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*Et al.*

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# IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice

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**Alcoholic liver disease (ALD) is characterized by steatosis and upregulation of proinflammatory cytokines, including IL-1 $\beta$ . IL-1 $\beta$ , type I IL-1 receptor (IL-1R1), and IL-1 receptor antagonist (IL-1Ra) are all important regulators of the IL-1 signaling complex, which plays a role in inflammation. Furthermore, IL-1 $\beta$  maturation is dependent on caspase-1 (Casp-1). Using IL-1Ra-treated mice as well as 3 mouse models deficient in regulators of IL-1 $\beta$  activation (Casp-1 and ASC) or signaling (IL-1R1), we found that IL-1 $\beta$  signaling is required for the development of alcohol-induced liver steatosis, inflammation, and injury. Increased IL-1 $\beta$  was due to upregulation of Casp-1 activity and inflammasome activation. The pathogenic role of IL-1 signaling in ALD was attributable to the activation of the inflammasome in BM-derived Kupffer cells. Importantly, *in vivo* intervention with a recombinant IL-1Ra blocked IL-1 signaling and markedly attenuated alcohol-induced liver inflammation, steatosis, and damage. Furthermore, physiological doses of IL-1 $\beta$  induced steatosis, increased the inflammatory and prosteatotic chemokine MCP-1 in hepatocytes, and augmented TLR4-dependent upregulation of inflammatory signaling in macrophages. In conclusion, we demonstrated that Casp-1-dependent upregulation of IL-1 $\beta$  and signaling mediated by IL-1R1 are crucial in ALD pathogenesis. Our findings suggest a potential role of IL-1R1 inhibition in the treatment of ALD.**

## Introduction

It is estimated that there are 17.6 million alcoholic individuals in the United States and 140 million worldwide. Although not all alcoholics develop symptomatic alcoholic liver disease (ALD), more than 12,000 deaths per year are attributed to ALD in the United States (1). The clinical spectrum of ALD includes alcoholic fatty liver, alcoholic steatohepatitis with or without fibrosis, cirrhosis, and hepatocellular cancer. Clinical observations suggest that steatosis and early phases of steatohepatitis are reversible; however, there is no definitive treatment available for any stages of the alcohol-related liver diseases. Short of abstinence, therapeutic options are limited, and even cessation of alcohol consumption may not prevent the progression of alcohol-induced liver damage (1).

It has been proposed that activation of Kupffer cells (KCs), the resident liver macrophages, has a pivotal role in the inflammation associated with ALD (2, 3). Numerous studies have reported on the crucial role of KC-derived inflammatory cytokines in the pathogenesis of alcohol-induced steatosis, inflammation, and injury and emphasized the role of TNF- $\alpha$  as a central mediator of ALD (reviewed in ref. 4). However, human clinical trials with TNF- $\alpha$  blockade using anti-TNF antibodies were discontinued due to infectious complications (5). These studies suggested the need to search for inflammatory pathways amenable to therapeutic intervention without compromising the immune status of the host.

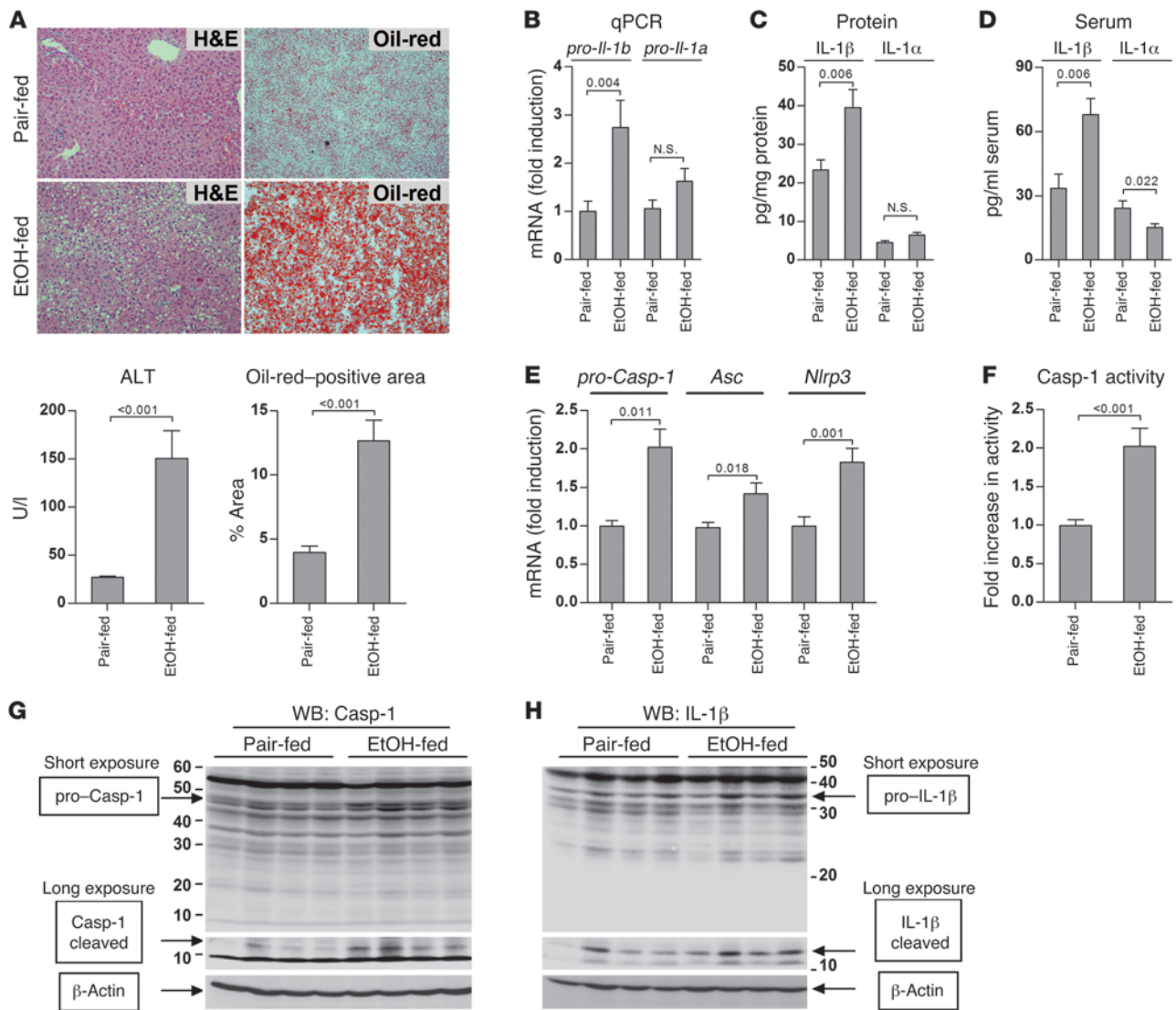
IL-1 $\beta$  is a potent proinflammatory cytokine (6), whose levels are increased in patients with ALD (7). IL-1 $\beta$  is produced as inac-

tive pro-IL-1 $\beta$  (encoded by *pro-Il-1b*) in response to inflammatory stimuli, including both microbial products and endogenous danger-associated molecules. IL-1 $\beta$  gene expression and synthesis of pro-IL-1 $\beta$  occurs after activation of pattern recognition receptors (PRRs). Inflammatory stimuli also drive activation of cytosolic CARD and PYHIN domain-containing PRRs that recruit ASC and caspase-1 (Casp-1) to assemble into the multiprotein complex inflammasome. Pro-Casp-1 (encoded by *pro-Casp-1*), activated by the inflammasome, cleaves pro-IL-1 $\beta$  into the bioactive IL-1 $\beta$  (8). IL-1 $\beta$  acts in an autocrine/paracrine manner via the type I IL-1 receptor (IL-1R1). Activation of IL-1R1 is inhibited by its binding to the IL-1 receptor antagonist (IL-1Ra), a naturally occurring cytokine whose function is to prevent the biologic response to IL-1. Treatment with IL-1Ra significantly improves symptoms in patients with rheumatoid arthritis (9) or autoinflammatory syndromes (6); unlike anti-TNF therapy, it is not associated with infectious complications (10).

Emerging data have provided evidence for the role of IL-1 signaling in acute and chronic liver injury of diverse origin, including acetaminophen-induced liver damage (11), nonalcoholic steatohepatitis (12), liver fibrosis (13), and immune-mediated liver injury (14). However, the significance of IL-1 signaling in ALD has yet to be evaluated. Considering the critical importance of inflammatory activation in ALD (4) and the role of IL-1 $\beta$  in innate immunity (6), we hypothesized that IL-1 signaling is critical in alcohol-induced liver pathology, including steatosis, inflammation, and hepatocyte damage. Given the differential input of parenchymal and nonparenchymal cells in the pathophysiology of ALD, we further hypothesized that activation of the inflammasome and IL-1 $\beta$  may be critical in alcoholic liver injury in a cell-specific manner.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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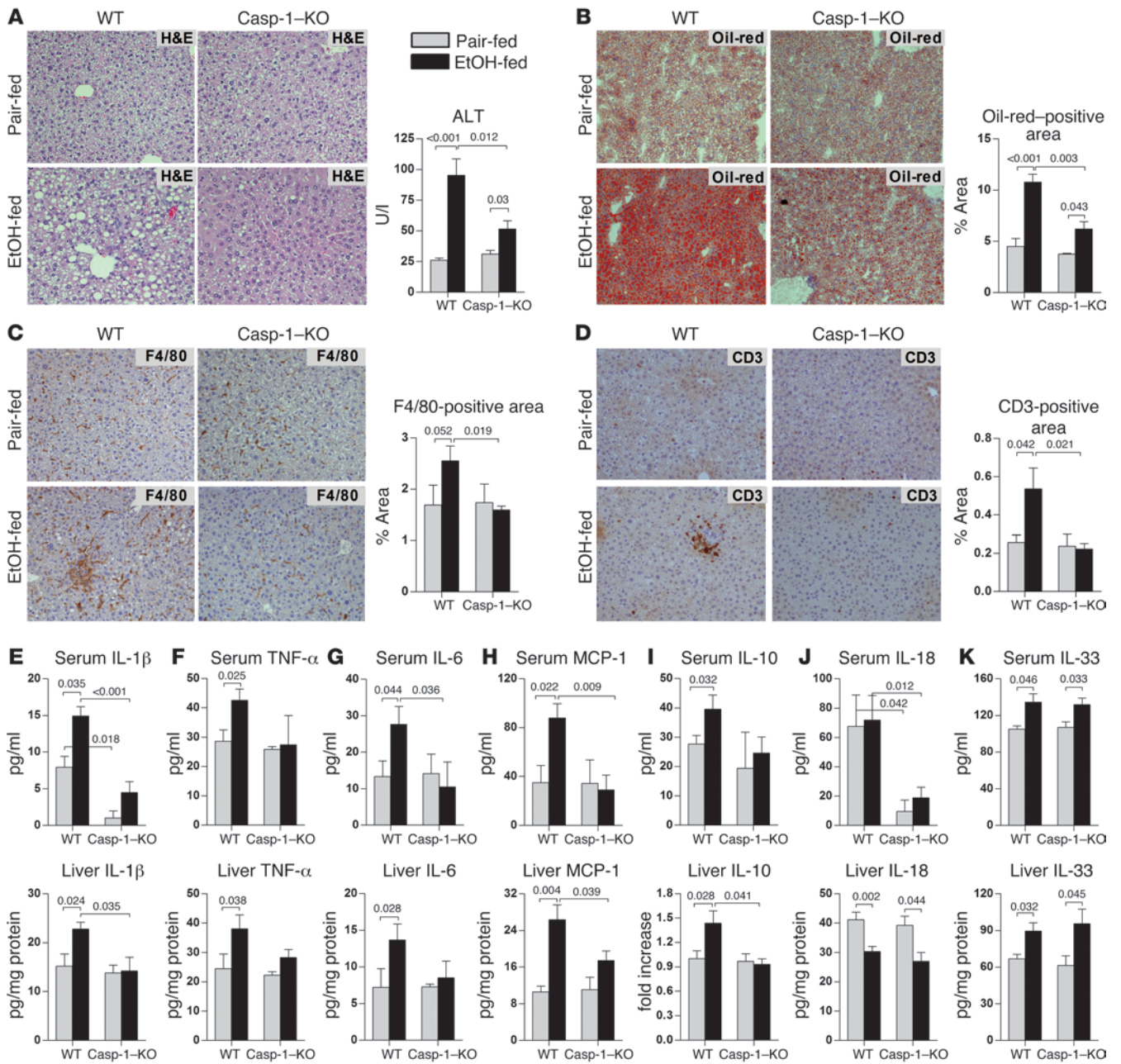
**Figure 1**

