GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Spliceosome-Associated Protein 130 Exacerbates Alcohol-Induced Liver Injury by Inducing NLRP3 Inflammasome—Mediated IL-1 β in Mice

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Excessive alcohol consumption leads to chronic liver diseases. Macrophage-inducible C-type lectin (Mincle) is a C-type lectin receptor that recognizes spliceosome-associated protein 130 (SAP130) known as an endogenous ligand released from dying cells. The aim was to examine the role of Mincle-SAP130 in the pathogenesis of alcoholic liver disease. Alcohol-induced liver injury was induced in wild-type (WT) and Mincle knockout (KO) mice by using a chronic-binge ethanol-feeding model. Mincle KO mice showed significant lower hepatic steatosis, inflammation with neutrophil infiltration, and fibrosis compared with WT mice after alcohol feeding. In contrast, Mincle activation exacerbated alcohol-induced liver injury. Kupffer cells (KCs) are major sources of Mincle. IL-1 β expression was significantly down-regulated in Mincle KO mice compared with that in WT mice after alcohol consumption. Interestingly, expression and production of IL-1 β were significantly decreased in SAP130-treated KCs isolated from leucine-rich—containing family pyrin domain containing-3—deficient mice compared with those in WT KCs. Such results were also observed in cells treated with SAP130 plus Syk inhibitor. Furthermore, infiltration of invariant natural killer T cells was decreased in livers of Mincle KO mice. Finally, inhibition of Syk signaling ameliorated alcohol-induced liver injury. Collectively, these results demonstrated that interaction between Mincle and SAP130 may promote the progression of alcoholic liver disease by IL-1 β production in KCs and consequently increase inflammatory immune cell infiltration. (*Am J Pathol* 2018, 188: 967–980; <https://doi.org/10.1016/j.ajpath.2017.12.010>)

Excessive and chronic alcohol intake is a leading cause of alcoholic liver disease (ALD) that includes a broad spectrum of hepatic disorders, ranging from simple steatosis to more severe hepatic damage such as steatohepatitis and cirrhosis, consequently resulting in hepatocellular carcinoma.¹ Alcoholic steatohepatitis (ASH) induced by prolonged alcohol consumption is an advanced stage of ALD characterized by ballooning of hepatocytes, neutrophil accumulation, inflammation, and fibrosis.² Intestinal bacterial dysbiosis and increased gut permeability involved in translocation of bacterial components such as lipopolysaccharide (LPS) from gut to liver have been observed in patients with ALD.³ Alcohol metabolism can also lead to accumulation of reactive oxygen species that promotes hepatocellular death, resulting in liver damage and subsequently facilitating

progression of ALD.⁴ Damage-associated molecular patterns released by necrotic hepatocytes combined with translocated LPS can synergistically activate innate immune response in hepatic immune cells by pattern recognition receptors such as toll-like receptors.⁵

Recently, mounting evidence suggests that hepatic infiltration of neutrophils is a prominent hallmark of patients

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with ALD.^{6,7} In addition, alcohol metabolites (such as acetaldehyde) and LPS can stimulate a variety of hepatic cells, including hepatocytes (HPs) and Kupffer cells (KCs), and subsequently induce the production of IL-8, CXCL1, and IL-17 that are directly or indirectly involved in the recruitment of neutrophils in the liver, thereby leading to ALD.^{8–10} Experimental studies have also suggested that chronic ethanol (EtOH) intake can activate hepatic invariant natural killer T (iNKT) cells and can lead to production of chemokines that recruit neutrophil, thus exacerbating the severity of ALD in mice.^{11,12} In addition, it has been found that the recruitment of iNKT cells in damaged liver induced by EtOH feeding is mediated by IL-1 β released from activated KCs in leucine-rich—containing family pyrin domain containing-3 (NLRP3) inflammasome—dependent manner.^{11,12}

Macrophage-inducible C-type lectin (Mincle) is expressed by several immune cells, including monocytes/macrophages, neutrophils, dendritic cells (DCs), and others. It can recognize pathogens, including fungi, *Mycobacterium tuberculosis*, and *Helicobacter pylori*.^{13,14} It has been well documented that trehalose-6,6'-dimycolate (TDM), a mycobacterial cell wall glycolipid, is an exogenous ligand of Mincle.¹⁵ Recent study has indicated that Mincle can also recognize spliceosome-associated protein 130 (SAP130), an endogenous ligand of Mincle released from necrotic cells. This suggests that Mincle signaling might be involved in innate immune response under sterile inflammatory condition. Once Mincle binds to its ligand, signaling cascade is mediated by immunoreceptor tyrosine-based activation motif—containing adaptor molecule Fc receptor γ -chain.¹⁶ Fc receptor γ -chain—induced Syk recruitment is involved in the activation of various signaling molecules such as NF- κ B through Card9-Bcl10-MALT1 signalosomes. This signaling pathway plays a pivotal role in connecting innate and adaptive immune response and activating the innate immune response itself.¹⁷

In addition to its role in inducing immune response against infection by pathogen, a growing body of evidence has indicated that Mincle signaling pathway is involved in noninfectious inflammatory diseases such as traumatic brain injury and subarachnoid hemorrhage.^{18,19} Furthermore, endogenous ligand released from damaged adipocytes can activate adipose tissue macrophage through ligation with Mincle and subsequently induce obesity-related adipose tissue fibrosis, resulting in hepatic steatosis and insulin resistance.²⁰ However, the precise role of Mincle-Syk signaling in inflammation-related chronic liver diseases has not been fully understood yet. Therefore, the objective of this study was to determine the role of Mincle in the pathogenesis of ALD with the use of a mouse model of chronic and binge EtOH feeding.

