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Interleukin-1 inhibition facilitates recovery from liver injury and promotes regeneration of hepatocytes in alcoholic hepatitis in mice

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Abstract

Background & Aims—Inflammation and impaired hepatocyte regeneration contribute to liver failure in alcoholic hepatitis (AH). Interleukin (IL)-1 is a key inflammatory cytokine in the pathobiology of AH. The role of IL-1 in liver regeneration in the recovery phase of alcohol-induced liver injury is unknown.

Methods—Here we tested IL-1 receptor antagonist to block IL-1 signaling in a mouse model of acute-on-chronic liver injury on liver inflammation and hepatocyte regeneration in AH.

Results—We observed that inhibition of IL-1 signaling decreased liver inflammation, neutrophil infiltration, enhanced regeneration of hepatocytes, and resulted in increased rate of recovery from liver injury in AH.

Conclusion—Our novel findings suggest that IL-1 drives sustained liver inflammation and impaired hepatocyte regeneration even after cessation of ethanol exposure.

Keywords

alcoholic liver disease; interleukin 1; interleukin 1 receptor antagonist; liver regeneration

Introduction

Impaired liver regeneration correlates with poor survival in patients with severe acute alcoholic hepatitis (AH).[1] The inability of patients with AH to restore fully functional hepatocyte mass and function has been reported in multiple clinical studies, [1,2] but the mechanisms of this phenomenon are yet to be elucidated. Studies using mouse models of

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Conflict of Interest: None

ALD have focused predominantly on the pathogenesis of ALD (reviewed in ref.[3,4]) but have not addressed hepatocyte regeneration during the recovery phase. The aim of this study was to propose a mouse model that would provide a tool to study liver recovery after exposure to chronic ethanol. We also used this model to investigate whether liver recovery can be facilitated by inhibition of IL-1, a cytokine crucially involved in the pathogenesis of ALD through NLRP3 activation inflammasome in mice.[5–7]

In our model, we attempted to mimic human drinking patterns of acute-on-chronic ethanol exposure in alcoholic patients. Wild-type mice were fed Lieber-DeCarli ethanol diet for 4 weeks. To enhance liver injury, mice received 3 acute doses of ethanol via an intragastric probe during the last 3 days of ethanol feeding (Maximal Injury). Subsequently, all mice were transitioned to the control diet with no ethanol and randomly assigned to daily treatment with Anakinra (IL-1Ra, an inhibitor of IL-1 signaling) or saline (Recovery phase), both delivered via intraperitoneal injections. Mice were sacrificed at baseline, at the point of maximum injury, and during the recovery phase, 1, 2, 3, 4 and 7 days after transition to control diet (Fig. 1A).

Materials and Methods

Animal Studies

6- to 8-week old female C57Bl/6 wild-type mice (purchased from Jackson Laboratory), were fed Lieber-DeCarli ad libitum diet (Dyets Inc.) with 5% (vol/vol) ethanol (36% ethanol-derived calories) for weeks; pair-fed control mice matched the ethanol-derived calories with maltose-dextrin. Mice received fresh Lieber-DeCarli diet in 50-mL feeders daily between 7 PM and 8 PM. At the conclusion of the experiment, we collected blood and harvested livers between 8 AM and 9 AM. To enhance liver injury, mice received 3 acute doses of ethanol (5 g/kg body weight) or isocaloric maltodextrin via an intragastric probe (a 22-gauge stainless steel feeding tube) during the last 3 days of ethanol feeding ('Max. Injury'). Subsequently, all mice were transitioned to the control liquid diet with no ethanol (Dyets Inc.). In the same time, mice were started on daily treatment with Anakinra (Amgen; 25 mg/kg) or saline delivered via intraperitoneal injections. We sacrificed the mice at the following time points: at baseline, at the point of maximum injury, and in the 'Recovery Phase', 1, 2, 3, 4 and 7 days after transition to control diet. 'Baseline' refers to mice sustained on iso-caloric control liquid diet for the duration of the feeding (Week 5, day 7). All animals received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School.

