

# Arginine Methylation of Hepatic hnRNP H Suppresses Complement Activation and Systemic Inflammation in Alcohol-Fed Mice

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Protein arginine methyl transferase 1 (PRMT1) is the main enzyme for cellular arginine methylation. It regulates many aspects of liver biology including inflammation, lipid metabolism, and proliferation. Previously we identified that PRMT1 is necessary for protection from alcohol-induced liver injury. However, many PRMT1 targets in the liver after alcohol exposure are not yet identified. We studied the changes in the PRMT1-dependent arginine methylated proteome after alcohol feeding in mouse liver using mass spectrometry. We found that arginine methylation of the RNA-binding protein (heterogeneous nuclear ribonucleoprotein [hnRNP]) H1 is mediated by PRMT1 and is altered in alcohol-fed mice. PRMT1-dependent methylation suppressed hnRNP H1 binding to several messenger RNAs of complement pathway including complement component C3. We found that PRMT1-dependent hnRNP H methylation suppressed complement component expression *in vitro*, and phosphorylation is required for this function of PRMT1. In agreement with that finding, hepatocyte-specific PRMT1 knockout mice had an increase in complement component expression in the liver. Excessive complement expression in alcohol-fed PRMT1 knockout mice resulted in further complement activation and an increase in serum C3a and C5a levels, which correlated with inflammation in multiple organs including lung and adipose tissue. Using specific inhibitors to block C3aR and C5aR receptors, we were able to prevent lung and adipose tissue inflammation without affecting inflammation in the liver or liver injury. **Conclusion:** Taken together, these data suggest that PRMT1-dependent suppression of complement production in the liver is necessary for prevention of systemic inflammation in alcohol-fed mice. C3a and C5a play a role in this liver–lung and liver–adipose interaction in alcohol-fed mice deficient in liver arginine methylation. (*Hepatology Communications* 2021;5:812–829).

**A**lcohol is a leading cause of preventable morbidity and mortality worldwide.<sup>(1–3)</sup> Although alcohol primarily affects the liver, it causes dysfunction of multiple organs including the brain, gut, muscle, lung, and adipose tissue.<sup>(1)</sup>

Complement is known to play a role in alcohol-associated liver disease.<sup>(4–7)</sup> Activation of the complement pathway can occur through the classical, lectin, or alternative pathways; all three pathways culminate in the activation of C3.<sup>(6)</sup> Following activation, C3 is

*Abbreviations:* AAV, adeno-associated virus; ADMA, asymmetric dimethyl arginine; ALT, alanine aminotransferase; H&E, hematoxylin and eosin; hnRNP, heterogeneous nuclear ribonucleoprotein; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; KO, knockout; MCP-1, monocyte chemoattractant protein 1; mRNA, messenger RNA; PBS, phosphate-buffered saline; PLA, proximity ligation assay; PRMT1, protein arginine methyl transferase 1; SA, dephosphorylated alcohol mimic; SD, phosphomimic; SDS, sodium dodecyl sulfate; TBG, thyroxine-binding globulin; TMT, tandem mass tag; TNF, tumor necrosis factor; WT, wild type.

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cleaved into C3a and C3b; C3b then acts in the activation/cleavage of C5 to C5a and C5b. These processes ultimately result in the formation of the membrane attack complex, which mediates lysis of target cells. In addition, the complement cleavage products C3a and C5a together contribute to several inflammatory responses, including regulation of cytokine production and chemotaxis.

Several studies suggest that the complement system contributes to alcohol-induced liver injury.<sup>(5,8-11)</sup> C3 contributed primarily to the accumulation of triglyceride in the liver, whereas C5 was reported to regulate inflammation and injury to hepatocytes.<sup>(4,7)</sup> However, the role of complement components in other organs in the context of alcohol has not been established.

Protein arginine methylation is a common post-translational modification catalyzed by protein arginine methyltransferases (PRMTs), which results in the addition of one or two methyl groups to arginine residues of proteins using S-adenosyl-L-methionine (AdoMet) as the methyl donor (reviewed in Bedford and Clark<sup>(12)</sup>). PRMT1 is the most conserved arginine methyltransferase and is responsible for over 80% of whole cell arginine methylation.<sup>(13)</sup>

Most of the arginine methylated substrates of PRMTs are involved in RNA processing, including a family of RNA binding proteins called heterogeneous nuclear ribonucleoprotein (hnRNPs; reviewed in Blackwell and Camen<sup>(14)</sup> and Yu<sup>(15)</sup>). Many of these factors contain "RGG" boxes, domains known to participate in both protein-protein and protein-RNA interactions.<sup>(12,14-16)</sup> One of the functional consequences of arginine methylation for many hnRNPs is their relocalization within the cell. Several other studies have shown that arginine methylation of an hnRNP compromises its ability to interact with nucleic acids.

PRMT1 regulates multiple aspects of liver biology including cell proliferation,<sup>(17)</sup> fatty acid metabolism,<sup>(18)</sup> glucose metabolism,<sup>(19)</sup> innate immune response,<sup>(20)</sup> oxidative stress response, and apoptosis.<sup>(21)</sup> Recently we identified that PRMT1 plays an important role in the liver as an alcohol protection factor. In alcohol-fed mice, PRMT1 prevents oxidative stress and promotes hepatocyte survival.<sup>(22)</sup> PRMT1 knockout in alcohol-fed mice results in a dramatic increase in hepatocyte death, inflammation and fibrosis, and increased serum alanine aminotransferase (ALT) levels. Additionally, we found that alcohol promotes PRMT1 dephosphorylation in the liver, which in turn affects PRMT1 target specificity.<sup>(22)</sup>

Here we identified hnRNP H1 as a target of PRMT1 in mouse liver. hnRNP H1 methylation is dependent on PRMT1, requires PRMT1 phosphorylation, and is consequently reduced by alcohol feeding. We found that PRMT1-dependent hnRNP H1 methylation was necessary for suppression of hnRNP H1 binding to several messenger RNAs (mRNAs) of the complement pathway, including complement component C3. Hepatocyte-specific PRMT1 knockout mice had an increase in complement component expression in the liver after alcohol feeding. Excessive complement expression in PRMT1 knockout mice fed alcohol resulted in further complement activation and increased serum C3a and C5a levels. This in turn resulted in inflammation-associated gene expression in other tissues such as lung and adipose tissue, which could be prevented by specific inhibitors of C3aR and C5aR receptors. Thus, the hepatic PRMT1/hnRNP H1/complement pathway is an important contributor to alcohol-mediated inflammatory changes in the lung and adipose tissue.

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## Materials and Methods

### MICE

Prmt1 floxed mice were described previously.<sup>(22)</sup> PRMT1 floxed mice were backcrossed seven generations to the C57BL6/J background. PRMT1 floxed littermates are randomly assigned to adeno-associated virus (AAV)–thyroxine-binding globulin (TBG)–control or AAV-TBG.CRE vector to induce knockout in hepatocytes. All mice were housed in a temperature-controlled, specific pathogen-free environment with 12-hour light–dark cycles and fed regular mouse chow and water *ad libitum*. All animal handling procedures were approved by the Institutional Animal Care and Use Committees at the University of Kansas Medical Center (Kansas City, KS).

Mice were fed Lieber-DeCarli alcohol liquid diet (4.8% alcohol). Control mice received control liquid diet without alcohol.

Mice were injected with C5aR antagonist (PMX53 Calbiochem, MilliporeSigma, Burlington, MA) at 2 mg/kg or C3aR Antagonist (SB290157 trifluoroacetate salt) at 5 mg/kg in 5% DMSO in phosphate-buffered saline (PBS), or vehicle control 5% DMSO in PBS, twice weekly during alcohol feeding.

### ANTIBODIES USED

#### Primary Antibodies

Anti-C3, anti-C1R, and anti-actin antibodies were from Santa Cruz Biotechnology (Dallas, TX). C3 antibody detects C3a and C3 C-terminal portion. C-terminal-specific anti-C3 antibodies were from Novus (St. Charles, MO). Anti-complement C3b/iC3b/C3dg antibody, clone 1H8, was from Sigma-Aldrich (St. Louis, MO). Rabbit anti-CD11b antibodies were from Abcam (Cambridge, United Kingdom). Anti-asymmetric dimethyl arginine (ADMA) antibodies were from Active Motif, Inc., Carlsbad, CA).

### CYTOKINE ARRAYS

Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems, Minneapolis, MN) detecting

40 mouse cytokines was used to detect C5a and other cytokines in mouse serum samples pulled from three mice each. Array was used according to the manufacturer's instructions. Mouse Cytokine Array C3 (RayBiotech, Inc., Norcross, GA) detecting 62 mouse cytokines and related proteins was used to analyze individual mouse lung extracts. Array was used according to the manufacturer's instructions.

### CELL CULTURE

Huh7 cells and were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 50U mL<sup>-1</sup> penicillin, and 50 mg mL<sup>-1</sup> streptomycin. Cells were transfected using Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

### VECTORS

pCMV6-PRMT1 and pCMV6-hnRNP H1 vectors were from OriGene (Rockville, MD). Plasmid-expressing C3 ORF was from Sino Biological (cat #HG13182-UT; Wayne, PA).

The R233K point mutation was generated by the Q5 mutagenesis kit from New England Biolabs (Ipswich, MA).

AAV8.TBG.PI.Null and AAV8.TBG.PI.Cre were from Vector BioLabs (Malvern, PA) and were used at 1–2 × 10<sup>11</sup> genome copies/mouse.

### HUMAN SPECIMENS

De-identified human specimens were obtained from the Liver Center Tissue Bank at the University of Kansas Medical Center. All studies using human tissue samples were approved by the Human Subjects Committee of the University of Kansas Medical Center.