Activation of the inflammasome and IL-1β in alcohol-induced liver injury. WT mice were fed control (pair-fed) or alcohol (EtOH-fed) diet and sacrificed 4 weeks later. Liver samples were stained by H&E or Oil-red-O, and liver injury and steatosis was quantified by measuring serum ALT and Oil-red-O-positive areas, respectively (A). Expression of pro-IL-1β and pro-IL-1α in the liver was analyzed by qPCR (B) and ELISA (C). Secreted forms of IL-1β and IL-1α were measured in the serum using specific ELISA (D). Expression of *pro-Casp-1*, *Asc*, and *Nlrp3* in the liver was measured using qPCR (E), and Casp-1 activity was measured using a colorimetric assay (F). Cleaved forms of Casp-1 (G) and IL-1β (H) in the livers were analyzed using antibodies that identify both full-length pro-form (short exposure, presented in linear contrast mode) and cleaved forms (long exposure, presented in sigmoidal contrast mode), and normalized to β-actin. See Supplemental Figure 1 for densitometric analysis. *n* = 5 (pair-fed); 10 (EtOH-fed). Numbers in graphs denote *P* values. Original magnification, ×200.

Here, we report that upregulation of cleaved active IL-1β in ALD was a Casp-1-dependent process and that inflammasome and IL-1 signaling were required for the pathogenesis of alcohol-induced inflammation, steatosis, liver damage, and fibrosis. We further showed that the requirement for inflammasome activation and IL-1β in the pathogenesis of ALD was due to the presence of the inflammasome in liver immune cells and that pharmacological inhibition of IL-1R1 using IL-1Ra ameliorated the development and progression of ALD. We also found that IL-1β amplified TLR4-induced inflammation. These findings, which we believe to be novel, suggest a therapeutic utility of IL-1 receptor blockade in the treatment of ALD.

**Results**

*Inflammasome and IL-1β are activated in alcohol-induced liver injury.* Although the role of proinflammatory cytokine production in ALD has been extensively investigated (reviewed in ref. 15), the importance of cleaved IL-1β is still unknown. To investigate whether IL-1β is activated in ALD, we fed ethanol or isocaloric control (pair feeding) diet to C57BL/6 WT mice. Histopathological analysis revealed that chronic alcohol feeding induced steatosis (Oil-red-O staining) and liver damage (H&E and serum alanine aminotransferase [ALT]) in ethanol-fed mice (Figure 1A), suggestive of ALD. Alcohol feeding significantly upregulated *pro-IL-1b*

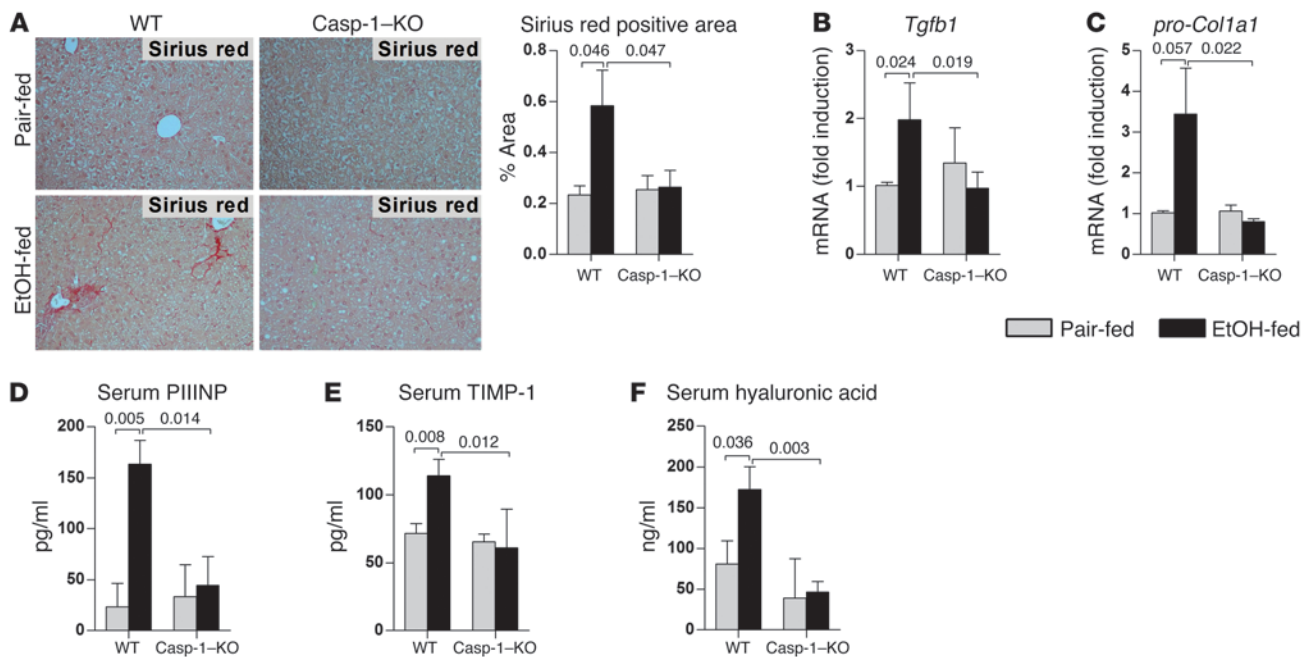


**Figure 2** Deficiency of Casp-1 attenuates alcoholic liver inflammation, steatosis, and damage. WT or Casp-1-KO mice were fed control or alcohol diet and sacrificed 4 weeks later. Liver injury was assessed by liver H&E staining and serum ALT (A). Steatosis was evaluated by Oil-red-O staining (B). Immunohistochemistry was used to evaluate recruitment of F4/80-positive macrophages (C) and CD3-positive lymphocytes (D). Serum and liver levels of IL-1 $\beta$  (E), TNF- $\alpha$  (F), IL-6 (G), MCP-1 (H), IL-10 (I), IL-18 (J), and IL-33 (K) were measured as described in Methods. Tissue levels of IL-10 (I; liver) were evaluated using immunoblotting (Supplemental Figure 3A). *n* = 6 (WT pair-fed); 11 (WT EtOH-fed); 3 (Casp-1-KO pair-fed); 7 (Casp-1-KO EtOH-fed). Numbers in graphs denote *P* values. Original magnification,  $\times 200$ .

mRNA (Figure 1B) and pro-IL-1 $\beta$  protein (Figure 1C) in the livers and increased secretion of IL-1 $\beta$  in the serum (Figure 1D) compared with controls. There was no increase of IL-1 $\alpha$  in the liver or serum of alcohol-fed mice (Figure 1, B-D).

IL-1 $\beta$  maturation and secretion is mediated by inflammasome-dependent activation of Casp-1 (16). Real-time quantitative PCR (qPCR) analysis demonstrated that alcohol feeding significantly

increased expression of the inflammasome components *pro-Casp-1*, *Asc* (which encodes ASC), and *Nlrp3* (which encodes NALP3) in the liver (Figure 1E). Importantly, we found increased activity of Casp-1, the effector protein of the inflammasome, in livers of alcohol-fed compared with pair-fed mice (Figure 1F). This finding was supported by significantly increased levels of the cleaved fragment of Casp-1 (Figure 1G and Supplemental Figure 1; supplemental material

**Figure 3**

Deficiency of Casp-1 attenuates alcoholic liver fibrosis. WT or Casp-1-KO mice were fed control or alcohol diet and sacrificed 4 weeks later. Liver fibrosis was evaluated by Sirius red staining (A) and by measuring expression of *Tgfb1* (B) and *pro-Col1a1* (C) by qPCR. Specific ELISA was used to evaluate serum markers of liver fibrosis, including PIIINP (D), TIMP-1 (E), and hyaluronic acid (F).  $n = 6$  (WT pair-fed); 11 (WT EtOH-fed); 3 (Casp-1-KO pair-fed); 7 (Casp-1-KO EtOH-fed). Numbers in graphs denote  $P$  values. Original magnification,  $\times 200$ .

available online with this article; doi:10.1172/JCI60777DS1) and of the cleaved form of IL-1 $\beta$  in the livers of alcohol-fed mice (Figure 1H and Supplemental Figure 1). Taken together, these findings suggest activation of the inflammasome and IL-1 $\beta$  in ALD.

