

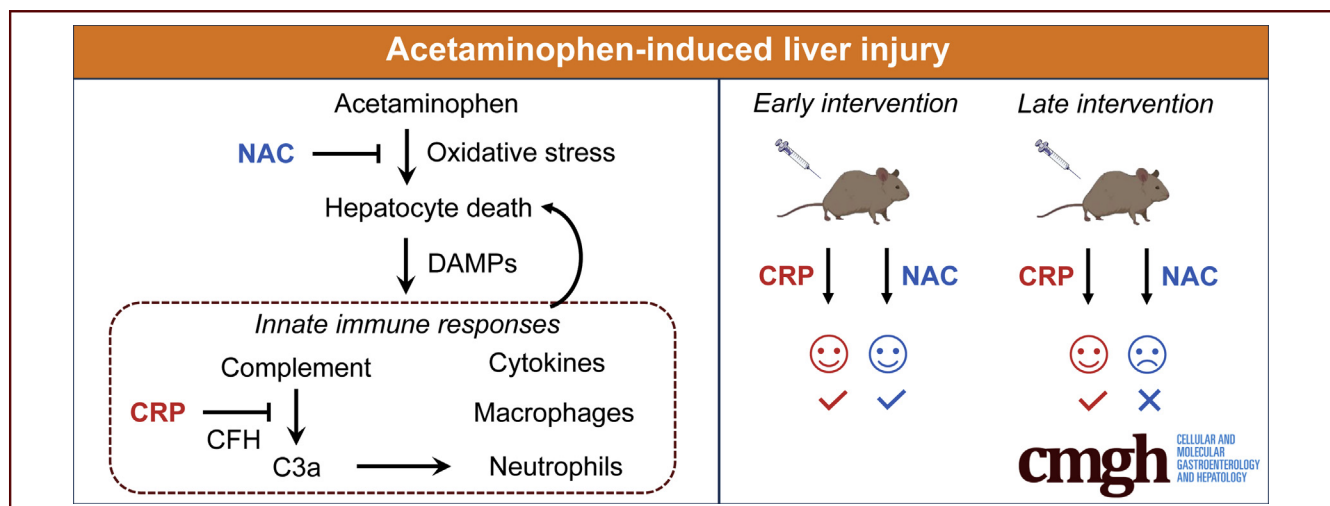
ORIGINAL RESEARCH

C-Reactive Protein Protects Against Acetaminophen-Induced Liver Injury by Preventing Complement Overactivation



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SUMMARY

C-reactive protein is a critical checkpoint that limits destructive activation of complement in acetaminophen-induced liver injury, and could be exploited as a promising therapeutic approach to treat hepatotoxicity caused by drug overdose.

BACKGROUND AND AIMS: C-reactive protein (CRP) is a hepatocyte-produced marker of inflammation yet with undefined function in liver injury. We aimed to examine the role of CRP in acetaminophen-induced liver injury (AILI).

METHODS: The effects of CRP in AILI were investigated using CRP knockout mice and rats combined with human CRP rescue. The mechanisms of CRP action were investigated in vitro and in mice with Fc γ receptor 2B knockout, C3 knockout, or hepatic expression of CRP mutants defective in complement interaction. The therapeutic potential of CRP was investigated by intraperitoneal administration at 2 or 6 hours post-AILI induction in wild-type mice.

RESULTS: CRP knockout exacerbated AILI in mice and rats, which could be rescued by genetic knock-in, adeno-associated virus-mediated hepatic expression or direct administration of

human CRP. Mechanistically, CRP does not act via its cellular receptor Fc γ receptor 2B to inhibit the early phase injury to hepatocytes induced by acetaminophen; instead, CRP acts via factor H to inhibit complement overactivation on already injured hepatocytes, thereby suppressing the late phase amplification of inflammation likely mediated by C3a-dependent actions of neutrophils. Importantly, CRP treatment effectively alleviated AILI with a significantly extended therapeutic time window than that of N-acetyl cysteine.

CONCLUSION: Our results thus identify CRP as a crucial checkpoint that limits destructive activation of complement in acute liver injury, and we argue that long-term suppression of CRP expression or function might increase the susceptibility to AILI. (*Cell Mol Gastroenterol Hepatol* 2022;13:289–307; <https://doi.org/10.1016/j.jcmgh.2021.09.003>)

Keywords: Biomarker; Hepatocytes; Inflammation; Pattern Recognition Receptor.

Acetaminophen (APAP)-induced liver injury (AILI) is a major cause of acute liver failure with limited therapeutic options.^{1–4} In the early phase of AILI, overdosed APAP is metabolized to N-acetyl-p-benzoquinone by cytochrome P450 (CYP450) enzymes in hepatocytes. Excessive

N-acetyl-p-benzoquinone depletes cellular stores of glutathione (GSH) and forms adducts with surrounding proteins, leading to dysfunction of mitochondria and generation of reactive oxygen species. These toxic events are further exacerbated by the activation of intracellular signaling, such as JNK, and eventually result in the necrosis of hepatocytes.¹⁻⁴ In the late phase of AILI, danger signals exposed by necrotic hepatocytes evoke innate immune responses by recruiting and activating resident Kupffer cells and circulating monocytes and neutrophils. Those inflammatory responses cause damages to neighboring hepatocytes, thereby aggravating the early phase, hepatocyte-autonomous toxicity induced by APAP.

Complement is a major branch of innate immunity,⁵ the dysregulated activation of which results in the assembly of membrane attack complex (MAC) that impairs cellular integrity, and the generation of anaphylatoxins that recruit and stimulate immune cells.⁵ One previous study shows that depleting complement with cobra venom factor (CVF) or C3 knockout (KO) alleviates AILI in mice.⁶ However, how complement activation is regulated, and what activation product play a major role in AILI remain elusive. C-reactive protein (CRP) is a marker of inflammation produced predominantly by the liver.⁷⁻¹¹ The production of CRP increases rapidly upon tissue injury wherein it may bind phosphorylcholine (PC) exposed on membranes of injured cells and regulate complement activation via recruiting C1q and factor H (CFH).^{12,13} In addition, CRP may also modulate responses of immune cells via Fcγ receptors (FcγRs).^{12,13} Nevertheless, it is unclear whether CRP plays any role in AILI.

In the present study, we demonstrate a critical and conserved function of CRP in protecting the liver from AILI by preventing complement overactivation. Importantly, endogenous CRP already confers an essential protection to mice and rats, while the defective protection in CRP KO animals can be fully rescued by genetic knock-in (KI), adeno-associated virus-mediated hepatic expression, or direct administration of human CRP (huCRP). Our findings thus indicate that mice and rats should not be considered as models naturally defective in CRP expression or function,^{7,14,15} arguing for a rethinking of how these animals should be manipulated to define the actions of CRP in vivo. Moreover, we suggest that autologous CRP administration might be exploited as a therapeutic approach to treat acute liver injury. This also raises the concern that persistent or long-term reduction of the hepatic expression,¹⁶⁻¹⁹ circulating levels,²⁰ or PC binding or complement regulatory activities of CRP^{21,22} might predispose the subjects to AILI.

Results

CRP Protects Against AILI in Mice and Rats

To examine the role of CRP in AILI, APAP was intraperitoneally injected into wild-type (WT) and CRP KO mice (Figure 1A) for 24 hours. APAP injection resulted in an overt liver injury in WT mice as manifested by drastically increased levels of circulating alanine transaminase (ALT) and aspartate transaminase (AST) (Figure 1B and C) and massive necrosis of hepatocytes (Figure 1D and E). All these manifestations were significantly exacerbated in CRP KO

mice (Figure 1B-E). The adverse consequence of CRP KO could even be noted upon visual inspection of intact organs (Figure 1F), leading to enhanced susceptibility to death induced by APAP at a higher dose sublethal to WT mice (Figure 1G). Importantly, intravenous administration of huCRP not only rescued the defects caused by mouse CRP KO, but also alleviated AILI in WT mice (Figure 1C-E). These results thus demonstrate that endogenous and huCRP are both protective in mouse AILI.


Corroborating the observations made in mice, CRP KO also exacerbated AILI in rats. Following APAP injection, CRP KO rats (Figure 2A) manifested much higher levels of circulating ALT and AST (Figure 2B), and significantly larger areas of hepatic damage than WT rats (Figure 2C and D). KI of huCRP (Figure 2A), however, corrected the defects caused by rat CRP KO (Figure 2B-D). Therefore, despite that distinct strategies were used to generate CRP KO animals (termination of transcription in mice vs deletion of exon in rats) and to rescue with huCRP (intravenous administration in mice vs genetic KI in rats), consistent or complementary phenotypes in AILI were nevertheless observed regardless of sex and possible substrain differences. Interestingly, patients diagnosed as drug-induced liver injury with higher CRP levels also appear to manifest lower ALT levels (Figure 3). We thus conclude that endogenous CRP is essential in protecting against AILI, and that this function is conserved across species.