### IMMUNOFLUORESCENCE

Immunostaining on formalin-fixed sections was performed as previously described.<sup>(23)</sup> Slides were observed in a Nikon Eclipse 800 upright epifluorescence microscope (Nikon Instruments, Melville, NY). Images were acquired using a Nikon CoolSNAP camera.

## RNA IMMUNOPRECIPITATION ASSAY

Huh 7 cells ( $1.5 \times 10^7$ ) were cross-linked by the addition of 1% formaldehyde for 10 minutes. Cells were lysed with 20-mM HEPES (pH 8.0), 150 mM NaCl, 0.5% NP-40, and 0.1% sodium dodecyl sulfate (SDS). Next, 50  $\mu$ g of extracts in 0.5 mL of 1% Triton X-100, 2 mM ethylene diamine tetraacetic acid, 20 mM Tris-HCl of pH 8.1, and 150 mM NaCl were immunoprecipitated overnight at 4°C with 4  $\mu$ g of antibody. A total of 20  $\mu$ L of magnetic beads (Dynabeads M-280; Invitrogen) were used to purify immunocomplexes. Following purification, RNA was purified with a Qiagen kit (cat # 27104; Hilden, Germany).

## TANDEM MASS TAG LABELING AND MASS SPECTROMETRIC ANALYSIS

Whole-liver extracts were lysed in 20-mM HEPES (pH 8.0), 150 mM NaCl, 0.5% NP-40, and 0.1% SDS. Trypsin digestion and tandem mass tag (TMT) labeling was performed following the manufacturer's instructions using the TMT-sixplexMass tagging kit (Thermo Fisher Scientific, Waltham, MA). Briefly, 100  $\mu$ g of protein per condition was digested with 2.5  $\mu$ g of trypsin (Promega, Madison, WI). After trypsin digestion, TMT label reagent was added. Equal amounts of labeled samples were combined; sample was then loaded into a high-pH reverse phase spin column previously conditioned following the manufacturer's instructions (Thermo Fisher Scientific). The peptides were eluted in nine fractions. Eluted samples were injected into the high-performance liquid chromatography coupled with the Orbitrap Fusion Lumos spectrometer (Thermo Fisher Scientific). For data analysis, all tandem mass spectrometry scans were searched using Protein Discoverer v.2.4 running Sequest HT and a mouse database downloaded from the National Center for Biotechnical Information NR repository (Bethesda, MD). Protein quantification was performed using unique peptides only.

## WESTERN BLOTTING

Protein extracts in radio immunoprecipitation assay buffer (50 mM Tris, pH 7.5, 150 mM sodium

chloride, 1% NP-40, and 1% protease and phosphatase inhibitors [Sigma-Aldrich]) were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Amersham Hybond ECL; GE Healthcare, Chicago, IL), and blocked in 3% bovine serum albumin/PBS at room temperature for 1 hour. Primary antibodies were incubated overnight at manufacturer-recommended concentrations. Immunoblots were detected with the ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ) or using near-infrared fluorescence with the ODYSSEY Fc, Dual-Mode Imaging system (Li-COR Biosciences, Lincoln, NE).

## ISOLATION OF MOUSE PRIMARY HEPATOCYTES

Primary mouse hepatocytes were isolated from 5-week-old male C57/B6 mice by a two-step collagenase perfusion method as described previously.<sup>(24)</sup> After the induction of anesthesia, the liver was perfused with Hank's balanced salt solution containing 0.025 mg/mL of Liberase TM (Cat #0540112001; Roche, Basel, Switzerland) until the liver revealed signs of digestion. The liver was then excised and chopped to release the isolated liver cells. The cell suspension was filtered through nylon gauze (100  $\mu$ m); hepatocytes were pelleted for 5 minutes at 50g at 4°C.

## PROXIMITY LIGATION ASSAY

Proximity ligation assays (PLAs) were carried out using a PLA kit (Sigma-Aldrich) according to manufacturer's instructions. Interactions were visualized using Duolink Brightfield detection reagent (Sigma-Aldrich). The PLA assay omitting one or both primary antibodies was used as a negative control.

## REAL-TIME PCR

RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen). Complementary DNA was generated using the RNA reverse-transcription kit (Cat #4368814; Applied Biosystems, Foster City, CA). Quantitative real-time reverse-transcription PCR was performed in a CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA) using specific



sense and antisense primers combined with iQ SYBR Green Supermix (Bio-Rad Laboratories) for 40 amplification cycles: 5 seconds at 95°C, 10 seconds at 57°C, and 30 seconds at 72°C.

## STATISTICS

Results are expressed as mean  $\pm$  SD. The Student *t* test, paired *t* test, Pearson's correlation, or one-way analysis of variance with Bonferroni *post hoc* test was used for statistical analyses. *P* value < 0.05 was considered significant.

## Results

### ALCOHOL AFFECTS LIVER ARGININE METHYLATED PROTEOME BY ALTERING THE ACTIVITY OF PRMT1

We used TMT mass spectrometry to identify and quantify proteins in whole-liver extracts of mice fed either Lieber-DeCarli alcohol liquid diet or control liquid diet for 4 weeks. A total of 5,215 proteins were identified; 3,204 of these were quantified. Top up-regulated and down-regulated proteins are presented in Fig. 1A. We found that several of these top-up or down-regulated proteins are mono or dimethylated at arginine residues (Fig. 1B).

We identified 411 arginine methylated proteins in the liver and were able to analyze the abundance of 135 arginine methylated peptides. Top peptides up-regulated or down-regulated by alcohol were analyzed for methylated peptide abundance relative to total protein abundance (Fig. 1C). We found seven individual methylated peptides for which relative methylation was decreased (Fig. 1C, above the line), suggesting that these proteins were particularly demethylated by the alcohol feeding. Among these proteins were several known PRMT1 targets, including the RNA binding protein hnRNP H1, which is methylated at R233.

Next, we confirmed the role of PRMT1 in protein methylation changes in alcohol-fed mice. We analyzed methylated peptide abundance in wild-type mice and hepatocyte-specific PRMT1 knockout mice fed alcohol (Fig. 1D). To induce hepatocyte-specific knockout, we injected AAV-TBG.CRE or control

AAV-TBG.control vector to PRMT1 floxed mice at the first day of alcohol feeding.<sup>(17,22)</sup> We found that PRMT1-dependent methylation is differentially regulated by alcohol. Some of the PRMT1 targets were more methylated in alcohol-fed mice, and some were less methylated. These data agree with our previous findings that alcohol alters PRMT1 substrate specificity.<sup>(22)</sup>

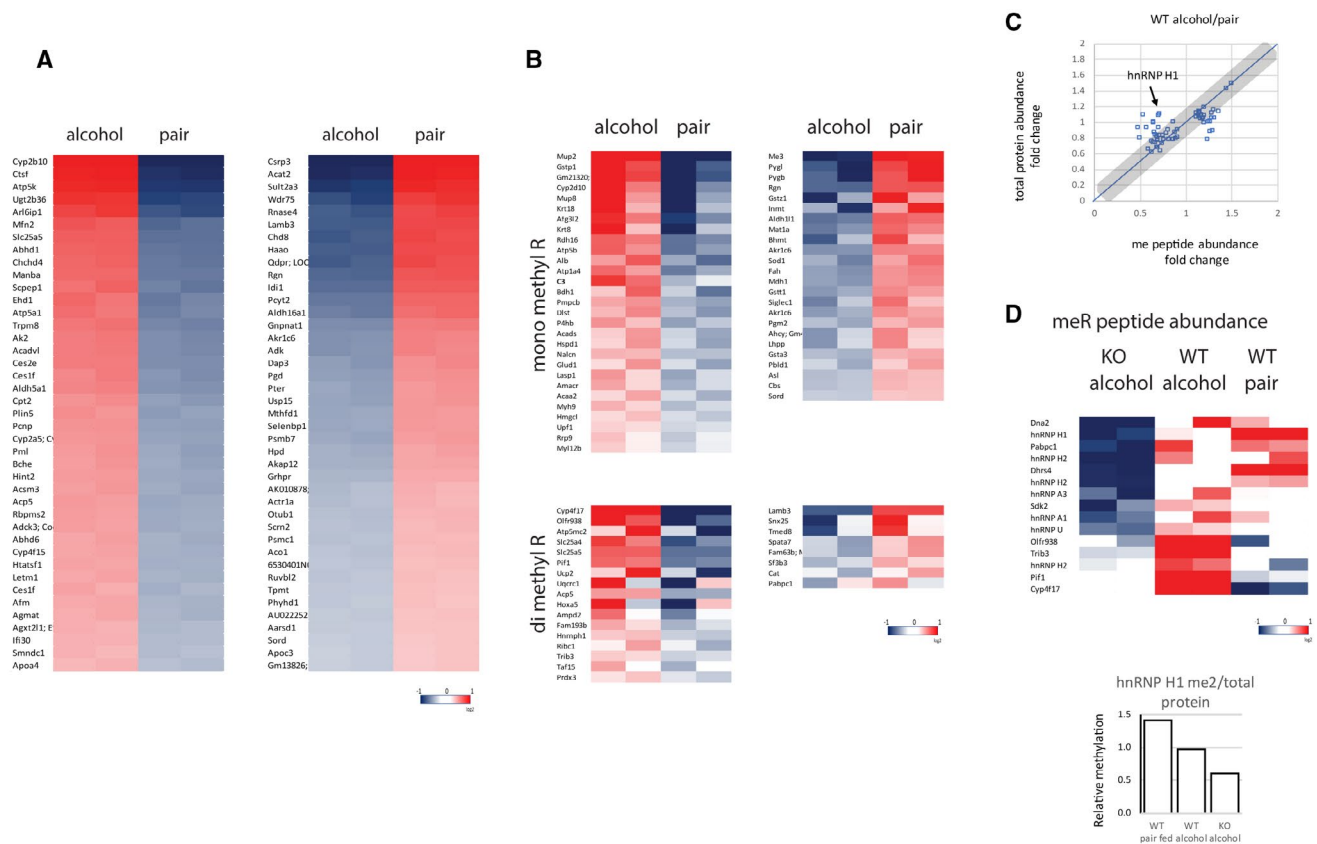
We found that hnRNP H1 R233 methylation further decreased in PRMT1 knockout mice (Fig. 1D), suggesting that PRMT1 methylated hnRNP H1 and that alcohol reduced PRMT1's ability to methylate hnRNP H1.