Serum and RNA Analysis

Serum alanine aminotransferase (ALT) was determined using a kinetic method (D-Tek LLC., Bensalem, PA). RNA was purified using the RNeasy kit (Qiagen Sciences) with on-column DNase digestion. cDNA was transcribed with the Reverse Transcription System (Promega, Corp.). qPCR was performed using Sybr-Green and iCycler from BioRad (BioRad Laboratories Inc.).

Histological analysis

A blinded, semi-quantitative analysis of H&E liver histology scoring was performed for hepatocellular ballooning (0–2), lobular inflammation (0–2) and steatosis (0–3).[19] Ki67 immunohistochemistry was performed with Ki67 polyclonal Ab (1:600, Vector Laboratories) and analyzed using Fiji software, as described previously;[20] Cyclin D1 immunohistochemistry was performed with Cyclin D1 mAb (clone SP4, 1:1000, Thermo Fisher Scientific). Myeloperoxidase (MPO) immunohistochemistry was performed with MPO polyclonal Ab (LS-B4741, LifeSpan Biosciences), as described in Ref.[8]. MPO and Cyclin D1 quantification was performed by counting the number of positive stained cells per visual field at 400x magnification, and averaging the values for five random images per individual sample.

Statistical Analysis

Statistical significance was determined by use of 2-sided t-test; ANOVA and Dunnett's multiple comparison post-test were used to compare the means of multiple groups. Data are shown as mean $\pm$ SEM, and were considered statistically significant at $P < 0.05$. We used Graphpad Prism 7 to perform statistical analyses.

Results

At the point of ethanol-induced maximum injury, H&E staining showed large inclusions of fat, some hepatocyte ballooning, and mild inflammatory infiltrate (Fig. 1B). In the recovery phase, ballooning slowly resolved within 72 hours (Fig. 1C), and scattered foci of inflammatory cells surrounding dead hepatocytes appeared within 24 hours but disappeared within 48 hours (Fig. 1D). Compared to saline-treated mice, the extent of liver inflammatory cell infiltration was significantly lower in mice treated with Anakinra (Fig. 1E). No significant differences were observed in the extent of ballooning (Fig. 1D). As ballooning provides only semi-quantitative assessment of hepatocyte damage, we used serum ALT for quantitation of hepatocyte damage. The mean ALT level at the point of maximal injury was 250 U/L (Fig. 1E), which is approximately two times higher compared to levels we observed in previous studies in mice on Lieber-DeCarli ethanol diet without acute ethanol binge.[8–10] ALT levels normalized by day 4 in saline-treated mice and by day 2 in mice treated with Anakinra, indicating that inhibition of IL-1 signaling facilitates recovery from liver injury (Fig. 1E). We further evaluated the role of Anakinra on inflammation by probing for neutrophil liver infiltration using myeloperoxidase (MPO) immunohistochemistry. We found levels of MPO staining had rapidly normalized by day 1 of recovery phase in mice treated with Anakinra and remained low, while saline-treated mice maintained elevated levels as late as day 4. Lastly, Oil-red-O staining of the liver, that was used to evaluate the accumulation of fat, showed significant steatosis at the point of maximal injury (Fig. 1H). In the recovery phase, fat droplets disappeared (Fig. 1B,H) and steatotic score dropped within 24 hours (Fig. 1I). We observed rapid regression of steatosis during the recovery phase which was so fast that we could not evaluate the role of Anakinra in this scenario.