CRP Does Not Affect the Early-Phase Injury of AILI

To understand the mechanisms of CRP protection, we first examined whether CRP affects the early phase of AILI, wherein CYP450-mediated metabolism of overdosed APAP injures hepatocytes by inducing toxic events such as GSH and activation of JNK signaling.¹⁻⁴ However, neither the expression of CYP450 isoenzymes (Figure 4A and B) nor the induction of GSH depletion (Figure 4C) and JNK activation (Figure 4D and E) was altered by CRP KO at 2 and 4 hours post-APAP administration. The inability of CRP to regulate these hepatocyte-autonomous events could be explained by the lack of FcγR expression on hepatocytes.²³ Indeed, knockout of FcγR2B, an inhibitory FcγR that mediates the anti-inflammatory activities of CRP in mice,^{12,13} showed only marginal effects on the protection of CRP in AILI (Figure 4F).

*Authors share co-first authorship.

Abbreviations used in this paper: AILI, acetaminophen-induced liver injury; ALT, alanine transaminase; APAP, acetaminophen; AST, aspartate transaminase; BSA, bovine serum albumin; CFH, complement factor H; CRP, C-reactive protein; CVF, cobra venom factor; CYP450, cytochrome P450; FcγR, Fcγ receptor; GSH, glutathione; huCRP, human C-reactive protein; IL, interleukin; KI, knock-in; KO, knockout; MAC, membrane attack complex; NAC, N-acetyl cysteine; PBS, phosphate-buffered saline; PC, phosphorylcholine; q-PCR, quantitative polymerase chain reaction; TNF-α, tumor necrosis factor α; WT, wild-type.

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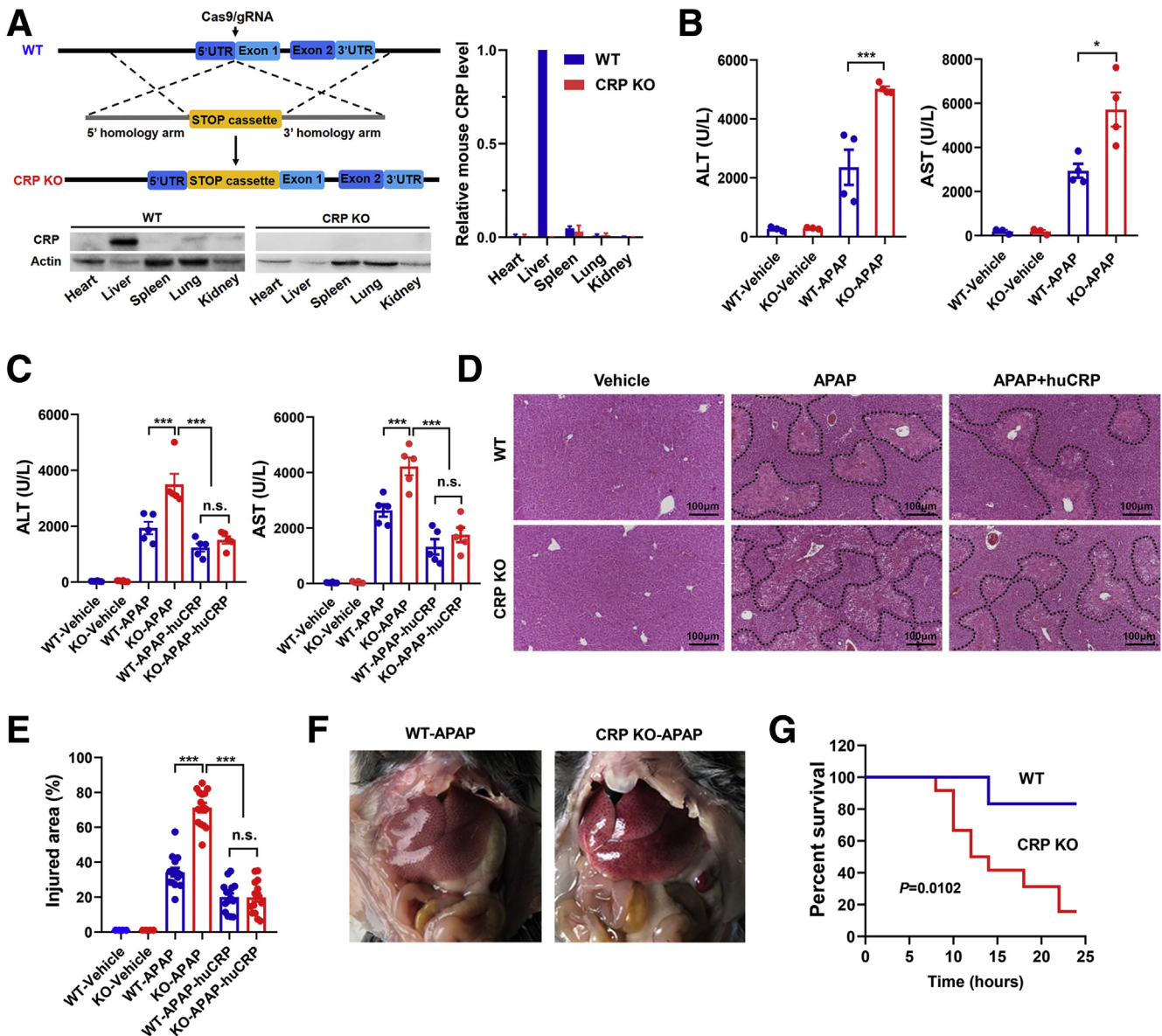


Figure 1. CRP protects against AILI in mice. (A) The strategy to generate CRP KO mice and their phenotypic identification ($n = 3$). (B) 300 mg/kg APAP was intraperitoneally injected into male WT or CRP KO mice for 24 hours. Circulating levels of ALT and AST ($n = 4$) were determined. (C–F) 350 mg/kg APAP was intraperitoneally injected into female WT or CRP KO mice for 24 hours without or with intravenous administration of 2.5 mg/kg huCRP both at 1 hour before and 4 hours post-APAP injection. The hepatic levels of huCRP 4 hours following injection were about 400 ng/mg tissue regardless of genotypes. (C) Circulating levels of ALT and AST ($n = 5$ –6), (D) hematoxylin and eosin staining of liver tissues, and (E) quantification of injured areas ($n = 15$) were then determined. (F) Representative images of intact livers of WT and CRP KO mice at 24 hours post-APAP injection. The liver of CRP KO mice exhibited more severe congestion. CRP KO exacerbated APAP-induced liver injury, whereas administration of huCRP alleviated disease severity in mice of both genotypes. (G) Survival of female WT and KO mice following intraperitoneal injection of 500 mg/kg APAP ($n = 12$). Survival analysis was performed using log-rank test. CRP KO mice were much more sensitive to death induced by high dose of APAP than WT mice. Data are presented as mean \pm SEM, * $P < .05$, *** $P < .001$, 1-way analysis of variance with Tukey post hoc. Data in Figure 1A–C and 1G are obtained from 1 individual experiment. Data in Figure 1E are pooled from 2– to 3 repetitive experiments.

Moreover, circulating markers of both liver injury (ie, ALT and AST) (Figure 4G) and overall inflammation (ie, interleukin [IL]-6 and tumor necrosis factor α [TNF- α]) (Figure 4H) were also comparable between CRP KO and WT mice at the early time points. These results together demonstrate that CRP has little effect in the early phase of AILI.

CRP Inhibits Complement Overactivation in the Late Phase of AILI

We then focused on how CRP acts in the late phase of AILI, wherein inflammatory injury induced by injured hepatocytes plays a major role. Danger signals exposed by injured hepatocytes can induce dysregulated production of