Materials and Methods

Animals

C57BL/6 mice (wild-type; WT), NLRP3, and Mincle knockout (KO) mice were used in this study. WT mice were purchased

from Taconic Farms, Inc. (Samtako Bio Korea, O-San, Republic of Korea). NLRP3 KO mice and Mincle KO mice were kindly provided by Prof. Jong-Hwan Park (Konyang University, Republic of Korea) and Prof. Young-Joon Kim (Yonsei University, Republic of Korea), respectively. Mice were maintained in pathogen-free environment with standard environmental condition ($24 \pm 2^\circ\text{C}$, $50 \pm 5\%$ humidity). They were provided sterile water and standard control diet. All experiments followed the criteria of requirements of Animal Care and Ethics Committees of Chonbuk National University. The animal facility of Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

Short- or Long-Term Chronic and Binge EtOH Feeding Model

Male WT or Mincle KO mice at 9 to 10 weeks of age were divided into two groups: control diet and Lieber-DeCarli diet (Bio-Server, Frenchtown, NJ) as previously described.²¹ To induce ALD in mice, published protocol of murine model of short-term chronic and binge EtOH feeding²² was used with slight modification. Briefly, each group of mice was fed a control liquid diet (pair-fed) *ad libitum* for 5 days to acclimatize to the feeding tube. After 5 days of acclimation, mice were fed Lieber-DeCarli diet that contained 6.3% EtOH (v/v) (EtOH-fed) for 10 days. To induce an advanced ALD, ASH mice were subjected to long-term chronic plus single binges of EtOH for 8 weeks as previously described.²³ On day 11 or after 8 weeks, mice were administered a single dose of binge EtOH (5 g/kg of body weight) in the morning. Nine hours after gavage, mice were anesthetized with an intramuscular injection of a combination of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil 50; Virbac, Carros, France) at a dose of 0.2 mL/kg body weight before collection of serum and liver tissues for further analysis.

For *in vivo* stimulation of Mincle signaling with the use of trehalose-6,6-dibehenate (TDB; InvivoGen, San Diego, CA), a synthetic analogue of TDM, emulsion that contained TDB (10 μg per mouse) was intraperitoneally injected every 10 days of EtOH-diet feeding. For *in vivo* inhibition of Syk signaling, mice received 10 mg/kg Syk inhibitor GS-9973 (Cayman Chemical, Ann Arbor, MI) or vehicle control by oral administration every 10 days of EtOH-diet feeding. To further demonstrate the effect of Syk on the pathogenesis of alcohol-induced liver injury more clearly, SKI-O-703 (another Syk-specific inhibitor) was used. SKI-O-703 was provided as a kind gift from Oscotec Inc. (Seongnam-si, Republic of Korea). After control diet feeding for 5 days, mice ($n = 3$ per group for pair-fed, $n = 6$ per group for EtOH fed) received 10 mg/kg SKI-O-703 or vehicle control by oral administration every 10 days during EtOH-diet feeding.

Histopathologic Examination

Livers were fixed in 10% phosphate-buffered formalin, routinely processed, and then embedded in paraffin. Tissue

sections (6 μm) were prepared with a microtome (HM-340E; Thermo Fisher Scientific Inc., Waltham, MA). Sections were placed onto glass slides. Hematoxylin and eosin staining was performed according to standard techniques.

For Oil Red O staining, frozen liver sections were prepared as usual. Oil Red O staining was performed with the use of the Oil Red O stain kit (ScyTek Laboratories, Logan, UT) according to the manufacturer's protocols. Data were expressed as percentage of Oil Red O—positive area per field. To observe fibrosis levels in liver tissues, liver sections were subjected to Sirius Red staining (saturated aqueous solution of picric acid containing Direct Red 80; Sigma-Aldrich, St. Louis, MO). Hepatic collagen deposition was quantified by measuring Sirius Red—positive area per total liver section (analysis TS; Olympus Corp., Tokyo, Japan). Total liver section images were analyzed for each animal with the use of a light microscope (BX-51; Olympus Corp.) and digital imaging software [analysis TS software version 5.1 (build 1692); Olympus Corp.].

Biochemical Measurements

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase were determined with the use of the AM101-K spectrophotometric assay kits (ASAN Pharmaceutical, Hwasung, Republic of Korea). Hepatic triglyceride (TG) and total cholesterol (TC) contents in liver were evaluated with AM202-K spectrophotometric assay kit (ASAN Pharmaceutical). Absorbance values of serum and hepatic biochemicals were measured at wavelengths of 490 and 550 nm, respectively, using an EMax spectrophotometer (Molecular Devices, Sunnyvale, CA).

Isolation of Liver Cell Fraction

Liver cells of WT mice were fractionated into various cell populations. Briefly, mouse livers were digested with collagenase (1 mL/min) perfusion followed by three-layer discontinuous density gradient centrifugation with 20%, 11.5% Optiprep (Sigma-Aldrich), and buffer to obtain HPs, mononuclear cell (MNC) fraction, and hepatic stellate cell fraction, respectively. DC and KC fractions were positively selected from the MNC fraction by magnetic cell sorting with the use of different antibodies (for DCs: anti-CD11c and anti-plasmacytoid DC antigen; for KCs: anti-F4/80 antibody; Miltenyi Biotec, Auburn, CA). The hepatic stellate cell fraction was purified by negative selection of contaminating KCs by magnetic cell sorting with appropriate antibody. Cell fractions were immediately homogenized for RNA extraction.

Hepatic KCs and DCs were isolated from EtOH-fed mice after mechanical disruption as described previously.²⁴ Briefly, livers of pair-fed or EtOH-fed WT and Mincle KO mice were minced into small pieces with surgical scissors and pressed through a 200-gauge stainless steel mesh. After centrifugation at $50 \times g$ for 4 minutes, supernatants that contained hepatic MNCs were collected, washed in

phosphate-buffered saline, and resuspended in 10 mL of 37.5% Percoll (GE Healthcare Life Science, Pittsburgh, PA) in RPMI-1640 media (Lonza, Basel, Switzerland), followed by centrifugation at $850 \times g$ with off-brake setting for 30 minutes at room temperature. KC and DC fractions were positively selected from the MNC fraction by magnetic cell sorting according to the protocol described above.