In addition to cleavage of IL-1 $\beta$ , inflammasomes are also involved in processing of IL-18 and IL-33 (8). We observed that alcohol-fed mice showed upregulation of *pro-IL-33* mRNA (Supplemental Figure 2A), pro-IL-33 protein, and the cleaved fragment of IL-33 in the liver (Supplemental Figure 2, C and D). In contrast to IL-1 $\beta$ , which was present mainly in the full-length form and underwent cleavage after ethanol administration (Figure 1H), the majority of IL-33 detected in the liver was cleaved, regardless of diet (Supplemental Figure 2C). In contrast to IL-1 $\beta$  or IL-33, we found no induction of IL-18 in the liver or serum of alcohol-fed mice (Supplemental Figure 2, B and D, and see below).

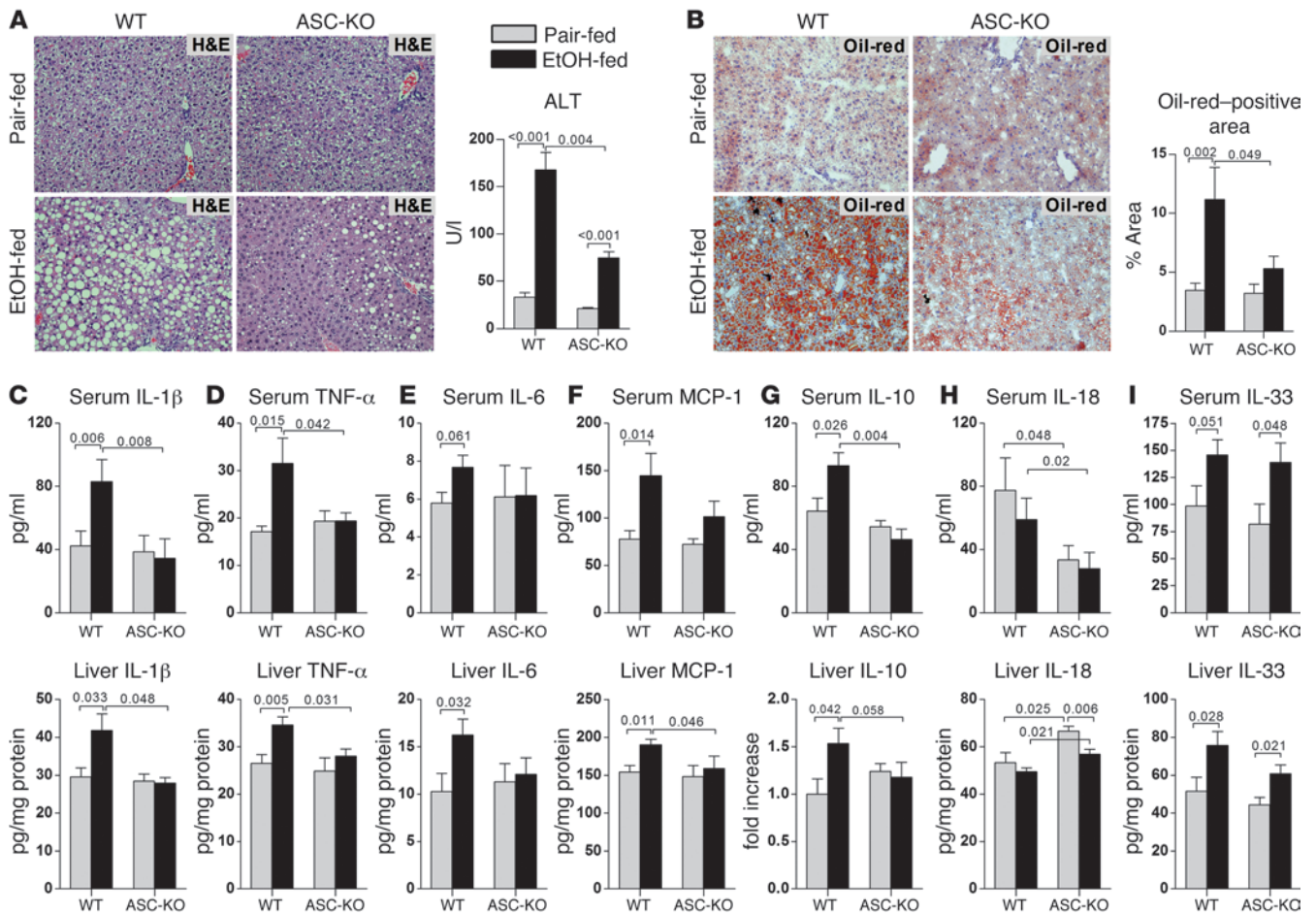
*Deficiency of Casp-1 ameliorates alcoholic liver steatosis and injury and prevents alcoholic hepatitis and fibrosis.* To further define the importance of Casp-1 activation in alcohol-induced liver injury, we fed ethanol or control diet to WT and Casp-1-KO mice. In contrast to WT mice, Casp-1-KO mice showed significant amelioration of the morphological features of ALD upon histological analysis (H&E and Oil-red-O; Figure 2, A and B) and demonstrated significant attenuation of liver injury, as documented by decreased serum ALT (Figure 2A).

Next, we examined the recruitment of KCs, a BM-derived cell population essential in the pathogenesis of ALD (2). Immunohistochemistry analysis showed that deficiency of Casp-1 completely prevented increased accumulation of F4/80-positive KCs (Figure 2C) and CD3-positive lymphocytes (Figure 2D) in the liver compared with alcohol-fed WT controls. This finding suggested a crucial role of Casp-1 in recruitment of myeloid and lym-

phoid cells to the liver. To further investigate liver inflammation, we analyzed cytokines involved in inflammation and cell recruitment. Consistent with the critical role of Casp-1 in the cleavage and release of bioactive IL-1 $\beta$ , we found significantly lower serum levels of IL-1 $\beta$  in Casp-1-KO mice and no alcohol-mediated induction of IL-1 $\beta$  in the liver compared with WT mice (Figure 2E). Further analysis demonstrated that deficiency of Casp-1 prevented increases in the proinflammatory cytokines TNF- $\alpha$  (Figure 2F) and IL-6 (Figure 2G), the chemokine MCP-1 (Figure 2H), and the antiinflammatory cytokine IL-10 (Figure 2I and Supplemental Figure 3A) compared with WT mice. These data support a central role of Casp-1 in alcohol-induced liver inflammation.

Consistent with our previous data (Supplemental Figure 2, A, B, and D), there was no increase of IL-18 in the serum or livers of alcohol-fed mice, although deficiency of Casp-1 globally decreased serum IL-18 compared with WT mice (Figure 2J). In contrast, we found that IL-33 was increased in both alcohol-fed WT and alcohol-fed Casp-1-KO mice (Figure 2K), which suggests that inflammasome-independent mechanisms cleave IL-33 in ALD.

Next, we analyzed the role of Casp-1 in alcohol-induced liver fibrosis. Casp-1-KO mice showed overt reduction of liver fibrosis, as indicated by Sirius red staining (Figure 3A) and by the expression of markers of fibrogenesis, including *Tgfb1* (which encodes TGF- $\beta$ ; Figure 3B) and *pro-Col1a1* (which encodes pro-collagen- $\alpha$ 1; Figure 3C). To confirm the involvement of Casp-1 in alcoholic liver fibrosis, we analyzed a panel of serum markers of liver fibrosis, including procollagen III N-terminal propeptide (PIIINP; Figure 3D), tissue inhibitor of matrix metalloproteinases 1 (TIMP-1; Figure 3E), and hyaluronic acid (Figure 3F). Alcohol feeding significantly upregulated these markers only in WT mice, not in Casp-1-KO animals (Figure 3, C-F).



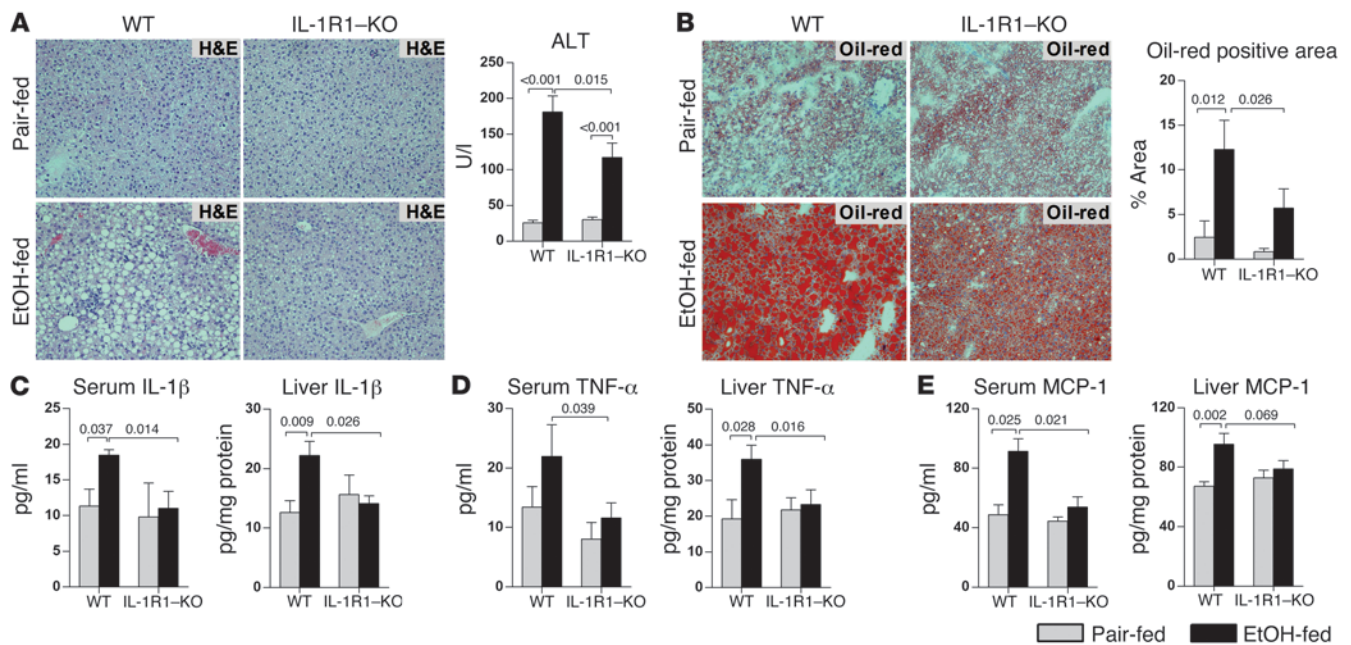
**Figure 4** Deficiency of ASC attenuates ALD. WT or ASC-KO mice were fed control or alcohol diet and sacrificed 4 weeks later. Liver injury was assessed by liver H&E staining and serum ALT (A). Steatosis was evaluated by Oil-red-O staining (B). Levels of IL-1 $\beta$  (C), TNF- $\alpha$  (D), IL-6 (E), MCP-1 (F), serum IL-10 (G), IL-18 (H), and IL-33 (I) were measured using specific ELISA; tissue levels of IL-10 (G; liver) were evaluated using immunoblotting (see Supplemental Figure 3B).  $n = 13$  (WT pair-fed); 19 (WT ETOH-fed); 6 (ASC-KO pair-fed); 9 (ASC-KO ETOH-fed). Numbers in graphs denote  $P$  values. Original magnification,  $\times 200$ .