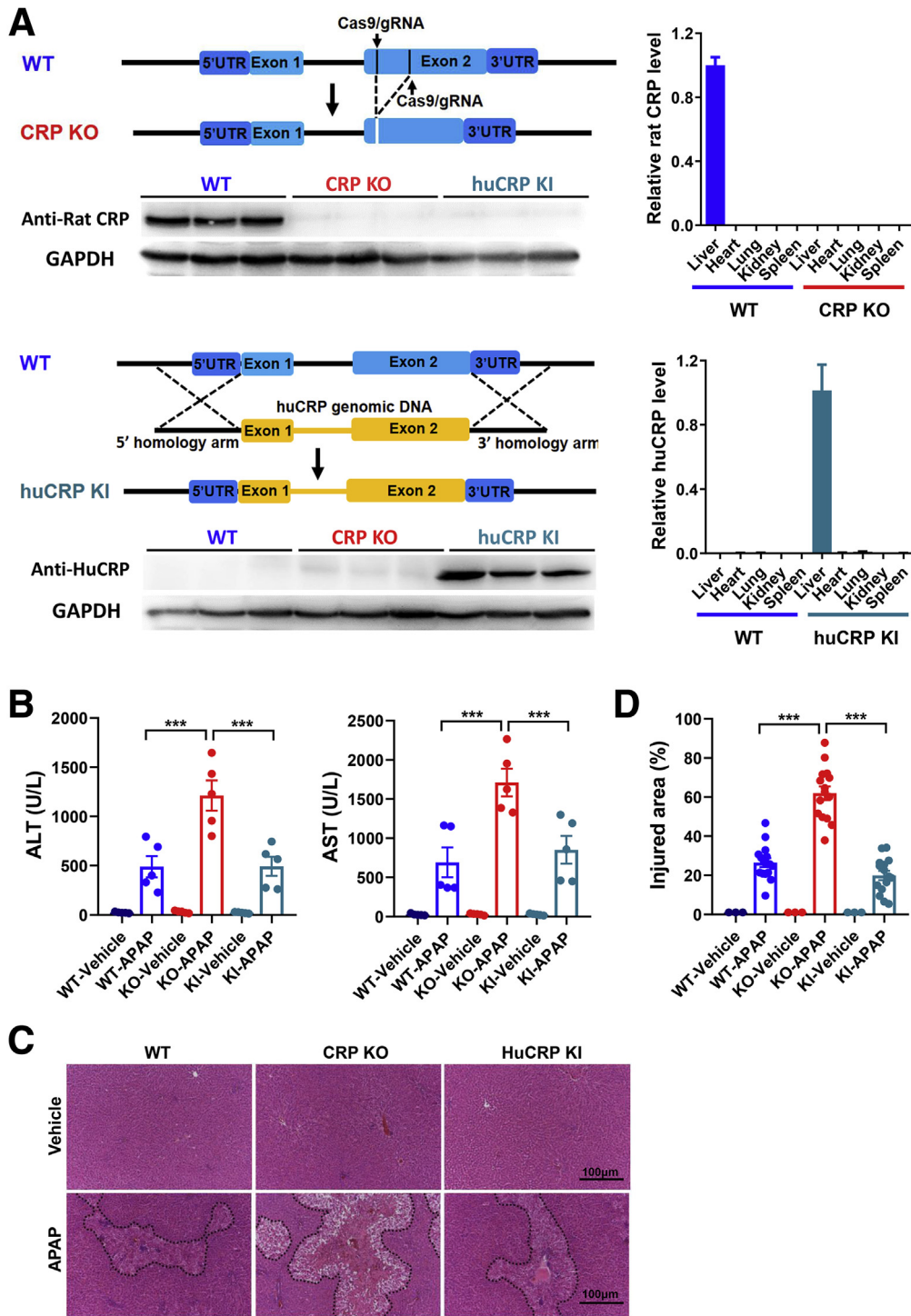


Figure 2. CRP protects against AILI in rats. (A) The strategies to generate CRP KO and huCRP KI rats and their phenotypic identification. A total of 750 mg/kg APAP was intraperitoneally injected into male WT, CRP KO, or huCRP KI rats for 24 hours. (B) Circulating levels of ALT and AST ($n = 6$), (C) hematoxylin and eosin staining of liver tissues, and (D) quantification of injured areas ($n = 15$) were then determined. CRP KO exacerbated APAP-induced liver injury, whereas huCRP KI rescued defects caused by CRP KO. Data are presented as mean \pm SEM, $**P < .01$, $***P < .001$, 1-way analysis of variance with Tukey post hoc. Data in Figure 2A and 2B are obtained from 1 individual experiment. Data in Figure 2D are pooled from 2 repetitive experiments.

cytokines from surrounding cells to exacerbate cell death and tissue injury.²⁴ Indeed, though liver injury was still comparable between the 2 genotypes at 6 hours post-AILI induction (Figure 5), circulating levels (Figure 6A) and hepatic expression of IL-1 β , IL-6, TNF- α , and IL-10 (albeit nonsignificantly) (Figure 6B) were already higher in CRP KO than in WT mice. However, CRP did not affect the expression of these cytokines in mouse hepatocytes

(Figure 6C), human hepatic cell line Hep3B (Figure 6D), and mouse bone marrow-derived macrophages (Figure 6E). These results suggest that the protection by CRP is not mediated through direct modulation of cytokine production in the late phase of AILI. The possibility that CRP may protect by promoting liver regeneration was also unlikely as CRP KO did not impair the proliferation of hepatocytes (Figure 7).

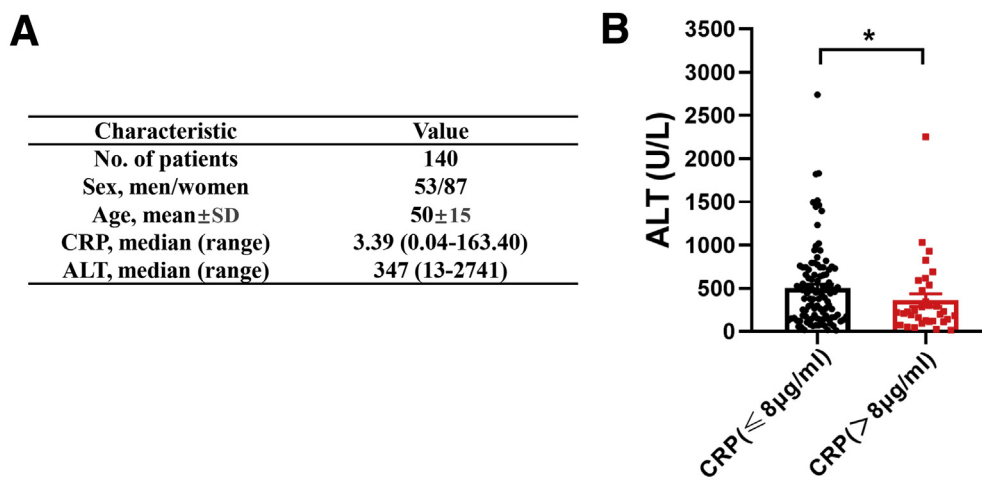


Figure 3. Drug-induced liver injury (DILI) patients with higher CRP levels manifest lower ALT levels. (A) Characteristics of DILI patients analyzed. These patients were diagnosed from November of 2016 to April of 2020 in the affiliated hospitals of Xi'an Jiaotong University according to following criteria: (1) manifestation of liver injury, (2) recent drug usage, and (3) exclusion of other liver diseases. (B) ALT levels of DILI patients divided into 2 groups according to their CRP levels. The Pearson's and Spearman's correlation coefficients between CRP and ALT levels are -0.113 ($p = 0.182$) and -0.107 ($p = 0.207$), respectively. * $P < .05$, 2-sided Kolmogorov-Smirnov test.

Injured hepatocytes can also induce complement overactivation that aggravates AILI.⁶ We and others have demonstrated that upon binding to membranes of injured cells, huCRP is converted to an activated conformation, which may both activate and inhibit complement by interacting with C1q and CFH, respectively.^{25–29} However, whether these interactions are conserved in rodents has been questioned by early studies.^{30–32} By using purified CRP coated on microtiter wells to mimic the membrane-bound, activated conformation,^{27,33} we showed that mouse, rat, and huCRP could all interact with C1q to activate exclusively the classic pathway of complement (Figure 8A–C). Moreover, they could bind CFH, resulting in a stronger recruitment of C1q but a weaker deposition of C3b in sera of WT than CRP KO mice after incubating with an artificial CRP ligand, ie, PC-KLH (Figure 8D). Importantly, CRP also reduced the generation of C3b and MAC (ie, C5b-9) on APAP-treated primary mouse hepatocytes and Hep3B cells (Figure 8E).

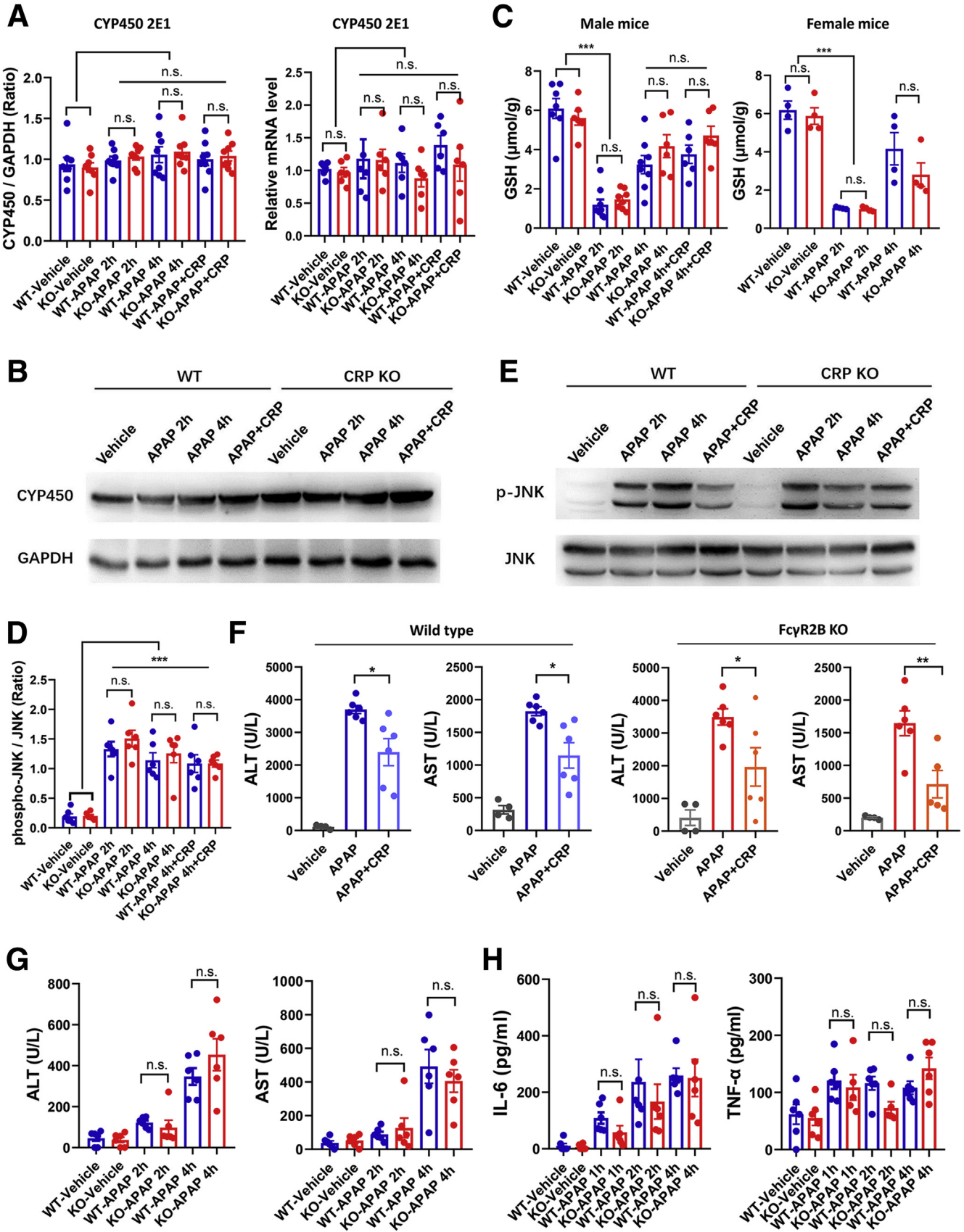
After confirming the interactions of CRP with complement are conserved across species, we further analyzed the activation of complement in livers of WT and KO mice with AILI. In WT mice, the initiation and activation of the classic pathway of complement in injured liver tissues was revealed by a focal deposition of C1q and C3b at 6 hours post-AILI induction (Figure 9A and B). At this time point, only a very weak signal of MAC could be observed, indicating that complement activation was still under tight control (Figure 9C and D). That control, however, was greatly impaired in CRP KO mice, as manifested by a much stronger hepatic deposition of C3b and MAC (Figure 9A–D). The dysregulated activation of complement in CRP KO mice persisted till 24 hours post-AILI induction, which could be explained by the nearly halved recruitment of CFH (Figure 9E and F). Indeed, administration of huCRP into WT mice with AILI conversely enhanced the recruitment of CFH

