

Title Page:**IL-1 receptor like 1 protects against alcoholic liver injury by limiting NF- κ B activation in hepatic macrophages**

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LIST OF ABBREVIATIONS:

ALD, Alcohol liver disease; **M Φ** , macrophage; **IL**, interleukin; **sST2**, soluble ST2; **IL1R**, interleukin 1 receptor; **TLRs**, Toll-like receptors; **Th2**, type 2 T helper cells; **TIR**, toll-interleukin-1 receptor domain; **Mal**, MyD88-adaptive-like; **IL-1RAcp**, Interleukin-1 receptor-associated protein; **ST2L**, full length of ST2; **PAMP**, pathogen-associated molecular pattern; **DAMP**, damage-associated molecular patten molecule; **EV**, extracellular vesicles;

Abstract:

Background & Aim: Alcohol consumption, through increasing intestinal permeability and causing hepatocytes damage, leads to the release of pathogen- and damage-associated molecular pattern molecules (PAMPs and DAMPs). These molecules stimulate hepatic macrophages (MΦs) and activate NF-κB, resulting in inflammation and exacerbating alcoholic liver disease (ALD). However, much less is known about the mechanisms attenuating inflammation and preventing disease progression in the majority of the heavy drinkers. Interleukin (IL)-33 is a DAMP (alarmin) released from dead cells and acts through its receptor, IL-1 receptor like 1 (ST2). ST2 signaling has been reported to either stimulate or inhibit NF-κB activation. The role of IL-33/ST2 in ALD has not been studied. **Methods:** Serum levels of IL-33 and its decoy receptor, soluble ST2 (sST2) were measured in ALD patients. Alcohol-induced liver injury, inflammation and hepatic MΦ activation were compared among WT, IL-33^{-/-} and ST2^{-/-} mice in several models. **Results:** Elevations of serum IL-33 and sST2 were only observed in patients with severe decompensated ALD. Consistently, in mice with mild ALD without significant amount of cell death and IL-33 release, IL-33-deletion did not affect alcohol-induced liver damage. However, ST2-deletion exacerbated ALD, through enhancing NF-κB activation in liver MΦs. In contrast, when extracellular IL-33 was markedly elevated, both IL-33^{-/-} and ST2^{-/-} mice developed attenuated liver injury and inflammation compared with WT mice. **Conclusion:** Our data revealed a dichotomous role of IL-33/ST2 signaling during ALD development. At early and mild stages, ST2 restrains the inflammatory activation of hepatic MΦs, through inhibiting NF-κB, and plays a protective function in an IL-33-independent fashion. During severe liver injury, significant cell death and marked IL-33 release occur, which triggers IL-33/ST2 signaling and exacerbates tissue damage.

Lay summary

In mild ALD, ST2 negatively regulates the inflammatory activation of hepatic MΦs, thereby protecting against alcohol-induced liver damage, whereas in case of severe liver injury, the release of extracellular IL-33 may exacerbate tissue inflammation by triggering the canonical IL-33/ST2L signaling in hepatic MΦs.

INTRODUCTION:

Alcoholic liver disease (ALD) is a major chronic liver disorder that can progress from mild hepatic inflammation/injury to more severe manifestations including alcoholic hepatitis, cirrhosis and hepatocellular carcinoma. (1) The severe forms of ALD result in end-stage decompensated liver disease in approximately one third of heavy drinkers. (1, 2) Ample evidence supports that hepatic inflammation plays a critical role in the underlying pathogenesis of ALD. (3) Alcohol consumption increases intestinal permeability, leading to the hepatic translocation of pathogen-associated molecular pattern molecules (PAMPs). In addition, damage-associated molecular pattern molecules (DAMPs, or alarmin) are released when severe injury and cell death occur (4, 5). These molecules activate NF- κ B and stimulate inflammatory responses in liver macrophages (M Φ s) and other innate immune cells. (6) A number of pro-inflammatory cytokines and chemokines such as TNF α , IL-1 β , IL-6 and MCP-1 are elevated in mice fed with ethanol and in patients with ALD. (7, 8) Targeting these cytokines has been employed as a strategy for the treatment of ALD, notably in alcoholic hepatitis. Neutralizing TNF α has been evaluated; however, it increased the risk of infection without survival benefits. (9, 10) In most inflammatory conditions, regulatory mechanisms play an important role in limiting the collateral tissue injury as result of surging in inflammatory responses. Thus, understanding the anti-inflammatory mechanisms that attenuate TLR/NF- κ B signaling during ALD is important in developing therapeutic strategies to prevent the progression of ALD from mild to severe forms.

Interleukin (IL)-33 is a member of the IL-1 family and it is constitutively expressed in endothelial and epithelial cells. (11) IL-33 normally resides in the nucleus, but is released as an alarmin (or DAMP) upon cell death during severe tissue injury.(12) The receptor of IL-33 is ST2 (suppression of tumorigenicity 2), also known as IL-1 receptor

like-1 (IL-1r1), is mainly expressed on various immune cells, including MΦs, type 2 T helper cells (Th2), mast cells and eosinophils (13-18) Similar to other IL-1 receptors, ST2 contains an intracellular toll-interleukin-1 receptor domain (TIR), which can bind to MyD88 and MyD88-adaptive-like (Mal). Aside from the transmembrane full length of the ST2 receptor (ST2L), a splicing variant gives rise to a soluble form (sST2) which acts as a decoy receptor neutralizing extracellular IL-33 and blocking IL-33/ST2L signaling. Interestingly, both ST2-mediated stimulation and inhibition of NF-κB activation have been reported. (13-17, 19) One reason for the discrepancy is the differential ST2 signaling in the presence or absence of IL-33. Once released, extracellular IL-33 binds to ST2 and recruits IL-1 receptor accessory protein (IL-1RAcP), followed by the activation of the MyD88/TRAF6/NF-κB pathway. However, in the absence of extracellular IL-33 and thus failure of IL-1RAcP recruitment, sequestering MyD88/Mal by ST2 actually inhibits NF-κB activation induced by TLR agonists. (13, 19)

The present study is the first to investigate the roles of IL-33 and ST2 in ALD. We observed elevations of IL-33 and sST2 in sera of patients with severe ALD. Moreover, our data revealed a dichotomous involvement of IL-33/ST2 signaling in animal models, depending on the presence or absence of substantial IL-33 release.