Isolation of Primary HPs and KCs

Primary HPs and KCs were isolated from mice as described previously.²⁵ Briefly, mouse livers were perfused (1 mL/min) and digested with collagenase 1 (Worthington Biochemical Corporation, Lakewood, NJ) after cannulation of the portal vein. Liver cell suspension was suspended and centrifuged at $50 \times g$ for 3 minutes. After centrifugation, the pellet representing HPs was re-suspended, filtered, and washed several times with Dulbecco's modified Eagle's medium (Lonza) supplemented with 5% fetal bovine serum (Thermo Fisher Scientific Inc.), 100 nmol/L dexamethasone (Sigma-Aldrich), 1% insulin-transferrin-selenium-X Supplement (Thermo Fisher Scientific Inc.), 100 IU/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. Viability of HPs was assessed with trypan blue (Sigma-Aldrich). It was $>80\%$. HPs were plated onto collagen-coated wells (Sigma-Aldrich). After 4 hours of attachment at 37°C , media were replaced with fresh serum-free media and incubated overnight at 37°C in a 5% CO_2 incubator for further experiments.

After centrifugation of the digested liver cell suspension at $50 \times g$ for 3 minutes, supernatant that contained hepatic MNCs was collected, washed with phosphate-buffered saline, and resuspended in 40% Percoll in RPMI-1640 media. The cell suspension was gently overlaid onto 70% Percoll and centrifuged at $750 \times g$ for 20 minutes with off-brake setting. MNCs were collected from the interface, washed twice with phosphate-buffered saline, and resuspended in RPMI-1640 media. KCs were positively selected from the MNC fraction by magnetic cell sorting according to the above-mentioned protocol.

In Vivo Depletion of KCs and SAP130 Treatment

Gadolinium chloride hexahydrate (Sigma-Aldrich) powder was reconstituted in sterile saline to a final concentration of 2 mg/mL. To deplete KCs, mice were intraperitoneally given gadolinium chloride hexahydrate (10 mg/kg) for 2 consecutive days before SAP130 treatment. The same volume of saline was injected as control. To stimulate *in vivo* Mincle signaling, mice were intraperitoneally injected with SAP130 or TDB (10 μg per mouse). After 2 hours, mice were anesthetized with Zoletil at a dose of 0.2 mL/kg body weight through intramuscular injection before collection of serum and liver tissues for further analysis.

Cell Culture and Treatment

KCs (1.0×10^6 cells/well) isolated from WT or NLRP3 KO mice were plated into 12-well plates and then cultured at

Table 1 Primer Sequence of Real-Time Quantitative PCR

Gene	Forward	Reverse
<i>TNFA</i>	5'-GTCTACTCCAGGTTTCTCTTCAAGG-3'	5'-GCAAATCGGCTGACGGTGTG-3'
<i>IL1B</i>	5'-CTCGCAGCAGCACATCAACAAG-3'	5'-CCACGGGAAAGACACAGGTAGC-3'
<i>IL6</i>	5'-ACAAAGCCAGAGTCCTTCAGAGAG-3'	5'-CCACGGGAAAGACACAGGTA-3'
<i>NLRP3</i>	5'-AGCCTTCCAGGATCCTCTTC-3'	5'-CTTGGGCAGCAGTTTCTTTTC-3'
<i>ASMA</i>	5'-TCAGGGAGTAATGGTTGGAA-3'	5'-CAGTTGGTGATGATGCCGTG-3'
<i>Col1</i>	5'-ACAGGCGAAACCGGTGACAG-3'	5'-GCCAGGAGAACCAGCAGAGC-3'
<i>TGFB</i>	5'-TGAACCAAGGAGACGGAATACAGG-3'	5'-GCCATGAGGAGCAGGAAGGG-3'
<i>Mincle</i>	5'-AACCCACTCTATCTGCTCAGTGCTT-3'	5'-CCAGCATGAATGGCATGGA-3'
<i>Ly6G</i>	5'-ACAAATGCCTGACAACAAACGTGTA-3'	5'-CTTGTCACAGCAGGTGGTGTGTA-3'
<i>Elane</i>	5'-ACAACCTGCTGAACGACATTGTGA-3'	5'-TGCACGTTGGCGTTAATGGTA-3'
<i>CXCL1</i>	5'-TGCACCCAAACCGAAGTC-3'	5'-GTCAGAAGCCAGCGTTTACC-3'
<i>CXCL2</i>	5'-GCCAAGGGTTGACTTCAAGAACA-3'	5'-AGGCTCCTCCTTCCAGGTCA-3'
<i>CXCR2</i>	5'-TTGGGAGCCACTCTGCTCAC-3'	5'-GGGACAGCATCTGGCAGAATA-3'
<i>RPLP0</i>	5'-CCACACTGCTGAACATGCTGAA-3'	5'-GTACCCGATCTGCAGACACACAC-3'

37°C in a 5% CO₂ incubator with RPMI-1640 media that contained 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. To stimulate Mincle signaling, cells were stimulated with 2 µg/mL recombinant SAP130 (MyBioSource, San Diego, CA) for 24 hours. To inhibit Syk signaling, cells were treated with GS9973 (100 nmol/L) in the presence or absence of recombinant SAP130 for 24 hours.

Primary HPs and KCs were co-cultured in 12-well *trans*-well plate (Sigma-Aldrich) at a ratio 1:4 of KCs/HPs. To mimic the *in vivo* condition, cells were cultured with 100 mmol/L EtOH in the presence or absence of LPS (100 ng/mL) for 24 hours.

Enzyme-Linked Immunosorbent Assay

Levels of IL-1β in the culture supernatants were quantified with the use of a mouse IL-1β enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

LDH Assay

Hepatocellular cytotoxicity was evaluated with lactate dehydrogenase (LDH) assay (Cytotoxicity Detection Kit; Sigma-Aldrich) by measuring the amount of LDH leaked into the culture medium according to the manufacturer's instruction. Absorbance of the sample was measured at a wavelength of 490 nm with the use of an EMax spectrophotometer (Molecular Devices).

Flow Cytometric Analysis

Hepatic immune cells obtained from liver were stained with CD11b and Ly6G antibodies (eBioscience) to identify neutrophils or CD3 and NK1.1 antibodies (eBioscience) to identify iNKT cells. They were then subjected to flow cytometric analysis with the use of a FACS Calibur (BD Biosciences, San Jose, CA). Data were analyzed with

CellQuest software version 3.1 (BD Biosciences) or FlowJo software version 7/8 (TreeStar Inc., Ashland, OR).