(Figure 9G and H). Additional experiments confirmed a comparable expression of major complement components between the 2 genotypes and the specificity of complement staining. These results together demonstrate that CRP inhibits complement overactivation in the late phase of AILI.

The Protection of CRP in AILI Depends on Complement-Regulatory Activities

To test whether CRP acts via regulating complement in AILI, we depleted complement by CVF administration or C3 KO (Figure 10A and B). While both manipulations alleviated AILI, co-treatment with CRP conferred no additional benefit, suggesting that the actions of CRP depend on complement. To exclude possible side effects caused by systemic depletion of complement, we further manipulated selectively the complement-regulatory activities of CRP (Figure 10C). Binding to PC exposed on injured cell membranes is the prerequisite for CRP to regulate complement.^{7–9,12,13} Accordingly, the expression of WT huCRP in livers of CRP KO mice greatly reduced the severity of AILI, whereas the expression of a PC binding mutant (D169A)^{34,35} was much less effective (Figure 10D–F). The initiators, effectors, regulators, and products of complement are recruited and generated in immediate proximity.³⁶ Therefore, even though the recruitment of C1q by CRP appears to be redundant in vivo (Figure 9A and B), it would still be required to effectively inhibit complement overactivation. This hypothesis is supported by the impaired capacity of a huCRP mutant defective in C1q binding (E88R)^{34,35} to alleviate AILI in CRP KO mice (Figure 10D–F).

To determine whether MAC or anaphylatoxins (ie, C3a and C5a) of overactivated complement plays a major role in aggravating AILI, we examined the effects of selective inhibitors against cellular receptors for anaphylatoxins. Inhibiting C3aR was protective in AILI (Figure 10G),



whereas inhibiting C5aR showed little effects (Figure 10H). Interestingly, co-treatment with CRP added no significant benefit to C3aR inhibition, but was still protective in the case of C5aR inhibition. These results suggest a major role of C3a but not C5a or MAC in amplifying the late phase injury of AILI. To determine the cell types that respond to C3a in AILI, we blocked the infiltration of neutrophils and monocytes with selective inhibitors. Blocking the infiltration of neutrophils but not monocytes alleviated AILI (Figure 10I and J). As co-treatment with CRP did not show further protection beyond neutrophile blocking, this cell type might be the major C3a responder that mediates tissue injury. Therefore, we conclude that CRP is an essential checkpoint to prevent complement overactivation, the detrimental effects of which in AILI might depend on C3a-dependent actions of neutrophils.

CRP Treatment Alleviates AILI

Having established a protective function of CRP in AILI, we asked whether it could be exploited as a treatment. Indeed, intraperitoneal administration of huCRP (2.5 mg/kg) into WT mice at 2 hours post-AILI induction markedly reduced liver injury, showing an efficacy comparable to 500 mg/kg N-acetyl cysteine (NAC) (Figure 11A–C), the sole available antidote approved to treat AILI through replenishing GSH depleted by overdosed APAP.^{1–3} However, NAC is effective only at the early phase of AILI,^{1–3} being unable to prevent liver injury when administered at 6 hours post-AILI induction (Figure 11D–F). By contrast, huCRP administered at this time point was still highly effective. Coadministration of NAC improved the therapeutic efficacy of huCRP at 2 hours but showed no improvement at 6 hours post-AILI induction. These again indicate that NAC and CRP differed in their mechanisms of protection. Given that people can tolerate high levels of circulating CRP, the administration of this protein might be a promising option to treat AILI with minimal side effects.

Discussion

AILI is a challenging clinical problem caused by APAP overdose. Metabolism of overdosed APAP in the liver initiates hepatocyte-autonomous injury, leading to exposure of danger signals that activate innate immune responses. This results in sterile inflammation that further amplifies hepatic injury.^{1–4} Complement is a major part of innate immune system activated immediately following tissue injury.⁵

Though complement overactivation has been shown to aggravate AILI,⁶ it is unclear which product of activated complement mediates the deleterious outcome. The present study suggests that instead of accelerating damage to already injured hepatocytes by MAC, recruiting or activating immune cells by anaphylatoxins, in particular C3a, play a dominant role in amplifying hepatic injury associated with complement overactivation. Neutrophils but not monocytes appear to be the main responders to C3a in this situation, consistent with their differential contributions to the late and early phases of AILI.^{37,38} As the numbers of neutrophils did not differ between WT and CRP KO livers 24 hours after AILI induction, C3a might mainly act via regulating the activation of neutrophils. It is thus worth noting that C3a can induce the release of DNA from neutrophils,³⁹ which is highly hepatotoxic.³⁷

Our results also uncover that CRP acts as a checkpoint that inhibits complement overactivation in AILI. CRP does not affect the initiation of AILI because its receptor Fc γ Rs are not expressed by hepatocytes.²³ Following the initiation of AILI, however, injured hepatocytes expose binding sites for CRP, licensing its conformational activation and regulation on complement. Intriguingly, mutations that impair complement-regulatory activities of CRP did not completely abrogate its effects. That could be due to residual activities of the examined mutants, or be explained by the multipath conversion of CRP to the activated conformation. As such, mutating PC binding site of CRP would prevent the membrane binding-dependent but not acidic pH- or oxidative stress-induced conformational activation.^{8–11} Mutating C1q binding site of CRP, on the other hand, might lower the regulatory efficiency of the mutant by impairing the colocalization of recruited CFH with its targets, ie, products of activated complement.

Most AILI cases are likely diagnosed well after the early, initiation phase. This would suggest that targeting innate immune responses in the late, amplification phase of AILI could lead to more effective, disease-modifying treatments.^{1–4} As such, both the previous study⁶ and our work indicate that complement might be a promising therapeutic target. There are currently available therapeutics, including specific monoclonal antibodies and protein or peptide drugs, that can inhibit complement overactivation.⁴⁰ However, systemic inhibition of complement may compromise innate defense to infection. By contrast, the complement regulatory activities of CRP should be more localized and selective because (1) the liver is the major organ where

Figure 4. (See previous page). CRP does not modulate APAP metabolism and JNK activation in the early phase of AILI. APAP was intraperitoneally injected into female or male WT and CRP KO mice for 2 or 4 hours. (A, B) The protein and messenger RNA (mRNA) levels of CYP450 2E1 (n = 6–8), (C) the levels of GSH (n = 4–8), and (D, E) the activation of JNK in liver tissues (n = 6) were then determined. (F) AILI was induced in female WT and Fc γ R2B KO mice by intraperitoneal injection of APAP. huCRP were administered 2 hours post-AILI induction. Circulating levels of ALT and AST were determined at 24 hours (n = 5–6). Circulating levels of (G) ALT and AST (n = 6), and (H) IL-6 and TNF- α (n = 5–6) were determined at 1, 2 or 4 hours post-AILI induction in WT and CRP KO mice. APAP metabolism, JNK activation, overall inflammation and hepatic injury were all comparable between the 2 genotypes at the early time points. Fc γ R2B KO also did not compromise the protection of CRP against AILI. Data are presented as mean \pm SEM, * P < .05, ** P < .01, *** P < .001, 1-way analysis of variance with Tukey post hoc. Data in Figure 4C (right) and 4F are obtained from 1 individual experiment. Data in Figure 4A, 4C (left), 4D, 4G, and 4H are pooled from 2 to 3 repetitive experiments.

