects. When neutrophils are depleted by an antibody in a healthy animal, most of the cells get stuck in capillary beds such as hepatic sinusoids and are functionally inactivated [118]. However, Kupffer cells will be activated and remove these cells by phagocytosis [118], which creates a substantial local stress in the liver leading to induction of a number of acute phase proteins in hepatocytes such as heat shock protein-70, heme oxygenase-1and especially metallothionein [119]. Each of these genes is protective against APAP toxicity, which suggests that their upregulation before APAP treatment causes the protection against APAP toxicity. Since this off-target effect depends on the depletion of neutrophils, it occurs only after treatment with neutropenia-inducing antibodies and not with control IgG [119]. As a consequence, this off-target effect is generally not recognized.

Elastase has recently been proposed as the primary mediator of neutrophil toxicity as reconstitution of wild-type murine bone marrow with neutrophils from elastase-deficient mice resulted in partial protection [52]. No mechanism has been demonstrated for how this might occur. As neutrophils kill hepatocytes largely through release of ROS [95], and the primary purpose of elastase is thought to be degradation of extracellular matrix to facilitate neutrophil migration, clarification of the elastase-dependent killing mechanism is needed. In addition, whole body elastase knockout mice are not protected against APAP-induced liver injury (Bajt and Jaeschke, unpublished). Although this result is consistent with the general understanding of neutrophil-dependent liver injury [95], the reason for this discrepancy to the previous study is not well understood and requires further experiments.

Both Kupffer cells and monocyte-derived macrophages have also been implicated as cytotoxic in the APAP model [53,120]. Although Kupffer cells can cause an oxidant stress and liver injury in the ischemia-reperfusion model [121], the predominant location of the most active Kupffer cells in the periportal region makes it difficult to cause a selective centrilobular injury characteristic of APAP toxicity. In addition, elimination of Kupffer cells by clodronate liposomes does not protect [83] and animals deficient in gp91phox, which are incapable of generating reactive oxygen by their phagocytes, show the same oxidant stress and are not protected [102,103]. The controversial results with CCR2-deficient mice regarding the role of monocyte-derived macrophages have already been discussed. As of yet, there exists little direct evidence that monocyte-derived macrophages or Kupffer cells are capable of directly killing cells during APAP toxicity *in vivo*, rather, a majority of studies have focused on the elimination of a cytokine or a DAMP and the assumption that the reduction in inflammation causes protection instead of also considering the possibility that the inflammatory response is attenuated due to less injury.

NK and NKT cells have also been implicated as major mediators of neutrophil recruitment and potentially cytotoxicity [78]; however, a number of problems have arisen with these data. Other groups have failed to reproduce the initial reported effect based on solvent issues with the APAP dosage [122]. In addition, modulation of NK/NKT cells through knockout of Jα-18 has given mixed results [123,124]. Currently, there is no conclusive study as to the effect of NK/NKT cells in APAP toxicity.

Thus, there is a considerable amount of data that raises concerns about the role of sterile inflammation and the importance of inflammasome activation during APAP hepatotoxicity. In particular, the cytotoxic cell type(s) responsible for the assumed secondary injury phase remains unclear. The stark differences and potential resolution to these issues are summarized in Table 2.

APAP-Induced ALF – Is Inflammation a Therapeutic Target during Human ALF?

Unquestionably, an inflammatory response occurs also after APAP overdose in human patients. A number of DAMPs are detectable in blood of these patients similar to the murine model including HMGB1, mitochondrial DNA, nuclear DNA fragments and many others [57,62,125]. In addition, both inflammatory and anti-inflammatory cytokines are released [69,73,74,96,126], however, pro-inflammatory cytokine levels including TNF-α and IL-1β (Figure 4) are only modestly increased during the first 5 days after APAP overdose [74]. Interestingly, human studies indicate that inflammation may actually be critical for *recovery* from APAP overdose [73,127], which are corroborated by studies in the mouse [68,76,100]. Regeneration of new hepatocytes is critically important for survival during APAP-induced ALF [128], and requires a healthy macrophage population for the removal of necrotic tissue [7396,129]. Patients with low monocyte counts or low levels of the monocyte proliferative stimulus colony stimulating factor-1 experience higher mortality rates, confirming the importance of these cells [96,129]. Recruited and endogenous macrophages during APAP-induced ALF are largely anti-inflammatory in nature due to the effect of secreted leukocyte protease inhibitor [127]. While this likely limits liver damage caused by this cell population and stimulates tissue turnover and regeneration, it may make the patients more susceptible to