### PRMT1-DEPENDENT REGULATION OF hnRNP H CONTROLS COMPLEMENT COMPONENTS LEVELS

To assess the PRMT1-dependent hnRNP H1-mediated changes in liver proteome, we compared previously reported genes that are hnRNP H1-dependent<sup>(25)</sup> with the proteomic data of proteins altered in the liver after PRMT1 knockout. We found 77 genes that are common between these two data sets (Fig. 2A). The top pathway that was enriched among common genes was the complement pathway (Fig. 2B).

Hepatocytes express large amount of complement components including C1r, C1s, C3, C5, and others. We tested whether hnRNP H1 binds mRNAs of these genes in Huh7 cells. We immunoprecipitated hnRNP H from cells overexpressing hnRNP H1 with or without PRMT1. We found that C3 and C1R mRNA were bound to hnRNP H1, and this binding was reduced in the presence of PRMT1 (Fig. 2C). We did not detect binding to C1S or C5 mRNA in these conditions. These data correlated with bioinformatic analysis of hnRNP H1 binding sites on these mRNA detected using the RBPmap tool.<sup>(26)</sup> The analysis predicted five clustered sites on C3 mRNA, three sites on C1R mRNA, and none or two single sites on C1S and C5, respectively (Fig. 2C).

Next, we studied how hnRNP H1 regulated complement component expression. We overexpressed hnRNP H1 or methylation-deficient mutant hnRNP H1 R233K in Huh 7 cells in the presence or absence of PRMT1 (Fig. 2D). hnRNP H1 wild-type or mutant overexpression resulted in an increase of



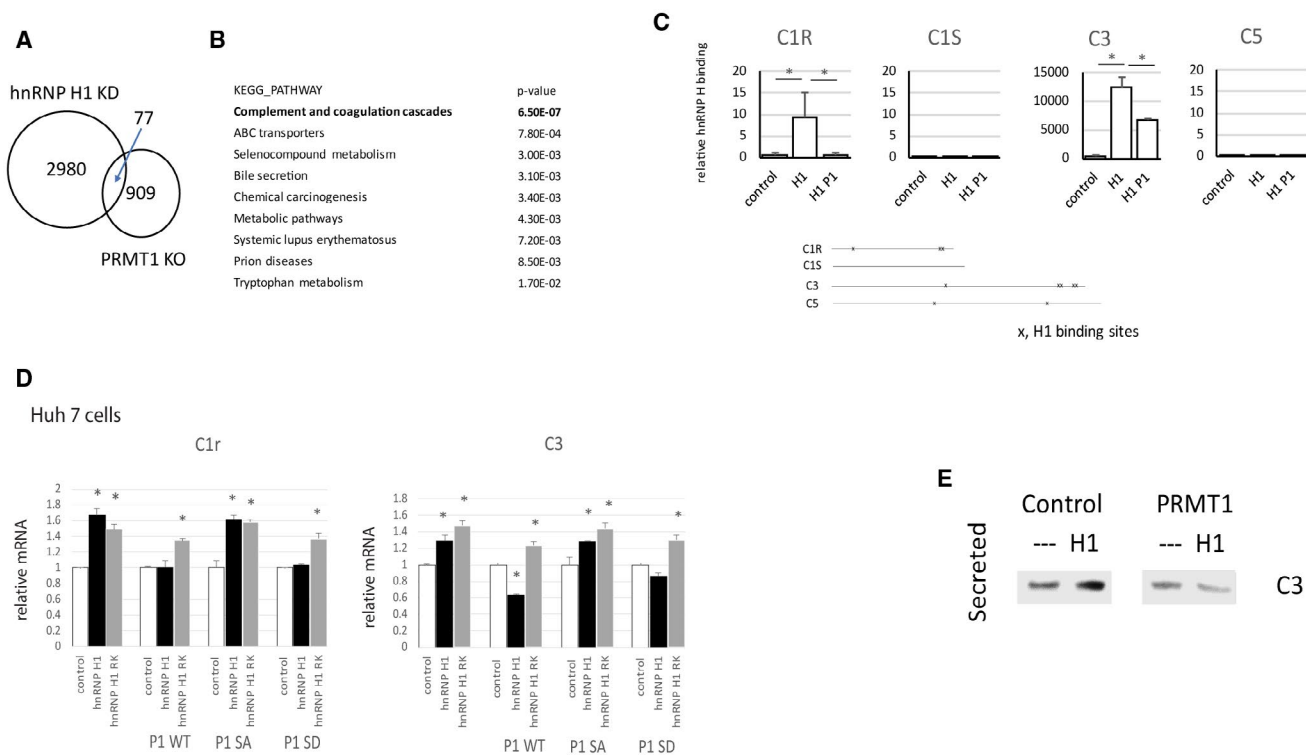
**FIG. 1.** Alcohol feeding alters arginine methylation of several PRMT1 substrates. (A–D) Mice were fed Lieber-DeCarli alcohol liquid diet or control liquid diet for 4 weeks. Whole-liver extracts were analyzed by TMT mass spectrometry. (A) Relative abundance of proteins up-regulated or down-regulated in alcohol-fed mouse livers. (B) Relative abundance of proteins containing arginine-methylated residues up-regulated or down-regulated in alcohol-fed mouse livers. (C) Relative abundance of arginine-methylated peptides plotted versus relative abundance of the protein. (D) PRMT1 floxed mice received AAV.TBG.Cre to induce PRMT1 knockout in hepatocytes or AAV.TBG.control vector after 10 days of alcohol feeding. Mice were fed Lieber-DeCarli alcohol liquid diet or control liquid diet for a total of 4 weeks. Whole-liver extracts were analyzed by TMT mass spectrometry. Figure shows relative abundance of arginine-methylated peptides. Abbreviations: KO, knockout; WT, wild-type.

mRNA abundance of C1R and C3 in Huh 7 cells as well as C3 protein secretion in the media (Fig. 2D,E). In contrast, hnRNP H1 overexpression in the presence of PRMT1 failed to increase mRNA expression of these genes. Overexpression of the methylation-deficient mutant of hnRNP H1 had the same effect on C1r and C3 mRNA, but it was not affected by PRMT1, suggesting that the PRMT1 effect on complement expression is mediated through hnRNP H1 methylation at R233.

Under normal conditions, PRMT1 is phosphorylated at S307.<sup>(22)</sup> In the presence of alcohol, PRMT1 is dephosphorylated at this site, which reduces its ability to catalyze the asymmetric dimethylarginine protein modification. We found that the PRMT1 S→A

mutant (a mimic of the dephosphorylated form present after alcohol) is not able to suppress hnRNP H1-mediated C1R and C3 expression. On the other hand, the S→D mutant, a mimic of the phosphorylated form present in the absence of alcohol, suppressed hnRNP H1-mediated expression. Taken together, these data suggest that PRMT1 phosphorylation is necessary for the methylation of hnRNP H1 in Huh 7 cells. These data agree well with reduced hnRNP H1 methylation in alcohol-fed mice compared with pair-fed control (Fig. 2D).

We further analyzed how PRMT1 level manipulation can affect complement component gene expression *in vitro*. PRMT1 wild-type overexpression reduced C3 mRNA and protein gene expression



**FIG. 2.** PRMT1 regulates methylation of hnRNP H1. (A) Genes up-regulated or down-regulated after hnRNP H1 knockdown and altered in the livers of PRMT1 knockout mice compared with wild-type mice fed alcohol. (B) Pathways enriched in the common genes. (C) Top: RNA immunoprecipitation assay from Huh 7 cells expressing wild-type hnRNP H1 in the presence or absence of PRMT1. Data are presented as average  $\pm$  SD of  $n = 3$  independent experiments.  $*P < 0.05$ . Bottom: hnRNP H1 binding sites identified on mRNA sequences of C1R, C1S, C3, and C5. (D) Relative mRNA expression of C1R and C3 mRNA in Huh 7 cells expressing hnRNP H1 wild-type or R233K mutant in the presence or absence of PRMT1 wild-type, S307A, or S307D mutant. Data are presented as average  $\pm$  SD of  $n = 3$  independent experiments.  $*P < 0.05$ . (E) C3 secreted protein in the media of Huh 7 cells expressing hnRNP H1 where indicated. Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; KD, knockdown.