Materials and methods:*Animal treatments*

C57Bl/6N (Taconic), IL-33^{-/-} and ST2^{-/-} mice (obtained from Dr. Robert B. Fick at Merck Research Laboratories) were maintained in ventilated cages under 12h light/dark cycle at the University of Colorado Anschutz Medical Campus (UCAMC) animal facility. All experiments were performed according to the guidelines of the IACUC at UCAMC. Ten-week-old female mice were fed with ethanol-containing Lieber-DeCarli liquid diet to study ALD, and the treatment details are described in supplementary methods.

Human subject cohort

All healthy controls, heavy drinkers and ALD patients provided informed consent and the study were approved by the Institutional Review Board at the Richard L. Roudebush Veterans Administration Medical Center, Fairbanks Alcohol Rehabilitation Center and Indiana University Purdue University Indianapolis (IUPUI). At the time of enrollment, blood samples were collected and stored in -80°C freezer, until the time of analysis. The details about these samples are described in supplementary methods.

Other methods

Additional methods and statistical analyses are described in the supplementary methods.

Results:

Serum levels of IL-33 and sST2 are significantly increased only in patients with severe ALD.

Associated with severe liver damage, increased serum levels of IL-33 and sST2 have been reported in patients with hepatitis B, acute liver failure, acute-on-chronic liver failure, liver cirrhosis, and hepatocellular carcinoma. (20, 21) However, the quantification of serum sST2 and IL-33 in ALD patients has not been reported. We obtained serum samples from 20 healthy individuals (age 33.2 ± 10.6 years, 90% male, and 70% White), 20 heavy drinkers (age 41.5 ± 13.3 years, 65% male, and 80% White), and 11 patients with ALD (age 56.7 ± 10.5 years, 90% male, and 100% White). Compared to healthy individuals, neither IL-33 nor sST2 was elevated in heavy drinkers, but both were markedly increased in ALD patients (Fig. 1A). Interestingly, there appeared to be two distinct clusters with differential levels of IL-33 and sST2 among subjects with ALD. Further analysis revealed that only patients with decompensated ALD (with Child-Pugh scores > 7) had dramatically higher levels of IL-33 and sST2, whereas the levels were similar among healthy individuals, heavy drinkers, and ALD patients with compensated livers (with Child-Pugh scores of 5-6, Fig. 1B). Our data demonstrate that the extracellular release of IL-33 and the elevation of sST2 occur in severe ALD.

The levels of serum IL-33 and sST2 correlate with the extent of liver damage in mice

To investigate the involvement of IL-33 and ST2 in ALD, we initially employed two murine models: i) chronic alcohol feeding-induced mild ALD, and ii) a recently developed model of more severe ALD, in which mice were fed with alcohol plus binge twice per week for 8 weeks (22). In agreement with the previous report (22), the ALT level remained modestly elevated in the model of chronic alcohol plus multiple binges (Fig. 2C), although higher than that in the mild ALD model (Fig. 2B&C). This suggests that dramatic cell death

is absent even in the advanced ALD model with worsened steatohepatitis and fibrosis. Consistent with the lack of marked cell death in both models, the serum levels of IL-33 (and sST2) were not significantly increased (Fig. 2E-F), and IL-33-deletion did not affect alcohol-induced liver injury (Fig. 2B&C). Interestingly, the ALT levels were significantly higher in the ST2^{-/-} than WT mice (Fig. 2B&C), suggesting a possible protective function of ST2 independent of IL-33.

Significant amount of cell death is a pre-requisite for IL-33 release, but it does not occur in ALD models currently available to most of the researchers in the field. As a proof-of-concept approach to investigate the role of extracellular IL-33, we produced more severe cell death by administering one low dose of CCl₄ after 4 weeks of alcohol feeding. In this case, serum ALT activities rose to more than 2000 IU/L in WT mice (Fig. 2D). As result, the levels of IL-33 and sST2 increased approximately 15-fold and 8-fold, respectively, compared with those in mice fed with alcohol alone or alcohol plus multiple binges (Fig. 2E-F). Interestingly, liver injury was significantly suppressed in both IL-33^{-/-} and ST2^{-/-} mice compared with WT mice (Fig. 2D). The levels of inflammatory mediators were lower in the two knockout strains than WT mice (Fig. 2G). Consistent with the notion that extracellular IL-33 binding to ST2L triggers NF- κ B activation (14-16), the data suggest that when there is substantial release of IL-33, the IL-33/ST2L axis exacerbates tissue inflammation and injury.

ST2 attenuates chronic alcohol feeding-induced liver injury and inflammation in the absence of extracellular IL-33

The clinical data showed that IL-33 was not released in patients with compensated ALD or in heavy drinkers without overt clinical manifestation (Fig. 1B). In mice treated with chronic alcohol or chronic alcohol plus multiple binges (Fig. 2E), the extracellular release of IL-33 was also minimal in the absence of marked cell death. As result, IL-33-deletion did

not affect liver injury in these two models. In contrast, ST2-deletion (in either male or female mice) exacerbated liver injury in both models (Fig. 2B-C and supporting Fig. S1). Moreover, hepatic lipid accumulation caused by chronic alcohol feeding was significantly higher in ST2^{-/-} than WT mice (Fig. 3A-B). Quantification of liver triglyceride levels confirmed that alcohol-induced steatosis is more severe in ST2^{-/-} than WT mice (Fig. 3C). Together, these data suggest that in the absence of extracellular IL-33, ST2 plays a protective role and perhaps serves as a regulator mechanism in attenuating ALD and slowing down the disease progression.

The TLR-induced NF- κ B activation is a major pathway contributing to ethanol-induced hepatic inflammation. ST2 has been reported to inhibit TLR-mediated NF- κ B activation. (13, 19) Therefore, we examined the role of ST2 in regulating alcohol-induced liver inflammation by comparing the levels of pro-inflammatory cytokines in WT and ST2^{-/-} mice. As shown in Fig. 4A, although the serum levels of TNF- α were similar, the levels of IL-1 β and IL-6 were more than 3-fold higher in ethanol-fed ST2^{-/-} mice (18.91 ± 3.695 pg/ml and 17.56 ± 4.149 pg/ml respectively) than in WT mice (6.077 ± 1.176 pg/ml and 5.186 ± 1.407 pg/ml). In addition, hepatic TNF- α and IL-1 β protein levels were significantly higher in ST2^{-/-} than WT mice, whereas hepatic IL-6 protein levels were comparable between both groups (Fig. 4B).

We further analyzed the hepatic mRNA expression levels of pro-inflammatory cytokines/chemokines, including IL-1 α , IL-1 β , TNF- α , MCP-1, IL-6 and IL-12p40. As shown in Fig. 4C, the levels of the majority of these pro-inflammatory mediators were dramatically increased in EtOH-fed compared with pair-fed ST2^{-/-} mice. However, EtOH feeding only up-regulated IL-1 β and IL-6 expression in WT mice. These results suggested that ST2-deletion augmented the inflammatory responses to chronic ethanol feeding, contributing to enhanced liver injury.