infection, as increased SLPI levels were associated with infection during hospitalization [127]. Accordingly, production of both neopterin and sCD163, markers of macrophage activation, were found to be elevated and associated with development of SIRS and poor outcomes in patients [130]; although, this may be more of an indirect marker of SIRS onset than APAP-induced liver injury. As infection/sepsis is responsible for up to 8% of all deaths due to ALF [47], blockade of inflammation, especially monocyte-derived macrophages, may actually be detrimental to patient outcomes. Serious adverse events have been seen in other models of liver injury when blocking inflammation while the patient is at a very high risk for sepsis or SIRS [131].

Similar arguments can be made for neutrophils in APAP overdose patients. Neutrophil activation (reactive oxygen formation, phagocytosis) in blood, which is a close surrogate of the liver neutrophil activation status [115], occurs only after the acute liver injury is over and regeneration starts in these recovering APAP overdose patients [103]. This is consistent with the limited pro-inflammatory cytokine formation during the injury and early post-injury phase [74] and a role of neutrophils in the clean-up of necrotic tissue during regeneration. However, in patients with APAP-induced ALF, there is increased TLR9 but decreased TLR4 expression on functionally exhausted neutrophils, which correlated with the degree of encephalopathy [132]. Thus, excessive activation of neutrophils during ALF can also increase the susceptibility to sepsis, which may contribute to the high mortality [133,134].

Summary and Conclusions

There is no question that the extensive necrotic cell death after an APAP overdose in mice and humans causes the release of DAMPs, which act on pattern-recognition receptors on macrophages and potentially other cells and transcriptionally induce cytokine and chemokine formation and also activate the inflammasome. These events result in recruitment of neutrophils and monocyte-derived macrophages into the liver. The primary purpose of this sterile inflammatory response is to remove the necrotic cell debris and make room for dividing hepatocytes to regenerate the lost liver tissue. This process is essential for the animal or person to survive. The controversial question discussed in this review is whether the inflammatory response contributes to the injury, and therefore may be considered a therapeutic target, or whether this inflammation is beneficial or even essential for survival. As discussed, the answer to this question based on a large number of different animal experiments is still controversial, although the evidence for a second inflammatory injury phase is very limited and the support for a cytotoxic role of inflammatory leukocytes is even less convincing. Nevertheless, it is concerning when more and more papers are published in support of one side without considering the contradicting data in the literature, and when manuscripts are published with exact opposite results as previously reported, using the same reagents and animals, even from the same vendor. In order to come to a consensus, move the field forward, and translate these data to humans, future studies in this area need to be mechanistically more detailed, address the controversies experimentally, and consider all aspects of the pathophysiology, which include the potential pro-injury and pro-regenerative aspect as well as the host defense function of inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

AIF	apoptosis-inducing factor
ALF	acute liver failure
ALT	alanine aminotransferase
APAP	acetaminophen
ASC	adaptor protein, apoptosis-associated speck-like protein containing a CARD
CARD	caspase recruitment domain
DAMPs	damage-associated molecular patterns
HMGB1	high mobility group box 1
ICAM-1	intercellular adhesion molecule-1
IL-1ß	interleukin-1ß
IL-1R	interleukin-1 receptor
iNOS	inducible nitric oxide synthetase
JNK	c-jun N-terminal kinase
LPS	lipopolysaccharide
KO mice	knockout mice
MCP-1	monocyte chemoattractant protein 1
NAC	N-acetylcysteine
NAPQI	N-acetyl p- benzoquinone imine
Nalp3	NACHT, LRR and PYD domains-containing protein 3
NOX2	NADPH oxidase

NLR	nucleotide-binding oligomerization domain (NOD) leucine-rich repeat (LRR)-containing protein
PRR	pattern recognition receptor
P2XR7	purinergic receptor P2X 7
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
TLR	toll-like receptor

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Key Point Box

- Acetaminophen (APAP)-induced acute liver failure remains a major clinical problem
- Sterile inflammation and inflammasome activation occurs in both mice and man after APAP overdose
- Data exists both for and against the idea that sterile inflammation contributes to APAP-induced liver injury
- Novel data in patients indicate monocyte recruitment may be necessary for survival
- Inflammation is likely a multi-component, time dependent factor, especially in human patients