Taken together, these data demonstrated that Casp-1 was required for the cleavage of IL-1 $\beta$  in alcoholic livers and that Casp-1 deficiency prevented immune cell recruitment as well as activation of inflammation and fibrosis and significantly ameliorated alcohol-induced liver steatosis and damage.

**Deficiency in IL-1 $\beta$  activation in ASC-KO mice ameliorates ALD.** In a majority of inflammasomes, activation of Casp-1 requires interaction with the adaptor protein ASC (8). Given our finding that *Asc* expression was increased in livers of alcohol-fed mice (Figure 1E), we investigated its role in ALD using mice with genetic deficiency of ASC. In contrast to WT mice, ASC-KO mice had attenuated alcohol-induced liver damage and steatosis, as demonstrated by H&E, Oil-red-O staining, and serum ALT (Figure 4, A and B), an outcome similar to that of Casp-1-KO mice (Figure 2, A and B). Moreover, deficiency of ASC prevented upregulation of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, MCP-1, and IL-10 in the liver and in serum (Figure 4, C-G, and Supplemental Figure 3B) in alcohol-fed mice compared with alcohol-fed WT controls, supporting the crucial role of ASC in the pathogenesis of alcohol-induced inflammation. Alcohol feeding did not

increase IL-18, whereas IL-33 showed upregulation in serum and liver in both WT and ASC-KO mice compared with pair-fed controls (Figure 4, H and I).

**Deficiency in IL-1 signaling in IL-1R1-KO mice ameliorates ALD.** Following its cleavage and release, IL-1 $\beta$  exerts its effects via IL-1R1 (16). To investigate whether IL-1 signaling was required for the pathogenesis of ALD, we fed ethanol or control diet to WT and IL-1R1-KO mice. Contrary to WT mice, IL-1R1-KO mice showed significant amelioration of steatosis and liver injury, as determined by histological analysis, Oil-red-O staining, and measurement of serum ALT (Figure 5, A and B). We also found no alcohol-induced increase of IL-1 $\beta$  (Figure 5C), TNF- $\alpha$  (Figure 5D), or MCP-1 (Figure 5E) in the serum and livers of IL-1R1-KO mice compared with pair-fed controls. This was in contrast to increased levels of all these cytokines in alcohol-fed WT mice (Figure 5, C-E). Taken together, these data demonstrated a critical role of IL-1R1 in the pathogenesis of alcohol-induced liver inflammation, damage, and steatosis. In the context of our data showing no induction of IL-1 $\alpha$  (Figure 1, B-D) and IL-18 (Supplemental Figure 2, A, B, and D, Figure 2J, and Figure 4H) in ALD as well as Casp-1- and

**Figure 5**

Deficiency in IL-1 signaling in IL-1R1-KOs ameliorates ALD. WT or IL-1R1-KO mice were fed control ( $n = 5$  per genotype) or alcohol ( $n = 10$  per genotype) diet and sacrificed 4 weeks later. Liver injury was assessed by liver H&E staining and serum ALT (A). Steatosis was evaluated by Oil-red-O staining (B). Serum and liver levels of IL-1 $\beta$  (C), TNF- $\alpha$  (D), and MCP-1 (E) were measured using specific ELISA. Numbers in graphs denote  $P$  values. Original magnification,  $\times 200$ .

ASC-independent upregulation of IL-33 (Figure 2K and Figure 4I) in alcohol-fed mice, our findings indicate that the predominant pathogenic effect of inflammasome in ALD is mediated by IL-1 $\beta$ .

*Pharmacologic intervention via inhibition of IL-1 signaling with IL-1Ra ameliorates ALD development and progression.* The activity of IL-1 signaling is tightly regulated by IL-1Ra, a natural endogenous antagonist of the proinflammatory IL-1 $\beta$ . IL-1Ra competes with both IL-1 $\alpha$  and IL-1 $\beta$  at the receptor level to block IL-1R1 signaling (16). We observed that alcohol feeding increased endogenous IL-1Ra protein levels in both liver (Figure 6A) and serum (Figure 6B) of alcohol-fed mice.

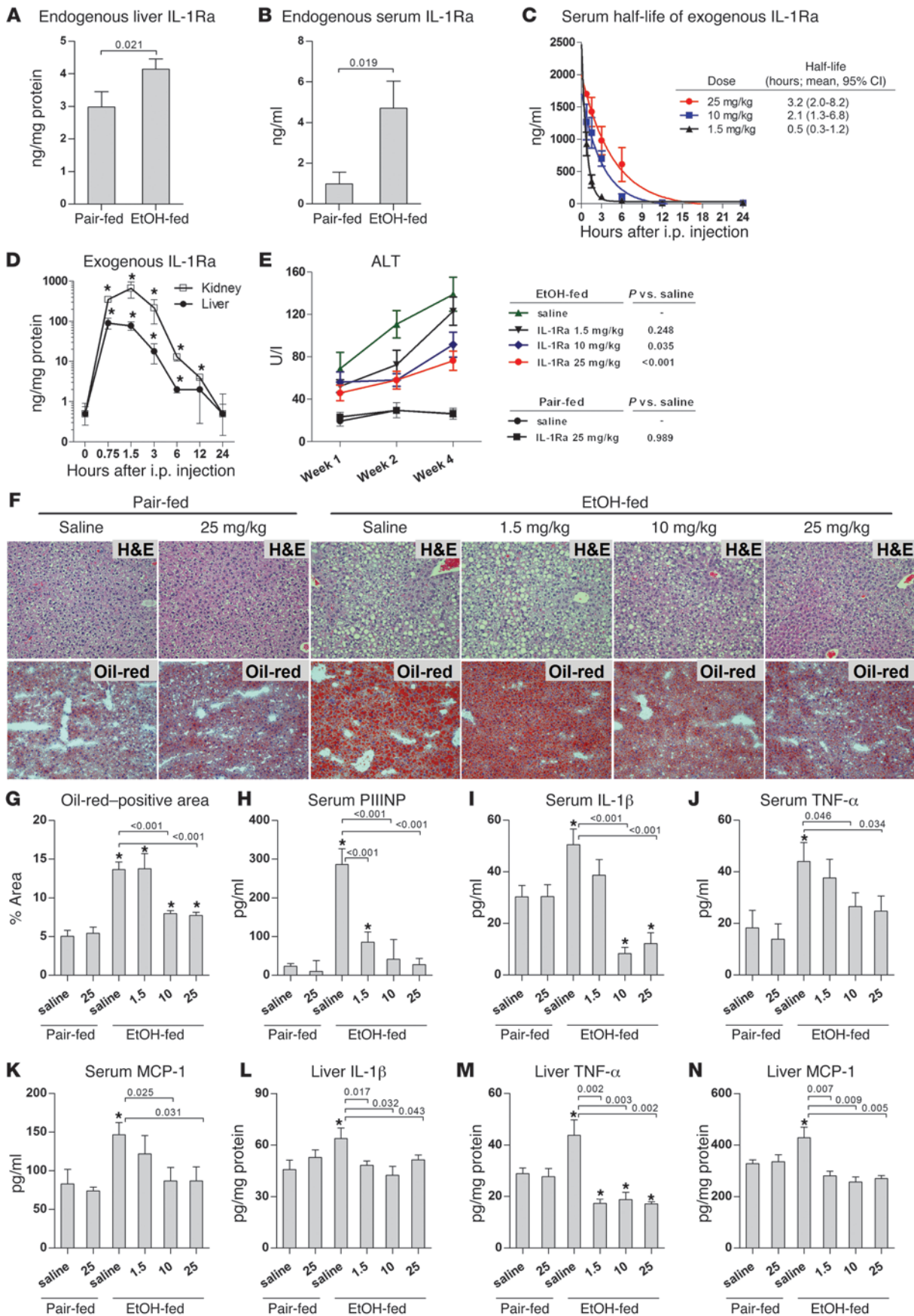
To evaluate whether pharmacological blocking of IL-1R1 has a protective effect in ALD, we took advantage of recombinant human IL-1Ra (anakinra). We sought to achieve pharmacokinetics of recombinant IL-1Ra similar to that observed in humans, in whom administration of IL-1Ra at 1.5 mg/kg demonstrates a half-life of 2–3 hours and therapeutic serum levels that are detectable 6–12 hours after injection (17). These parameters were achieved after administration of 10 or 25 mg/kg i.p. in our mouse model (Figure 6, C and D). The dose of 1.5 mg/kg, used in humans, resulted in a 30-minute half-life after i.p. administration (Figure 6C), most likely due to the pronounced renal accumulation (Figure 6D) and rapid elimination of IL-1Ra in rodents (18).

Next, we treated WT mice with daily i.p. injections of 1.5, 10, or 25 mg/kg IL-1Ra along with a 4-week feeding with alcohol diet and found that 10 or 25 mg/kg/d recombinant IL-1Ra significantly ameliorated alcohol-induced liver steatosis and damage (Figure 6, E–G). All 3 doses of IL-1Ra significantly attenuated serum levels of the fibrosis marker PIIINP (Figure 6H). Treatment with IL-1Ra at 25 mg/kg showed superior protection from alcohol-induced ALT increase compared with 10 or 1.5 mg/kg

( $P = 0.045$  vs. 10 mg/kg,  $P = 0.007$  vs. 1.5 mg/kg; Figure 6E). Administration of IL-1Ra also dose-dependently decreased levels of IL-1 $\beta$  (Figure 6I), TNF- $\alpha$  (Figure 6J), and MCP-1 (Figure 6K) in the serum of alcohol-fed mice compared with saline-treated alcohol-fed controls. All 3 tested doses of IL-1Ra prevented upregulation of IL-1 $\beta$ , TNF- $\alpha$ , and MCP-1 protein in the liver of alcohol-fed mice (Figure 6, L–N). These data demonstrated that administration of IL-1Ra could achieve the extent of protection from ALD observed in Casp-1-, ASC-, and IL-1R1-KO mice (Figures 2–5). This protective effect required administration of active IL-1Ra, as administration of heat-inactivated IL-1Ra did not ameliorate alcohol-induced liver steatosis, damage, or inflammation (Supplemental Figure 4).