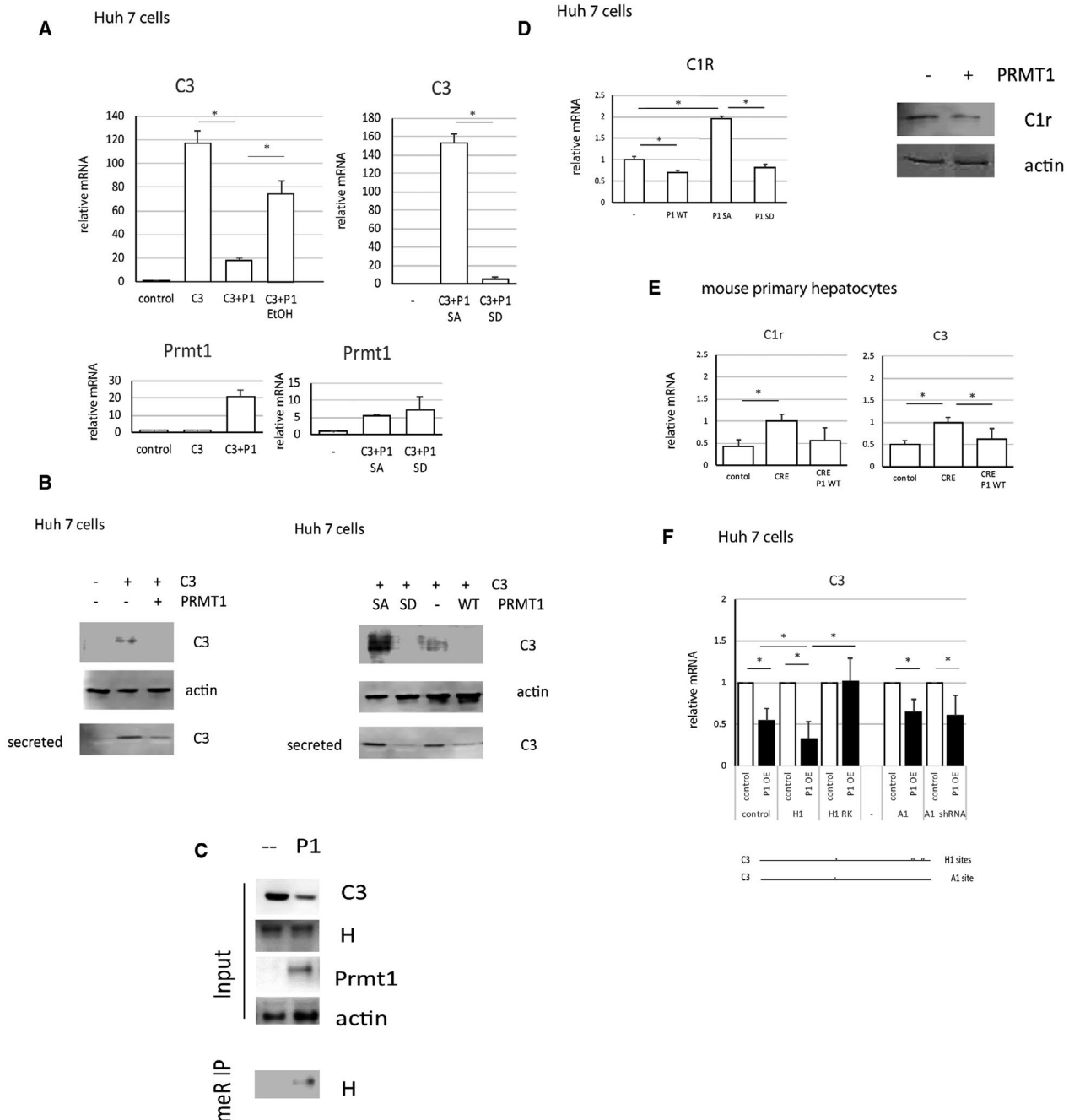
in Huh 7 cells as well as secreted C3 in the media (Fig. 3A,B), and the ability of PRMT1 to suppress C3 expression was reduced in the presence of alcohol. The dephosphorylated “alcohol” mimic overexpression did not reduce C3 expression, whereas the phosphomimic was able to suppress C3 efficiently (Fig. 3A,B). Additionally, we expressed hnRNP H1 in the presence or absence of PRMT1 and immunoprecipitated arginine-methylated proteins. We found that Prmt1 overexpression increases methylation of H1, which correlates with a decrease in C3 levels (Fig. 3C).

Similarly, PRMT1 overexpression reduced C1R mRNA and protein levels in Huh 7 cells (Fig. 3D). SD mimic expression resulted in a two-fold decrease in C1R levels compared with SA expression.

This mechanism was relevant in primary cells as well. Primary mouse hepatocytes were isolated

from livers of PRMT1 floxed mice and treated with either a Cre recombinase-expressing vector to induce PRMT1 knockout (CRE) or a control vector (control). Additionally, we re-expressed PRMT1 in knockout cells (CRE P1 WT). We found that loss of PRMT1 results in about a two-fold increase in C1R and C3 mRNA levels. PRMT1 overexpression in knockout cells resulted in a decrease in expression to the level of the wild-type cells (Fig. 3E).

Finally, we examined whether PRMT1-dependent hnRNP H1 methylation is required for PRMT1-mediated complement gene regulation. We expressed PRMT1 in Huh 7 cells in the presence of hnRNP H1, wild-type or mutant. H1 overexpression significantly increased PRMT1-induced C3 suppression. In contrast, hnRNP H1 R233K mutant expression prevented PRMT1-mediated C3 suppression (Fig. 3F).



**FIG. 3.** PRMT1 regulates C1R and C3 mRNA abundance through methylation of hnRNP H1. (A,B) Relative mRNA expression of C3 mRNA (B) and protein (C) in Huh 7 cells overexpressing C3 in the presence or absence of PRMT1 wild-type, S307A, or S307D mutant. Cells were treated with 50 mM alcohol for 48 hours where indicated. Data are presented as average  $\pm$  SD of  $n = 3$  independent experiments.  $*P < 0.05$ . (C) hnRNP H1 was overexpressed in Huh 7 cells in the presence or absence of PRMT1 and immunoprecipitated using arginine-methylated specific antibodies. (D) Relative mRNA expression of C1R mRNA and protein in Huh 7 cells in the presence or absence of PRMT1 wild-type, S307A, or S307D mutant. Data are presented as average  $\pm$  SD of  $n = 3$  independent experiments.  $*P < 0.05$ . (E) Relative mRNA expression of C1R and C3 mRNA in mouse primary hepatocytes treated with Cre recombinase-expressing vector or control vector in the presence of PRMT1 wild type, where indicated. Data are presented as average  $\pm$  SD of  $n = 3$  independent experiments.  $*P < 0.05$ . (F) Relative mRNA expression of C3 mRNA in Huh 7 cells overexpressing C3 in the presence or absence of PRMT1 wild type, and hnRNP A1, hnRNP A1 short hairpin RNA, hnRNP H1, or hnRNP H1 R233K, where indicated. Bottom. Position of hnRNP protein binding sites on C3 mRNA. Data are presented as average  $\pm$  SD of  $n = 3$  independent experiments.  $*P < 0.05$ . Abbreviations: EtOH, ethanol; IP, immunoprecipitation; OE, overexpression; SA, dephosphorylated alcohol mimic; SD, phosphomimic.



Additionally, we expressed PRMT1 in Huh 7 cells in the presence of another well-known PRMT1 target hnRNP A1, which is predicted to bind C3 mRNA (Fig. 3F), or in the presence of short hairpin RNA specific to hnRNP A1. We found that hnRNP A1 overexpression or knockdown did not affect the ability of PRMT1 to suppress C3 expression. Taken together, these data suggest that hnRNP H1 methylation is required for PRMT1-dependent regulation of the complement component C3 expression.

### hnRNP H METHYLATION CORRELATES WITH COMPLEMENT COMPONENT C3 GENE EXPRESSION IN HUMANS

To assess the relevance of the proposed mechanism in humans, we analyzed the expression of PRMT1 and complement components in human liver tissues using the GEPIA tool.<sup>(27)</sup> We found that PRMT1 strongly negatively correlated with upstream complement components C1R, C1s, and C3 expression, whereas no correlation was found between PRMT1 and the downstream complement component C5 (Fig. 4A). Additionally, we found no negative correlation of PRMT1 with C1qa and C1qb expression, complement components that are produced primarily by macrophages (Fig. 4A).

Next, we measured hnRNP H1 methylation in human liver specimens from the KUMC Liver Bank using proximity ligation assay, and assessed PLA signal levels in relation to C3 staining intensity in these specimens (Fig. 4B). PLA allows detection of post-translational modifications; in this case, hnRNP H and aDMA-specific antibodies bound in close proximity on methylated hnRNP H protein produce a positive signal indicated by brown/red dots, which can be readily seen against the counterstain but not in the absence of the primary antibodies (Fig. 4B,C).

We found that there is a significant negative correlation between hnRNP H methylation (average number of PLA dots per high power field) and C3 levels in the liver samples from patients with cirrhosis and a weak negative correlation with C3b levels, suggesting that PRMT1-dependent hnRNP H methylation controls C3 expression in humans (Fig. 4D). We found no significant difference in C3 levels between liver samples from patients with cirrhosis due to alcohol and patients with cirrhosis due to other

causes. Similarly, we found no significant differences between groups in hnRNP H methylation, and both groups of cirrhosis samples had significantly reduced hnRNP H methylation levels compared with control donor samples (Fig. 4E), which correlates with previously published data on PRMT1 activity changes in cirrhosis.<sup>(20,28)</sup>