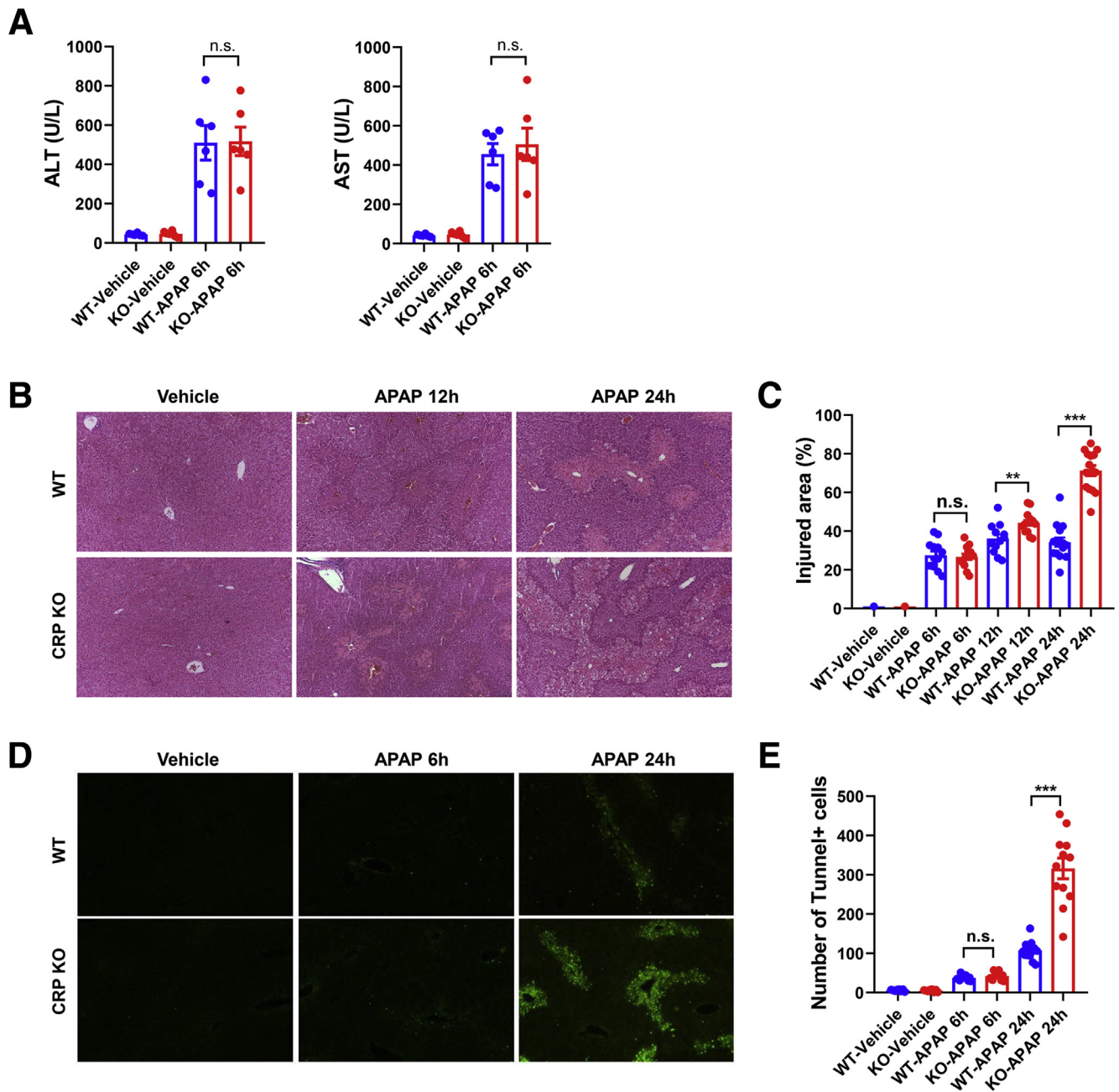
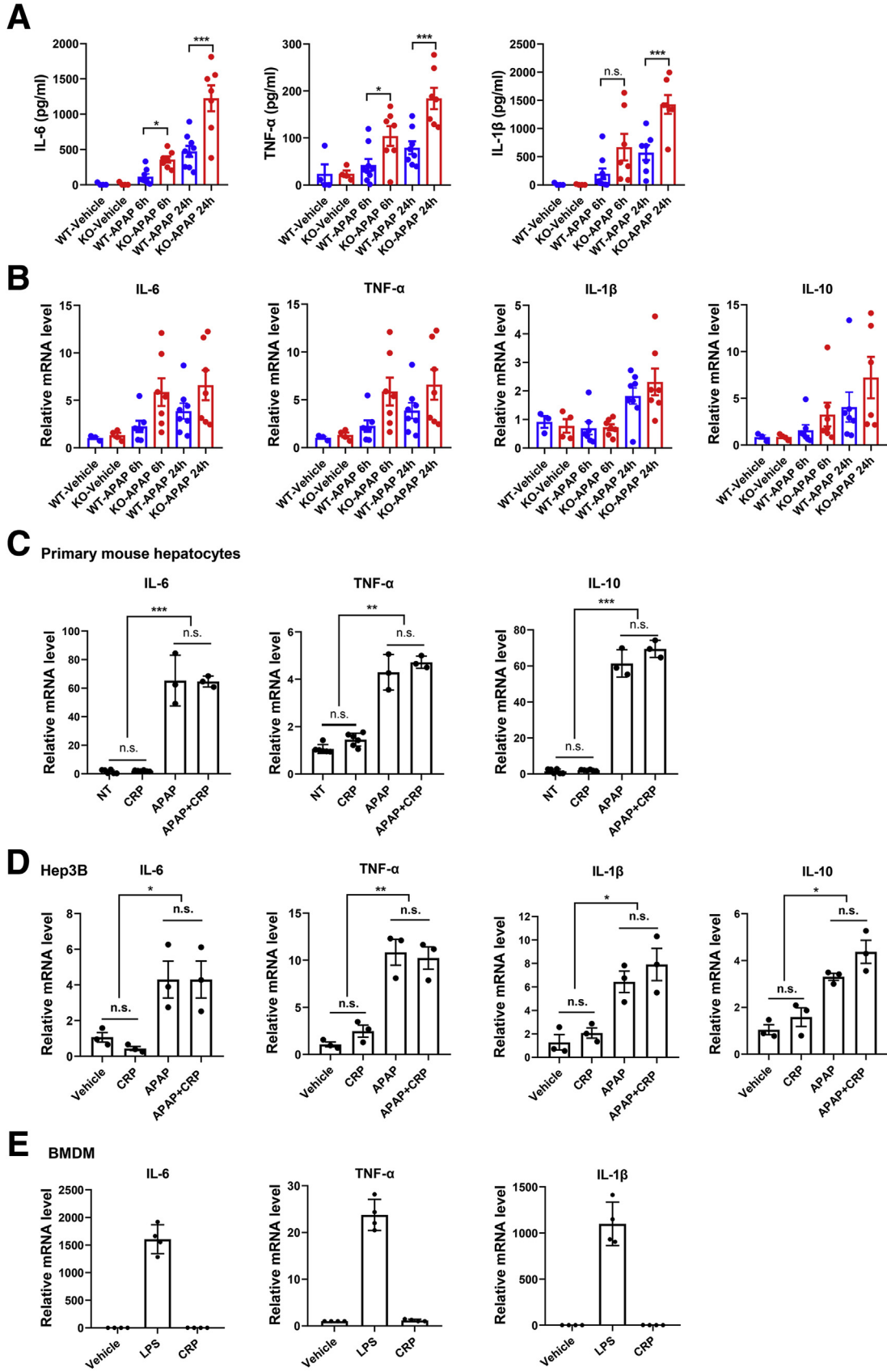


Figure 5. CRP does not affect tissue injury and cell death in the early phase of AILI. A total of 350 mg/kg APAP was intraperitoneally injected into WT and CRP KO mice for the indicated times. (A) Circulating levels of ALT and AST ($n = 6$), (B) hematoxylin and eosin staining of liver tissues, (C) quantification of injured areas ($n = 12-15$), (D) TUNEL staining of liver tissues, and (E) quantification of TUNEL-positive cells ($n = 8-12$). Tissue injury and cell death were comparable between the 2 genotypes even at 6 hours post-AILI induction, but were more exacerbated in CRP KO mice at the later time point, ie, 24 hours. Data are presented as mean \pm SEM, ** $P < .01$, *** $P < .001$, 1-way analysis of variance with Tukey post hoc. Data in Figure 5A is obtained from 1 individual experiment. Data in Figure 5C and 5E are pooled from 2 to 3 repetitive experiments.

administrated CRP accumulates^{41,42} and (2) conformational activation driven by conditions enriched at sites of tissue injury is the prerequisite for CRP to regulate complement.^{7-9,12,13} Therefore, CRP might be exploited as a safe and effective therapeutic approach to treat AILI as well as other liver diseases where complement overactivation plays an adverse role.