To reflect the clinical scenario in which most patients present with alcoholic hepatitis superimposed on established ALD (1), we first fed WT mice with Lieber-DeCarli ethanol or control diet for 2 weeks to allow development of ALD and then initiated i.p. treatment with 25 mg/kg/d IL-1Ra or saline for the last 2 weeks of feeding, while still maintaining the alcohol diet. Treatment of established ALD with IL-1Ra prevented progression of alcohol-induced liver damage (ALT increase) and steatosis (Figure 7, A and B). Similar protection was achieved when the 2-week IL-1Ra treatment was initiated after the initial 4 weeks of alcohol feeding (Figure 7C).

Next, we asked whether IL-1Ra can prevent progression of ongoing alcohol-induced liver injury for a prolonged period of time. We fed WT mice with ethanol or control diet for 2 weeks, then initiated daily treatment with IL-1Ra or saline and continued the alcohol diet for 5 additional weeks (7 weeks total duration). Alcohol-fed mice treated with IL-1Ra showed significantly lower levels of serum ALT at all investigated time points (Figure 7D) and showed significantly improved survival compared with saline-treated, alcohol-fed







## Figure 6

Pharmacologic intervention via inhibition of IL-1 signaling ameliorates ALD development. (A and B) WT mice were fed control ( $n = 5$ ) or alcohol ( $n = 10$ ) diet and sacrificed 4 weeks later. Endogenous IL-1Ra was measured in the liver (A) and in the serum (B) using mouse-specific ELISA. (C and D) WT mice ( $n = 3$  per time point and dose) were injected with human recombinant IL-1Ra, and pharmacokinetics was evaluated using human-specific ELISA in the serum (C) and in the liver or kidney extract (D).  $*P < 0.05$  vs. baseline. (E–N) WT mice were fed with control ( $n = 5$ ) or alcohol diet ( $n = 10$  per IL-1Ra dose), treated daily with indicated doses of recombinant human IL-1Ra (anakinra) or saline i.p., and sacrificed 4 weeks later. Liver injury was quantified by serum ALT (E) and H&E (F), and steatosis was evaluated by Oil-red-O staining (F and G). Fibrosis was estimated by PIIINP in the serum (H). Serum and liver levels of IL-1 $\beta$  (I and L), TNF- $\alpha$  (J and M), and MCP-1 (K and N) were measured using specific ELISA. Numbers in graphs denote  $P$  values.  $*P < 0.05$  vs. pair-fed saline. Original magnification,  $\times 200$ .

controls (48% survival at 7 weeks in alcohol-fed, IL-1Ra-treated, vs. 23% in alcohol-fed, saline-treated;  $P = 0.036$ ). At the conclusion of the experiment, IL-1Ra-treated mice showed a substantially improved histological picture of ALD (H&E and Oil-red-O) compared with alcohol-fed, saline-treated mice (Figure 7E). Daily administration of IL-1Ra significantly inhibited progression of alcohol-induced liver injury for 4 of the 5 weeks of IL-1Ra treatment ( $P = 0.243$ ,  $P = 0.851$ ,  $P = 0.311$ , and  $P = 0.006$  for ALT weeks 4, 5, 6, and 7, respectively, vs. ALT week 2 in alcohol-fed, IL-1Ra-treated mice; Figure 7D).

Finally, we investigated the role of IL-1R1 blockade on the recovery from acute-on-chronic alcohol liver injury. In this model, WT mice were fed Lieber-DeCarli ethanol or control diet for 4 weeks to allow development of ALD and received 3 acute doses of ethanol via gastric gavage during the last 3 days of alcohol feeding (Figure 8A). Subsequently, all mice were transferred to the control diet and started on daily treatment with IL-1Ra or saline. Saline-treated mice recovered from liver injury at day 4, whereas animals treated with IL-1Ra showed normal ALT after only 2 days (Figure 8A). Similarly, administration of IL-1Ra was associated with substantially improved liver histology on day 2 (H&E; Figure 8B) compared with saline-treated mice. We could not evaluate the role of IL-1Ra on lipid accumulation in this model due to the fact that all mice showed almost complete regression of steatosis on Oil-red-O staining in less than 48 hours after conversion to the control diet (Figure 8B).

Collectively, these data suggested that inflammasome and IL-1 $\beta$  drive the pathogenesis of alcohol-induced liver inflammation, steatosis, damage, and fibrosis and that therapy with IL-1Ra attenuates the development of ALD, protects from progression of alcohol-induced steatosis and liver damage, and facilitates recovery from acute-on-chronic alcoholic liver injury.

**Cell-specific role of the inflammasome in ALD.** The liver represents a complex coexistence of parenchymal and nonparenchymal cells. To identify the cell population responsible for the pathogenic effects of Casp-1 and IL-1 $\beta$  in ALD, we isolated primary hepatocytes and liver mononuclear cells (LMNCs) from the livers of WT mice. Baseline levels of Casp-1 were substantially higher in LMNCs than in hepatocytes (Figure 9A). Similarly, baseline expression of *pro-Casp-1*, *Asc*, *Nlrp3*, and *pro-Il-1b* mRNA was approximately 20-fold higher in LMNCs than in primary hepatocytes (Figure 9B). Analysis of LMNCs and primary hepatocytes isolated from alcohol-fed mice treated with saline or LPS prior to sacrifice showed that alcohol or LPS induced increases in the cleaved fragment of Casp-1

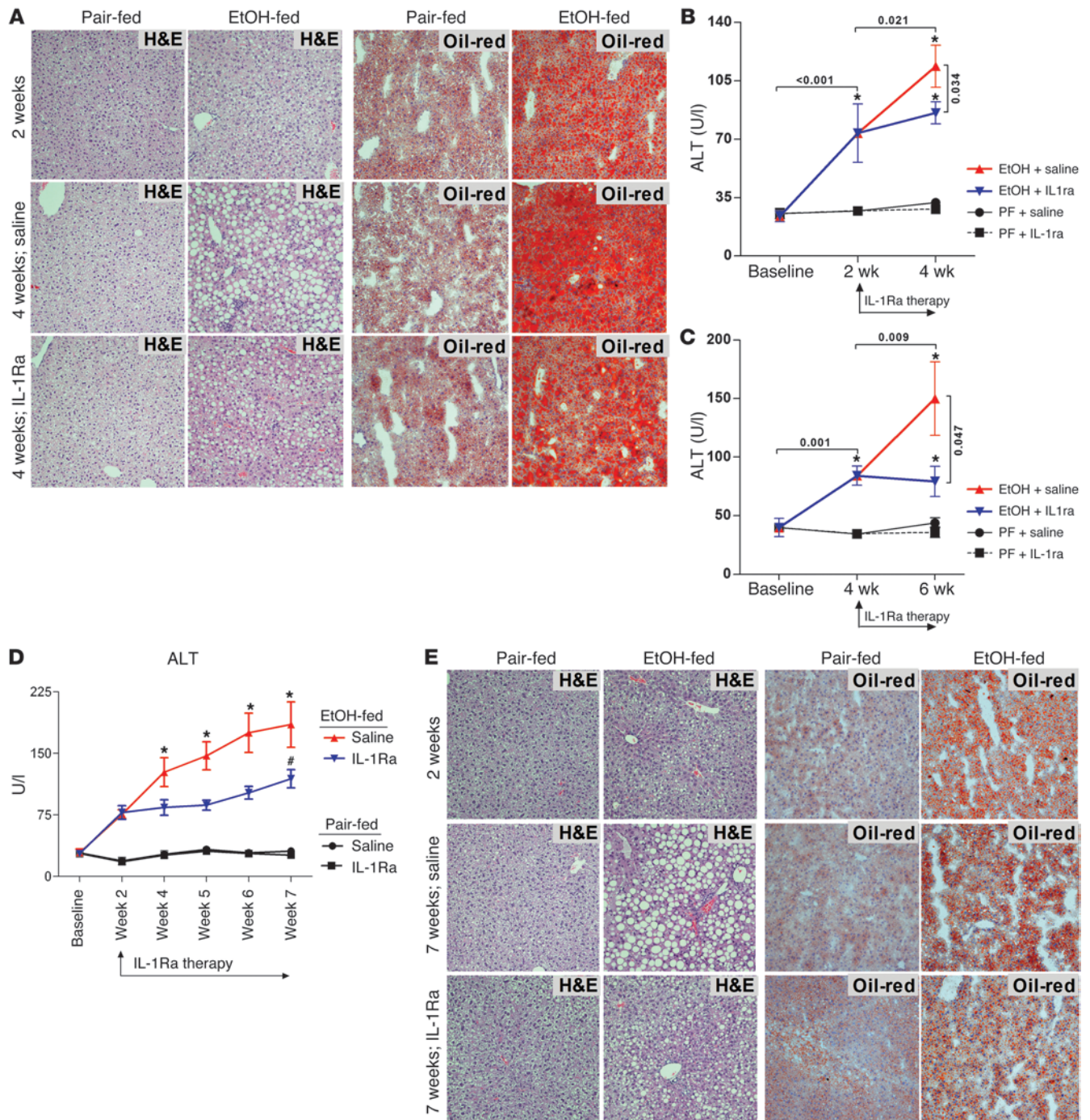
and IL-1 $\beta$  in LMNCs, but not in primary hepatocytes (Figure 9, C–E). These data suggest that LMNCs are the predominant cells that activate Casp-1 and IL-1 $\beta$  in ALD.

To explore whether KCs mediate the pathogenic effects of the inflammasome in ALD, we generated Casp-1-chimeric mice using a combination of clodronate-mediated KC depletion, irradiation, and BM transplantation (BMT). This protocol achieves full depletion of KC in acceptor livers (Supplemental Figure 5) and greater than 90% reconstitution of BM-derived cells (19). Successful BMT was further supported by serum levels of IL-1 $\beta$  that were significantly lower in recipients of Casp-1-KO BM compared with recipients of WT BM (Figure 9J). Using this protocol, we generated reciprocal chimeras by transplanting WT BM into clodronate-pretreated, irradiated Casp-1-KO mice (referred to herein as Casp-1-KO/WT-BM) or by transplanting Casp-1-KO BM into clodronate-pretreated, irradiated WT mice (WT/Casp-1-KO-BM). Clodronate-pretreated, irradiated WT mice transplanted with WT BM (WT/WT-BM) served as controls.