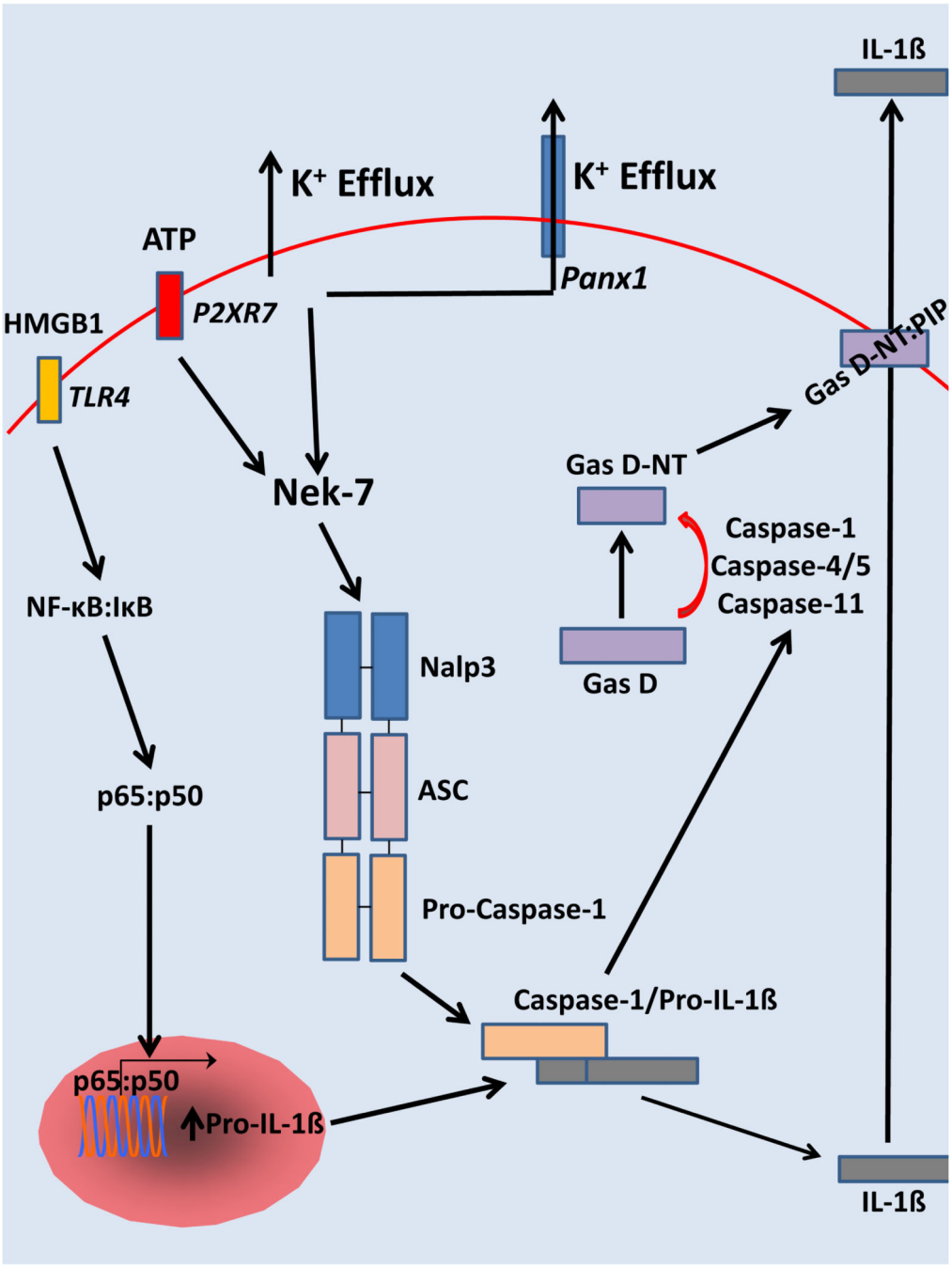

Figure 1.