LPS causes a more severe exacerbation of alcohol-induced liver injury in ST2^{-/-} than WT mice

Alcohol-induced hepatic translocation of bacterial products is important in triggering hepatic inflammation through TLR signaling. As a typical TLR4 agonist, LPS has been used as a model system to investigate the TLR/NF- κ B pathway in ALD. (23, 24) Previous studies have shown that LPS challenge in addition to chronic alcohol feeding exacerbates the degree of liver injury. (25) Based on the data described above, we hypothesized that ST2^{-/-} mice might be more susceptible to liver injury and inflammation caused by chronic alcohol feeding plus LPS.

To test this hypothesis, we treated EtOH-fed and pair-fed mice with 0.5 mg/kg of LPS after 4 weeks of feeding. Consistent with the results shown in Fig. 2B, alcohol-feeding alone induced higher levels of ALT in ST2^{-/-} than WT mice. LPS challenge further elevated the ALT levels in both WT and ST2^{-/-} mice. Interestingly, the effect of LPS was more dramatic in ST2^{-/-} mice, as the ALT levels were almost 2-fold higher in ST2^{-/-} mice than WT mice (Fig. 5A). There were no significant differences of ALT levels in pair-fed WT and ST2^{-/-} mice regardless of the additional LPS stimulation.

Furthermore, compared with WT mice treated with ethanol plus LPS, the ST2^{-/-} mice showed significantly higher serum levels of IL-1 β and TNF- α (Fig. 5B). The hepatic mRNA expression levels of TNF- α , IL-1 β , and IL-6 were compared among WT and ST2^{-/-} mice treated with either pair-feeding plus LPS or EtOH-feeding plus LPS (Fig. 5C). In ST2^{-/-} mice, alcohol ingestion markedly augmented LPS-induced upregulation of these cytokines. Although a similar phenomenon was observed in WT mice, the combination of ethanol and LPS caused a much greater inflammatory response in ST2^{-/-} mice. The data further confirmed the inhibitory effect of ST2 on TLR/NF- κ B signaling.

Hepatic MΦs from ST2^{-/-} mice exhibit an enhanced pro-inflammatory phenotype and NF-κB activation

Hepatic MΦs represent a major source of pro-inflammatory mediators and contribute to ALD. (26-28) Thus, we hypothesize that the enhanced hepatic inflammation observed in ethanol-treated ST2^{-/-} mice may be due to exacerbated inflammatory responses of hepatic MΦs. To examine this hypothesis, we purified liver MΦs from ethanol-fed WT and ST2^{-/-} mice and compared the expression levels of pro-inflammatory cytokines and chemokines. The levels of IL-1α, IL-1β, TNF-α, MCP-1, IL-6 and IL-12p40 were all increased more than 10-fold in ST2^{-/-} hepatic MΦs than WT MΦs (Fig. 6A). In mice treated with ethanol plus LPS, the expression levels of TNF-α, MCP-1 and IL-6 in hepatic MΦs from ST2^{-/-} mice were also significantly higher than those from WT mice (Fig.6B). These data suggest that ST2 negatively regulates inflammatory response of hepatic MΦs during ALD.

Hepatic MΦs are well-recognized as a major cell type responding to PAMPs translocate from the gut, as well as to DAMPs released from hepatocytes. We hypothesized that hepatic MΦs from ST2^{-/-} mice are more sensitive to PAMPs/DAMPs stimulation than WT MΦs. Recent studies have shown that extracellular vesicles (EVs) which are released from hepatocytes during alcohol-induced liver injury, exert a pro-inflammatory effect on MΦs. (29, 30) Thus, we isolated EVs from sera of WT mice subjected to the Gao-binge model, in which the mice were binged with EtOH after 10 days EtOH feeding. (31) Hepatic MΦs were isolated from chronic EtOH-fed WT and ST2^{-/-} mice and stimulated with EVs. In some experiments, the cells were treated with EVs plus LPS, as a surrogate for PAMP. Our data showed that EVs alone or in combination with LPS

caused enhanced inflammatory responses in ST2^{-/-} hepatic MΦs than WT-MΦs, evident by increased expression levels of IL-1β, TNFα, and MCP-1 (Fig. 6C).

It has been shown that ST2 is not expressed in naïve MΦs, but can be induced by inflammatory stimulation. (13) We purified hepatic MΦs from naïve, pair-fed and ethanol-fed WT mice to determine ST2 expression. Our data show that pair-feeding slightly induced ST2 expression in hepatic MΦs, but ethanol-feeding dramatically upregulated ST2 levels in these cells (Fig. 7A). Interestingly, ethanol-feeding also induced ST2 expression in CD45-negative non-immune cells of the liver (Fig. 7A). However, the mRNA expression levels of pro-inflammatory mediators were markedly lower in CD45-negative cells than in hepatic MΦs (data not show), suggesting that the CD45-negative cells are not major players in alcohol-induced hepatic inflammation.

NF-κB is the key transcription factor regulating the pro-inflammatory mediators that were significantly upregulated in ST2^{-/-} MΦs. To determine whether ST2 attenuates the inflammatory response of hepatic MΦs through suppressing NF-κB activation, we purified hepatic MΦs from ethanol-fed WT and ST2^{-/-} mice. As shown in Fig. 7B, the ratio of phosphorylated p65 to total p65 was significantly higher in ST2^{-/-} MΦs than WT MΦs. Moreover, compared with WT liver MΦs, those from EtOH-fed ST2^{-/-} mice showed dramatically higher nuclear DNA binding activity of NF-κB p65 (Fig. 7C). To further examine whether the inhibition of ST2 promotes NF-κB-driven promoter activity, we co-transfected WT bone marrow derived MΦs with a ST2-targeting siRNA and a NF-κB-luciferase reporter plasmid. The data demonstrated that LPS-induced NF-κB promoter activity was significantly increased when ST2 was knocked down (Fig. 7D) Together, these data suggest that ST2 is an important negative regulator of NF-κB activation, thereby limiting inflammatory responses of hepatic MΦs and attenuating ALD.

