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The role of Intrahepatic CD3+/CD4–/CD8– double negative T (DN T) cells in enhanced acetaminophen toxicity

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Abstract

The role of the immune system, specifically NK, NKT and CD3 cells, in acetaminophen (APAP) induced liver injury remains inconsistently defined. In the present study, wild type (C57BL/6J) mice and granzyme B deficient (GrB $-/-$) mice were treated with acetaminophen to assess the role of the immune system in acute liver injury. Doses of acetaminophen that induced sub lethal liver injury in wild type mice unexpectedly produced fatal hepatotoxicity in granzyme B deficient (GrB $-/-$) mice. Analysis revealed that GrB $-/-$ mice had an increased population of intrahepatic CD3 (+), CD4 (–), CD8 (–) lymphocytes expressing the CD69 activation marker and Fas Ligand. Depletion of these cells in the GrB $-/-$ and wild type mice made them less susceptible to APAP injury, while depletion of NK1.1 (+) cells or both CD4 (+) and CD8 (+) T cells failed to provide the same hepatoprotection. Transfer of the GrB $-/-$ IHLs further exacerbated liver injury and increased mortality in wild type mice but not in LRP/LPR mice, lacking fas expression.

Conclusions—Acetaminophen toxicity is enhanced by the presence of activated, FasL expressing intrahepatic CD3 (+), CD4 (–), CD8 (–), NK1.1 (–) T cells. Depletion of these cells from GrB $-/-$ mice and wild type mice greatly reduces mortality and improves the course of liver injury recovery.

Keywords

Drug-induced liver injury; immune system; Granzyme B; CD3; T lymphocytes; Intrahepatic Lymphocytes

1. Introduction

Acetaminophen (APAP) is one of the most widely used pharmaceuticals in the world and is now a leading cause in acute liver failure in both the U.S. and many Western nations (Arundel and Lewis, 2007; Lee and Seremba, 2008). The role of APAP metabolism and the

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production of its toxic metabolite n-acetyl-benzoquinone imine (NAPQI) are well characterized. The degree of injury is directly proportional to the dose of APAP ingested and the amount of NAPQI produced. The efficiency in stabilizing this toxic metabolite NAPQI, by hepatic glutathione, directly correlates to the amount of injury. In the absence of hepatic glutathione, NAPQI binds to cysteine groups on various proteins, forming acetaminophen protein adducts. The role of inflammatory or immune responses to APAP protein products or to injured hepatocytes in determining the evolution of liver injury has been less clearly defined (Dahlin *et al.*, 1984).

The role of the adaptive immune system in drug induced liver injury (DILI) has been suggested in cases associated with a systemic manifestation of hypersensitivity and/or association with specific MHC alleles (Ju and Reilly, 2012). Amplification of DILI by natural killer (NK) cells and cytotoxic T lymphocytes (CTL) has been noted for a variety of drugs (Seguin and Uetrecht, 2003; Liu *et al.*, 2004; Martin-Murphy *et al.*, 2013). To carry out these functions, cytotoxic T lymphocytes utilize multiple cytotoxic effector mechanisms including the Fas/Fas ligand (FasL), other death receptor-mediated pathways, and the degranulation of cell mediated cytotoxic proteases. The granule exocytosis pathway utilizes perforin-dependent, granzyme (Gr)-mediated proteolysis of specific host cell proteins to initiate target cell death (Liu and Kaplowitz, 2002; Seguin and Uetrecht, 2003). The Granzymes, of which Gr B has been well characterized, comprise of a family of closely related neutral serine proteases that are expressed almost exclusively in CTL and NK cells. They are stored in lysosome-like secretory granules along with the pore-forming protein perforin. In addition, pro-inflammatory cytokines released by Kupffer cells and T cells in response to injury have also been seen in the progression of APAP hepatotoxicity (Blazka *et al.*, 1995; Ishida *et al.*, 2002; Numata *et al.*, 2007).