Proposed mechanism of inflammasome activation by ATP:P2XR7 interaction in macrophages. Elevated ATP levels in serum released from dying cells activates P2XR7 causing pannexin-1 pore opening and potassium release. In addition, activation of P2XR7 causes activation of the protein Nek-7, which has a currently undefined function, but leads to formation of the Nalp3 inflammasome with activation of pro-caspase-1. Stimulation of toll like receptors, e.g. TLR4, by substrates such as high mobility group box 1 (HMGB1) protein causes NF-κB activation and transcriptional induction of pro-IL-1β formation. The active

caspase-1 cleaves pro-IL-1β and the mature cytokine is being released. Activation of pro-inflammatory caspases, either directly by LPS in the case of caspase-4/5 or caspase-11, or through the inflammasome in the case of caspase-1, results in cleavage of Gasdermin D into the N-Terminal cleaved form of Gasdermin D. The N-terminal form mediates cell death via perforation of the plasma membrane after binding plasma membrane components such as phosphatidyl inositol or cardiolipin. Pore formation results in cellular collapse in cells undergoing pyroptosis and passive release of constituents such as IL-1ß. P2XR7 – purinergic receptor P2X 7, Nek-7 – NIMA-related kinase 7, Nalp3 – NACHT, LRR and PYD domains-containing protein 3, ASC - Apoptosis-associated speck-like protein containing a CARD, IL-1ß – interleukin-1ß, Gas D – Gasdermin D, Gas D-NT – Gasdermin D N-Terminal cleavage product. PIP – phosphatidylinositol phosphate

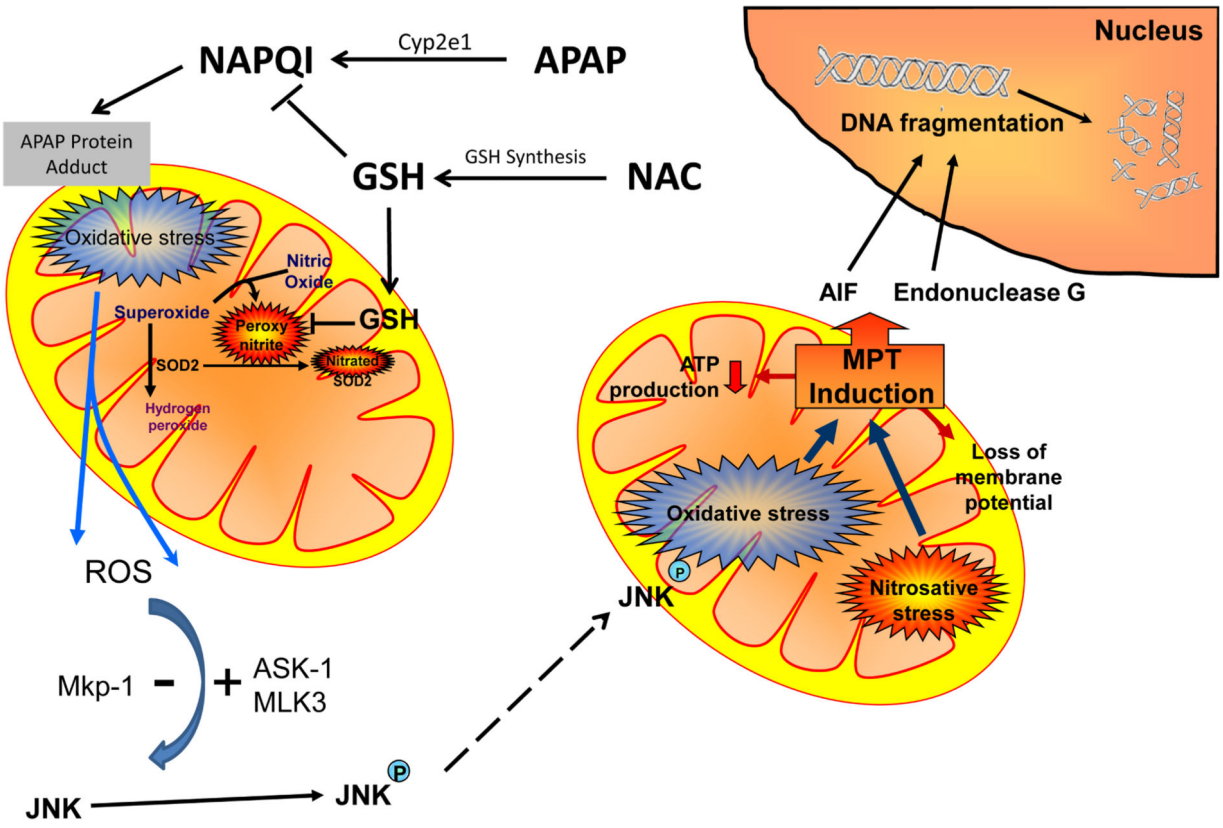

Figure 2.