Discussion:

One of the important mechanisms of alcohol-induced liver injury is that alcohol or its metabolite, acetaldehyde, enhances gut permeability and thus promotes the translocation of bacterial products. In the liver, PAMPs can activate the innate immune cells, such as MΦs, causing pro-inflammatory responses and liver injury. The TLR and IL-1R signaling pathways play an important role in mediating hepatic inflammatory responses in alcohol-induced liver injury. (23, 26) However, only about one third of heavy drinkers develop severe ALD, (1, 2) suggesting that there exist compensatory mechanisms preventing the disease progression. This study provides the first evidence that ST2 acts as a negative regulator of the pro-inflammatory cascades and attenuates alcohol-induced liver damage. Consistent with our finding, an anti-inflammatory effect of ST2 on MΦs has been described. (13, 19) It is reported that ST2-deficient MΦs are much more sensitive to bacterial lipoprotein stimulation of TLR2, evident by increased pro-inflammatory cytokine production and enhanced formation of TLR2-MyD88 and MyD88-IRAK complexes. (19) Another study demonstrates that MΦs from ST2-deficient mice produce significantly higher levels of pro-inflammatory cytokines in response to stimulation by a number of TLR agonists. (13) As result, ST2-deficient mice developed more severe inflammatory responses to LPS stimulation and were unable to mount endotoxin tolerance. (13)

Regarding the underlying mechanism accounting for the anti-inflammatory function of ST2 during mild ALD, our data demonstrate that ST2 suppresses NF-κB activation in hepatic MΦs. ST2 shares a similar TIR domain as other members of the TIR family. Upon binding to its ligand IL-33, ST2L forms a heterodimer with IL-1RAcP, which is indispensable for activating NF-κB. (14-16, 32) In contrast, in the absence of extracellular IL-33, ST2 inhibits NF-κB activation stimulated by agonists of TLR2, TLR4 and TLR9. (13) This inhibition is due to sequestration of MyD88 and Mal by ST2, thereby negatively

regulating TLR/NF- κ B signaling (Fig. 8). ST2 does not inhibit TLR3 signaling because it does not interact with Trif, the TLR3 adaptor. (13) These findings suggest that in the absence of IL-33, ST2L not only cannot signal to activate NF- κ B, but also competes for MyD88 binding and inhibits TLR-induced NF- κ B activation. (13, 19) The protective function of ST2 in ALD is consistent with two reported studies investigating the role of ST2 in other models of liver injury (33, 34). It has been shown that ST2^{-/-} mice develop significantly more severe liver injury after concanavalin A treatment, with higher number of hepatic infiltrating leukocytes that produce higher levels of pro-inflammatory cytokines (TNF α , IFN γ , and IL-17). (34) A hepato-protective function of ST2 was also reported in liver ischemia-reperfusion (IR) injury, as blocking ST2 with anti-ST2 antibody significantly worsened liver damage. (33) In contrast to the marked increase of ethanol-induced hepatic inflammation and injury in ST2^{-/-}, we did not observe a phenotype in IL-33-deficient mice. This discrepancy between IL-33^{-/-} and ST2^{-/-} mice can be explained by the lack of release of extracellular IL-33 during mild ALD. Consistent with our results, other studies have reported phenotypes of ST2^{-/-} mice when extracellular release of IL-33 is negligible. (35, 36) These findings suggest that ST2 has functions independent of IL-33. The intracellular IL-33 is a chromatin-associated nuclear factor, and it has been reported to act as a transcriptional repressor. (37) However, the fact that we do not observe a phenotype in IL-33^{-/-} mice suggests that the intracellular functions of IL-33 do not play a role in mild ALD either.

Consistent with the notion that IL-33 is released when there is substantial tissue damage, we found that extracellular IL-33 was only significantly elevated in severe ALD patients with decompensated liver, but not in ALD patients with compensated liver nor in heavy drinkers. Similarly, significant IL-33 release was only observed in mice treated with chronic alcohol plus one low dose of CCl₄, which markedly increased ALT levels, but not in

mouse models with low levels of ALT. Moreover, compared with WT mice, attenuated liver injury and inflammation were observed in both IL-33^{-/-} and ST2^{-/-} mice treated with alcohol plus a single dose of CCl₄. The data suggest that when there is substantial release of IL-33, the IL-33/ST2L axis exacerbates alcohol-induced liver injury. Taken together, our results suggest that ST2 signaling in MΦs differs between mild and severe ALD (Fig. 8). During mild ALD, extracellular IL-33 is not release. ST2L predominantly inhibits TLR-mediated NF-κB activation and acts as a negative regulator of inflammatory responses of hepatic MΦs to PAMPs. This mechanism is important in attenuating ALD and may be in preventing/ delaying the progression of ALD from mild to severe forms. In transition from mild to severe ALD, the extracellular IL-33 is significantly elevated. Through binding to ST2L, IL-33 can trigger the formation of IL-33/ST2L/IL-1RAcP/MyD88 complex and activate the canonical IL-33/ST2 signaling pathway (Fig. 8), resulting in NF-κB activation and the production of pro-inflammatory mediators. Nonetheless, the decoy receptor, sST2 is also produced in this case. The sST2 acts as a negative regulator of IL-33 action by neutralizing IL-33. In a model of liver fibrosis, it has been demonstrated that the extracellular IL-33 activates type 2 innate lymphoid cells through ST2L, thereby inducing pro-fibrogenic cytokine production and causing fibrosis. (38) Interestingly, sST2 administration blocks the IL-33/ST2L signaling and attenuates liver fibrosis. (38) Elevated serum levels of IL-33 and sST2 have been reported in numerous inflammatory diseases. (39-41) There are eleven on-going clinical trials (clinicaltrial.gov) evaluating sST2 as a biomarker for several disease conditions, as many single nucleotide polymorphisms (SNPs) of ST2 are significantly associated with circulating sST2 levels. (42) Our study is the first to demonstrate significant elevations of serum levels of sST2 and IL-33 in ALD patients. Future studies are warranted to determine whether serum levels of IL-33, sST2 and/or the IL-33/sST2 ratio correlate with the severity of ALD.

In summary, this is the first study to elucidate the role of ST2 in the pathogenesis of ALD and provide insights into the differential effects of ST2 signaling in mild versus severe ALD (Fig. 8). Our data suggest that, during mild ALD, ST2 negatively regulates the inflammatory activation of hepatic macrophages, thereby protecting against alcohol-induced liver damage. In cases of severe liver injury, the release of extracellular IL-33 may exacerbate tissue inflammation by triggering the canonical IL-33/ST2L signaling in hepatic MΦs. Although in our study, the IL-33/ST2L-antagonizing effect of sST2 appeared to be overridden, varying levels of sST2 in patients may be associated with disease severity. Thus, the ratio of IL-33/sST2 could be used a biomarker to stratify disease severity and predict survival outcomes in patients with ALD.