Analysis of Relative Gene Expression Using qPCR

Total RNA was isolated from tissue with the use of the Easy-Spin Total RNA extraction kit (GeneAll, Seoul, Republic of Korea). After treatment with gDNA Remover (Toyobo Co., Osaka, Japan), reverse transcription of total RNA to cDNA was performed with a random primer and MultiScribe MuLV Reverse Transcriptase (Toyobo Co.) according to the manufacturer's instructions. cDNA was subjected to real-time quantitative PCR (qPCR) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the use of SYBR Green I as double-stranded DNA-specific binding dye. After the reaction was completed, specificity was verified with the melting curve analysis. Quantification was performed by comparing Ct values of each sample normalized with ribosomal protein large P0 as an internal control. Sequences of PCR primers are listed in [Table 1](#).

Immunohistochemical Evaluation

For immunohistochemical staining, livers were fixed in 10% phosphate-buffered formalin, routinely processed, and then embedded in paraffin. Liver sections were cut and placed onto glass slides. Before proceeding with the staining protocol, slides were deparaffinized, rehydrated, and submerged in Antigen retrieval solution (Dako, Seoul, Republic of Korea) for 30 minutes at 100°C. Nonspecific binding was blocked with 3% peroxidase solution, followed by blocking with 10% normal goat serum. These sections were then incubated with rabbit anti-mouse myeloperoxidase antibody (Abcam, Cambridge, UK) at 4°C overnight. Negative control slides were incubated with nonimmune Ig under the same condition. These sections were further incubated with biotin-conjugated rat or rabbit Ig antibody as secondary antibodies. Immune complexes were detected with the use of the DAB Substrate Kit (Vector Laboratories,

Burlingame, CA) according to the manufacturer's instructions. Finally, tissues were counterstained with hematoxylin and mounted. Images were analyzed under a light microscope (BX-51; Olympus Corp.) and digital imaging software (analySIS TS; Olympus Corp.).

Immunoblot Analysis

Liver tissues were directly homogenized on ice with an extraction buffer (T-PER; Thermo Fisher Scientific Inc.) for 5 minutes. After centrifugation at $13,000 \times g$ for 15 minutes at 4°C , protein concentration in the supernatant was measured with the use of the Pierce BCA Protein Assay kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Equal amounts of protein were then subjected to SDS-PAGE. After transferring to polyvinylidene difluoride membrane, blocking was performed with 5% bovine serum albumin in Tris-buffered saline (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) that contained 0.05% Tween-20 at room temperature for 1 hour. The membrane was then incubated with primary antibodies diluted 1:1000 in blocking buffer at 4°C overnight. The following antibodies were used: anti-Mincle antibody (MBL International Corporation, Woburn, MA), anti-pSyk antibody (Cell Signaling Technology, Danvers, MA), anti-SAP130 antibody (GeneTex Inc., Irvine, CA), and anti- β -actin (Santa Cruz Biotechnology Inc., Dallas, TX). To detect antigen-antibody complexes, peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) were diluted 1:2000 in blocking buffer and incubated at room temperature for 1 hour. Protein bands were visualized with enhanced chemiluminescence detection system with the use of ImageQuant LAS 500 (GE Healthcare Life Science). Expression levels of protein were quantified with ImageQuant TL software version 8.1.

Statistical Analysis

All data are expressed as means $\pm$ SEM. Differences between two groups were compared with the *t*-test. Differences were considered statistically significant when two-tailed *P* value was <0.05 .

Results

Mincle Deficiency Reduces the Severity of Alcohol-Induced Liver Injury

To determine whether the Mincle signaling pathway was involved in ALD development, Western blot analysis was performed to determine protein levels of Mincle, its ligand SAP130, and signaling molecule. Results are shown in Figure 1A. EtOH-fed mice showed increased protein levels of Mincle and SAP130 in the liver. Protein levels of pSyk, an active form and downstream of Mincle signaling pathway, were also significantly elevated in livers of EtOH-fed mice compared with those in pair-fed mice. Alcohol

consumption—associated hepatic steatosis was markedly decreased in livers of EtOH-fed Mincle KO mice compared with that in EtOH-fed WT mice. To further clarify hepatic fat deposition, liver tissue sections were stained with Oil Red O. A similar pattern was observed in livers between EtOH-fed Mincle KO and EtOH-fed WT mice (Figure 1, B and C). In accordance with these results, lower levels of serum ALT (Figure 1D) and hepatic TG levels except for TC (Figure 1E) were found in EtOH-fed Mincle KO mice compared with those in EtOH-fed WT mice, confirming reduced liver injury in Mincle KO mice. This finding indicated that Mincle signaling exerted pathologic effects on the progression of alcohol-induced liver injury. To assess hepatic inflammation and fibrosis, qPCR was performed to determine levels of proinflammatory cytokines such as tumor necrosis factor α , IL-1 β , and IL-6. Hepatic expression levels of these genes were significantly down-regulated in EtOH-fed Mincle KO mice compared with those in EtOH-fed WT mice (Figure 1F). Levels of α -smooth muscle actin were also decreased in EtOH-fed Mincle KO mice (Figure 1G). However, no significant difference in other fibrotic genes such as collagen1 α 1 or transforming growth factor β was observed between the two types of mice with alcohol consumption (Figure 1G). These results suggested that Mincle signaling was essential for progression of alcohol-induced liver injury.

Mincle Deficiency Inhibits Neutrophil Infiltration in the Liver of EtOH-Fed Mice

It has been established that neutrophils massively infiltrated in alcohol-induced hepatic liver contribute to the progression of alcohol-induced liver injury, resulting in severe inflammatory and fibrogenic liver injury.¹⁰ Therefore, hepatic neutrophil infiltration induced by chronic alcohol intake was evaluated in this study. Immunohistochemical and flow cytometric analyses revealed that infiltrated neutrophils in livers were significantly decreased in EtOH-fed Mincle KO mice compared with those in EtOH-fed WT mice (Figure 2, A and B). Consistently, myeloperoxidase-positive area and percentage of infiltrated neutrophils were markedly decreased in EtOH-fed Mincle KO mice compared with those in EtOH-fed WT mice (Figure 2C). In line with these results, reduced expression of neutrophil markers (Elane and Ly6G) accompanied by decreased expression of neutrophil-recruiting chemokines and its receptor (Figure 2, D–F) were observed. Taken together, these results indicated that Mincle signaling pathway was involved in the recruitment of neutrophils in injured livers.