Central role of mitochondrial dysfunction in the intracellular signaling mechanisms of acetaminophen (APAP)-induced cell death. APAP is converted to NAQPI mainly by cytochrome P450 2E1. NAPQI binds to proteins, including mitochondrial proteins, causing a mitochondrial oxidant stress, which leads to activation of a mitogen-activated protein kinase cascade ultimately causing JNK phosphorylation. The translocation of p-JNK to mitochondria triggers an amplification of the oxidant stress resulting in the opening of the mitochondrial permeability transition (MPT) pores with loss of the membrane potential and declining ATP production. The MPT triggers matrix swelling with rupture of the outer member and release of intermembrane proteins including apoptosis-inducing factor (AIF) and endonuclease G, which translocate to the nucleus and cause nuclear DNA fragmentation. The injury process can be attenuated by treatment with N-acetylcysteine (NAC), which stimulates GSH synthesis. GSH can scavenge NAPQI, and reactive oxygen species and peroxynitrite in mitochondria. Figure adapted from [48]. ASK-1, apoptosis signal-regulating kinase 1; Mkp-1, mitogen-activated protein kinase phosphatase; MLK3, Mixed-lineage protein kinase 3; NAPQI, *N*-acetyl-*p*-benzoquinone imine; JNK, c-Jun *N*-terminal kinase; ROS, reactive oxygen species.