The potential role of cytotoxic lymphocytes in amplifying APAP liver injury has been suggested before when the observation was made that animals deficient in Fas expression are resistant to APAP toxicity (Zhang *et al.*, 2000). NK and NKT cells were previously reported to play a critical role in APAP induced liver injury (Liu *et al.*, 2004). However, the use of DMSO in the dissolution of APAP was subsequently questioned as a confounding variable and was found to activate innate immune cells, thereby enhancing hepatotoxicity. Subsequent studies performed without the use of DMSO found that NK and NKT did not amplify APAP hepatotoxicity (Masson *et al.*, 2008) while other studies have suggested that the absence of NK T cells increased sensitivity to APAP induced liver injury (Martin-Murphy *et al.*, 2013).

Thus, the role of the immune system in APAP induced liver injury still remains poorly defined and controversial. In the present study, the hypothesis that immune cells exacerbate APAP DILI was tested. Mice with targeted mutations in genes encoding granzyme B as well as animals treated with specific antibodies to deplete NK1.1, CD4 and CD8 expressing, or Thy 1 expressing T and NK cells were assessed for susceptibility to APAP toxicity. Our results indicate that thymus derived intrahepatic CD3+ T cells lacking CD4, CD8 or NK1.1 expression exacerbates APAP induced liver injury while depletion of these cells ameliorates the injury. The genetic absence of granzyme B expression was associated with increased numbers and activity of these intrahepatic CD3+ T cells lacking CD4, CD8 or NK1.1

expression. These findings affirm the contribution of the immune system to APAP induced liver injury. Defects in the granzyme B mediated pathways of induced cell death shows an increased population of intrahepatic lymphocytes and reduced hepatoprotection from APAP induced liver injury.

2. Materials and Methods

2.1 Mice

Male C57BL/6J and C57BL/6.MRL-*Fas^{lpr}*/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Granzyme B deficient (GrB^{-/-}) mice backcrossed on the B6 background for >20 generations (B6.gzmb^{-/-}) were generously provided by Dr. T. Ley (Washington University, St. Louis, MO) (Revell *et al.*, 2005). Both GrB^{-/-} and C57BL/6J (Wt) mice were bred in house under the same environmental conditions. Male mice used in experiments were between 8 and 12 weeks of age. All animal studies were carried out in compliance with accepted standards of humane animal care as described in the Guide for the Care and Use of Laboratory Animals (Council., 1996) and were approved by the UT Southwestern Institutional Animal Care and Use Committee.

2.2 Acetaminophen Administration and serum alanine aminotransferase (ALT) assay

Following an overnight fast, mice were injected intraperitoneally (i.p.) with doses of acetaminophen (APAP) dissolved in DPBS varying from 200 to 300 mg/kg body weight, as indicated, or an equal volume of DPBS. Serum levels of alanine transaminase (ALT) were determined as previously described (Getachew *et al.*, 2010).

2.3 APAP protein adduct measurement

Following euthanasia, blood samples were drawn to obtain serum for measurement of acetaminophen protein adducts; livers were harvested, snap frozen using liquid nitrogen cooled aluminum tongs, and powdered under liquid nitrogen. APAP protein adducts were quantified in plasma and powdered liver homogenates by high-pressure liquid chromatography and electrochemical detection (HPLC-ECD) as previously described (Muldrew *et al.*, 2002).

2.4 Liver glutathione content and Immunoblotting of liver Cyp2E1 and Cyp1A2

Glutathione levels and immune blotting techniques were performed previously described (Getachew *et al.*, 2010).

2.5 Quantitative RT-PCR of select liver mRNA

RNA isolation and PCR methods have been previously described (Getachew *et al.*, 2010). Primer sequences used are listed in supplemental Table 1 and unless indicated all primers are noted to be of mouse origin: 18S and GAPDH were ordered from Sigma Aldrich Life Science (The Woodlands, TX) and B-Actin from IDT Integrated DNA Technologies (Eugene, OR). Relative mRNA levels were quantified using the delta delta CT method (Stout-Delgado *et al.*, 2007).