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Figure legend:

Figure 1: Serum levels of sST2 and IL-33 are significantly increased in patients with severe ALD. (A) Serum samples from healthy controls, heavy drinkers without liver diseases and ALD patients were collected. The levels of IL-33 (13 controls, 13 heavy drinkers and 10 ALD patients) and sST2 (20 controls, 20 heavy drinkers and 11 ALD patients) were measured by ELISA. (B) Correlation of the levels of sST2 and IL-33 with ALD severity based on Child Pugh classification. Those with the scores of 5-6 were classified as compensated ALD and those with score >7 were considered as decompensated liver disease. All data shown represent mean \pm SEM. * $p < 0.05$ (One-way ANOVA).

Figure 2: Measurement of serum IL-33 and sST2 and comparing alcohol-induced liver injury among WT, IL-33^{-/-} and ST2^{-/-} female mice. (A) The dosing regimens of the three mouse models. Serum ALT activities (B-D), serum levels of IL-33 (E) and sST2 (F) were compared (n=5-15/group). * $P < 0.05$ (One-way ANOVA) compared to various groups as indicated. (G) Liver NPC mRNA expression of cytokines (n=5/group). * $p < 0.05$ (One-way ANOVA) compared to WT mice. All data shown represent mean \pm SEM.

Figure 3: Chronic ethanol feeding caused increased hepatic lipid accumulation in ST2^{-/-} than WT mice. Female WT and ST2^{-/-} mice were pair-fed or EtOH-fed for 4 weeks. (A-B) Liver Oil Red-O staining images and quantification (n=6/group). (C) Liver triglyceride levels were measured (n=5-13/group). Data were analysis by 2-way ANOVA, Tukey's HSD. ^a $p < 0.05$ compared with EtOH-fed WT mice. ^b $p < 0.05$ compared with pair-fed mice of the same genotype. All data shown represent mean \pm SEM.

Figure 4: ST2^{-/-} mice exhibit an increased inflammatory response to chronic alcohol treatment. Female mice were treated as described in Figure 3. Proteins levels of pro-inflammatory cytokines in serum (**A**) and EtOH-fed liver (**B**) were measured. Unpaired *t*-test **p*<0.05 compared to WT mice. Hepatic mRNA levels (**C**) were measured (n=12/group). Data were analysis by 2-way ANOVA, Tukey's HSD. ^a*p*<0.05 compared with pair-fed mice of the same genotype. ^b*p*<0.05 compared with WT EtOH-fed mice. All data represent mean ± SEM

Figure 5: LPS causes more severe exacerbation of alcohol-induced liver injury in ST2^{-/-} than WT mice. Female WT and ST2^{-/-} mice were treated with pair-fed, EtOH-fed, pair-fed plus LPS or EtOH-fed plus LPS. (**A**) Serum ALT activities were measured (n=5-15/group). Data were analysis by 2-way ANOVA, Tukey's HSD. ^a*p*<0.05 compared with WT mice of the same treatment group. ^b*p*<0.05 compared with pair-fed mice of the same genotype. ^c*p*<0.05 compared with mice of the same genotype and treated with the same feeding regimen but without LPS challenge. (**B**) Serum cytokine levels in WT and ST2^{-/-} mice treated with EtOH plus LPS (n = 5-8/group). **p*<0.05 (Unpaired *t*-test) compared with WT mice. (**C**) Hepatic mRNA levels of were measured (n = 5-15/group). Data were analysis by 2-way ANOVA, Tukey's HSD. ^a*p*<0.05 compared with pair-feeding plus LPS-treated mice of the same genotype. ^b*p*<0.05 compared with EtOH-feeding plus LPS-treated WT mice. All data shown represent mean ± SEM.

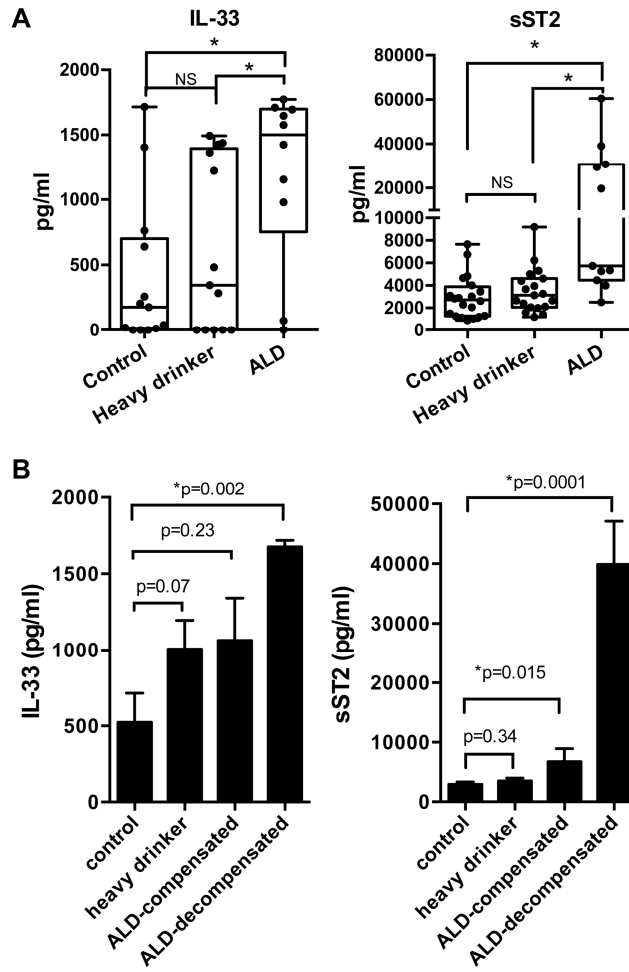
Figure 6: ST2^{-/-} hepatic MΦs exhibit an enhanced pro-inflammatory phenotype. Female WT and ST2^{-/-} mice (n=9/group) were fed with EtOH for 4wks (**A**) and some mice were further challenged with LPS (**B**) Hepatic MΦs were isolated and mRNA expressions of pro-inflammatory mediators were measured. **p*<0.05 (Unpaired *t*-test) compared with