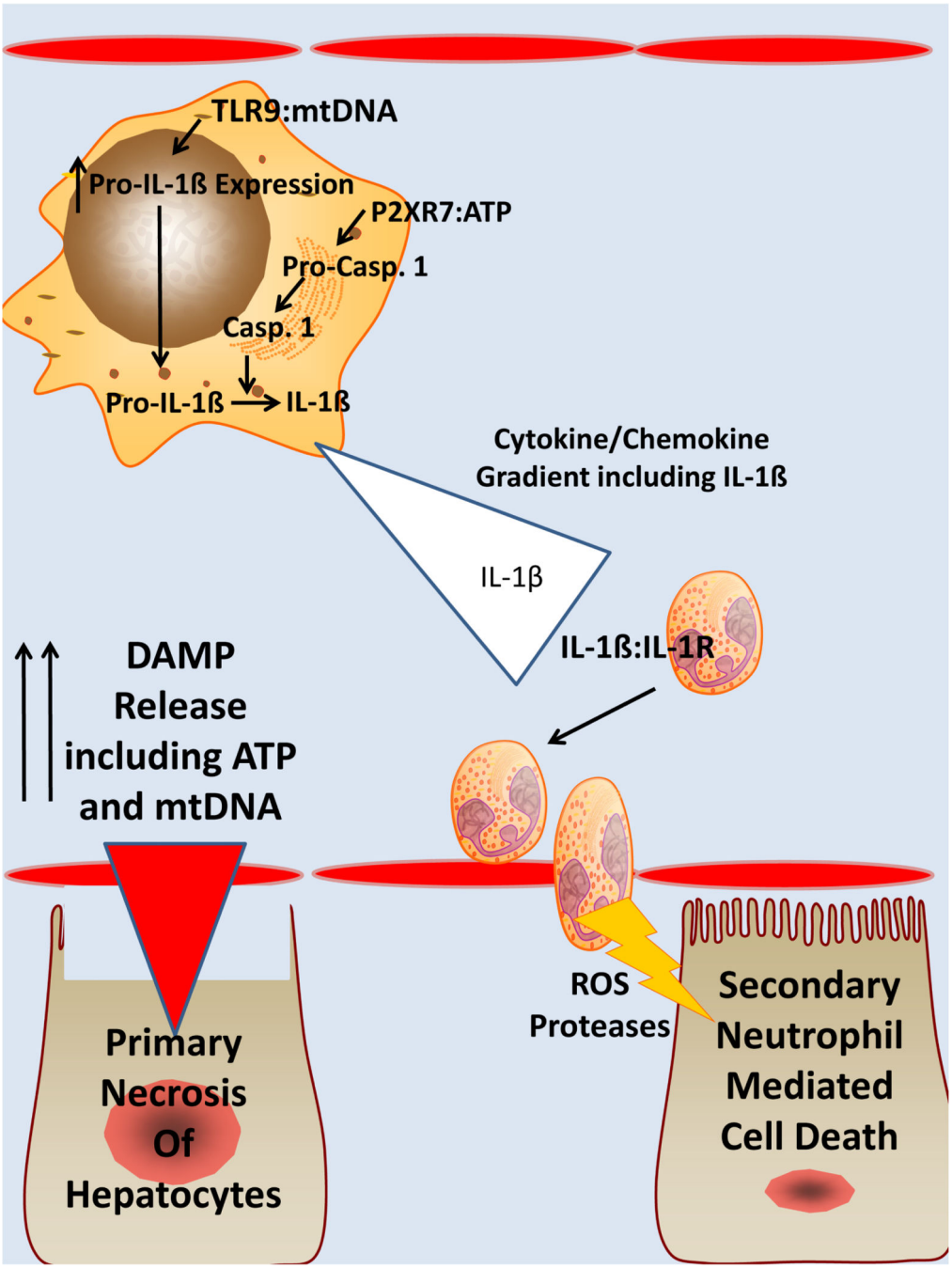

Figure 3.

Proposed schematic of APAP-induced inflammatory liver injury. DAMPs released from hepatocytes during the first phase of necrosis, including ATP, HMGB1, and mtDNA, cause transcriptional activation of cytokine formation and activation of the inflammasome in resident macrophages such as Kupffer cells. This causes formation and release of cytokines including IL-1ß, which then amplify the immune signal by activating and recruiting of neutrophils thereby causing more liver damage through release of cytotoxic reactive oxygen species or proteases. This is the second (inflammatory) phase of necrotic cell death mediated

by macrophages and neutrophils. DAMP, damage associated molecular pattern; mtDNA, mitochondrial DNA; IL-1ß, interleukin-1ß; Casp 1, caspase-1; IL-1R, interleukin-1 receptor; P2XR7, purinergic receptor P2X 7; TLR, toll-like receptor.

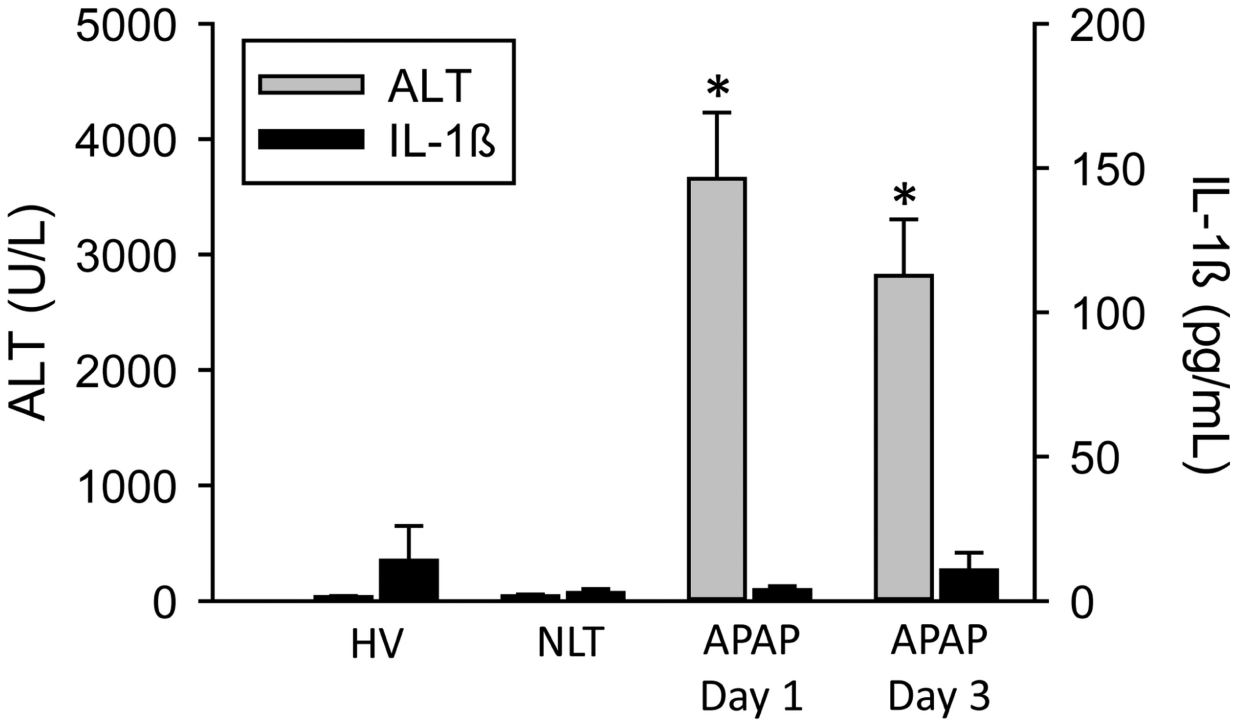

Figure 4.