WT/WT-BM mice developed significant liver injury, steatosis, and inflammatory cytokine activation after 4 weeks of alcohol diet feeding compared with pair-fed controls (Figure 9, F–L). Compared with WT/WT-BM mice, WT/Casp-1-KO-BM mice showed significant amelioration of ALD, as indicated by liver histology (H&E; Figure 9F), Oil-red-O staining for lipids (Figure 9, G and I), and decreased serum ALT (Figure 9H). The attenuated liver injury and steatosis in WT/Casp-1-KO-BM mice was accompanied by a significant decrease in serum concentrations of IL-1 $\beta$  (Figure 9J), TNF- $\alpha$  (Figure 9K), and MCP-1 (Figure 9L). The extent of protection from alcohol-induced liver injury, steatosis, and inflammation in WT/Casp-1-KO-BM mice (Figure 9, F–I) was comparable to the extent of protection observed in the global Casp-1-KO animals (Figure 2, A and B), which supports the hypothesis that the pathogenic role of inflammasome in ALD is specific to KCs. In contrast, Casp-1-KO/WT-BM mice showed no significant protection from alcohol-induced liver damage, steatosis, and induction of inflammatory cytokines compared with WT/WT-BM mice (Figure 9, F–L).

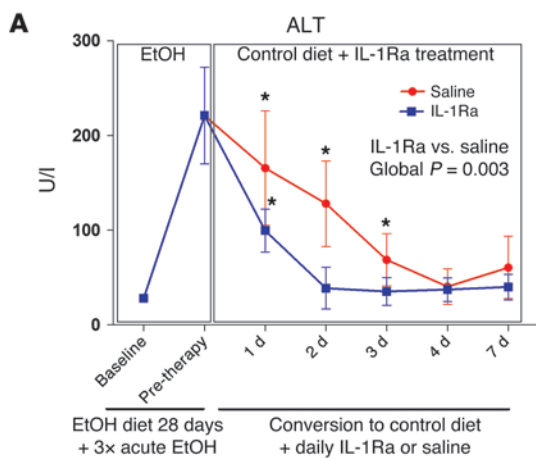
These data suggested that Casp-1 expressed in KCs was involved in alcohol-induced liver inflammation, steatosis, and injury and did not support a significant pathogenic role for Casp-1 in parenchymal cells in the development of ALD. In the context of our data demonstrating a crucial role of IL-1R1 in ALD, the absence of ethanol-mediated induction of IL-1 $\alpha$  or IL-18 in the liver or serum, and inflammasome-independent induction of IL-33, our findings collectively demonstrated that the pathogenic effect of the inflammasome in ALD is driven by KCs and mediated by IL-1 $\beta$  in an autocrine/paracrine manner.

**Physiological concentrations of IL-1 $\beta$  exert biological effects in macrophages and hepatocytes.** Finally, we asked whether the low concentrations of IL-1 $\beta$  observed in the serum of alcohol-fed mice (ranging 15–80 pg/ml; Figure 1D, Figure 2E, Figure 4C, Figure 5C, Figure 6I, and Figure 9J) were sufficient to exert pathogenic effects. We investigated the role of IL-1 $\beta$  in the context of TLR4-mediated induction of inflammatory cytokines. Essential for the pathogenesis of ALD (20), TLR4 signaling is activated by gut-derived LPS that increases in the serum after alcohol feeding (21), activates nuclear transcription factors including NF- $\kappa$ B, and upregulates production of inflammatory mediators (reviewed in ref. 15). Given the partial overlap of signaling pathways downstream of TLR4 and IL-1R1 (22), we hypothesized that IL-1 $\beta$  augments TLR4-dependent signaling in immune cells.



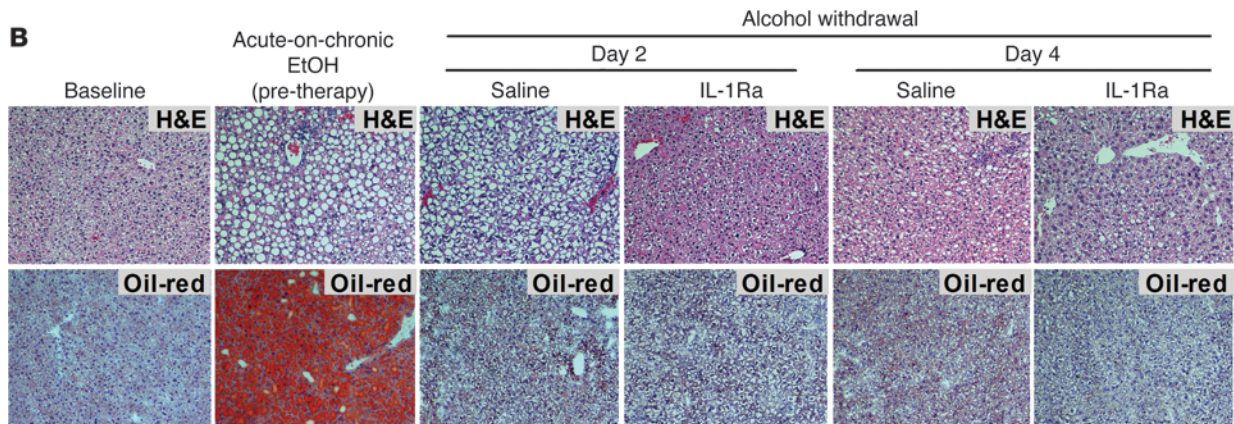
**Figure 7**

Pharmacologic intervention via inhibition of IL-1 signaling ameliorates ALD progression. (A–C) WT mice were fed control or alcohol diet. At day 14 (A and B) or day 28 (C), mice were started on daily injections of IL-1Ra (anakinra; 25 mg/kg) or saline i.p. and sacrificed 2 weeks later. Liver histologies from days 14 and 28 were stained with H&E and Oil-red-O (A), and ALT was measured in the serum (B and C). (A and B)  $n = 5$  (pair-fed saline and pair-fed IL-1Ra); 17 (EtOH-fed saline and EtOH-fed IL-1Ra). (C)  $n = 5$  (pair-fed saline and pair-fed IL-1Ra); 9 (EtOH-fed saline and EtOH-fed IL-1Ra). \* $P < 0.05$  vs. pair-fed. (D and E) WT mice were fed control or alcohol diet. After day 14, mice were started on daily injections of IL-1Ra or saline i.p. and sacrificed 5 weeks later. The progression of liver injury was evaluated by measuring serum ALT (D). Liver histologies from days 14 (week 2) and 49 (week 7) were stained with H&E and Oil-red-O (E).  $n = 5$  (pair-fed saline and pair-fed IL-1Ra); 40 (EtOH-fed saline); 29 (EtOH-fed IL-1Ra). At the end of experiment (week 7), survival was 23% in the EtOH-fed saline group and 48% in the EtOH-fed IL-1Ra group ( $P = 0.036$ ). \* $P < 0.05$  vs. EtOH-fed IL-1Ra; # $P < 0.05$  vs. week 2. Numbers in graphs denote  $P$  values. Original magnification,  $\times 200$ .



**Figure 8**

Pharmacologic intervention via inhibition of IL-1 signaling facilitates recovery from acute-on-chronic alcoholic liver injury. WT mice were treated with alcohol diet for 4 weeks and received 3 intragastric gavages of EtOH (5 g/kg) during the last 3 days of alcohol feeding. On day 28, all mice were switched to control diet, and daily treatment with IL-1Ra or saline was initiated. Liver injury was analyzed using serum ALT (A) and H&E staining (B), and steatosis was evaluated by Oil-red-O staining (B). *n* = 3–5 per time point and treatment. \**P* < 0.05 vs. baseline. Original magnification, ×200.



Using immortalized murine macrophages, we observed that recombinant IL-1 $\beta$  dose-dependently increased production of the NF- $\kappa$ B-dependent inflammatory cytokine TNF- $\alpha$ , and a significant effect of IL-1 $\beta$  was observed at concentrations as low as 5 pg/ml (Figure 10A). Furthermore, very low doses (1–25 pg/ml) of IL-1 $\beta$  synergistically augmented production of TNF- $\alpha$  in murine macrophages stimulated with low doses of LPS (Figure 10B). This finding is supported by previous studies showing that IL-1 signaling is required for the full expression of NF- $\kappa$ B-induced inflammatory cytokines in human leukocytes (17, 23) and consistent with our *in vivo* data that KC-specific deficiency of Casp-1 (Figure 9, J–L), global deficiency of ASC (Figure 4, C–I) or IL-1R1 (Figure 5, C–E), or pharmacological blocking of IL-1R1 (Figure 6, I–N) inhibited upregulation of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-10, and MCP-1 in ALD. Taken together, our results strongly support the crucial role of the inflammasome and IL-1 signaling in positive feed-forward induction of IL-1 $\beta$  and other LPS-inducible inflammatory cytokines (17) in the context of ALD.