Activation of Mincle Signaling Accelerates the Progression of Alcohol-Induced Liver Injury

Whether stimulation of Mincle by treatment with its synthetic ligand TDB affected the severity of alcohol-induced liver injury in mice was determined next. EtOH-fed WT

Figure 1 Macrophage-inducible C-type lectin (Mincle) deficiency attenuates alcohol-induced liver injury. Wild-type (WT) and Mincle knockout (KO) mice were fed with control and Lieber-DeCarli diets. This *in vivo* experiment was repeated twice. **A:** Western blot analysis reveals that Mincle, its ligand spliceosome-associated protein 130 (SAP130), and signaling molecule pSyk were significantly increased in livers of ethanol (EtOH)-fed mice compared with those in pair-fed mice. **B:** Representative images of hematoxylin and eosin (H&E)—and Oil Red O—stained liver sections are shown. **C:** Percentage of Oil Red O—positive area is decreased in the liver of EtOH-fed Mincle KO mice compared with that in WT mice. **D:** Mincle KO mice show more decreased liver injury than WT mice based on serum alanine aminotransferase (ALT) levels. **E:** Hepatic levels of triglyceride (TG), but not total cholesterol (TC), are decreased in livers of Mincle KO mice with alcohol consumption. **F:** Hepatic mRNA expression levels of proinflammatory cytokines are significantly decreased in livers of EtOH-fed Mincle KO mice compared with those in WT mice. **G:** Expression levels of fibrogenic genes are similar except for α -smooth muscle actin (α -SMA). Data are expressed as means $\pm$ SEM per group. $n = 5$ mice fed the control diet; $n = 6$ mice fed the Lieber-DeCarli diet; $n = 4$ pair-fed mice per group. * $P < 0.05$, ** $P < 0.01$. Original magnification: $\times 400$ (H&E); $\times 200$ (Oil Red O). Col1, collagen1 α 1; TGF- β , transforming growth factor β ; TNF α , tumor necrosis factor α .

Figure 2 Infiltration of neutrophil is decreased in livers of macrophage-inducible C-type lectin (Mincle)-deficient mice after alcohol feeding. Immunohistochemical staining (A) and flow cytometric analysis (B) were performed to evaluate neutrophil accumulation in steatohepatic livers of wild-type (WT) and Mincle knockout (KO) mice. C and D: Representative images exhibit the proportion of hepatic infiltrated neutrophils. E and F: Expression levels of neutrophil markers, chemokine, and chemokine receptor in livers of WT or Mincle KO mice fed with alcohol diet, based on real-time quantitative PCR. Data are expressed as means $\pm$ SEM per group (C–F). * P < 0.05, ** P < 0.01. Original magnification, $\times 400$ (A). MPO, myeloperoxidase.

mice treated with TDB showed significant increase of hepatocellular lipid accumulation with augmented liver injury according to increased serum ALT levels (Figure 3, A–C). Similarly, histologic results revealed that hepatic TG levels, but not TC, were markedly increased in livers of EtOH-fed WT mice after treatment with TDB (Figure 3D). However, such effects were not observed in EtOH-fed Mincle KO mice treated with TDB compared with those in EtOH-fed Mincle KO mice (Supplemental Figure S1), indicating that harmful effects of TDB stimulation were caused by Mincle signaling. Furthermore, hepatic expression levels of proinflammatory cytokines were significantly up-regulated in EtOH-fed WT mice treated with TDB compared with those in EtOH-fed WT mice without TDB stimulation (Figure 3E). Regarding inflammatory responses, neutrophil infiltration was also augmented in livers of EtOH-fed WT mice treated with TDB (Figure 3, F and G). These results were further supported by increased expression of neutrophil markers in livers of EtOH-fed WT mice treated with TDB (Figure 3H). Collectively, our findings suggested that

stimulation of Mincle signaling exerted detrimental effects on the pathogenesis of alcohol-induced liver injury.

Mincle Deficiency Inhibits IL-1 β Production in KCs

To ascertain cellular source of Mincle, several hepatic cell populations were isolated from WT mice, and Mincle expression was subsequently measured by qPCR. Results showed that KCs and DCs were main cellular sources of Mincle (Figure 4A). In addition, expression levels of Mincle were significantly increased in KCs and DCs isolated from livers of EtOH-fed mice compared with those in pair-fed mice. Interestingly, the expression level of Mincle in KCs was higher than that in DCs of EtOH-fed mice (Figure 4B). This finding suggested that Mincle signaling pathway in KCs played a critical role in the pathogenesis of alcohol-mediated hepatic inflammatory responses. Because the NLRP3–IL-1 β axis plays critical roles in inflammation of steatohepatic livers,^{11,12} mRNA expression levels of *NLRP3* and *IL1B* in KCs isolated from livers of WT and Mincle KO mice after

Figure 3 Macrophage-inducible C-type lectin (Mincle) stimulation accelerates alcohol-induced liver injury. Wild-type mice were fed control (Cont) or Lieber-DeCarli diet with or without trehalose-6,6-dibehenate (TDB) injection. **A:** Representative images of hematoxylin and eosin (H&E)— and Oil Red O—stained liver sections are shown. **B:** Percentage of Oil Red O—positive area is increased in steatohepatic livers of TDB-treated mice compared with that in nontreated mice. **C:** Serum alanine aminotransferase (ALT) analysis showing that hepatic injury induced by alcohol consumption is increased by TDB injection in mice. **D:** Hepatic levels of triglyceride (TG), but not total cholesterol (TC), are increased in livers of TDB-injected mice with alcoholic steatohepatitis (ASH). **E:** Hepatic mRNA expression of proinflammatory cytokines is up-regulated in steatohepatic livers of mice treated with TDB compared with that in nontreated mice. **F and G:** Increased neutrophil infiltration is observed in steatohepatic livers of TDB-treated mice compared with that in nontreated mice, based on Immunohistochemical staining. **H:** Increased levels of neutrophil markers in livers of TDB-injected mice with ASH. Data are expressed as means $\pm$ SEM per group. $n = 4$ per group of pair-fed mice; $n = 8$ per group of EtOH-fed mice. $*P < 0.05$, $**P < 0.01$. Original magnification: $\times 400$ (H&E and MPO), $\times 200$ (Oil Red O). MPO, myeloperoxidase; TNF α , tumor necrosis factor α .