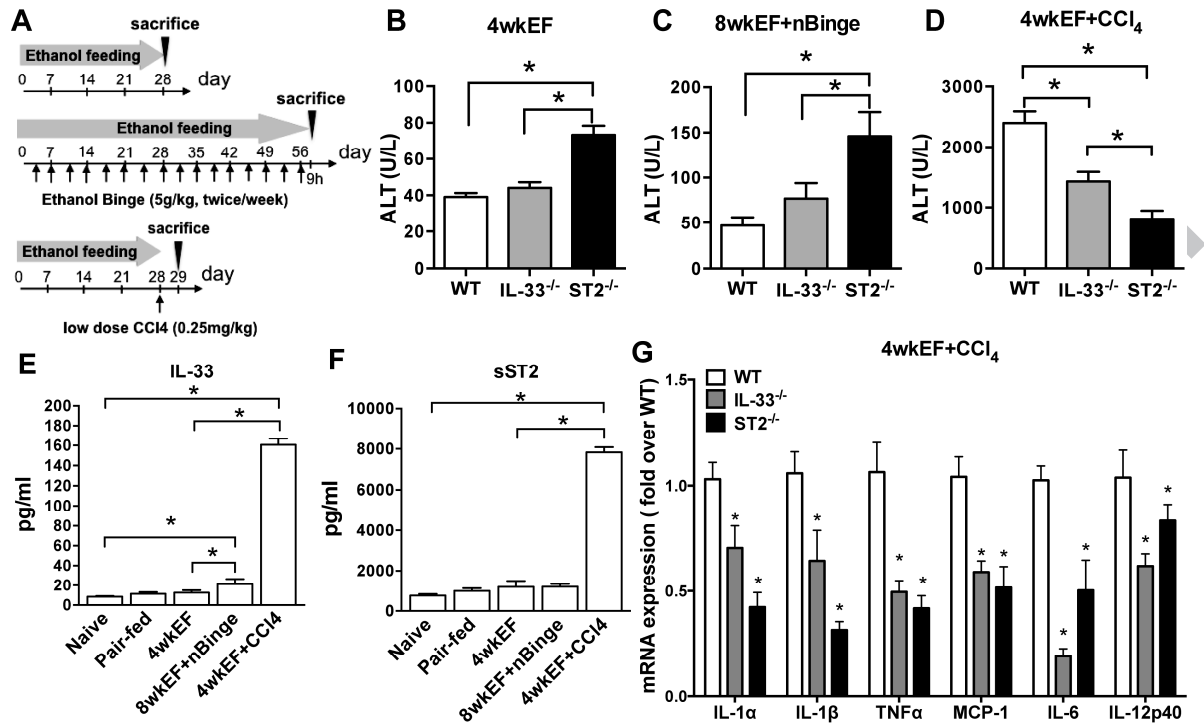
WT-MΦs. **(C)** Hepatic MΦs isolated from EtOH-fed female WT and ST2^{-/-} mice were treated with EVs in the presence or absence of LPS. EVs were isolated from sera of EtOH-treated WT mice. mRNA expressions of pro-inflammatory mediators were measured. Data were analysis by 2-way ANOVA, Tukey's HSD. ^a p<0.05 compared to WT-MΦs subjected to the same treatment. ^b p<0.05 compared to non-treated control MΦs of the same genotype. ^c p<0.05 compared to EV alone-treated MΦs of the same genotype. All data shown represent mean ± SEM.

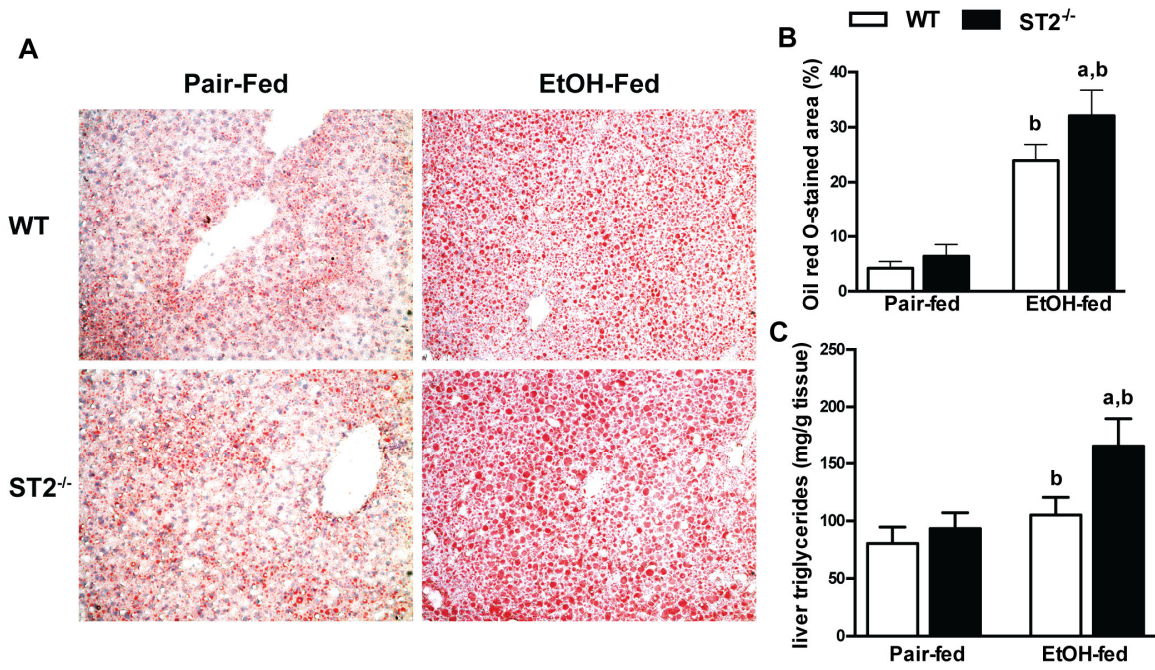
Figure 7: ST2^{-/-} hepatic MΦs exhibit enhanced NF-κB activation. **(A)** ST2 message levels were measured in hepatic MΦs and CD45-negative cells by RT-PCR. Hepatic MΦs were isolated from female EtOH-fed WT and ST2^{-/-} mice. **(B)** The expression levels of p65 and phosphorylated p65 were measured by Western blot. *p<0.05 (Unpaired t-test) compared with WT-MΦs. Results shown represent 3 independent experiments of total 9 mice per group. **(C)** DNA-binding activities were measured using an NF-κB p65 transcription factor activity kit. Control samples (CTL) include a negative CTL without proteins, a positive CTL using pure NF-κB p65, and another CTL using dsDNA to compete for DNA-binding. *p<0.05 (Unpaired t-test) compared with WT-MΦs (n=4/group). **(D)** WT bone marrow derived-MΦs were co-transfected with a NF-κB luciferase reporter gene construct and either scrambled siRNA or ST2 siRNA. Luciferase activities in the absence or presence of LPS stimulation were measured. Results are from three separate experiments, each performed in triplicate. Data were analysis by 2-way ANOVA, Tukey's HSD. *p<0.05 compared with scrambled siRNA-treated controls. All data shown represent mean ± SEM.