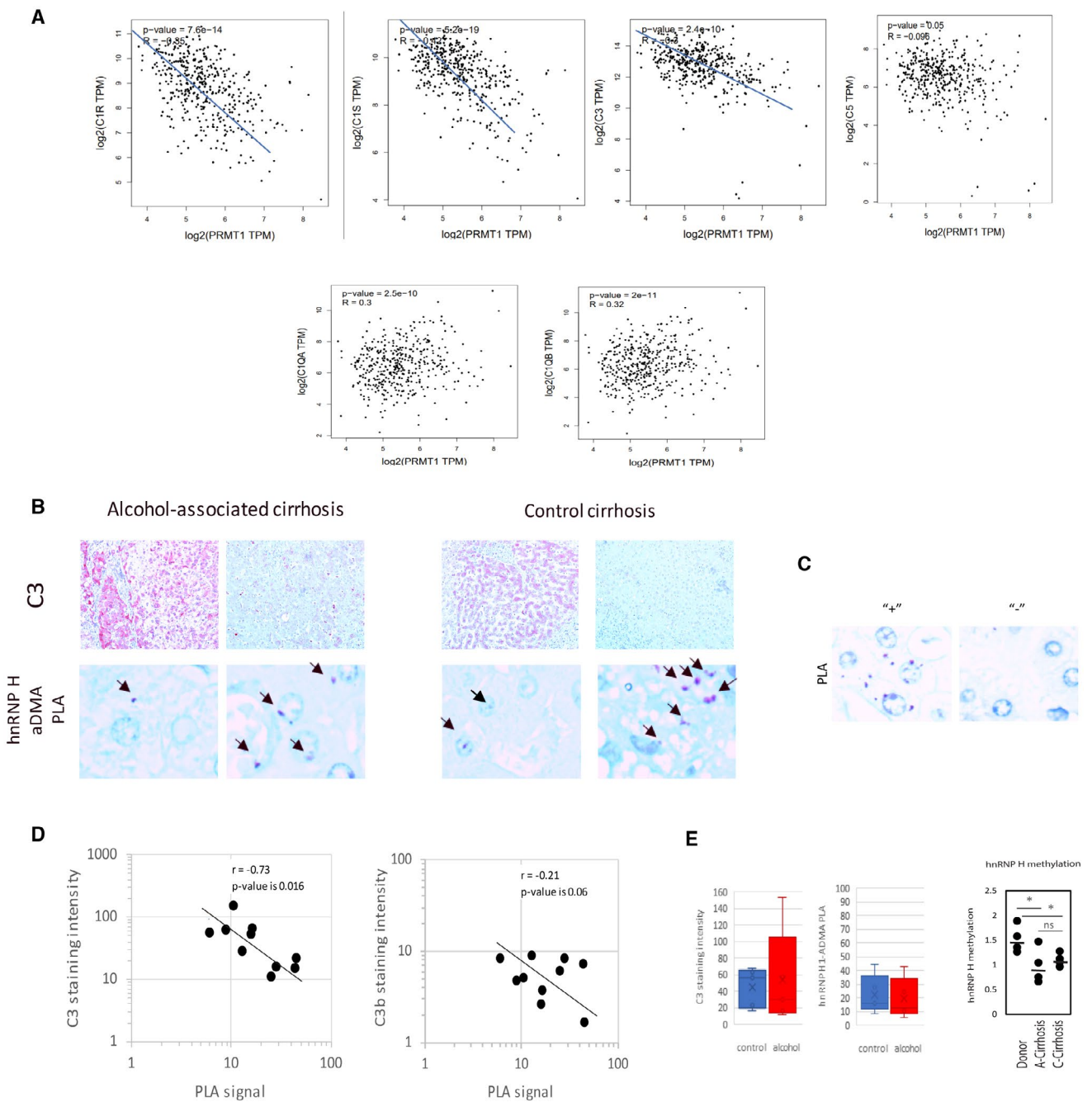
Taken together, data from human samples indicate that the PRMT1-hnRNP H-C3 relationship is present in human disease as well.

### PRMT1 REGULATES COMPLEMENT ACTIVATION IN ALCOHOL-FED MICE

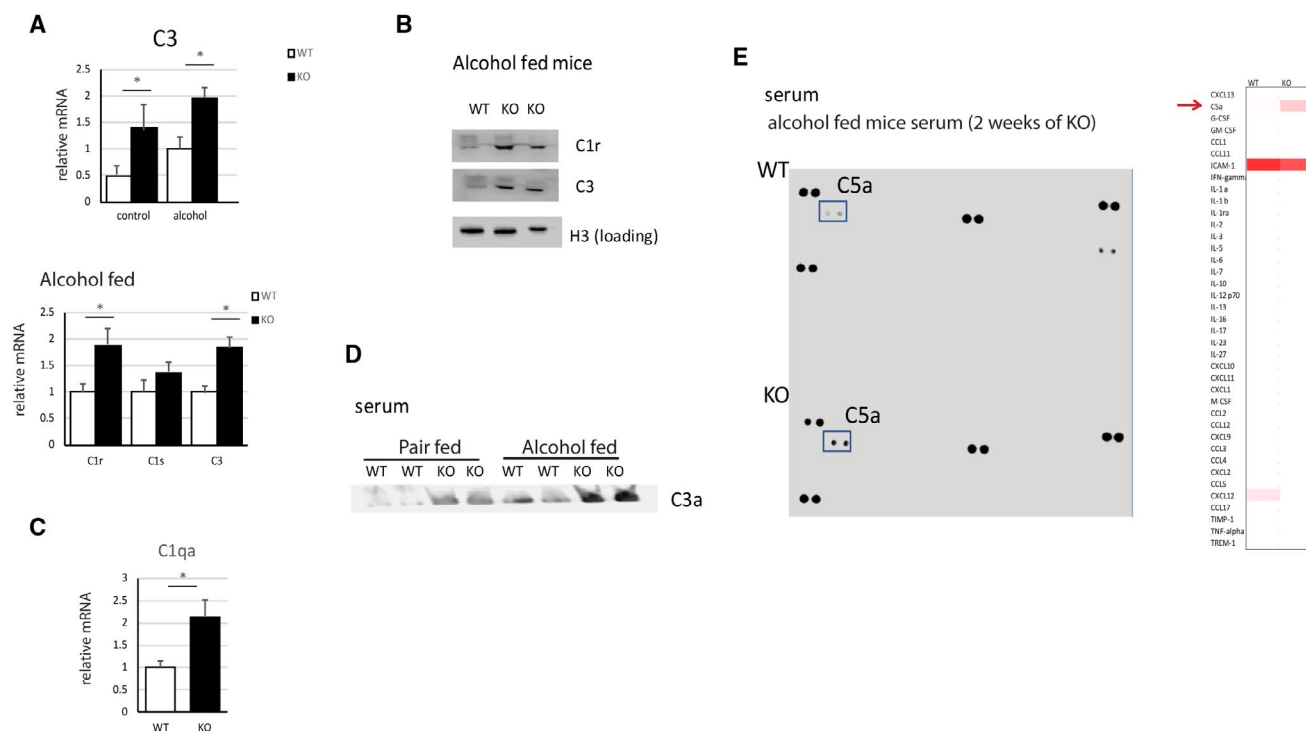
To assess the role of this mechanism *in vivo*, we measured complement gene expression and complement activation in wild-type and hepatocyte-specific PRMT1 knockout mice. We found that PRMT1 knockout results in about a three-fold increase in C3 mRNA expression in the absence of alcohol and about two-fold increase in the presence of alcohol, in agreement with *in vitro* data (Fig. 5A). In alcohol-fed mice, PRMT1 knockout results in about a two-fold increase in C1r mRNA, but not C1s mRNA expression, in agreement with *in vitro* data (Fig. 5A).

Likewise, we detected an increase in C1r and C3 protein levels in livers of knockout mice (Fig. 5B). Previously, we found that PRMT1 knockout results in an increase of hepatocyte death and inflammation in the alcohol-fed liver.<sup>(22)</sup> Additionally, we found that this inflammation is associated with an increase in complement component C1qa expression, likely through indirect mechanism, as it is primarily expressed in macrophages (Fig. 5C). Thus, the PRMT1 knockout results in an increase in expression of several complement components during alcohol feeding, suggesting that complement pathway is activated in these mice. We found that knockout mice fed alcohol have dramatically elevated C3a levels in the serum compared with wild-type controls (Fig. 5D). Interestingly, we did not find elevated levels of other cytokines or chemokines in the serum of these mice by array, except an elevation in C5a, a downstream component of the complement pathway (Fig. 5E).

Taken together, these data suggest that PRMT1 knockout results in a specific elevation of complement C3a and C5a levels during alcohol exposure.



**FIG. 4.** PRMT1 expression and hnRNP H methylation correlates with complement component expression in humans. (A) Correlation between PRMT1 mRNA expression and complement component mRNA expression in human liver samples from the Cancer Genome Atlas and GTEx projects plotted using GEPIA.<sup>(27)</sup> (B) Top: Human liver sections from patients with cirrhosis were stained using anti-C3 antibodies. Bottom: Proximity ligation assay using a combination of anti-hnRNP H and anti-aDMA antibodies. PLA signal (dots) was detected using 3,3'-diaminobenzidine substrate. Arrows indicate positive signal. (C) Positive and negative controls for PLA detection using validated set of antibodies (“+”) or in the absence of primary antibodies (“-”) in human liver cirrhosis section. (D) Left: Correlation between C3 staining intensity and PLA signal (average number of dots per high power field);  $n = 10$ ;  $P < 0.05$ . Right: Correlation between C3b levels and PLA signal;  $n = 10$ . (E) Left: C3 staining intensity and PLA signal in patients with cirrhosis associated with alcohol (alcohol) or not associated with alcohol (control);  $n = 5$  per group. Right: Relative hnRNP H methylation levels detected as in Fig. 3C;  $n = 4$  per group;  $*P < 0.05$ . Abbreviations: ns, not significant; TPM, transcripts per million.