Figure 4 Macrophage-inducible C-type lectin (Mincle) deficiency decreases the induction of IL-1 β in Kupffer cells (KCs). **A:** Cellular source of Mincle in isolated liver cell fractions, including hepatocytes (HPs), hepatic stellate cells (HSCs), KCs, and dendritic cells (DCs) in wild-type (WT) mice was evaluated by real-time quantitative PCR. **B:** mRNA expression level of Mincle is higher in KCs than that in DCs isolated from pair-fed or ethanol (EtOH)-fed WT mice. **C:** Increased expression levels of leucine-rich-containing family pyrin domain containing-3 (NLRP3) and IL-1 β are observed in isolated KCs of EtOH-fed WT mice compared with those in Mincle KO mice. **D:** Hepatic NLRP3 expression is decreased in EtOH-fed WT mice compared with that in EtOH-fed Mincle KO mice. **E:** Mincle activation induced by treatment with spliceosome-associated protein 130 (SAP130; 2 μ g/mL) increased IL-1 β expression or production in KCs isolated from WT mice. Such effects are totally abrogated in KCs isolated from NLRP3 KO mice. **F:** Elevated expression and production of IL-1 β in KCs by Mincle activation are decreased by treatment with 100 nmol/L GS9973, a specific inhibitor of Syk signaling. **G** and **H:** HPs and KCs were co-cultured and treated with 100 mmol/L EtOH or EtOH plus lipopolysaccharide (LPS; 100 ng/mL). Hepatocellular damage and inflammation were determined by measuring released lactate dehydrogenase (LDH) and expression levels of IL-1 β in KCs, respectively. Decreased hepatocellular damage and inflammation are observed in Mincle lacking the co-culture system. Similar results were observed after treatment with Syk inhibitor. Values presented are results from one image of at least three independent experiments. Data are expressed as means $\pm$ SEM per group. $n = 4$ per group of pair-fed or EtOH-fed WT mice (**B**); $n = 4$ per group of EtOH-fed WT and Mincle KO mice (**C**); $n = 3$ per group (**G** and **H**). * $P < 0.05$, ** $P < 0.01$. Cont, control.

chronic alcohol feeding were assessed. Notably, the expression level of IL-1 β was significantly decreased in KCs isolated from EtOH-fed Mincle KO mice compared with that in KCs isolated from EtOH-fed WT mice (Figure 4C). Similar to our previous result (Figure 1F) on decreased hepatic expression of IL-1 β , expression levels of NLRP3 were significantly decreased in livers of EtOH-fed Mincle KO mice (Figure 4D). To further support these results, KCs were depleted by injection of Gdcl₃ and then mice were treated with SAP130 or TDB (10 μ g per mouse). Depletion of KCs was confirmed by significant down-regulation of hepatic F4/80 expression level (Supplemental Figure S2A). KC-depleted mice showed a significant decrease in hepatic expression level of IL-1 β compared with KC-nondepleted mice at 2 hours after SAP130 or TDB injection (Supplemental Figure S2B). These results strongly indicated that KCs were the main cells involved in Mincle signaling pathway of alcohol-induced steatohepatitis.

Previous study has showed that activation of NLRP3 inflammasome is mediated by Syk signaling for antifungal host defense.²⁶ Therefore, whether NLRP3 inflammasome and Syk signaling were involved in SAP130-mediated Mincle activation was examined. In addition, IL-1 β expression level was determined. Expression or production levels of IL-1 β were increased in primary KCs treated with SAP130 (Figure 4, E and F). Its levels were decreased in NLRP3-deficient KCs or KCs treated with specific inhibitor of Syk. However, SAP130 treatment did not induce *IL1B* mRNA expression in Mincle-deficient KCs (Supplemental Figure S2C). These findings suggested that Mincle stimulation by treatment with SAP130 was involved in the production of IL-1 β in KCs by the Syk-NLRP3-dependent pathway.

Next, whether Mincle signaling affected hepatocellular cytotoxicity by EtOH was evaluated. Because no significant difference was found in cytotoxicity between WT HPs and Mincle KO HPs after treatment with EtOH (Supplemental

Figure 5 Macrophage-inducible C-type lectin (Mincle) deficiency leads to reduced infiltration of invariant natural killer T (iNKT) cells in injured livers. **A:** Flow cytometric analysis was performed to detect infiltrated iNKT cells in livers of pair-fed or ethanol (EtOH)-fed wild-type (WT) and Mincle knockout (KO) mice. **B:** Analysis of hepatic iNKT cell percentage. Data are expressed as means $\pm$ SEM per group. $n = 3$ per group of pair-fed mice; $n = 4$ per group of EtOH-fed mice. $**P < 0.01$.

Figure S3), co-culture system of HPs and KCs isolated from WT or Mincle KO mice was used. Cytotoxicity (measured by released LDH) in Mincle KO HPs co-cultured with Mincle KO KCs treated with EtOH or EtOH plus LPS was significantly decreased compared with that in WT HPs co-cultured with WT KCs (Figure 4G). In line with this result, decreased expression levels of IL-1 β were observed in co-cultured Mincle KO KCs compared with those in co-cultured WT KCs, suggesting that SAP130 released from damaged HPs may induce the inflammatory milieu by affecting KCs in a Mincle signaling-dependent manner, subsequently facilitating hepatocellular damage. Consistently, cell death and inflammation induced by treatment with EtOH or EtOH plus LPS were markedly decreased when Syk signaling was inhibited (Figure 4H). Collectively, these results indicated that Mincle-Syk signaling was involved in the pathogenesis of alcohol-mediated hepatocellular damage and inflammation.