2.6 Depletion of CD3, CD4, CD8, and NK cells

Depletion of the mouse Thy-1.2 (CD90.2) expressing cell population is effective in depletion of CD3 (+) T cells in vivo (Sugai *et al.*, 1998). Anti-Thy-1.2 protein was produced by cloning it in a pET23b vector that was translated and purified via Ni-NTA affinity (Ledbetter and Herzenberg, 1979). Antibody production occurred by successive injections of Thy-1.2 into a host rabbit; crude serum was purified via a Thy-1.2 protein-affinity column to isolate out the Anti-Thy-1.2 monoclonal antibodies (Harlow and Lane, 1988) performed by Pierce Custom Services and Thermo Scientific (Rockford, IL) (Burgess and Deutscher, 2009). Mice were treated with 20.5 µg of Anti-Thy-1.2 every 48 hours starting 7 days before APAP administration with the last injection occurring 18–24 hours before APAP administration. This protocol depleted > 90% of CD3 (+) IHL without affecting the absolute number of CD3 (–) NK1.1 (+) IHL. Control mice received equivalent amounts of rabbit polyclonal serum antibody (Pierce Custom Services and Thermo Scientific, Rockford, IL) prior to APAP treatment in all the depletion experiments. Depletion of CD4 and CD8 were carried out in a similar fashion using GK 1.5 and YTS 169 antibodies as previously described (Thiele *et al.*, 1992). This protocol depleted > 95% of CD4 (+) and CD8 (+) T cells without affecting the absolute number of CD3 (–) NK1.1 (+) IHL. Similarly, depletion of hepatic NKT and NK cells was achieved by intraperitoneal injections of 300-µg mouse anti-NK1.1 antibody (PK136, purchased from Applied Biosystems). This protocol depleted > 95% of CD3 (–) NK1.1 (+) IHL.

2.7 Isolation of IHL, infusion of IHL and Flow cytometry

Liver intrahepatic lymphocyte isolation has been described in detail (Crispe, 2001). The frequency of positive cells was determined using a CyAn™ LX flow cytometer (Dako Cytomation, Carpinteria, CA) and FlowJo software (TreeStar, Ashland, OR). Cell gating was performed on singlets, while excluding doublets, on CD45 (+) leukocytes live cells. Several staining antibodies were used to assess the different cell populations: anti-CD45 (BD Biosciences), anti-NK1.1 (PK 136, PE or APC; BD Biosciences), anti-CD3 (145-2C11, FITC or PE; BD Biosciences), anti-CD 69 cells (FITC), anti-CD4 and anti-CD8 (PerCP), anti-LY6C (FITC), anti-CD 11a (PE), anti-CD19 purchased from BD Biosciences and 7-aminoactinomycin D (7-AAD; BD Biosciences). Intra cellular FasL staining was done using anti-FasL (APC) as described by Baum *et al.* (Baum *et al.*, 2005). IHL's that were isolated from GrB –/– mice were injected via tail vein to wild type B6 strain and LPR/LPR strain. The number of IHL's infused per mouse was 2–3 × 10⁶ cells. Administration of APAP at the indicated dosed proceeded after - 48 hrs of tail vein injection.

2.8 Histology

Formalin-fixed liver sections were embedded in paraffin, and 5-µm sections were cut and stained with hematoxylin and eosin. Slides were graded in a blinded manner by a pathologist, and images were captured at ×4, and ×20 magnifications.

2.9 Statistical evaluation

Data are presented as mean ± SE. To detect differences among groups, statistical significance was evaluated by t-test (for comparison of two groups) or one-way ANOVA

(followed by a posttest using Bonferroni for comparison of multiple groups; Graph Pad Prism Version 5 [Graph Pad Software, San Diego, CA]); differences at the level of $p < 0.01$, $p < 0.02$ and $p < 0.05$ are noted separately.