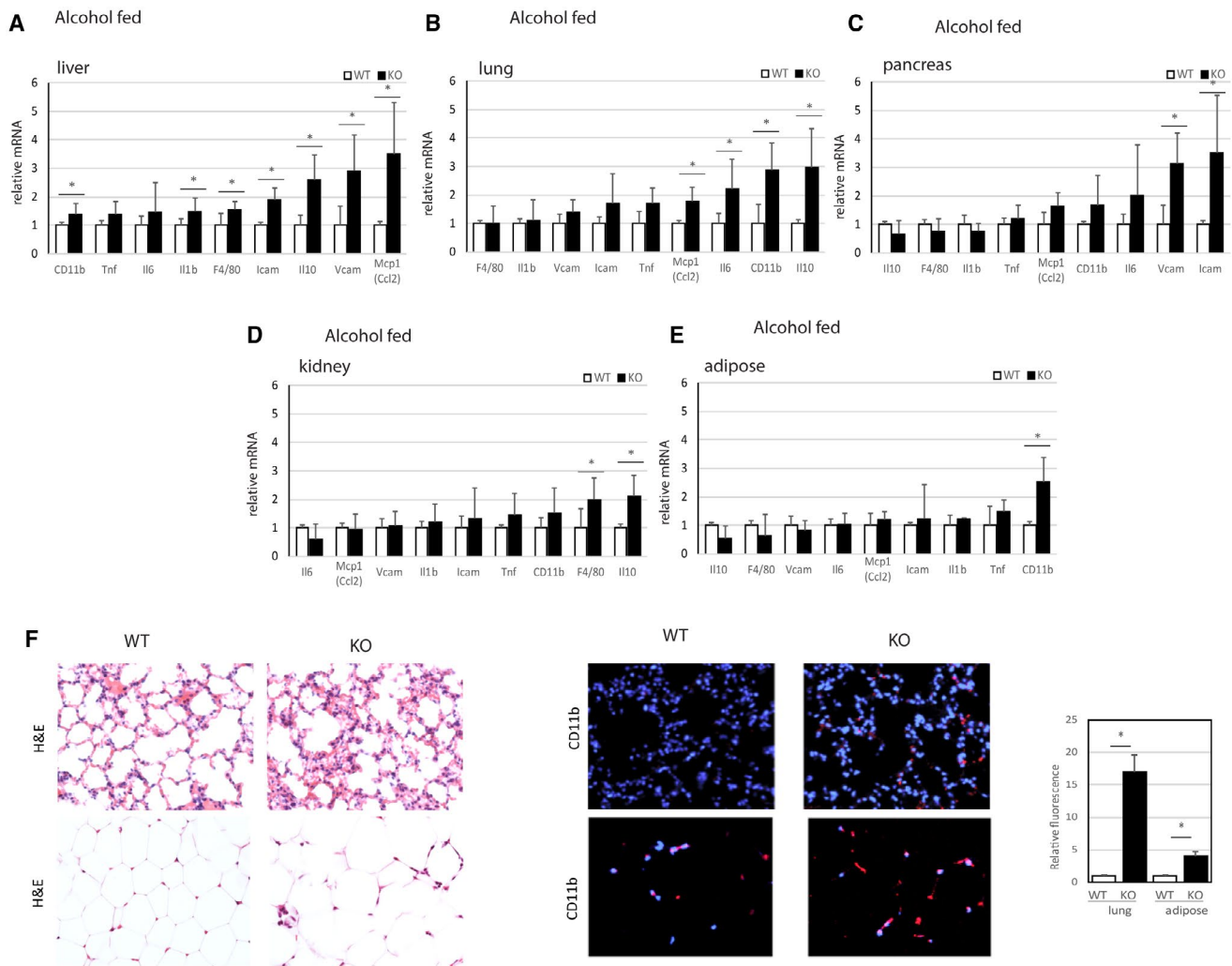
**FIG. 5.** Hepatocyte-specific PRMT1 knockout mice show elevated complement gene expression and complement activation in the presence of alcohol. (A-D) PRMT1 floxed mice received AAV.TBG.Cre (KO) or AAV.TBG.control (WT) on the first day of alcohol feeding and fed alcohol or control liquid diet for total of 3 weeks. (A) Relative mRNA levels in the livers of wild-type and knockout mice. Data are presented as average  $\pm$  SD of  $n = 8-10$  mice per group.  $*P < 0.05$ . (B) Protein levels in whole-liver extracts of wild-type and knockout mice. (C) Relative mRNA levels in the livers of wild-type and knockout mice. Data are presented as average  $\pm$  SD of  $n = 8-10$  mice per group.  $*P < 0.05$ . (D) Serum C3a levels of wild-type and knockout mice. (E) Left; Serum samples of wild-type and knockout mice (pulled from  $n = 3$  mice) were used for cytokine array experiment. Right: Levels of cytokines in wild-type and knockout mouse serum. Abbreviations: CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM, intercellular cell adhesion molecule 1; M-CSF, macrophage colony-stimulating factor; TIMP-1, tissue inhibitor of metalloproteinase 1.

## LOSS OF HEPATIC PRMT1 RESULTS IN SYSTEMIC INFLAMMATION

As mentioned previously, hepatocyte-specific PRMT1 knockout mice are more susceptible to alcohol-induced liver injury and liver inflammation, at least partly due to increased oxidative stress in response to alcohol. Several inflammation-associated genes had elevated expression in the livers of these mice compared with wild-type mice after 3 weeks of Lieber-DeCarli alcohol liquid diet (Fig. 6A).

Interestingly, we detected elevated inflammatory gene expression in other tissues as well, including lung, pancreas, kidney, and adipose tissue (Fig. 6B-E). Elevated gene expression correlated with elevated levels of CD11b-positive cells in the lungs and adipose tissue of the knockout mice, compared with the wild-type mice (Fig. 6F).

One of the explanations for the elevated inflammatory gene expression in distant organs is that PRMT1-dependent ADMA production is reduced in knockout mice. Hepatic PRMT1 is responsible for 30%-50% of serum ADMA,<sup>(22)</sup> which can affect the immune cell phenotype in other organs, due to ADMA's potent anti-inflammatory properties. ADMA treatment of primary macrophages reduced pro-inflammatory (M1) gene expression and increased expression of Mrc1 (M2 marker) gene (Fig. 7A). We tested whether elevated inflammation was due to a decrease in serum ADMA levels. To do so, we restored circulating ADMA levels by injecting knockout mice with 0.2 mg/kg of synthetic ADMA daily. Resulting serum ADMA levels were comparable with the levels of the wild-type mice (Fig. 7B). Unexpectedly, ADMA supplementation increased, rather than decreased, inflammatory gene expression in the knockout mice (Fig. 7C), suggesting



**FIG. 6.** PRMT1 knockout in the liver results in systemic inflammation. PRMT1 floxed mice received AAV.TBG.Cre (KO) or AAV.TBG.control (WT) on the first day of alcohol feeding and fed alcohol for a total of 3 weeks. Relative mRNA levels in the liver (A), lung (B), pancreas (C), kidney (D), and adipose tissue (E). Data are presented as average  $\pm$  SD of  $n = 4-6$  mice per group.  $*P < 0.05$ . (F) Representative images of hematoxylin and eosin staining (left) and CD11b staining (right) of lung and adipose tissue of wild-type and knockout mice fed alcohol. Abbreviations: H&E, hematoxylin and eosin; ICAM, intercellular cell adhesion molecule 1.

that loss of ADMA is not involved in the systemic inflammation in these mice.

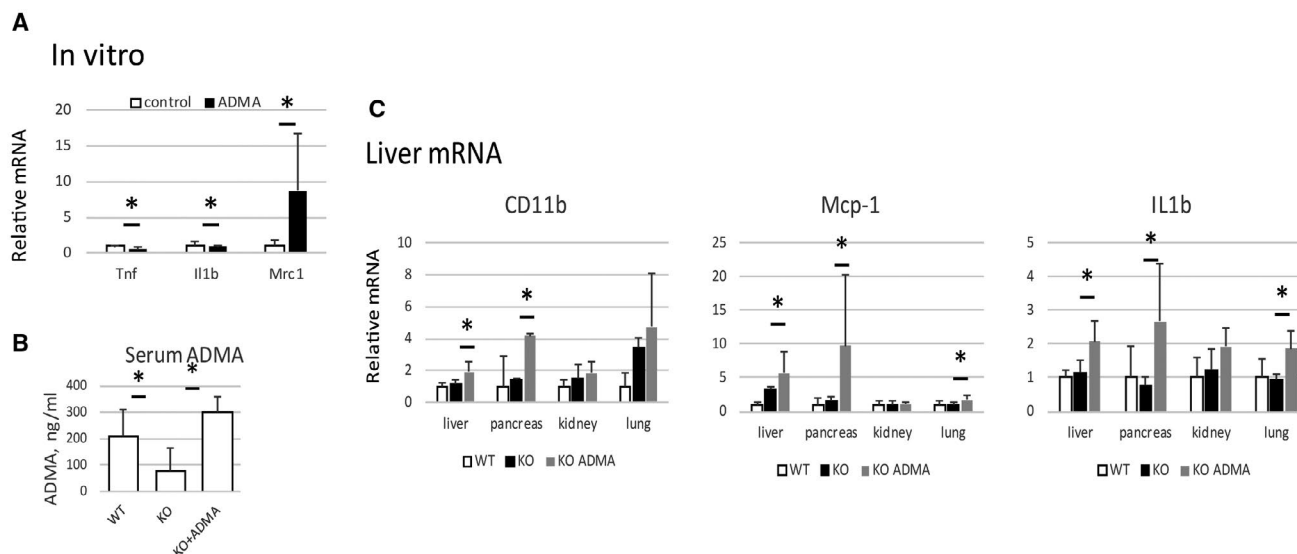
### C3aR OR C5aR INHIBITION ABROGATES THE EFFECT OF PRMT1 KNOCKOUT ON THE INFLAMMATION IN THE LUNG AND ADIPOSE TISSUE OF ALCOHOL-FED MICE

We hypothesized that increased systemic inflammation is due to the elevated complement C3a and

C5a levels, as both C3a and C5a can act as chemokines and were previously reported to promote inflammation in lung and kidneys.<sup>(29-32)</sup> To test this hypothesis, we injected knockout mice either with vehicle control or small molecule inhibitors of C3aR or C5aR receptors twice weekly during duration of alcohol feeding.