Figure 8: Schematic for the roles of ST2 in mild versus severe ALD.

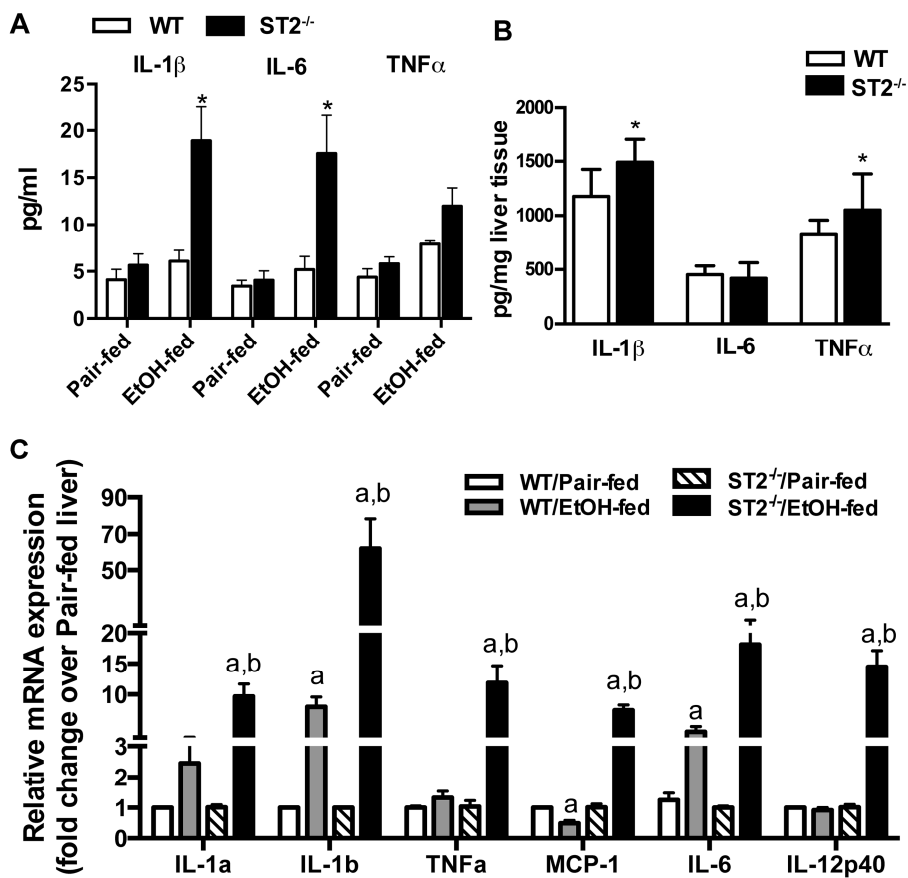
In mild ALD, extracellular release of IL-33 is minimal. In the absence of IL-33, ST2L does not form heterodimers with IL-1RAcP (interleukin-1 receptor-associated protein) and thus does not activate NF- κ B. Instead, ST2L sequesters MyD88 and competes with TLR for MyD88 binding, thereby attenuating TLR-induced signaling and NF- κ B activation. In severe ALD, substantial cell death causes significant accumulation of extracellular IL-33. Binding of IL-33 to ST2L recruits IL-1RAcP and induces down-stream signaling, thereby activating NF- κ B and promoting pro-inflammatory responses. However, during severe ALD, sST2 is also released, which acts as a decoy receptor blocking the IL-33/ST2L signaling. Therefore, the ratio of IL-33/sST2 may be an important indicator for the degrees of hepatic inflammation and injury during severe ALD. (PAMP, pathogen-associated molecular pattern; IRAK, IL-1R-associated kinase; TRAF6, TNFR-associated factor 6, IL-1RAcP, interleukin-1 receptor-associated protein)





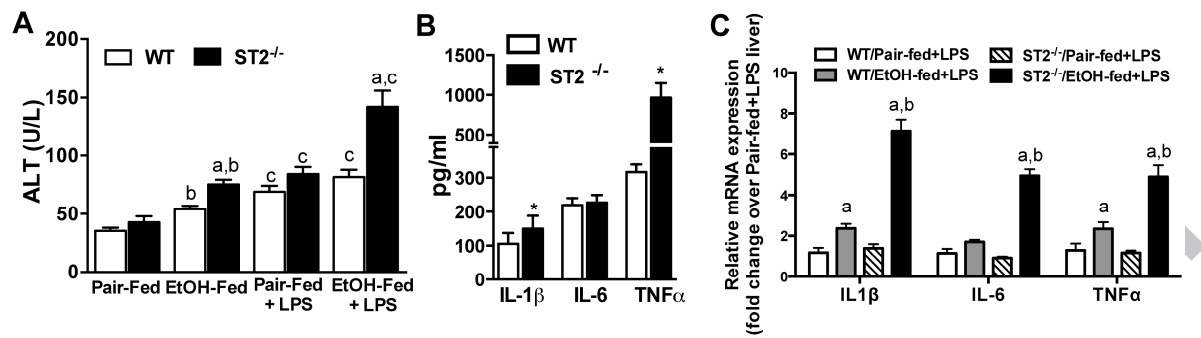


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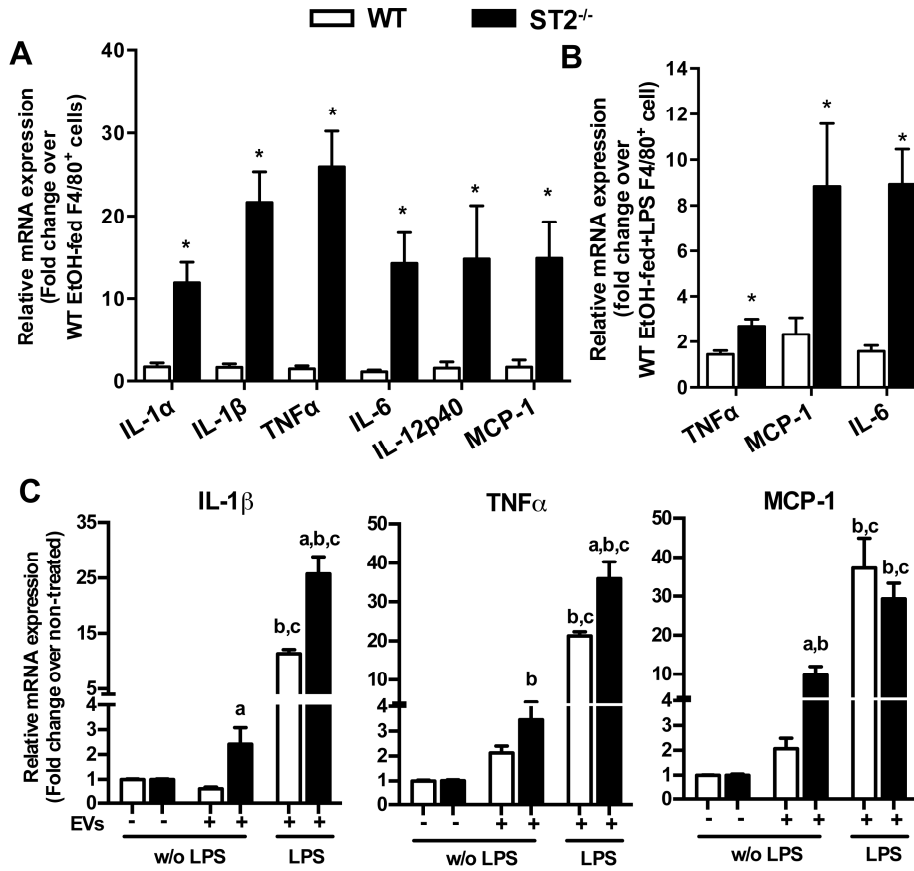