To evaluate liver regeneration, we stained livers for Ki67, a marker expressed by proliferating cells but absent in quiescent cells. Livers harvested from mice at baseline and at

the point of maximal injury showed minimal Ki67 signal, indicating minimal proliferative activity and there was no significant difference between these two time-points (Fig. 2A). Compared to the point of maximal injury, increased Ki67 nuclear staining was present in hepatocytes from saline-treated mice on days 1 and 2 of recovery. Furthermore, the increase in Ki67 was even greater in mice treated with Anakinra (Fig. 2A,C). Image analysis showed a significant increase in the proportion of Ki67-positive hepatocytes on days 1 and 2 of recovery (Fig. 2A,C), and this proportion was significantly higher in mice treated with Anakinra compared to saline treatment. We further tested our hypothesis by evaluating Cyclin D1 levels in the liver. Cyclin D1 is a nuclear protein required for progression through the G1 phase of the cell cycle. Cyclin D1 liver immunohistochemistry staining in murine liver sections 24 hours post partial hepatectomy served as positive control (Fig. 2B). We found that hepatocellular size increased after partial hepatectomy, with distinct nuclear and cytoplasmic expression in controls, while ethanol administration resulted in a different pattern of expression similar to that observed with Ki67 staining. Mice treated with Anakinra had increased levels of Cyclin D1 protein expression per visual field compared to saline-treated controls (Fig. 2B,D). This data indicated that inhibition of IL-1 signaling facilitates liver regeneration in ethanol-induced liver injury. The proportion of Ki67-positive hepatocytes significantly decreased by day 3 and returned to baseline by day 4 of recovery both in the Anakinra and saline treated groups (Fig. 2C), while Anakinra-treated mice had consistently higher levels of Cyclin D1 throughout the recovery phase (Fig. 2D).

Next, we evaluated expression of TNF- α (an inflammatory cytokine), MCP-1 (an inflammatory chemokine), KC (an inflammatory chemokine equivalent to human IL-8) and IL-6 (an inflammatory cytokine involved in hepatocyte regeneration) in the livers. Expression of TNF- α , MCP1 and KC showed maximal upregulation at 24 hours after withdrawal of ethanol, followed by return to baseline expression at day 2 of recovery (Suppl. Fig. 1A–C). In contrast, upregulation of IL-6 was significant delayed, and peaked at day 4 of recovery (Suppl. Fig. 1D). Importantly, mice treated with Anakinra showed ameliorated expression of TNF- α , MCP1 and KC during recovery compared to saline-treated mice (Suppl. Fig. 1A–C). In contrast, the expression of IL-6, the cytokine essential in liver regeneration,[11,12] was significantly higher in the livers of mice treated with IL-1Ra compared to saline (day 4 in Suppl. Fig. 1D). It is notable that pro-inflammatory cytokine expression was the highest on day 1 of recovery during ethanol withdrawal.

Finally, to assess liver size, we analyzed liver mass as a function of bodyweight of mice at the time of sacrifice (Suppl. Fig. 2). We found that saline-treated mice had increased liver size at day 2, the same time-point associated with increased hepatocellular ballooning (Fig. 1B,C). Importantly, Anakinra-treated mice had an increase in liver size at day 7 of the recovery phase, compared to the saline-treated controls. These findings correlate positively with the increased enhanced hepatocyte regeneration of mice treated with Anakinra (Fig. 2).

Discussion

In summary, we are proposing a mouse model that may be used to study recovery from acute-on-chronic liver injury induced by ethanol. Although this model involves an acute-on-chronic exposure, commonly seen in patients in AH,[13] it is not necessarily equivalent in

complexity to severe acute AH in humans, where 80% of patients have underlying cirrhosis. Our model lacks Mallory hyaline, cholestasis, cirrhosis and ductular reaction.[1] However, we envision this model as a tool to address some aspects of impaired hepatocyte regeneration, steatosis, lobular inflammation and neutrophil infiltration, which pose key unresolved questions in severe alcoholic hepatitis. Our data has also demonstrated that treatment with Anakinra, an inhibitor of IL-1 signaling, facilitates recovery from acute-on-chronic liver injury induced by ethanol.