CRP is frequently claimed to be a soluble pattern recognition receptor.^{12,13} This claim, however, appears to be based largely on in vitro evidence, lacking unambiguous in vivo support. The exact contribution of neither pattern ligand (ie, PC) recognition, nor complement activation/regulation of CRP in inflammation and host defense has been firmly established using CRP KO animals with



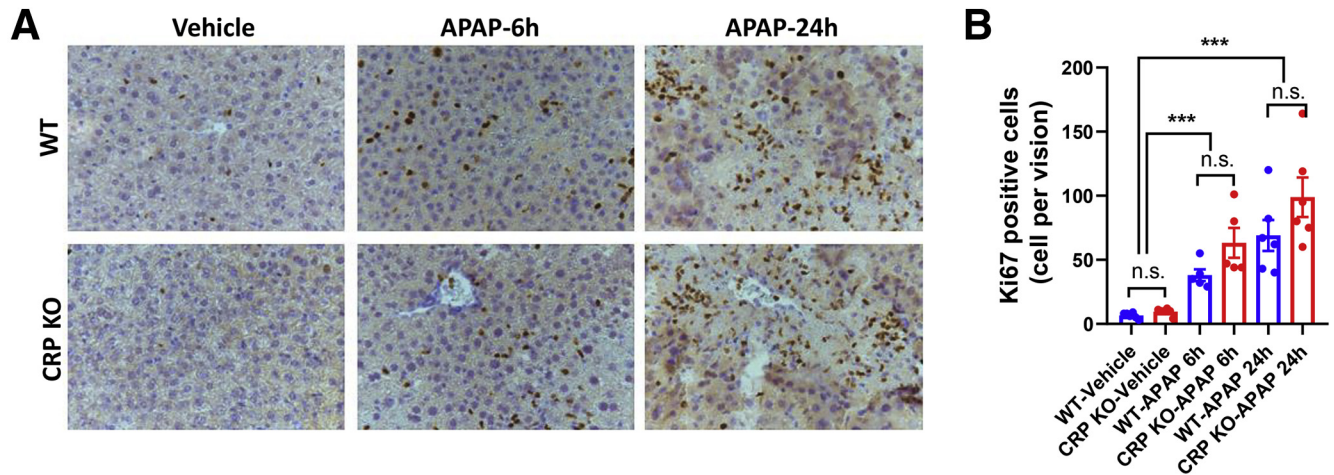


Figure 7. CRP does not modulate liver regeneration. A total of 350 mg/kg APAP was intraperitoneally injected into female WT and CRP KO mice for the indicated times. (A) Ki67 staining of liver tissues and (B) quantification of Ki67-positive cells ($n = 5-6$) are shown. CRP KO showed little effect on Ki67-positive cells. Data are presented as mean \pm SEM, *** $P < .001$, 1-way analysis of variance with Tukey post hoc. Data in Figure 7 are obtained from 1 individual experiment.

reconstituted WT or mutant huCRP. Indeed, most in vivo studies were conducted in mice and rats with transgenic expressed or administrated huCRP.^{12,13} That is probably due to the dogma that mice and rats are natural models with their endogenous CRP defective in expression or complement activation.^{7,14,15} But even so, the functions of transgenic or administrated huCRP would still be interfered by its endogenous counterparts, as mouse and rat CRP possess intact PC-binding capacity and can therefore compete with huCRP for ligands. Making the issue even more complicated, huCRP has been reported to be incapable of activating the classic pathway of mouse complement.^{31,32}

These would argue that mice and rats, the most popular animal models for basic and preclinical research, are not suitable to define the functions of (hu)CRP,^{7,14,15,31,32} thereby posing a strong barrier to investigating its actions and therapeutic potential in health and disease. The present study carefully reevaluated this issue by using CRP KO animals reconstituted with WT or mutant huCRP, and by using highly purified proteins in in vitro assays. As CRP is mainly produced by the liver, we chose to examine the actions of CRP in this organ to rule out possible interferences cause by the origin of CRP.⁴² Acute tissue injury, ie, AILI, was chosen to minimize differences between actions of distinct CRP conformations, which interact in a converging fashion with complement.⁷⁻¹¹ As such, both in vivo and in vitro experiments clearly demonstrate that rodent and huCRP behave

similarly in regulating complement and in protecting against AILI. We thus argue that when appropriately manipulated and controlled, mice and rats are suitable models to investigate the functions of CRP in inflammation and host defense.

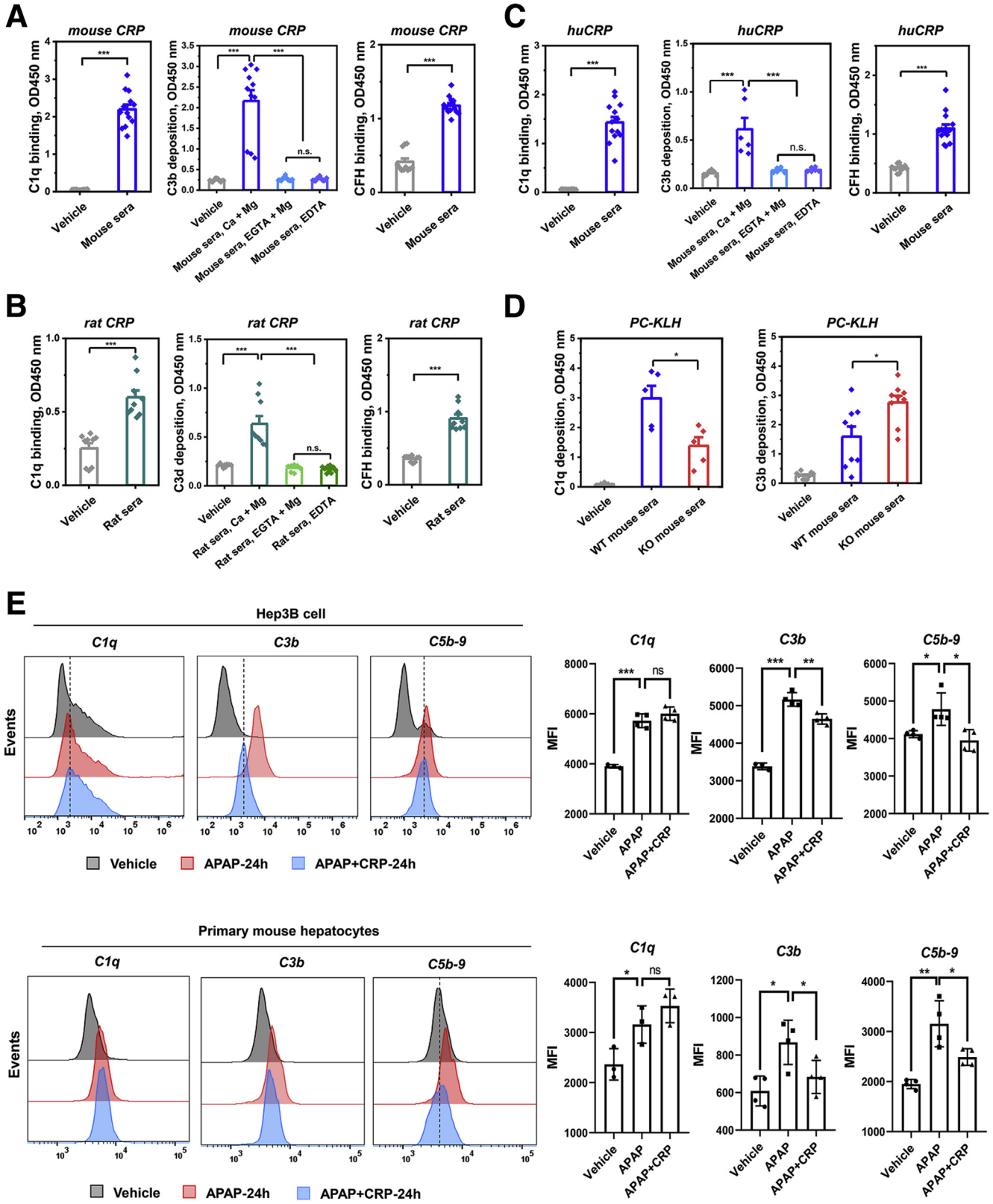
Materials and Methods

Preparation of Human and Rodent CRP

HuCRP (purity > 97%) purified from ascites was purchased from the BindingSite (Birmingham, United Kingdom; catalog number: BP300.X; lot number: 404353, 434009). The purchased proteins were re-purified with p-Amino-phenyl Phosphoryl Choline Gel (Thermo Fisher Scientific, Rockford, IL; catalog number: 20307; lot number: SF251770), eluted with TBS-Ca (10 mM Tris, 140 mM NaCl, 2 mM CaCl₂, pH 7.4) containing 10 mM PC, and dialyzed into TBS-Ca. The repurified CRP was passed through Detoxi-Gel Columns (Thermo Fisher Scientific; catalog number: 20344) to remove endotoxin when necessary. Mouse anti-huCRP monoclonal antibodies were generated as described.⁴³

Coding sequences of mouse and rat CRP were fused to a N-terminal His-tag. These sequences were cloned into pPick9k vector immediately following the signal peptide of α -factor and expressed in *Pichia pastoris* strain GS115 cells. CRP secreted into conditioned media were first purified with HisTrap FF (BioCatal, Beijing, China; catalog number:

Figure 6. (See previous page). CRP does not directly modulate cytokine production. A total of 350 mg/kg APAP was intraperitoneally injected into female WT and CRP KO mice for the indicated times. (A) Circulating levels of IL-6, TNF- α , and IL-1 β ($n = 6$) and (B) hepatic mRNA levels of IL-6, TNF- α , IL-1 β , and IL-10 ($n = 5-9$) were then determined. The levels of these cytokines were already higher in CRP KO mice at 6 hours post-AILI induction, and occurred before the aggravation of tissue injury. (C) Mouse primary hepatocytes ($n = 3$) or (D) human hepatic Hep3B cells ($n = 3$) were incubated with 5 mM APAP in the absence or presence of 20 μ g/mL CRP for 24 hours. (E) Mouse bone marrow-derived macrophages ($n = 4$) were incubated with 20 μ g/mL CRP or 100 ng/mL LPS for 24 hours. After incubation, mRNA levels of IL-6, TNF- α , IL-1 β , and IL-10 were determined with q-PCR. CRP did not affect the expression of these cytokines. Data are presented as mean \pm SEM, * $P < .05$, ** $P < .01$, *** $P < .001$, 1-way analysis of variance with Tukey post hoc. Data in Figure 6A and 6B are obtained from 1 individual experiment.