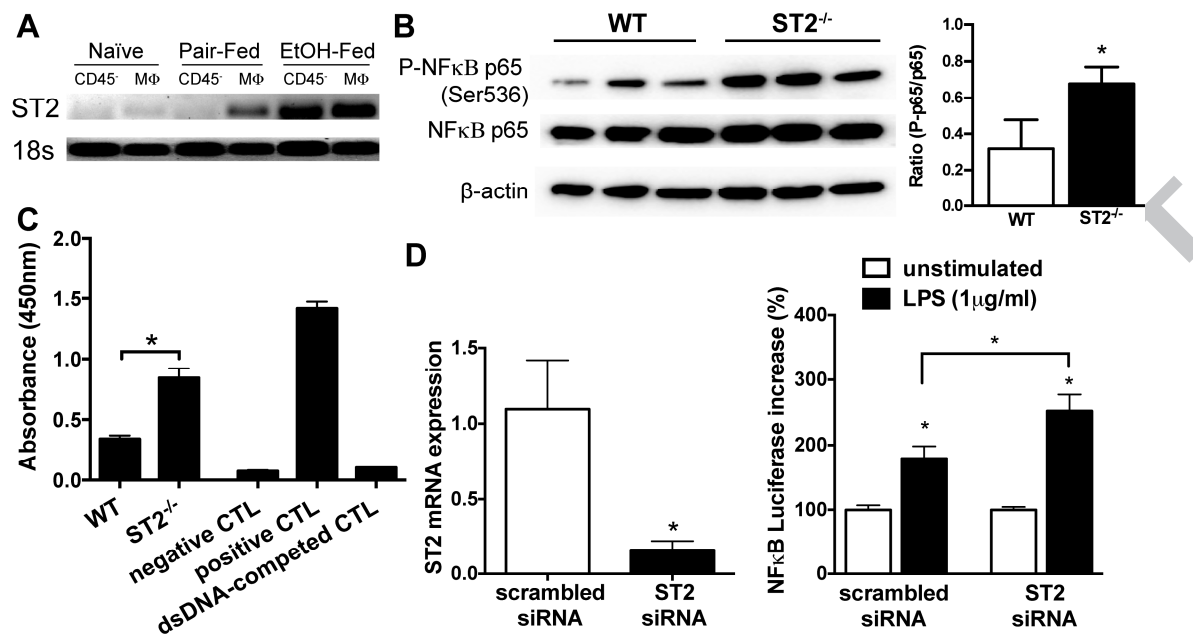
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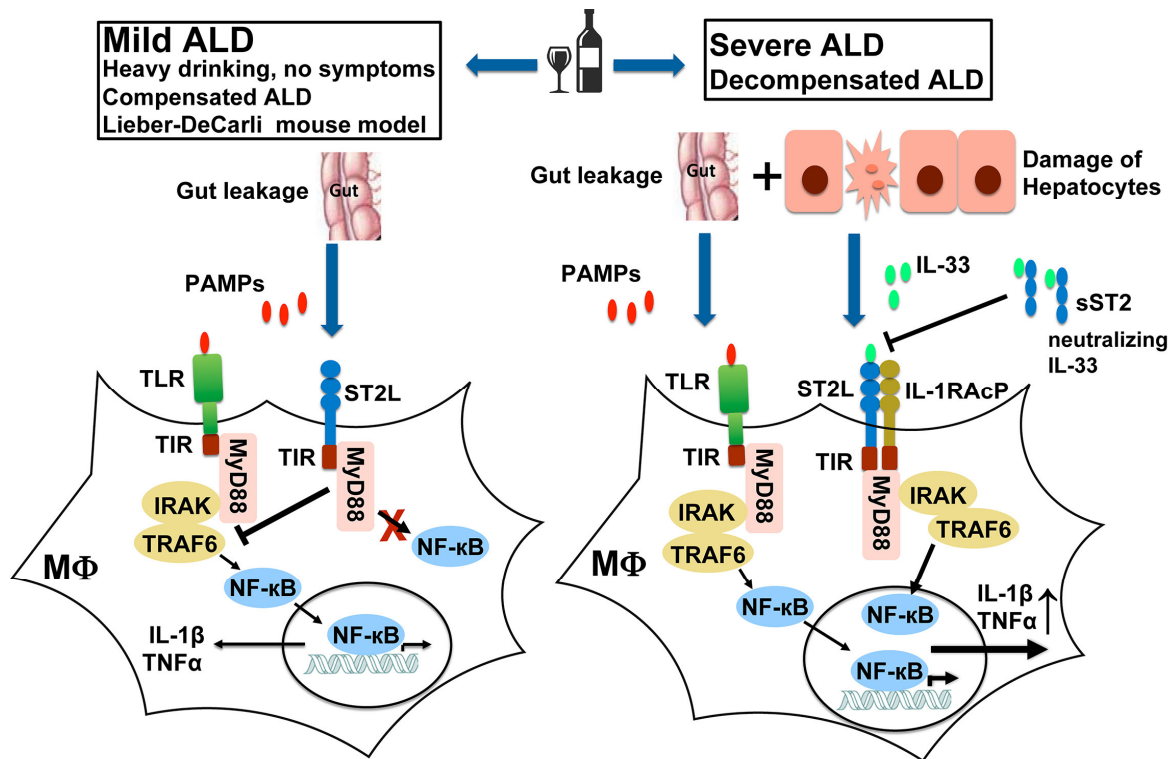
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Graphical abstract