Results

GrB $-/-$ mice exhibit enhanced liver injury and mortality after APAP treatment

To further characterize the importance of the Granzyme B dependent pathways in lymphocyte mediated cytotoxicity, GrB $-/-$ mice and wild type mice were treated with 200–300 mg/kg of APAP. Toxicity was assessed by ALT serum measurements. Administration of APAP resulted in marked ALT elevations in both groups of mice. However, there was a marked difference noted in the extent of injury exhibited after the administration of 200mg/Kg or 300mg/Kg of APAP in the different strains. During the first 12 hours after APAP administration, ALT levels in GrB $-/-$ mice were 2–3 fold higher and the extent of centrilobular (zone 3) and mid-zonal hepatocyte necrosis was greater than in wild type B6 mice receiving the same dose of APAP (Fig 1A – D). In addition, at 300mg/Kg, whereas liver injury in APAP treated wild type mice resolved over 36–48 hours, GrB $-/-$ mice exhibited 100 % mortality within 24 hours (Fig 1E). At high doses of APAP (700 mg/kg), 100% mortality was also achieved in wild type mice (data not shown).

APAP metabolism is comparable in wild type and GrB $-/-$ mice at baseline and post APAP administration

Levels of mRNA transcripts for the major P450 isoforms responsible for APAP metabolism were measured at baseline and 8 hours post APAP administration. Messenger RNA levels for Cyp2e1, Cyp1a2 and Cyp3a11 were similar in both strains of mice (Fig. 2A). Immunoblot assays of Cyp2e1, Cyp1a2 and Cyp3a11 protein levels were similar at baseline and 8 hours, consistent with the mRNA results (data shown only for Cyp2e1 Fig. 2B and C) in both mouse strains. Quantification of acetaminophen protein adducts is recognized as a surrogate marker of APAP-metabolism; an important mechanistic component of the toxicity (Muldrew *et al.*, 2002). To investigate whether the increased liver injury in GrB deficient mice was related to metabolism APAP protein adducts were determined in liver samples at 90 min and 3 hours post-APAP administration (Fig. 2D). As depicted in the figure, hepatic APAP protein adducts were comparable between the two groups of mice. In addition, hepatic glutathione depletion was comparable between the two groups of mice (Figure 2E). These findings indicated that the greater extent of liver injury exhibited by GrB $-/-$ mice was due to mechanisms independent to the CYP P450 metabolism of APAP.

Intrahepatic lymphocytes from Granzyme B deficient mice exhibit increased numbers of CD3+ / CD4 (–) / CD8 (–) T cells

In additional experiments, the livers of GrB $-/-$ and wild type mice were examined for levels of various lymphoid and myeloid cells likely to be involved in drug induced inflammatory responses. No differences were noted in numbers of peripheral blood mononuclear cells (PMN), B Cells, and Macrophages. However as depicted in the figure 3A, higher levels of mRNA encoding CD3 and FasL transcripts were noted at baseline in GrB $-/-$ mice. Levels of CD4 and CD8 mRNA transcripts were comparable in the GrB $-/-$ and the

wild type mice, suggesting that the differences in CD3 mRNA are not due to differences in the numbers of CD4 or CD8 expressing cells. Using 4-color flow analysis of intrahepatic lymphocytes (Fig. 3B and E), increased numbers of CD3 (+) cells were observed in the GrB $-/-$ mice compared to the wild type mice. Further analysis of the CD3 (+) cells revealed a higher level of CD3 (+), CD4 (-), CD8 (-) intrahepatic lymphocytes in granzyme B deficient mice (Fig 3C and E). The numbers of NK and NKT cells, measured by enumeration of NK 1.1 expressing cells, were noted to be comparable in the two strains (Fig. 3D).

Intrahepatic lymphocytes from Granzyme B deficient mice exhibit enhanced Fas Ligand expression and increased numbers of activated CD3 (+) CD4 (-) CD8 (-) T cells

Further analysis of the intrahepatic CD3 (+) double negative (DN) lymphocytes in GrB $-/-$ and wild type mice revealed a high percentage of CD69 (+) that are FasL (+) (Fig 4A and B). In both GrB $-/-$ and wild type mice, the majority of CD3 (+) CD4 (-) CD8 (-) IHL expressed TCR $\alpha\beta$, however greater than 2/3 of both TCR $\alpha\beta$ and TCR $\gamma\delta$ expressing IHLs expressed FasL. Assessment of PBMCs or spleen cells in wild type or GrB $-/-$ mice showed that a similar, small percentage (<5%) of the lymphocyte population was DN T cells (data not shown). In contrast, a higher proportion of lymphocytes in the mesenteric lymph nodes from wild type and an even larger number of mesenteric lymph node cells in granzyme B deficient mice were noted to have these activated DN T cells (Fig 4C).