170901H5) and then with p-Aminophenyl Phosphoryl Choline Gel.

Induction of AILI in Mice and Rats

CRP KO mice of C57BL/6 background were generated by CRISPR/Cas9-mediated insertion of a STOP cassette at the translation start site of CRP gene via homologous recombination (Figure 1A) (Shanghai Biomodel Organism Science & Technology Development, Shanghai, China). CRP KO mice are fertile and grossly healthy. Fc γ R-2B KO mice were purchased from the Jackson Laboratory (Sacramento, CA; stock number: 002848). C3 KO mice were kindly provided by Prof. Ke Li (Xi'an Jiaotong University). WT C57BL/6 mice were obtained from the Animal Center of Xi'an Jiaotong University. Mice 8–9 weeks of age were fasted for 12 hours before injected intraperitoneally (intraperitoneal) with 300 or 350 mg/kg APAP (Macklin, Shanghai, China; catalog number: A800441) in phosphate-buffered saline (PBS). Fasting has little effect on protein levels of endogenous CRP in WT mice. When indicated, mice were treated with CVF (Quidel, San Diego, CA; catalog number: A600, lot number: 139349; 7.5 U/mice, intraperitoneally injected at 24 and 3 hours before AILI induction), C3aR antagonist (SB 290157; Tocris Bioscience, Bristol, United Kingdom; catalog number: 5473, lot number: 6A; 10 mg/kg, intraperitoneally injected at 1 hour before and 6 hours after AILI induction), C5aR antagonist (PMX53; Merck, Darmstadt, Germany; catalog number: L1003, lot number: 3231001; 1 mg/kg, intraperitoneally injected at 1 hour before and 8 hours after AILI induction), CXCR1/2 antagonist (SCH 527123; APEX BIO, Houston, TX; catalog number: A3802, lot number: 473727-83-2; 40 mg/kg, oral gavage at the same time of AILI induction), or CCR2/5 antagonist (Cenicriviroc; Selleckchem, Houston TX; catalog number: S8512, lot number: S851201; 15 mg/kg, subcutaneously injected at 1 hour after AILI induction). In rescue experiments, 2.5 mg/kg huCRP (diluted with PBS to 100 μ L at a final calcium concentration of 0.2 mM) were administered intravenously (intravenous) into WT and KO mice at both 1 hour before and 4 hours post-APAP injection. In treatment experiments, 2.5 mg/kg huCRP, 500 mg/kg N-Acetyl Cysteine (NAC) (Amresco, Solon, OH; catalog number: 0108) or their combination were intraperitoneal administrated into WT mice at 2 or 6 hours post-APAP

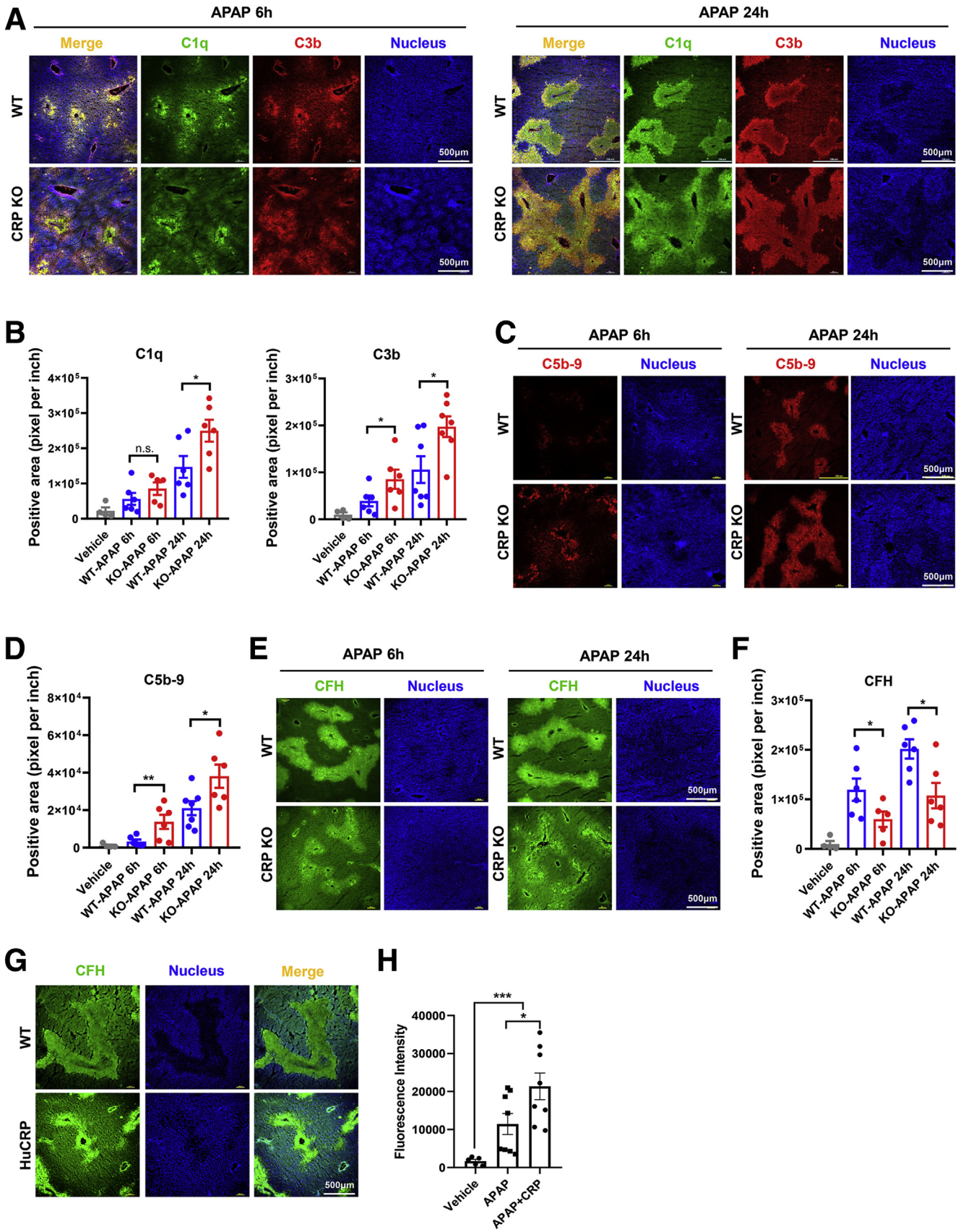
injection. intraperitoneal administration of CRP was tested in treatment experiments for 2 purposes: (1) exclude the possibility that the effects of CRP depend on route of infusion and (2) the circulating half-life of CRP is extended upon intraperitoneal vs intravenous administration. Tissues and sera were sampled at 6, 12 or 24 hours post-APAP injection.

CRP KO rats of SD background were generated by CRISPR/Cas9-mediated truncation of exon 2, while huCRP KI rats of SD background were generated by CRISPR/Cas9-mediated replacement of the exons and intron of rat CRP with that of huCRP (Figure 2A) (Shanghai Model Organisms, Shanghai, China). CRP KO rats are fertile and grossly healthy. WT SD rats were obtained from the Animal Center of Xi'an Jiaotong University. Male WT, CRP KO, and huCRP KI rats of 8 weeks of age were fasted for 12 hours before intraperitoneally injected with 750 mg/kg APAP in PBS. Tissues and sera were sampled at the indicated time points post-APAP injection.

Liver tissues were formaldehyde-fixed, paraffin-embedded, and cut into 4- μ m-thick sections with a Leica RM2235 HistoCore BIOCUT (Wetzlar, Germany). Liver histology was evaluated using tissue sections stained with a hematoxylin and eosin staining kit (Beyotime Biotechnology, Shanghai, China; catalog number: C0105; lot number: 040419190522). Liver regeneration was evaluated by Ki67 staining. Antigen-retrieved tissue sections were incubated with an anti-Ki67 antibody (Abcam, Cambridge, United Kingdom; catalog number: ab15580; lot number: GR3293897-1; 1:600) overnight at 4°C followed by incubation with a horseradish peroxidase-labeled Goat Anti-Rabbit IgG H&L (Beyotime Biotechnology; catalog number: A0208; 1:50) for 1 hour at room temperature. The sections were then developed with DAB and counterstained with hematoxylin for 40 seconds.

Hepatic levels of GSH (catalog number: A006-2-1; lot number: 20200109), circulating levels of ALT (catalog number: C009-2-1; lot number: 20190530) and AST (catalog number: C010-2-1; lot number: 20190531) were determined with kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Circulating levels of IL-6 (Catalog number: 431301; lot number: B282978), TNF- α (Catalog number: 430901; lot number: B265617) and IL-1 β (Catalog number: 432601; lot number: B284776) were determined with kits from BioLegend (San Diego, CA).