Mincle Deficiency Reduces Hepatic Recruitment of iNKT Cells in Injured Livers

It is known that infiltrated iNKT cells through IL-1 β are closely associated with pathogenesis of ALD.¹¹ Therefore, hepatic infiltration of iNKT cells was evaluated by flow cytometric analysis. Hepatic recruitment of iNKT cells was significantly increased in the livers of mice with chronic alcohol consumption compared with that in the livers of pair-fed mice. Such effect was markedly decreased in Mincle KO mice compared with that in WT mice after alcohol feeding (Figure 5). These results combined with our previous results (Figure 2, A and B) were consistent with a previous study showing that EtOH-feeding can trigger accumulation of iNKT cells in livers and consequently increase neutrophil infiltration in livers in the ALD condition.¹² Overall, these results together with our earlier results indicated that Mincle signaling was responsible for IL-1 β production, resulting in recruitment of iNKT cells and consequent infiltration of neutrophils. Such effects can exacerbate the severity of alcohol-induced liver injury.

Inhibition of Mincle Downstream Signaling Ameliorates the Severity of Alcohol-Induced Liver Injury

To determine the therapeutic effect by modulating Mincle signaling pathway, EtOH-feeding mice were orally treated with Syk inhibitor (GS9973). EtOH-fed mice administered 10 mg/kg GS9973 showed decreased hepatic steatosis compared with mice fed with EtOH alone (Figure 6, A and B). Similar to histopathologic observation, serum ALT and hepatic TG levels were reduced by treatment with Syk inhibitor except for hepatic TC (Figure 6, C and D). Consistently, expression levels of proinflammatory cytokines and fibrosis-related genes were significantly reduced by treatment with Syk inhibitor in mice with chronic alcohol feeding (Figure 6, E and F). Finally, infiltration of neutrophil in the injured liver induced by alcohol consumption was evaluated. Results showed that inhibition of Syk induced a decrease in accumulation of neutrophil in the liver based on immunohistochemical analysis (Figure 7, A and B). In addition, treatment with Syk inhibitor reduced expression levels of neutrophil markers accompanied by decreased expression of neutrophil-recruiting chemokines and its receptor in livers of EtOH-fed mice (Figure 7, C and D). Similarly, decreased liver injury and steatosis were observed in livers of EtOH-fed mice treated with SKI-O-703, another Syk inhibitor (Supplemental Figure S4, A and B). In addition, EtOH-fed mice treated with SKI-O-703 showed reduced hepatic neutrophil infiltration compared with EtOH-fed mice without SKI-O-703 treatment (Supplemental Figure S4, C and D). Furthermore, increased liver injury induced by TDB treatment was significantly abrogated by inhibition of Syk signaling (Supplemental Figure S5). These results suggested that Mincle-Syk signaling pathway played a pivotal role in the pathogenesis of alcohol-induced liver injury.

Discussion

It is well known that endogenous ligands released from necrotic dying cells mediate inflammatory and

Figure 6 Inhibition of Syk signaling ameliorates the severity of alcohol-induced liver injury. **A:** Histopathologic observation and Oil Red O staining were performed to evaluate whether Syk inhibition by GS9973 (daily i.p. injection at a dose of 10 mg/kg) affects liver injury and lipid accumulation induced by alcohol consumption in wild-type (WT) mice. **B:** Percentage of Oil Red O—positive area is decreased by inhibiting Syk signaling pathway. **C:** Ethanol (EtOH)-fed mice treated with GS9973 show more decreased liver injury than vehicle-treated mice with alcohol consumption, based on serum alanine aminotransferase (ALT) levels. **D:** Hepatic levels of triglyceride (TG), but not total cholesterol (TC), are decreased in livers of EtOH-fed mice treated with GS9973 compared with those in vehicle-treated mice. **E** and **F:** Hepatic mRNA expression of proinflammatory and fibrogenic genes is down-regulated in livers of EtOH-fed mice after inhibiting Syk signaling pathway. Data are expressed as means $\pm$ SEM per group. $n = 3$ per group of pair-fed mice; $n = 6$ per group of EtOH-fed mice. * $P < 0.05$, ** $P < 0.01$. Original magnification: $\times 400$ (H&E); $\times 200$ (Oil Red O). α -SMA, α -smooth muscle actin; H&E, hematoxylin and eosin; TNF α , tumor necrosis factor α ; TGF- β , transforming growth factor β .

fibrogenic responses in chronic liver diseases, including ALD. Over the past decades, numerous studies have provided compelling evidence that the toll-like receptor signaling pathway is involved in the pathogenesis of

ALD through binding to its ligand such as pathogen-associated molecular patterns translocated from intestine or damage-associated molecular patterns. However, little is known about how liver diseases are affected in

Figure 7 Inhibition of Syk signaling reduces hepatic neutrophil infiltration in ethanol (EtOH)-fed mice. **A:** Immunohistochemical staining was performed to evaluate neutrophil accumulation in steatohepatic livers of wild-type (WT) mice with or without GS9973 treatment. **B:** Representative images showing significant decrease of myeloperoxidase (MPO)-positive area in livers of EtOH-fed mice after inhibiting Syk signaling pathway. **C and D:** Decreased expression levels of neutrophil markers, chemokine, and chemokine receptor in livers of EtOH-fed mice are observed after inhibiting Syk signaling pathway, based on real-time quantitative PCR. Data are expressed as means $\pm$ SEM per group. * P < 0.05, ** P < 0.01. Original magnification, $\times 400$.

response to other pattern recognition receptors, including Mincle.