Plasma IL-1ß levels and alanine aminotransferase (ALT) activities in patients with acetaminophen-induced acute liver injury. IL-1ß levels were measured as part of a multiplex cytokine array and ALT activities were determined with a kinetic enzyme assay in healthy volunteers (HV), patients with acetaminophen overdose but no major increase in serum transaminases (NLT), and in patients with severe APAP-induced liver injury. The data show the very low levels of IL-1β in APAP overdose patients with severe liver injury suggesting limited inflammasome activation in patients. Data represent means ± SE of n = 8-10 patients per group. *P<0.05 versus HV. Data adapted from [74].

Table 1

Critical Experimental Design Aspects in Drug-Induced Liver Injury

	Common Flaws	**Proposed Solution**
Metabolism	Altered APAP metabolism by drugs or other interventions	- Measure depletion of hepatic GSH between 0 and 1 hour - Measure hepatic and/or plasma APAP protein adducts at multiple time points between 30 minutes and 6 hours post APAP
Mouse Strain Problems	Strain or Substrain differences in APAP metabolism or gene expression that affects cell death	- Use of littermate controls - Extended acclimation to normalize potential microbiome issues - Co-housing of mice to further control against variation
Incomplete Time Courses	Data are only examined at 1 or 2 time points	- Evaluate injury and other parameters over extended time course: injury (0-24 h); inflammation (6-24 h); regeneration (24-96 h)
Drug treatment	Preferred use of drug pretreatment regimen	- Drug pretreatment can be useful for mechanistic studies but drug metabolism needs to be carefully evaluated - Use posttreatment (therapeutic) regimen
Repetition of Previous Studies	Previous experiments, especially with controversial results, are not considered or repeated	- More complete consideration of previous similar work and discussion of observed differences - Repeat previous experiments, especially when results are controversial - Attempts should be made to explain potential differences in results (strains/substrains/housing/etc)
Inflammatory Cells	Neutrophils/Monocytes/ Lymphocytes assumed to be cytotoxic	- More direct studies need to be performed on how these inflammatory cells cause cell death - Use more than a single intervention
Inflammatory Mediators	Cytokines/chemokines assumed as cytotoxic	- More studies are needed to directly assess the target cells of the mediators - More studies are required to evaluate how the inflammatory mediator(s) actually affect cell death and liver injury mechanism

Table 2

Critical Unresolved Issues Regarding the Role of Inflammation and the Inflammasome in Drug-Induced Liver Injury

Topic	Conflicting Papers			Potential Resolution
Role of Inflammasome	Refs. 22, 65	Vs	Refs. 72, 90	- Further delineation of KO mouse models - Understanding of IL-18 and secondary effects of the inflammasome
Role of Monocytes and Macrophages	Refs. 53, 120	Vs	Refs. 68, 83,102	- More specific treatments for targeting macrophage function
Role of NKT Cells	Ref. 123 Ref. 124	Vs Vs	Ref. 122 Ref. 124	- Better understanding of NKT cell biology in the liver - Inhibition of specific NKT functions
Role of Neutrophils	Refs. 50,51,54	Vs	Refs. 67,75,103, 115, 119	- Mechanistic assessment of specific neutrophil components - Specific, targeted removal of neutrophils without secondary effects
Are Neutrophils or Monocytes Pathogenic?	Ref. 52	Vs	Ref. 53	- Resolution of which mediator(s) affect injury
Role of TLR4	Ref. 52	Vs	Ref. 46	- Better understanding of the off-target effects of TLR knockout - Increased understanding of HMGB1 as a mediator of APAP induced liver injury
Is Inflammation Detrimental or Beneficial?	Refs. 22, 46, 52, 53, 78	Vs	Refs. 68,72, 73, 83, 90, 102 103, 119, 127	- Refined, mechanistic studies in both humans and mice designed to define mediators, and understand the pro-inflammatory and anti-inflammatory environment in a time dependent context