We found no effect of the inhibitor treatment on liver histology, liver/body weight ratios, or weight change during feeding (Fig. 8A) or liver injury assessed by serum ALT and aspartate aminotransferase levels (Fig. 8B). In contrast, we found that both inhibitors





**FIG. 7.** Systemic inflammation in PRMT1 knockout is not due to the lack of circulating ADMA. (A) Peritoneal macrophages were treated with 100 ng/mL of ADMA for 48 hours. Data are presented as average  $\pm$  SD,  $n = 5$  independent experiments (macrophages from 5 mice). \* $P < 0.05$ . (B,C) PRMT1 floxed mice received AAV.TBG.Cre (KO) or AAV.TBG.control (WT). Knockout mice received daily injections of ADMA 0.2 mg/kg or saline control. (B) Serum ADMA levels. (C) Relative mRNA in indicated tissues. Data are presented as average  $\pm$  SD,  $n = 3$ –4 mice. \* $P < 0.05$ .

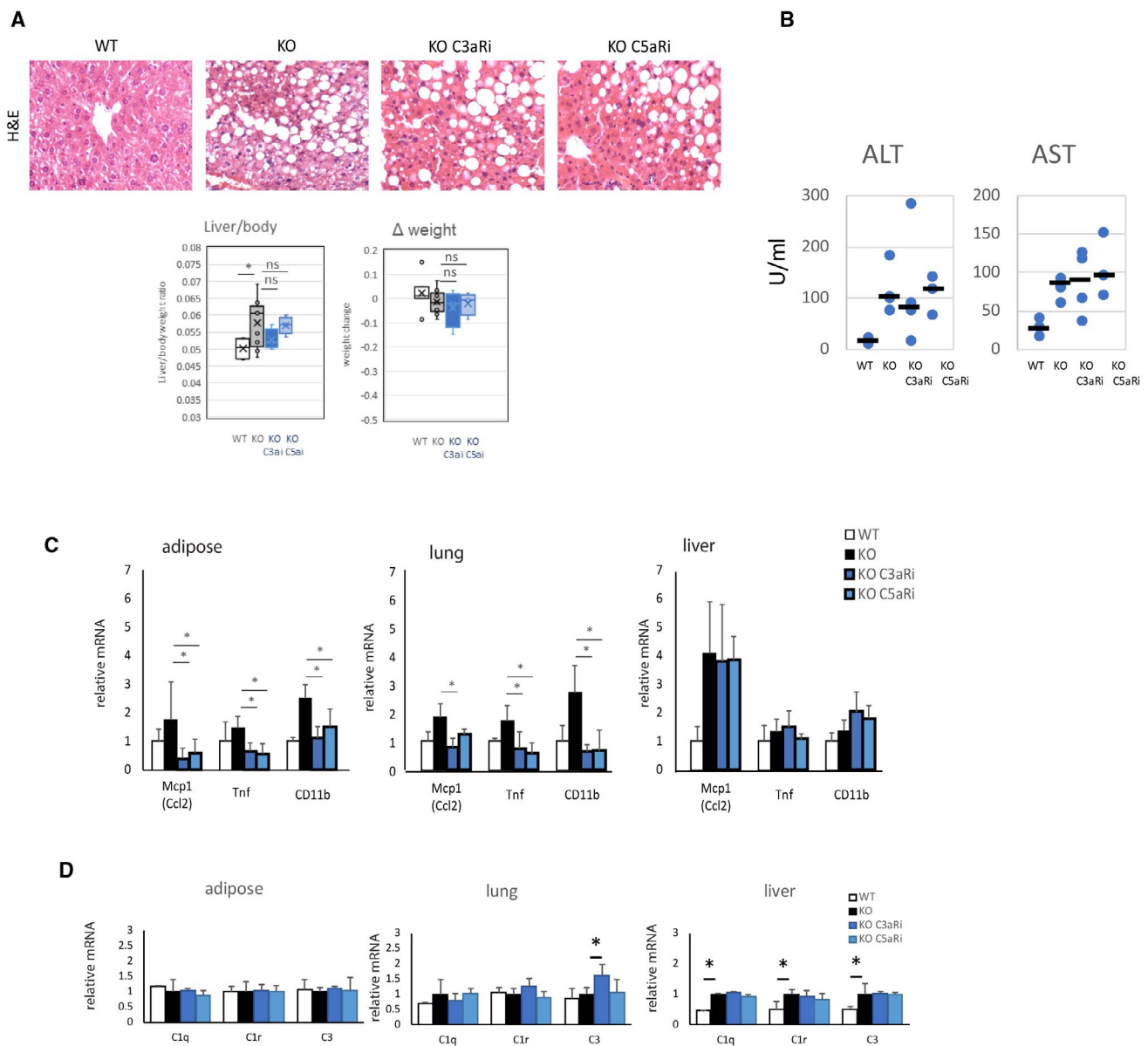
were efficient in reducing inflammation in adipose tissue and in lung tissue (Fig. 8C), while we did not see significant differences in the livers of knockout mice treated with inhibitors or controls (Fig. 8C). The observed effect was not due to the decrease in complement components gene expression in these organs (Fig. 8D).

In agreement with expression data, we found that inhibitor treatment reduced the number of CD11b-positive cells in the lungs (Fig. 9A). In addition, we found that C3a inhibitor was efficient in reducing interleukin (IL)-6 and IL-10 as well as vascular cell adhesion molecule 1 (VCAM1) mRNA levels in lung tissue (Fig. 9B). Next, we analyzed 62 mouse cytokines, chemokines, and related proteins in lung tissues of wild-type, hepatocyte-specific PRMT1 knockout mice treated with vehicle control, C3aR, or C5aR inhibitors (Fig. 9C). We found that knockout mice had elevated levels of several cytokines (both pro-inflammatory and anti-inflammatory) and chemokines. Two of the studied proteins were significantly reduced (macrophage inflammatory protein 1 $\gamma$  and PF-4 or chemokine [C-X-C motif] ligand 4). Inhibitor treatment significantly reduced abundance of elevated cytokines and chemokines including IL-4, IL-13, IL-6, IL-10, interferon- $\gamma$

(IFN- $\gamma$ ), IL-12, IL-17, IL-2, eotaxin-2 (chemokine [C-C motif] ligand 24), fractalkine (CX3CL1), monocyte chemoattractant protein 1 (MCP-1) (chemokine [C-C motif] ligand 2), and adhesion molecule VCAM1 (CD106). We noted that C3aR inhibitor had better efficacy compared with C5aR inhibitor (Fig. 9B). Taken together, these data suggest that hepatic PRMT1 plays a role in liver–lung and liver–adipose tissue crosstalk through regulation of complement activation.

## Discussion

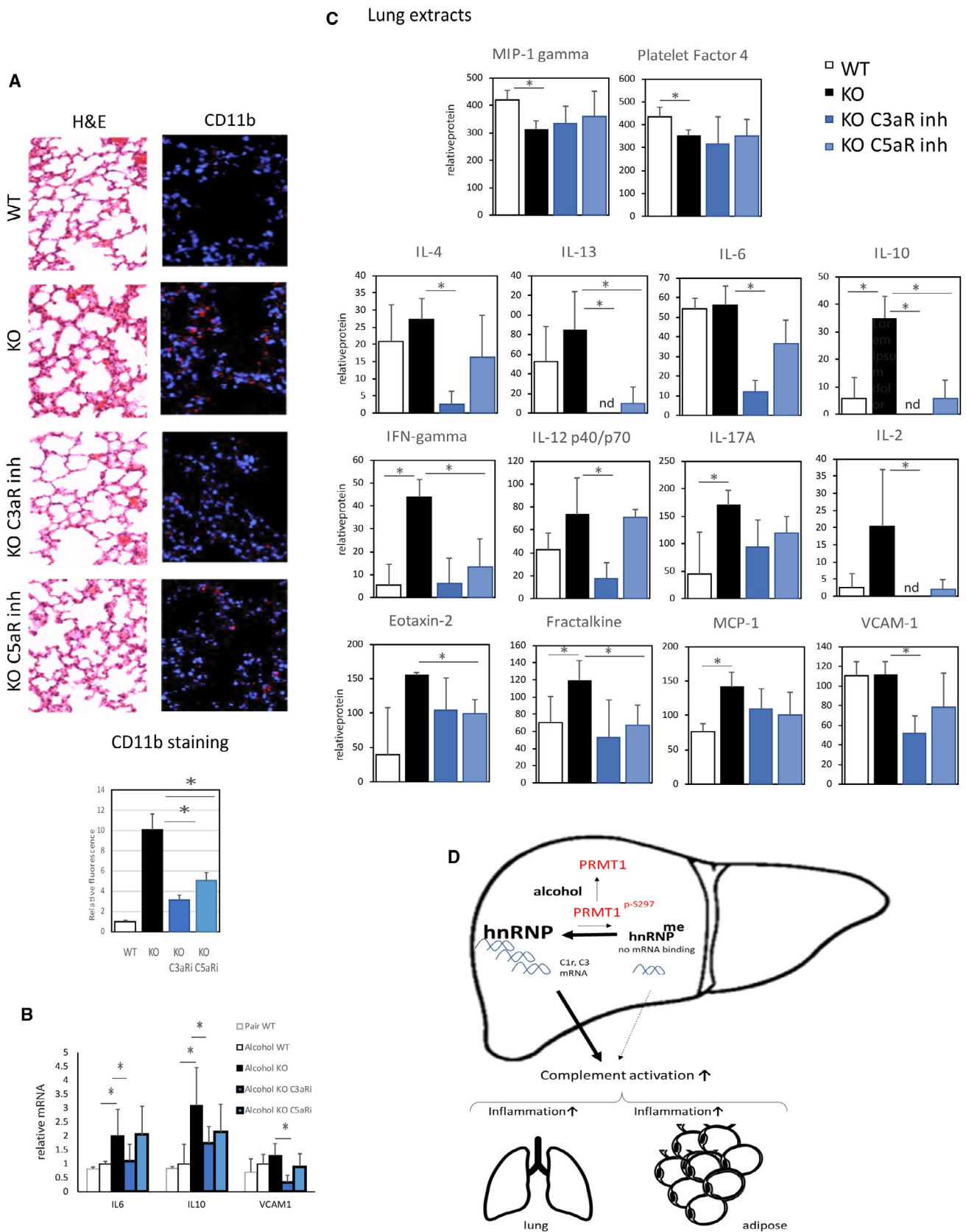
Excessive ethanol consumption affects many organs, both by indirect and direct mechanisms. Considerable research in the last two decades has focused on the importance of proinflammatory cytokines and oxidative damage in the pathogenesis of many of the systemic manifestations of alcoholism (reviewed in Gonzalez-Reimers et al<sup>(33)</sup>). In addition to alcohol-associated liver disease, cytokines may also induce damage in remote organs such as brain, bone, muscle, heart, lung, adipose tissue, peripheral nerve, and pancreas. These effects are seen in alcoholics, even without significant liver disease.<sup>(33)</sup>