In the present study, it was demonstrated that Mincle and its endogenous ligand SAP130 were increased in injured livers induced by alcohol consumption and that Mincle signaling exacerbated alcohol-induced liver injury. Because the lack of Mincle signaling alleviated the severity of alcohol-induced liver injury, whether Mincle activation affected alcohol consumption-mediated hepatic inflammatory responses was determined in this study. Our findings showed that Mincle activation by TDB treatment significantly increased hepatic steatosis and inflammation induced by alcohol intake. Furthermore, increased neutrophil infiltration in livers was observed in TDB-treated mice with alcohol consumption. In addition, our results demonstrated that KCs were a primary cellular source of the Mincle signaling pathway in the inflammatory milieu in injured livers induced by alcohol consumption. KCs are known as residential and tissue-fixed macrophages as the major source of IL-1 β in livers of ALD.¹¹ Similarly, our results showed reduced IL-1 β expression in livers of KC-depleted mice treated with SAP130 or TDB, strongly suggesting that KCs were the main cell type of the Mincle signaling pathway that resulted in IL-1 β expression. Consistent with these findings together with our *in vivo* results, KCs isolated from EtOH-fed Mincle KO mice showed a significant decrease in

the expression of NLRP3 and IL-1 β compared with KCs isolated from EtOH-fed WT mice. From these results, the effect of Mincle signaling on activation of NLRP3 and IL-1 β was examined by using murine primary KCs. Our findings showed that KCs were activated by treatment with the endogenous ligand SAP130 through the Mincle signaling pathway, resulting in IL-1 β production. Such effects were abrogated in NLRP3-deficient KCs. Recently, Lee et al²⁷ have demonstrated that TDM-induced Mincle activation can promote nitric oxide production by enhancing translational regulation, subsequently inhibiting NLRP3-dependent IL-1 β production. They mentioned that these opposite results were because of different types of cells and different methods of TDM treatment.²⁷ Contrary to this result but similar to our findings, previous studies have indicated that TDM and TDB have a stimulatory effect on NLRP3 inflammasome by ligation of Mincle, resulting in IL-1 β secretion in bone marrow-derived macrophages or bone marrow-derived DCs.^{28,29} In addition, TDM stimulation can induce the expression of inflammatory and fibrogenic genes in peritoneal macrophages.²⁰ Furthermore, EtOH-fed NLRP3 KO mice have shown decreased serum and hepatic protein levels of IL-1 β and tumor necrosis factor α as well as reduced hepatic lipid accumulation.³⁰ In the present study, increased IL-1 β expression or production was markedly reduced in KCs when Syk signaling was inhibited.

These results were further supported by a previous study¹⁹ showing that administration of SAP130 may significantly increase the production of IL-1 β in an animal model of subarachnoid hemorrhage. Such effect was also decreased by treatment with Mincle siRNA or Syk inhibitor.¹⁹ Therefore, chronic alcohol consumption—mediated hepatocellular damages can induce the release of SAP130 and subsequently activate Mincle signaling, leading to IL-1 β production in KCs by Syk-NLRP3—dependent manners.

Fibrosis is one hallmark of ASH induced by chronic alcohol consumption. Because fibrosis increases the risk of ASH-related complications in patients with portal hypertension and ascites,³¹ management of fibrosis is a pivotal therapeutic strategy to treat ASH and its complications. Herein, our results showed that alcoholic injury—related hepatic fibrogenic response was reduced in mice deficient in Mincle or treated with Syk inhibitor. To more clearly demonstrate the effects of Mincle signaling on ASH severity and its related liver fibrosis, the long-term alcohol feeding model was used as previously described.²³ Consistently, reduced ASH severity and its related hepatic inflammation, steatosis, and fibrosis were observed in Mincle-lacking mice compared with WT mice (Supplemental Figure S6). These results are consistent with results of a recent study showing that Syk inhibition can suppress renal fibrosis through transforming growth factor β 1—mediated myofibroblast activation and down-regulation of mitogen-activated protein kinase—p38 pathway.³² Another recent study has provided convincing evidence that treatment with Syk inhibitor can attenuate thioacetamide-induced liver fibrosis by increasing the production of hepatocyte growth factor and IL-10.³³

Numerous studies have provided evidence that NKT cells play pivotal roles in lipid metabolic disorder-related disease, including non-ASH and ASH because of their specific recognition of CD1d/glycolipid-antigen complex.^{34,35} A previous study has demonstrated that iNKT cells are significantly increased at 9 hours after EtOH gavage.¹¹ These effects depend on the activation NLRP3, resulting in IL-1 β secretion in KCs.¹¹ Infiltrated iNKT cells may promote the recruitment of neutrophils in the liver by the production of cytokines and chemokines, resulting in acceleration of liver injury and inflammation.¹² Results of the present study revealed that Mincle KO mice had lower infiltration of iNKT cells compared with WT mice after chronic alcohol consumption. In accordance with this result, neutrophil infiltration was significantly decreased in Mincle KO mice. Therefore, the severity of ALD was reduced in Mincle KO mice compared with that in WT mice by decreased production of IL-1 β in KCs, resulting in reduction of infiltrated iNKT cells and neutrophils in the liver.

A recent study has indicated that toll-like receptors signaling mediates IL-1 receptor-associated kinase M activation and regulates Mincle expression.³⁶ In addition, increased Mincle expression is closely associated with the progression of ASH by ligation of SAP130, resulting in activation of Syk-NLRP3 inflammasome.³⁶ Similar to this

finding, Syk inhibition can ameliorate the severity of ASH by decreasing inflammation and steatosis in murine models of ASH.³⁷ Although results of the present study were similar to those of the two previous studies,^{36,37} the present study further demonstrated the effect of Mincle-Syk signaling on the pathogenesis of ALD through evaluating ALD severity by TDB stimulation. In addition, the present study demonstrated that hepatic infiltration of iNKT cells in EtOH-fed Mincle KO mice was decreased compared with that in EtOH-fed WT mice. Therefore, our *in vivo* and *ex vivo* findings support previous studies^{36,37} and provide a broad understanding on the effect of Mincle signaling on the pathogenesis of ALD. Because Mincle is also expressed in other immune cells, including neutrophils with cellular functions,³⁸ it will be valuable to verify the role of Mincle signaling in neutrophils on the progression of ALD-related inflammatory immune responses.

Conclusions

Our findings demonstrated that Mincle-Syk signaling pathway exacerbated the progression of ALD by increasing IL-1 β production, consequently increasing inflammatory immune cells, including iNKT cells and neutrophils. Thus, inhibition of Mincle-Syk signaling pathway may be a therapeutic strategy to control inflammation-induced chronic liver diseases.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.12.010>.

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