Depletion of CD3 (+) T cells but not of NK1.1 (+) or CD4 (+) and CD8 (+) T cells ameliorates APAP induced liver injury and death

The presence of an increased number of activated FasL expressing CD3(+) T cells in GrB $-/-$ B6 mice suggested their potential role in the greater susceptibility of these mice to APAP induced liver injury. To further assess this potential role, mice were treated with a neutralizing anti-Thy1.2 antibody prior to APAP treatment (300 mg/kg). CD3 (+) T cells were depleted without affecting other cell lines such as NK or B cells. Following depletion of T cells, mice were tested for their susceptibility to APAP injury. As shown in figure 5A, both the GrB $-/-$ and the wild type mice treated with the anti-Thy1.2 antibody prior to APAP had markedly lower levels of ALT. Specifically, T cell depleted GrB $-/-$ mice survived treatment with a dose of APAP that caused 100% mortality in previous experiments GrB $-/-$ mice (Fig 5B). To determine whether NK T cells account for the enhanced APAP injury in granzyme B deficient mice, GrB $-/-$ and B6 wild type mice were treated with APAP after depletion of NK 1.1 positive cells. As shown in the figure 5C, depletion of NK 1.1 cells did not alter the degree of APAP induced ALT elevations in wild type or GrB $-/-$ B6 mice. To further investigate which subset of T cells was responsible for amplifying APAP toxicity in GrB $-/-$ mice, wild type and GrB $-/-$ mice were depleted of CD4 and CD8 cells as described in the materials and methods section. ALT levels were measured at 8 hours interval post APAP administration. There was no significant reduction in ALT levels in wild type or GrB $-/-$ groups after depletion of both CD4+ and CD8+ T cells (Fig. 5D). These findings indicate a unique role of CD3 (+) CD4 (-) CD8 (-) NK1.1-T cells in accentuating APAP induced liver injury.

Infusion of GrB $-/-$ IHL's in wild type but not in Fas deficient LPR/LPR mice resulted in enhanced liver injury and mortality after APAP treatment

To further characterize the importance of the IHL in enhancing APAP toxicity, IHLs isolated from Granzyme B deficient mice were infused via tail vein into wild type and Fas deficient LPR mice 48 hours before the treatment of APAP (300 mg/kg). Toxicity was assessed by ALT measurements in serum. As previously reported, the degree of ALT rise in LPR mice is significantly lower than in wild type mice. In contrast, IHL infused wild type mice exhibited significantly higher ALT elevation 8 hrs after APAP while limited toxicity was noted in APAP treated LPR mice after transfer of GrB $-/-$ IHL (Fig 6A). In addition, whereas liver injury in APAP treated LPR mice that had received GrB $-/-$ IHL resolved over 36–48 hours, wild type mice infused with GrB $-/-$ IHL exhibited 100 % mortality within 24 hours similar to the results seen with GrB $-/-$ mice (Fig 6B).

Discussion

The role of the immune system in drug induced liver injury has been suggested by a number of observations (Ju and Reilly, 2012). The involvement and recruitment of liver nonparenchymal cells (NPC) and Kupffer cells (KC) has also been shown to amplify APAP induced liver injury through the release of HMGB1 and HSP-70 molecules by damaged hepatocytes (Martin-Murphy *et al.*, 2010). The mechanistic role of neutrophils (Liu *et al.*, 2004) in APAP DILI is unclear and conflicting data has been published (Cover *et al.*, 2006; Williams *et al.*, 2010a; Williams *et al.*, 2010b). As in the present studies, multiple studies have found animals deficient in Fas expression to be resistant to APAP hepatotoxicity (Zhang *et al.*, 2000; Liu *et al.*, 2004; Williams *et al.*, 2013). Some have found that LPR mice with lifelong Fas deficiency exhibit faster recovery of hepatic GSH levels, reduced iNOS induction, and enhanced expression of Hsp70; suggesting that these are off target effects of the LPR gene mutation accounting for the reduced susceptibility to APAP-induced cell death (Williams *et al.*, 2013). However, the short term reduction of Fas mRNA and protein expression following siRNA administration also affords similar protection from APAP induced liver injury supporting a true role for this death receptor pathway in modulating this form of drug induced liver injury (Zhang *et al.*, 2000).