Interestingly, analysis of the recovery phase after this acute-on-chronic model of ethanol-induced liver injury revealed a potentially important role of IL-1 signaling in neutrophils. Recently it has been shown by various groups that P2×7 receptor-activated NLRP3 inflammasome signaling in neutrophils results in release IL-1 β and IL-18, but not IL-1α and IL-33,[14–16] and it relies on the same receptors and inflammasome sensor that have been shown to play important roles in mouse models of ALD (reviewed in Ref.[17]). The decreased MPO staining in Anakinra-treated mice suggests an IL-1 feedforward loop resulting in neutrophil recruitment to the liver. Similarly, an important role of neutrophils in liver recovery post-injury is further supported in a model of acetaminophen-induced liver injury.[18]

The ongoing clinical trial with Anakinra in severe alcoholic hepatitis (NCT01809132 at www.clinicaltrials.gov) may provide an answer as to the validity of this finding in a human disease. The mechanisms by which IL-1 signaling impairs hepatocyte regeneration in AH are yet to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AH	alcoholic hepatitis
IL	interleukin
ALD	alcoholic liver disease
NRLP3	NOD-like receptor family, pyrin domain-containing 3 protein
IL-1Ra	interleukin 1 receptor antagonist
ALT	alanine aminotransferase
TNF	tumor necrosis factor

MCP macrophage chemotactic protein

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Key points

- Impaired liver regeneration is a key determinant of mortality in alcoholic liver disease; however, mechanisms that drive recovery of the liver damaged by ethanol are largely unknown
- Here we propose a mouse model that provides a tool to study some aspects of liver recovery after acute-on-chronic exposure to ethanol
- Treatment with Anakinra, an inhibitor of IL-1 signaling, facilitates recovery from acute-on-chronic liver and inflammation induced by ethanol
- Treatment with Anakinra, enhances liver regeneration from ethanol-induced acute-on-chronic liver injury

Figure 1. Treatment with Anakinra facilitates recovery from acute-on-chronic alcoholic liver injury

WT mice were treated with ethanol diet for 4 weeks and received 3 intragastric gavages of EtOH (5 g/kg) during the last 3 days of ethanol feeding ('Max. Injury', red). After day 28, all mice were switched to control diet, and daily treatment with Anakinra or saline was initiated (Recovery Phase, blue). Baseline (green) refers to mice sustained on iso-caloric control liquid diet for the duration of the feeding (Week 5, day 7) (A). Liver injury was analyzed using H&E staining (B) and hepatocyte death was quantitatively evaluated using serum ALT (C). Semi-quantitative scoring was performed for histological features of

hepatocellular ballooning (0–2) (D) and lobular inflammation (0–2) (E). Neutrophil liver infiltration was assessed by MPO immunohistochemistry (F) with LPS-injected mouse liver (0.5 mg/kg, 12hrs) used as positive control (Pos.Control), and positive cells per visual field were quantified, average of 5 images per sample (G). Steatosis was evaluated by Oil-red-O staining (H) and scored semi-quantitatively (0–3). n = 3–5 per time point and treatment. Numbers in graphs indicate p-values. *P = 0.05 vs. baseline.

Figure 2. Treatment with Anakinra enhances liver regeneration from acute-on-chronic alcoholic liver injury

WT mice were treated with ethanol diet for 4 weeks and received 3 intragastric gavages of EtOH (5 g/kg) during the last 3 days of ethanol feeding (Max. Injury). After day 28, all mice were switched to control diet, and daily treatment with Anakinra or saline was initiated (Recovery Phase). Liver regeneration was analyzed using Ki67 (A) and Cyclin D1 staining (B), with murine liver 24-hours post partial hepatectomy used as positive control (Pos.Control). Quantitation of Ki67 signal was performed using ImageJ (C). Cyclin D1

positive cells were counted per visual field, average of 5 images per sample (D). n = 3–5 per time point and treatment. Numbers in graphs indicate p-values. *P < 0.05 vs. baseline

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