**FIG. 8.** C3aR and C5aR receptor inhibitors treatment reduced inflammation in lung and adipose tissue but not in the livers of knockout mice. PRMT1 floxed mice received AAV.TBG.Cre (KO) or AAV.TBG.control (WT) on the first day of alcohol feeding and fed alcohol for a total of 3 weeks. Mice were injected with C5aR Antagonist (PMX53) at 2 mg/kg or C3aR Antagonist (SB290157) at 5 mg/kg in 5% DMSO in PBS, or vehicle control 5% DMSO in PBS, twice weekly during alcohol feeding. (A) Hematoxylin and eosin staining of livers. (B) Serum ALT and aspartate aminotransferase levels. (C,D) Relative mRNA levels in adipose tissue, lung, and liver. Data are presented as average  $\pm$  SD of  $n = 4-6$  mice per group. \* $P < 0.05$ . Abbreviation: ns, not significant.

Several studies have reported involvement of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), IL-1 $\beta$ , IL-6, and transforming growth factor  $\beta$ , and activation of nitric oxide synthases in the process of alcohol-induced systemic inflammation.<sup>(33)</sup> Complement activation is also known to play a role in these processes.<sup>(5,8-11)</sup> In

this work we describe a mechanism of liver to distant organs crosstalk that involves regulation of the complement pathway in the liver. We found that complement component mRNAs and protein expression in the liver are regulated through PRMT1-dependent suppression, which can be altered by alcohol's effects on PRMT1.



**FIG. 9.** C3aR and C5aR receptor inhibitors treatment reduced inflammation in lung and adipose tissue of the knockout mice. PRMT1 floxed mice received AAV.TBG.Cre (KO) or AAV.TBG.control (WT) on the first day of alcohol feeding and fed alcohol for total of 3 weeks. Mice were injected C5aR Antagonist (PMX53) at 2 mg/kg or C3aR Antagonist (SB290157) at 5 mg/kg in 5% DMSO in PBS, or vehicle control 5% DMSO in PBS, twice weekly during alcohol feeding. (A) Representative images of H&E staining and CD11b staining of lung tissue of wild type and knockout mice fed alcohol as in A. (B) Relative mRNA levels in lung. Data are presented as average  $\pm$  SD of  $n = 4-6$  mice per group. \*,  $P < 0.05$ . (C) Lung tissue extracts were used for cytokine array experiment. Levels of proteins in wild type and knockout mice as in A. Data are presented as average  $\pm$  SD of  $n = 3-4$  mice per group. \*,  $P < 0.05$ . (D) Model of PRMT1-dependent regulation of complement activation. Abbreviation: nd, none detected.

PRMT1 methylates multiple targets in the liver, and its activity is affected by alcohol, as previously reported by us and others,<sup>(21,34,35)</sup> resulting in an increase in methylation of a subset of targets and a decrease in methylation of another set of targets. One of the PRMT1 targets that is affected by alcohol-mediated PRMT1-activity changes is hnRNP H1, specifically its methylation at R233. We identified a detailed mechanism of a PRMT1–hnRNP H1 complement regulation that is involved in complement pathway activation in alcohol-fed mice (Fig. 9D). PRMT1 methylates hnRNP H1, and the methylation results in a decrease of binding to two of the complement component mRNAs: C1r and C3. In contrast, we did not detect hnRNP H1 binding to complement components C1s and C5.

Data from our study suggest that mRNA expression of C3, a vital component of the pathway, is regulated specifically by PRMT1-dependent methylation of hnRNP H1. We detected several thousand-fold enrichment for H1-C3 mRNA binding by RNA immunoprecipitation (Fig. 2C), suggesting that this is a strong and specific interaction. On the other hand, hnRNP H1 R233K mutant expression completely prevented PRMT1-mediated effects on C3 abundance. Thus, regulation of C3 expression through this mechanism likely contributes to the complement activation seen in alcohol-fed mice.

In patients with liver disease, a similar mechanism is present. We found a strong correlation between PRMT1 or hnRNP H1 methylation and expression of the complement components C1R and C3. Interestingly, hnRNP H1 methylation in livers from patients with cirrhosis from alcohol and nonalcohol etiologies were the same. We found no reduction of methylation in the alcohol group. We hypothesize that PRMT1 activity can be altered by other causes of liver disease in a similar way as alcohol. For example, HCV infection is known to produce an effect on PRMT1 activity that is very comparable to that of alcohol.<sup>(21,36,37)</sup> Previously, we showed that PRMT1

activity is reduced in livers of patients with cirrhosis regardless of etiology.<sup>(28)</sup>

We found that PRMT1 knockout in hepatocytes results in a specific elevation of C3a and C5a in the serum of alcohol-fed mice. This effect is likely due to increased C1r and C3 protein levels in the liver in combination with increased C1qa and other complement components produced by immune cells that results from liver inflammation in these mice. Our study does not define the relative contribution of these two pathways to C3a and C5a elevation. We hypothesize that they can act synergistically.

Short-term Lieber-DeCarli feeding of wild-type mice as presented in this work usually does not result in inflammation. However, we previously found that hepatic PRMT1 deficiency results in significant liver injury, inflammation, and even some fibrosis in feeding conditions when wild-type mice barely react to alcohol.<sup>(22)</sup> Interestingly, despite the elevated cytokine and chemokine gene expression previously observed in livers of hepatocyte-specific PRMT1 knockout mice,<sup>(22)</sup> we did not find detectable levels of other cytokines and chemokines, such as TNF $\alpha$  or IL-1 $\beta$ , commonly held responsible for alcohol-induced systemic inflammation.

Several studies suggest that the complement pathway is important in alcohol-induced fatty liver and alcohol-induced liver injury.<sup>(5,6)</sup> C3 whole-body knockout mice are protected from alcohol-mediated accumulation of triglyceride in the liver without affecting liver injury or inflammation<sup>(7)</sup>; however, the impact of C3a–C3aR interaction to the phenotype of these mice was not studied. Our data suggest that inhibiting C3aR receptor had no effect on liver fat accumulation or liver injury and inflammation, but we did see a trend toward lower liver/body weight ratios in these mice. In contrast, inhibiting C3aR receptor did influence inflammatory gene expression in lung and adipose tissue.

Because adipose and other tissues express the complement components, we also considered complement



pathway activation in other organs as well. However, we did not see C3 elevation or C1qa elevation in lung or adipose tissue. Interestingly, in the lung after C3aR inhibitor treatment, we noted elevated levels of complement component expression, suggesting a complex interaction between circulating C3a and complement gene expression in the lung.

C5 has previously been reported to regulate liver inflammation in alcohol-fed mice. These studies with cell type-specific C5aR knockout mice revealed that C5aR on immune cells can contribute to inflammation in alcohol-fed mice.<sup>(8)</sup> In agreement with that data, our results using a C5aR inhibitor showed that C5aR contributes to nonhepatic organ inflammation in alcohol-fed mice. We did not, however, observe an effect of the C5aR inhibitor treatment on liver inflammation. This discrepancy could possibly be due to excessive inflammation in PRMT1 knockout livers resulting from hepatocyte death.

Taken together, our data demonstrate that complement pathway regulation controlled by liver arginine methylation capacity determines the excessive levels of complement C3a and C5a in the circulation of alcohol-fed mice and contributes to liver–lung and liver–adipose tissue crosstalk.

Our work has revealed several potential therapeutic targets for future treatment of alcohol-associated liver disease. It further suggests that the liver might be a key target for prevention of alcohol effects on other organs. We know from previous work that many patients with alcohol-associated liver disease have reduced levels of PRMT1 in the liver. Thus, they may benefit from complement directed therapy. On the other hand, hnRNP H1 and its paralog hnRNP H2 can potentially be used to regulate complement levels and systemic inflammation. Genome-wide association studies reveal that single-nucleotide polymorphisms associated with these genes are associated with phenotypes such as type 2 diabetes and alcohol dependence (<https://www.ebi.ac.uk/gwas/>). Further studies are necessary to evaluate the role of these genes in disease progression and potential value of targeting these genes in the treatment or prevention of alcohol-associated tissue injury.

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