The present study, using APAP dissolved in DMSO-free phosphate buffered saline, demonstrates that the presence of activated, CD4 $(-)$, CD8 $(-)$ (DN) T cells in the livers of granzyme B deficient mice leads to enhanced APAP induced liver injury and mortality. Previous studies have suggested that the metabolism of APAP may be altered in genetically modified mice (Lee *et al.*, 1996). The present study demonstrates that the metabolism of APAP, as reflected by protein expression of Cyp2E1, hepatic glutathione, and hepatic levels of APAP protein adducts, was comparable between the wild type and the GrB $-/-$ mice. The increased sensitivity of the GrB $-/-$ mice was associated with increased numbers of intrahepatic activated DN T cells, suggesting that the immune response significantly impacted the final degree of liver injury. Depletion of all T cells with an anti-Thy1 antibody treatment, not depleting the CD4 $(+)$ and CD8 $(+)$ T cells or NK1.1 $(+)$ NK and NKT cells, afforded protection from APAP induced liver injury in wild type mice as well as in granzyme B deficient mice. Thus, resident intrahepatic DN T cells played a role in

amplifying APAP hepatotoxicity in the wild type mice that was even more apparent in the granzyme B deficient mice due to the increased population of intrahepatic DN T cells.

Analysis of mesenteric lymph nodes demonstrated that more than 50% of the T cells in this compartment of the mucosal immune system lacked CD4 or CD8 expression in the wild type and GrB $-/-$ mice. These cells are rarely seen in the spleen or in peripheral blood. While DN T cells in the mesenteric lymph nodes of wild type mice rarely express additional activation markers such as CD69, higher percentages of CD69 positive DN T cells were present in mesenteric lymph nodes of GrB $-/-$ mice. The presence of even higher numbers of activated CD69 (+) Fas Ligand expressing DN T cells in the livers of granzyme B deficient mice likely relates to the previously noted role of granzyme B in T cell activated cell death (Zhang *et al.*, 2006a) as well as the propensity of activated T cells to infiltrate and accumulate in the liver (John and Crispe, 2004; Tu *et al.*, 2007). Granzyme B mediates T cell receptor - induced cell death (TCR-ICD) in Th2 cells, while CD95L is required for T cell receptor - induced cell death (TCR-ICD) in T helper 1 cells. In addition, granzyme B has been demonstrated to be involved in homeostatic regulation of CD8 (+) cytotoxic T cells (Devadas *et al.*, 2006) and to regulate the function of Treg that in turn suppress NK and CTL activity. The increased numbers of intrahepatic activated DN T cells in GrB $-/-$ mice suggests a role for granzyme B in TCR-ICD in these cells similar to what has been described for CD8 (+) cytotoxic T cells and Th2 cells. Previous studies have shown that DN T cells can exhibit an immune-regulatory role via either Fas/FasL interactions or via perforin-dependent mechanisms (Priatel *et al.*, 2001; Zhang *et al.*, 2006b; Ford *et al.*, 2007; Zhang *et al.*, 2007). The fact that intrahepatic DN T cells from granzyme B deficient mice are capable of enhancing APAP induced liver injury in wild type but not in fas deficient mice supports an additional role for DN T cells in exacerbating liver injury, via FasL/Fas effector pathways. These current observations are consistent with findings from studies by Abougergi et al which have shown that virally infected hepatocytes also are cleared predominantly via FasL/Fas pathways rather than granule-dependent cytolytic mechanisms (Abougergi *et al.*, 2005).