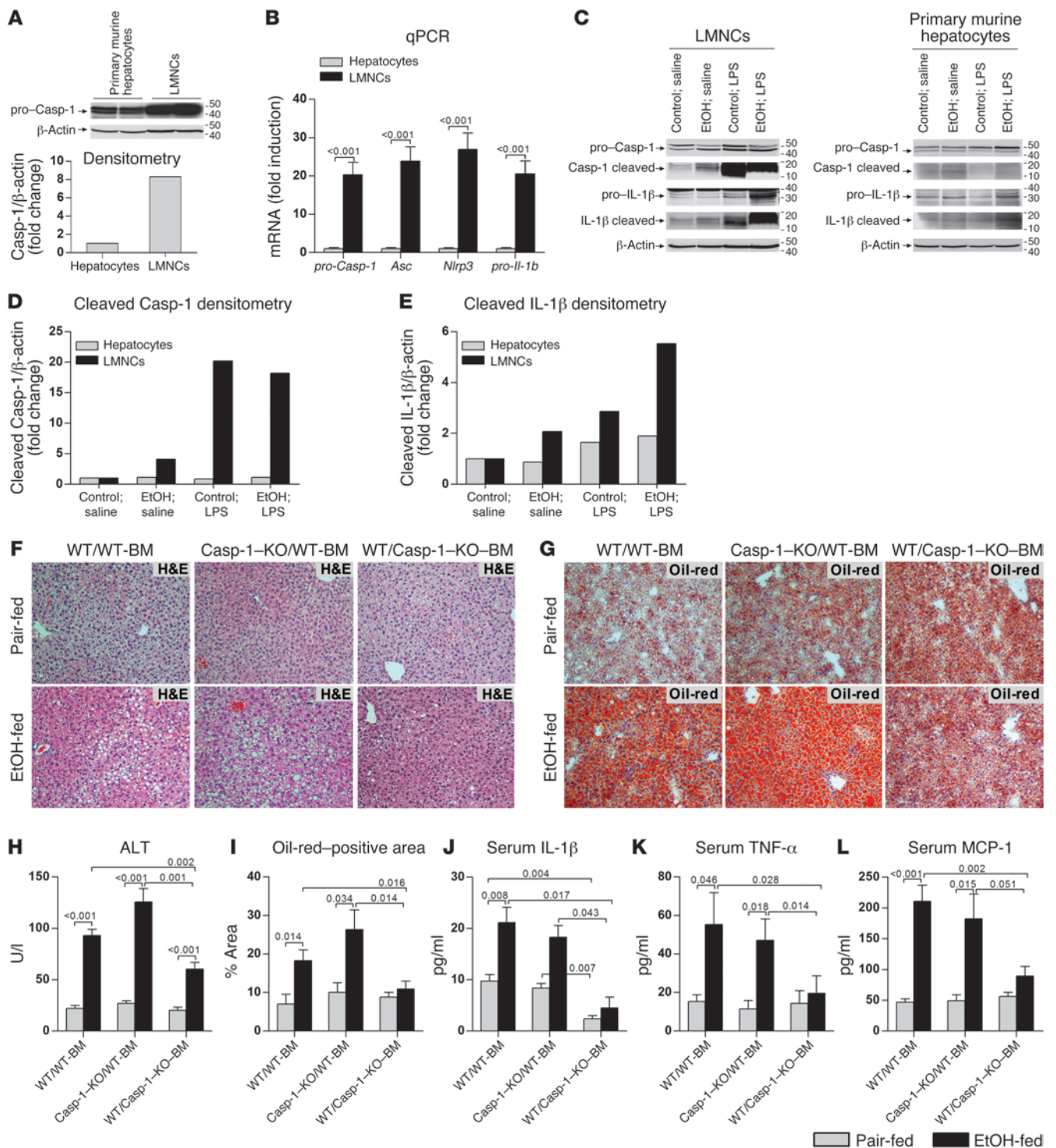
Our previous data demonstrated that low doses of IL-1 $\beta$  sensitize primary mouse hepatocytes to TNF- $\alpha$ -induced cytotoxicity (14). We also found that stimulation of primary WT hepatocytes with IL-1 $\beta$  induced a significant increase in the production of MCP-1 (Figure 10C). MCP-1 is a chemokine required for the pathogenesis of ALD (24) that, when present in high doses, induces lipid accumulation in hepatocytes (12). To assess the steatogenic potential of IL-1 $\beta$  and MCP-1, we evaluated their respective effects on triglyceride accumulation in isolated hepatocytes and observed that the

maximum extent of lipid accumulation was substantially higher in cells stimulated with MCP-1 compared with IL-1 $\beta$  (Figure 10D). However, the steatogenic effect of MCP-1 was observed only at supraphysiological concentrations (greater than 10,000 pg/ml), whereas IL-1 $\beta$  induced lipid accumulation at doses comparable to the concentrations of IL-1 $\beta$  observed in the serum *in vivo* after chronic alcohol administration (less than 100 pg/ml; Figure 1D, Figure 2E, Figure 4C, Figure 5C, Figure 6I, and Figure 9J).

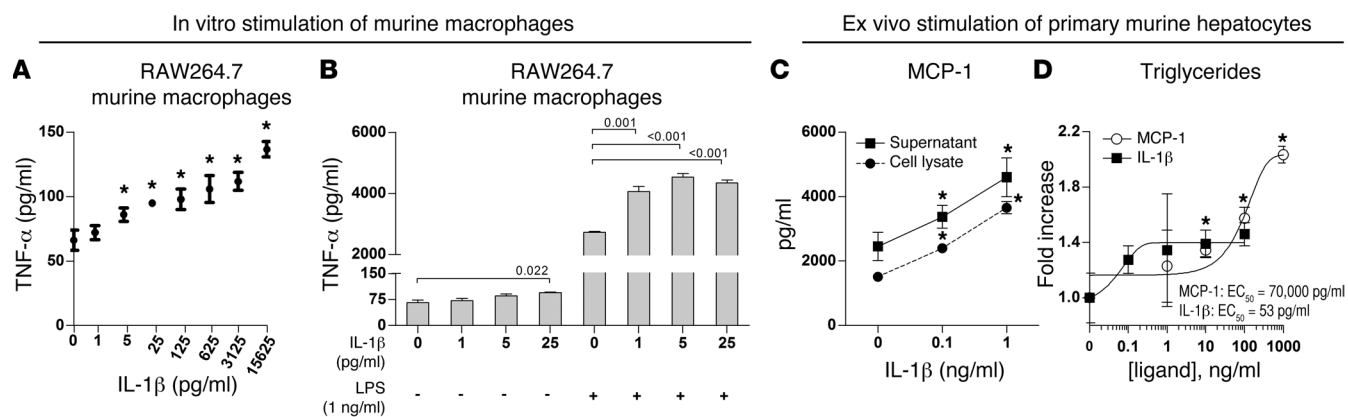
Taken together, these data further support the hypothesis that IL-1 $\beta$  exerts significant effects on immune cells and hepatocytes at concentrations observed *in vivo* and therefore represents a crucial determinant in the pathogenesis of alcohol-induced liver inflammation, steatosis, and damage.

### Discussion

ALD is tightly linked to liver inflammation and steatosis in humans as well as in experimental models (25). Here we have shown that activated inflammasome and IL-1 $\beta$  drive the pathogenesis of alcohol-induced liver inflammation, steatosis, injury, and fibrogenesis and that the pathogenic effect of inflammasome and IL-1 $\beta$  is KC specific. We demonstrated, for the first time to our knowledge, that Casp-1 was involved in IL-1 $\beta$  activation in ALD and that deficiency in Casp-1, ASC, or IL-1R1 consistently prevented alcoholic hepatitis and significantly attenuated alcohol-induced steatosis and liver damage. In addition, we demonstrated that pharmacological inhibition of IL-1 signaling had protective effects in various stages of ALD, including development and pro-



**Figure 9** BM-derived cells mediate pathogenic effects of Casp-1 in ALD. (A–E) LMNCs or primary hepatocytes were isolated from the livers of chow-fed WT mice as described in Methods. Pro-Casp-1 levels in cell lysate were evaluated using immunoblotting and normalized to  $\beta$ -actin (A). Expression of *pro-Casp-1*, *Asc*, *Nlrp3*, and *pro-Il-1b* was measured using qPCR (B). WT mice received 1 dose of intragastric EtOH (5 g/kg body weight) or isocaloric dextran-maltose per day on 3 consecutive days. 12 hours after the third intragastric gavage, LMNCs or primary hepatocytes were isolated. Cleaved forms of Casp-1 and IL-1 $\beta$  in cell lysates (C) were analyzed using antibodies that identify both full-length (short exposure, presented in linear contrast mode) and cleaved forms (long exposure, presented in sigmoidal contrast mode) and normalized to  $\beta$ -actin (D and E). LMNCs or hepatocytes were pooled from 5 (A and C–E) or 11 (B) mice per group. (F–L) WT/WT-BM, Casp-1-KO/WT-BM, and WT/Casp-1-KO-BM mice were fed control ( $n = 4$ –5 per genotype) or alcohol ( $n = 8$  per genotype) diet and sacrificed 4 weeks later, as described in Methods. Liver injury was assessed by liver H&E staining and serum ALT (F and H). Steatosis was evaluated by Oil-red-O staining (G and I). Serum levels of IL-1 $\beta$  (J), TNF- $\alpha$  (K), and MCP-1 (L) were measured by specific ELISA. Numbers in graphs denote  $P$  values. Original magnification,  $\times 200$ .

**Figure 10**

Physiological doses of IL-1 $\beta$  elicit biological response in macrophages and hepatocytes. **(A and B)** Immortalized murine RAW264.7 macrophages were stimulated with the indicated doses of recombinant mouse IL-1 $\beta$  **(A)**, or concurrently with recombinant mouse IL-1 $\beta$  and/or LPS **(B)**, and levels of TNF- $\alpha$  were evaluated 12 hours later using specific ELISA. **(C and D)** Primary hepatocytes isolated from WT mice were treated with recombinant IL-1 $\beta$ . MCP-1 in hepatocyte culture supernatant and in hepatocyte lysate was measured using specific ELISA **(C)**. Triglycerides were measured in primary hepatocytes stimulated for 24 hours with MCP-1 or IL-1 $\beta$  at the indicated doses **(D)**. All stimulations were performed in triplicate. Numbers in graphs denote  $P$  values. \* $P < 0.05$  vs. baseline.

gression of ALD and recovery from acute-on-chronic alcoholic liver injury. Based on these findings, we have identified inflammasome and IL-1 $\beta$  as potential therapeutic targets in ALD.

Although Casp-1 cleaves IL-1 $\beta$ , IL-18, and IL-33 (8), and IL-1R1 recognizes IL-1 $\alpha$ , IL-1 $\beta$ , and IL-1Ra (16), our study provides several lines of evidence that IL-1 $\beta$  – not IL-18, IL-33, or IL-1 $\alpha$  – is the primary cytokine that mediates the pathogenic effects of activated inflammasome in ALD. First, alcohol administration in mice significantly increased IL-1 $\beta$  and IL-33, whereas levels of IL-1 $\alpha$  and IL-18 remained unchanged. Furthermore, our studies showed that Casp-1 and ASC were required for cleavage and activation of IL-1 $\beta$  in ALD, in contrast to inflammasome-independent cleavage of IL-33. Second, IL-1 $\beta$  exerted its effects through paracrine signaling, which is considered to be the primary mode of interaction between immune cells and parenchymal cells in the liver (4, 15, 26). In contrast, IL-1 $\alpha$  is a membrane-bound autocrine growth factor that does not require inflammasome activation (16). Consistent with this, we found no upregulation or secretion of IL-1 $\alpha$  in ALD. Finally, genetic deficiency or pharmacological blockade of IL-1R1 ameliorated alcohol-induced liver steatosis, inflammation, and damage to the same extent as global deficiency of Casp-1 or ASC or KC-specific deficiency of Casp-1, strongly supporting the evidence that the pathogenic effect of inflammasome in ALD is mediated via KC-derived IL-1 $\beta$  by way of paracrine signaling. However, we cannot completely exclude an IL-1R1-independent contribution of IL-1 $\alpha$  or an inflammasome-independent effect of IL-33 in ALD. For example, nuclear translocation of IL-1 $\alpha$  that serves as an intracrine activator of proinflammatory responses (27), or induction of Th2-immune responses by IL-33 (28), may contribute to ALD.