Another interesting implication of the current findings is the potential recognition and ability to directly or indirectly injure non-lymphocyte, self-targets, i.e. hepatocytes by DN T cells. CD3+ DN T cells also have been shown to play a role in amplifying early ischemia reperfusion injury (Eggenhofer *et al.*, 2013). The ability of DN T cells to respond to ischemic or APAP induced liver injury and amplify the course of hepatotoxicity that evolves over the first 3–24 hours suggests that these T cells have insufficient time to develop adaptive immune responses to non-self antigens and thus are more likely to be responding via innate immune response mechanisms or TCR responses to auto- or neo-antigens exposed or produced as part of hepatocyte injury. In the present study, we were not able to confirm previous observations suggesting that NKT cells expressing NK1.1 mediate such immune amplification of APAP hepatotoxicity. Only a minor fraction (30 %) of the DN T cells present in expanded numbers in the livers of GrB $-/-$ mice co-expressed NK1.1 and depletion of these cells by anti-NK1.1 treatment from GrB $-/-$ or wild type mice prior to administration of APAP did not prevent nor ameliorate hepatotoxicity.

In summary, the present studies demonstrate an important role for a unique population of activated, FasL expressing intrahepatic DN T cells in the amplification of APAP induced liver injury. These cells are increased in number and in the degree of activation leading to enhanced susceptibility to APAP hepatotoxicity in mice lacking the granzyme B lymphocyte expression. Further characterization of this population of intrahepatic lymphocytes and study of the immunoregulatory role of granzyme B in modulating the functions of intrahepatic DN T cells should provide additional insight into the role of immune responses in the amplification of drug induced liver injury.

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Abbreviations used in this article

GrB	granzyme B
DN T	double negative T
APAP	acetaminophen
DILI	Drug-induced liver injury
NAPQI	n-acetyl-benzoquinone imine
NK	natural killer
CTL	cytotoxic T lymphocytes
FasL	Fas ligand
Gr	granzyme
i.p.	intraperitoneally
ALT	alanine transaminase
HPLC-ECD	high-pressure liquid chromatography and electrochemical detection
NPC	non parenchymal cells
KC	kupffer cells
PBMC	peripheral blood mono-nuclear cells

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Highlights

- Intrahepatic lymphocytes (IHL's) from GrB $-/-$ mice harbor activated DNT cells.
- IHL's from GrB $-/-$ mice exhibit enhanced Fas Ligand expression.
- Acetaminophen toxicity is enhanced by activated, FasL expressing DNT cells.

Fig. 1.

GrB ^{-/-} mice exhibit increased sensitivity to APAP. (A) ALT levels were measured at the indicated time points after APAP was administered to wt B6 and GrB ^{-/-} mice at 200 mg/kg. Data represent the mean $\pm$ standard error of all mice (N= 10). (B) Liver damage was assessed in wt B6 and GrB ^{-/-} mice at 300 mg/Kg by measuring ALT levels at the different time points. Data represent the mean $\pm$ standard error of all mice (N= 10). Liver histology (H&E stain) shown at 4 $\times$ and 20 $\times$ magnification 8 hours after administration of 300 mg/Kg APAP in (C) wild type and (D) GrB ^{-/-} mice. (E) GrB ^{-/-} mice with 100% mortality 16 hours of the 300 mg/Kg APAP administration (N=10). Each figures' legend depicts an independently performed 3 experiments.

Fig. 2. APAP metabolizing enzymes and hepatic APAP protein adduct levels are comparable in wild type and GrB^{-/-} mice treated with APAP (300 mg/kg). **(A)** Histogram shows the mean $\pm$ standard error of the mean (SEM; $n = 10$ mice per group) of CYP 2A1, CYP 2E1 and CYP 3A11 messenger RNA (mRNA) expression, relative to GAPDH, as analyzed by real-time PCR. **(B)** Representative western blotting showing CYP 2E1 and GAPDH in proteins extracted from the livers of mice at baseline. **(C)** Relative amount of protein expression determined by densitometry. **(D)** Liver APAP protein adduct levels were measured at the indicated times. **(E)** Hepatic glutathione levels in wt B6 and GrB^{-/-} mice at the indicated time points. Data represent the mean $\pm$ standard error of all mice ($N = 10$). Each experiment was performed in duplicate.

Fig. 3.

Granzyme B deficient mice exhibit increased numbers of CD3 (+) CD4 (-) CD8 (-) T cells in their intrahepatic lymphocytes and enhanced expression of CD3 and FasL in the whole liver. (A) Baseline mRNA levels of GrB^{-/-} B6 vs. wild type B6 show significantly higher levels of CD3 and FasL mRNA. Data represents mean $\pm$ SEM (n = 20 per group). (B) Absolute number of flow cytometry of CD3 (+) IHL. Data represents mean $\pm$ SEM (n = 20 per group). (C) Absolute number of flow cytometry of CD4 and CD8 expression by CD3 (+) IHL. Data represents mean $\pm$ SEM (n = 20 per group). (D) Absolute number of NK1.1 (+) CD3 (-) versus NK1.1 (-) CD3 (+) IHL. Data represents mean $\pm$ SEM (n = 20 per group). (E) Flow cytometry analysis of phenotypes of CD45 (+) CD3 (+) and CD45 (+) CD3 (+) CD4 (-) CD8 (-) IHL cells (a representative of density plot) *p = 0.04, **p < 0.01, *** p < 0.05. Each figure legend depicts an independently performed 2 experiments.

Fig. 4.

Intrahepatic lymphocytes from Granzyme B deficient mice exhibit enhanced Fas Ligand expression and increased numbers of activated CD3 (+) CD4 (-) CD8 (-) T cells, while mesenteric lymph node lymphocytes from Granzyme B deficient mice exhibit increased numbers of CD3 (+) CD4 (-) CD8 (-) T cells. (A) Absolute number of flow cytometry of CD3 (+) CD4 (-) CD8 (-) IHL. Data represents mean $\pm$ SEM (n = 20 per group). (B) Flow cytometry analysis of phenotypes of CD45 (+) CD3 (+) CD4 (-) CD8 (-) IHL cells (a representative of density plot). (C) Baseline flow studies evaluating absolute number of CD3 (+) vs. CD3 (+) CD4 (-) CD8 (-) in mesenteric lymph node cells. Data represents mean $\pm$ SEM (n = 20 per group). *** p < 0.05. Each figure legend depicts an independently performed 2 experiments.

Fig. 5.

Depletion of CD3 (+) T cells using anti-Thy1 or lack of functional CD3 (+) T cells ameliorates APAP induced liver injury and death while depletion of either NK1.1 (+) or CD4 (+) and CD8 (+) cells does not alter susceptibility to APAP induced liver injury. **(A)** Mice that were treated with anti- thy 1 and control mice that were treated with sham polyclonal rabbit serum were assessed for serum levels of ALT at the indicated time points after administration of 300mg/Kg APAP. Data indicate the mean $\pm$ standard error of the mean (SEM) of all mice (N = 5 per group). Special symbol (*) indicates all animals were deceased at this time point in GrB $-/-$ control group. **(B)** Percent survival for GrB $-/-$ control mice and Thy 1.1 treated GrB $-/-$ mice is depicted after 300 mg/Kg APAP administration (N=10). **(C)** Mice that were treated with anti- NK1.1 and control mice were assessed for serum levels of ALT at 8 hours after APAP treatment. Data indicate the mean $\pm$ standard error of the mean (SEM) of all mice (N = 5 per group). **(D)** Mice that were treated with GK 1.5 / YTS 169 and control mice were assessed for serum levels of ALT at 8 hours after APAP treatment. Data indicate the mean $\pm$ standard error of the mean (SEM) of all mice (N = 5 per group). Each figure legend depicts an independently performed 2 experiments.

Fig. 6.

Infusion of GrB $-/-$ IHLs in wild type mice resulted in enhanced liver injury. (A) Wild type and LPR/LPR mice were treated with 300 mg/Kg (APAP) 48 hours after they were infused with 2–3 million GrB $-/-$ IHLs via tail vein per mouse. Serum ALT levels were measured at the indicated time points. Special symbol (*) indicates all animals were deceased at this time point. Data indicate the mean $\pm$ standard error of the mean (SEM) of all mice (N = 5 per group). (B) Wild type mice infused with IHL exhibited significantly higher mortality in 24 hours after 300 mg/Kg APAP administration (N=5). Each figure legend depicts an independently performed 2 experiments.