A number of cell types, including epithelial and immune cells, express Casp-1. We showed that the protection from alcohol-induced liver inflammation, steatosis, and damage observed in mice with global deficiency of Casp-1 was attributable to Casp-1 expressed in KCs. This finding was consistent with reports demonstrating that Casp-1 is indispensable for cleavage and activation of IL-1 $\beta$  in macrophages (reviewed in ref. 8) and with data demonstrating a crucial role of KCs in response to gut-derived LPS

and in the pathogenesis of ALD (2, 3, 20). Our data and reports of others demonstrate that the mechanisms by which IL-1 $\beta$  exerts its pathogenic effects involve sensitization of hepatocytes to cytotoxicity induced by TNF- $\alpha$  (14, 29), upregulation of lipid synthesis in hepatocytes (12), activation of hepatic stellate cells (30), maintenance of macrophages in inflammatory state (31), and feed-forward induction of LPS-inducible inflammatory cytokines (17), such as TNF- $\alpha$  or MCP-1, that are crucial in ALD (24, 32). Our results also demonstrated that even very low concentrations of IL-1 $\beta$  exerted significant biological effects on hepatocytes and macrophages, thus giving additional support to the crucial role of IL-1 $\beta$  in ALD, even at the relatively low absolute serum levels of IL-1 $\beta$  in vivo. Moreover, our findings suggest a novel IL-1 $\beta$ -dependent self-sustaining mechanism that amplifies inflammatory signaling in the liver after alcohol abuse.

The liver is a main target of gut-derived bacterial products, and the rate of translocation of bacterial endotoxin (LPS) increases in individuals drinking alcohol (ref. 21 and G. Szabo, unpublished observations) and in animal models of ALD (33). Thus, translocated LPS provides the first signal for TLR4-mediated upregulation of inflammatory mediators, such as TNF- $\alpha$ , pro-IL-1 $\beta$ , and MCP-1. However, the nature of the second signal required for the activation of inflammasome and IL-1 $\beta$  in ALD is unclear. Our data demonstrated the crucial role of ASC in ALD, narrowing the inflammasome candidates to the ASC-dependent NALP3 and AIM2 inflammasomes (8). Activators of the NALP3 inflammasome involve host-derived molecules indicative of injury (such as extracellular ATP and hyaluronic acid), signs of metabolic stress (including elevated extracellular glucose), and particulate substances (such as uric acid crystals), whereas activators of AIM2 inflammasome include cytosolic double-stranded DNA (reviewed in ref. 8). Indeed, mitochondrial dysfunction with disturbances of ATP homeostasis have been implicated in the pathogenesis of ALD (34, 35), which is consistent with our animal data showing alcohol-induced increase in serum ATP and upregulation of the ATP-conducting channel pannexin-1 in the liver (Supplemental Figure 6). In addition, we showed here that the extracellular concentration of hyaluronic acid



increased in alcohol-fed mice (Figure 3F). AIM2 inflammasome activation by modified self DNA from apoptotic hepatocytes as a damage-associated endogenous signal in ALD cannot be ruled out. Detailed investigation of mechanisms involved in inflammasome activation in ALD is beyond the scope of this study and will be the subject of our future research.

The presence of IL-1Ra, an endogenous inhibitor of IL-1R1, is a unique regulatory feature that has not been observed in other cytokines (16). Our finding that IL-1Ra was increased in ALD, which we believe to be novel, suggests that IL-1 signaling in the liver is under a tight endogenous control. Our data showed a protective effect of IL-1Ra in the development of ALD. A protective effect of the blockade of IL-1 signaling has also been shown in TLR9-associated liver injury (14), recovery after partial hepatectomy in mice (36), and liver failure induced by D-galactosamine (37). Moreover, we demonstrated, for the first time to our knowledge, that administration of IL-1Ra inhibited progression of already established ALD and facilitated recovery from acute-on-chronic alcoholic liver injury, a scenario observed in the majority of patients presenting with acute alcoholic hepatitis (1). In contrast to anti-TNF approaches, whose utility was compromised by infectious complications (1, 5), administration of IL-1Ra has demonstrated an excellent safety profile, and its use has not been associated with adverse reactions or superinfections with long-term treatment in patients with rheumatoid arthritis (38) or in acute settings in patients with sepsis (10).

In conclusion, our data demonstrated that signaling mediated by IL-1 $\beta$  was crucial in the pathogenesis of alcohol-induced liver inflammation, steatosis, and injury in a cell-specific manner. Furthermore, our findings suggest that inflammasome activation may represent a new pathway in the pathogenesis of ALD. Finally, our data support the potential role of IL-1Ra in the treatment of ALD.

## Methods

**Animal studies.** 6- to 8-week-old female C57BL/6 WT and IL-1R1-KO mice (Jackson Laboratory), and Casp-1-KO and ASC-KO mice (gift of A. Hise, Case Western Reserve University, Cleveland, Ohio, USA), were used. Some animals were fed the Lieber-DeCarli ad libitum diet (Dyets Inc.) with 5% (vol/vol) ethanol (36% ethanol-derived calories) for 4–7 weeks; pair-fed control mice matched the alcohol-derived calories with dextran-maltose. Specifically, 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. Intra-gastric administration of ethanol (5 g/kg body weight), or isocaloric dextran-maltose, was performed in some mice using 22-gauge stainless steel feeding tubes. Some mice were treated with recombinant human IL-1Ra (1.5, 10, or 25 mg/kg) or saline i.p. every 24 hours (anakinra; Amgen), and IL-1Ra treatment was ongoing until the end of experiment. In a pilot experiment, nonspecific effects were excluded by heat inactivation of IL-1Ra (39).

**BMT protocol.** BM was collected from the long bones of 8-week-old donor male mice by flushing with a 25-gauge needle, then passed through a cell strainer to remove clumps. Female mice were injected with 200  $\mu$ l liposomal clodronate (purchased from N. van Rooijen, Free University Amsterdam, Amsterdam, the Netherlands) via tail vein injection. 24 hours later, mice were irradiated (9 Gy) from a Cesium irradiator (Gammacell 40; Atomic Energy of Canada), then 4 hours later transplanted with  $5 \times 10^6$  freshly isolated donor BM cells via a single tail vein injection. Transplanted mice were housed in microisolator cages and placed on medicated water (sulfamethoxazole/trimethoprim; Hi-Tech Pharmacal Inc.) until engraftment was complete 6 weeks later. After engraftment, feeding with Lieber-DeCarli diet was initiated. Depletion of KCs in acceptor livers was assessed

48 hours after clodronate injection using F4/80 staining (CI:A3-1; AbD Serotec) and compared with that in mice injected with control PBS liposomes (Supplemental Figure 5). Success of BMT was confirmed by measuring IL-1 $\beta$  in the serum (Figure 9J), serving as a marker for Casp-1 activity.

**Sample storage.** Serum was stored at  $-80^\circ\text{C}$ . Livers were snap-frozen in liquid nitrogen for proteins, frozen in TissueTek for Oil-red-O analysis (Sakura Finetek USA Inc.), stored in RNAlater (Qiagen GmbH) for RNA extraction, or fixed in 10% neutral-buffered formalin for histopathological analysis.

**Biochemical assays.** Serum ALT was determined using a kinetic method (D-Tek LLC). Serum ATP was measured using the bioluminescence kit from Promega. Colorimetric assay (R&D Systems Inc.) was used to measure the activity of Casp-1 in the liver.

**Cytokine measurement.** Levels of IL-1 $\beta$ , IL-1 $\alpha$ , IL-33, TIMP-1, hyaluronic acid, and endogenous IL-1Ra in the tissues and serum were measured using specific anti-mouse ELISAs, and the exogenously administered IL-1Ra was measured using specific ELISA recognizing human IL-1Ra (R&D systems Inc.). TNF- $\alpha$ , IL-6, and MCP-1 in the serum and in the livers as well as serum IL-10 was measured using ELISA from Biolegend Inc. Due to high background signal, ELISA could not be used for detection of IL-10 in liver. Serum and liver IL-18 was measured using ELISA from MBL International; TNF- $\alpha$  in cell culture supernatants was measured using ELISA from BD Biosciences; and we used ELISA from Novatein Biosciences to measure PI3NP.

**Protein quantification.** Whole-cell lysates were extracted from liver, as described previously (40). Equal amounts of proteins were separated on polyacrylamide gel, and transferred to a nitrocellulose membrane. Target proteins were detected by Western blot and immunostaining with specific primary antibody, followed by horseradish peroxidase-labeled secondary antibody. The specific immunoreactive bands of interest were detected by chemiluminescence (Amersham). Digital system (ImageQuant LAS 4000; GE Healthcare) was used for image acquisition. Antibodies specific for IL-1 $\beta$  (catalog no. 30311) and for IL-33 (catalog no. 396118) were from R&D Systems Inc. The IL-18 antibody (catalog no. 39-3F) was purchased from MBL International, and antibodies specific for IL-10 (catalog no. NYRmIL-10) and Casp-1 (catalog no. M20) were from Santa Cruz Biotechnology.  $\beta$ -Actin antibody (catalog no. 8229) was from Abcam.

**RNA analysis.** RNA was purified using the RNeasy kit (Qiagen Sciences) and on-column DNA digestion. cDNA was transcribed with the Reverse Transcription System (Promega Corp.). qPCR was performed using Sybr-Green and iCycler from BioRad (Bio-Rad Laboratories Inc.); primer sequences and reaction conditions are shown in Supplemental Table 1.

**Histopathological analysis.** Liver sections were stained with H&E, Oil-red-O, or Sirius red and analyzed by microscopy, as we previously described (40). Immunohistochemistry staining for F4/80 (CI:A3-1; AbD Serotec) or CD3 (SP7; ThermoFisher) was performed in formalin-fixed, paraffin-embedded livers according to the manufacturer's instructions. ImageJ (NIH) was used for image analysis.

**Isolation of primary mouse hepatocytes and LMNCs.** Anesthetized animals were perfused by way of portal vein with saline solution followed by enzymatic digestion as described previously (40). Hepatocytes were separated by centrifugation; LMNCs were purified by centrifugation in Percoll gradient.

**In vitro experiments.** Primary hepatocytes were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum and 1% insulin, transferrin, selenium (ITS) solution. Primary hepatocytes were seeded in 6-well collagen-coated plates. Before starting stimulation experiments, hepatocytes were rested for 4 hours. Subsequently, culture media was replaced, and stimulation was performed as indicated in the figure legends. RAW264.7 macrophages were stimulated with LPS (Sigma-Aldrich). Recombinant murine IL-1 $\beta$  and MCP-1 proteins were purchased from R&D Systems.



**Statistics.** Statistical significance was determined using 2-sided *t* test; ANOVA and Dunnett multiple-comparison post-test were used to compare the means of multiple groups. Data are shown as mean  $\pm$  SEM. A *P* value less than 0.05 was considered significant.

**Study approval.** 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.

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