Figure 8. (See previous page). CRP prevents complement overactivation on injured hepatocytes. Microtiter wells with immobilized mouse (n = 11–16) (A) or rat CRP (n = 9) (B) were incubated with mouse or rat sera (10% or 1%) at the indicated conditions. The deposition of C1q, C3b, C3d, and CFH onto immobilized ligands was then determined with enzyme-linked immunosorbent assay. Mouse and rat CRP could both bind autologous C1q and CFH, and result in generation of C3b or C3d in a calcium-dependent manner, indicating the exclusive activation of the classic pathway of complement. (C) Microtiter wells with immobilized huCRP were incubated with mouse sera at the indicated conditions (n = 11–16). huCRP could bind mouse C1q and CFH, and activate exclusively the classic pathway of complement. (D) Microtiter wells with immobilized PC-KLH were incubated with sera of WT or CRP KO mice (n = 5–9). CRP KO reduced C1q binding but enhanced C3b deposition on PC-KLH. (E) Primary mouse hepatocytes or Hep3B cells treated with or without 20 or 6 mM APAP and 20 or 50 μ g/mL huCRP for 24 hours were incubated with 10% normal human sera. C1q, C3b, and C5b-9 deposition on cell surfaces were determined with flow cytometry (n = 3–4). Representative plots and MFI quantification were shown. The presence of CRP inhibited surface deposition of C3b and C5b-9. Data are presented as mean \pm SEM, **P* < .05, ****P* < .001, 1-way analysis of variance with Tukey post hoc or 2-tailed *t* test. Data in Figure 8D are obtained from 1 individual experiment. Data in Figure 8A–C are pooled from 2 repetitive experiments.



The hepatic levels of CYP450 2E1 and JNK activation were determined by immunoblotting. Tissues (0.08 g) were homogenized in 1 mL RIPA buffer containing 10 μ L PMSF (Solarbio, Beijing, China; catalog number: R0020), 10 μ L phosphatase inhibitor cocktail 3 (Sigma-Aldrich St. Louis, MO; catalog number: P0044; lot number: 108M4001V) and 10 μ L protease inhibitor cocktail (Sigma-Aldrich; catalog number: I3786; lot number: 962M4093V). The supernatants (100 μ g total protein) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with an anti-CYP450 2E1 antibody (Abcam; catalog number: ab28146; lot number: GR3260945-9; 1:5000), an anti-JNK antibody (CST, Danvers, MA; catalog number: 9252; lot number: 17; 1:1000), an anti-p-JNK antibody (CST; catalog number: 4668; lot number: 15; 1:1000), or an anti-GAPDH antibody (CST; catalog number: 2118; lot number: 14; 1:5000). Membranes were blocked with 5% fat-free milk and were visualized with ECL.

Total RNA of liver tissues was extracted with RNAiso Plus (Takara, Shiga, Japan; catalog number: 9109; lot number: AKA3402, AKA5802). cDNA was synthesized from 3 μ g total RNA using PrimeScript RT Master Mix system (Takara; catalog number: RR036A; lot number: AK4102, AK4403). Gene expression was determined with quantitative polymerase chain reaction (q-PCR) using RealStar Green Power Mixture (Genestar, Beijing, China; catalog number: A311; lot number: 7AB01) in a StepOne Plus real-time PCR system (Thermo Fisher Scientific). The gene expression levels were normalized to that of GAPDH. The primer sequences used were: huCRP (forward: 5'-AATTCAGGCCCTTGATCACT-3'; reverse: 5'-TCCATGTGGCAAACAAGATGGCTA-3'); mouse CRP (forward: 5'-CATGATCAGCTTCTCTCGGACT-3'; reverse: 5'-TGTTCAGTGGCTTCTTTGACTCT-3'); rat CRP (forward: 5'-TGTCTCTATGCCACGCTGA-3'; reverse: 5'-AAACCCITGACCCCTAGTCCA-3'); mouse IL-6 (forward: 5'-ACCACGGCCTCCCTACTT-3'; reverse: 5'-CATTTCCACGATTTCCAGA-3'); mouse TNF- α (forward: 5'-CAGGCGGTGCCTATGTCTC-3'; reverse: 5'-CGATCACCCCGAAGTTCAGTAG-3'); mouse IL-1 β (forward: 5'-GCAACTGTTCTGAACTCAACT-3'; reverse: 5'-ATCTTTTGGGGTCCGTCAACT-3'); mouse IL-10 (forward: 5'-GCTTFACTGACTGGCATGAG-3'; reverse: 5'-CGCAGCTCTAGGAGCATGTG-3'); mouse C1q (forward: 5'-AAAGGCAATCCAGGCAATATCA-3'; reverse: 5'-TGGTTCTGGTATGGACTCTCC-3'); mouse C3 (forward: 5'-CCAGCTCCCCATTAGCTCTG-3'; reverse: 5'-GCACTTGCCCTTTAGGAAGTC-3'); mouse CFH (forward: 5'-AGGCTCGTGGTCA-GAACAAAC-3'; reverse: 5'-GTTAGACGCCACCCATTTTCC-3');

mouse C5 (forward: 5'-GAACAAACCTACGTCATTTTCAGC-3'; reverse: 5'-GTCAACAGTGCCCGGTTTT-3'); mouse CYP450 2E1 (forward: 5'-CGTTGCCTTGCTTGTCTGGA-3'; reverse: 5'-AAGAAAGGAATTGGGAAAGGTCC-3'); mouse CYP450 1A2 (forward: 5'-AGTACATCTCCTTAGCCCCAG-3'; reverse: 5'-GGTCCGGTGGATTCTTCAG-3'); CYP450 1A1 (forward: 5'-GACCCTTACAAGTATTTGGTGGT-3'; reverse: 5'-GGTATC-CAGAGCCAGTAACCT-3'); CYP450 1B1 (forward: 5'-CACCAGCCT-TAGTGCAGACAG-3'; reverse: 5'-GAGGACCACGGTTTCCGTTG-3'); CYP450 2B10 (forward: 5'-AAAGTCCCGTGGCAACTTCC-3'; reverse: 5'-TTGGCTCAACGACAGCAACT-3'); mouse GAPDH (forward: 5'-GGGCTACTGAGGACCAGGTT-3'; reverse: 5'-TGCTGTAGCCGTATTCATTGTCA-3').

Animals (5 mice or 8 rats per cage) were maintained under specific pathogen-free conditions with a 12-hour light-dark cycle, and had ad libitum access to water and normal diet. All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by NIH, and were conducted according to the protocols approved by the Ethics Committee of Animal Experiments of Xi'an Jiaotong University.

Fluorescence Imaging

To visualize the deposition of complement components, 8- μ m-thick cryosections of mouse liver tissues were prepared, fixed in cold acetone for 15 minutes, washed with PBS and then blocked with 3% bovine serum albumin (BSA) for 1 hour at room temperature. The sections were incubated with an anti-C1q antibody (Abcam; catalog number: ab11861; lot number: GR271687-5; 1:50), an anti-CFH antibody (Abcam; catalog number: ab8842; lot number: GR305836-10; 1:200), an anti-C3b antibody (Hycult Biotech, Uden, the Netherlands; catalog number: HM1065; lot number: 24277M0518-A; 1:200), or an anti-C5b-9 antibody (Abcam; catalog number: ab55811; lot number: GR3210984-1; 1:1000) at 4°C overnight followed by an FITC-labeled rabbit anti-rat IgG H&L (Abcam; catalog number: ab6730; lot number: GR309441-15; 1:1000), an DyLight 650-labeled goat anti-mouse IgG H&L (Abcam; catalog number: ab96882; lot number: GR207688-1; 1:200), or an Alexa Fluor 555-labeled goat anti-rabbit IgG H&L (Abcam; catalog number: ab150078; lot number: GR3184964-2; 1:500) for 1 hour at room temperature. Quality controls were prepared by omitting the primary antibodies or using an irrelevant primary antibody, ie, monoclonal antibody 8D8 against huCRP. The nucleus were counter-stained with 2 μ g/mL DAPI (Solarbio; catalog

Figure 9. (See previous page). CRP KO prevents complement overactivation in AILI. A total of 350 mg/kg APAP was intraperitoneally injected into female WT and CRP KO mice for the indicated times. The hepatic deposition of (A, B) C1q/C3b (n = 4–9), (C, D) C5b–9 (n = 4–6), and (E, F) CFH (n = 4–6) in tissue cryosections were imaged and quantified. CRP KO mice exhibited much stronger deposition of C3b and C5b–9 but markedly reduced recruitment of CFH compared with WT mice. (G, H) Liver tissues were collected 24 hours after AILI induction with or without huCRP administration into WT mice (2.5 mg/kg). The deposition of CFH in tissue cryosections were imaged and quantified (n = 4–8). Tissue injury was markedly reduced by CRP administration, whereas intensities of deposited CFH were significantly enhanced. Staining signals were lost when omitting primary antibodies. Data are presented as mean \pm SEM, * P < .05, ** P < .01, 1-way analysis of variance with Tukey post hoc. Data in Figure 9B, 9D, and 9F are pooled from 3 repetitive experiments. Data in Figure 9H are obtained from 1 individual experiment.

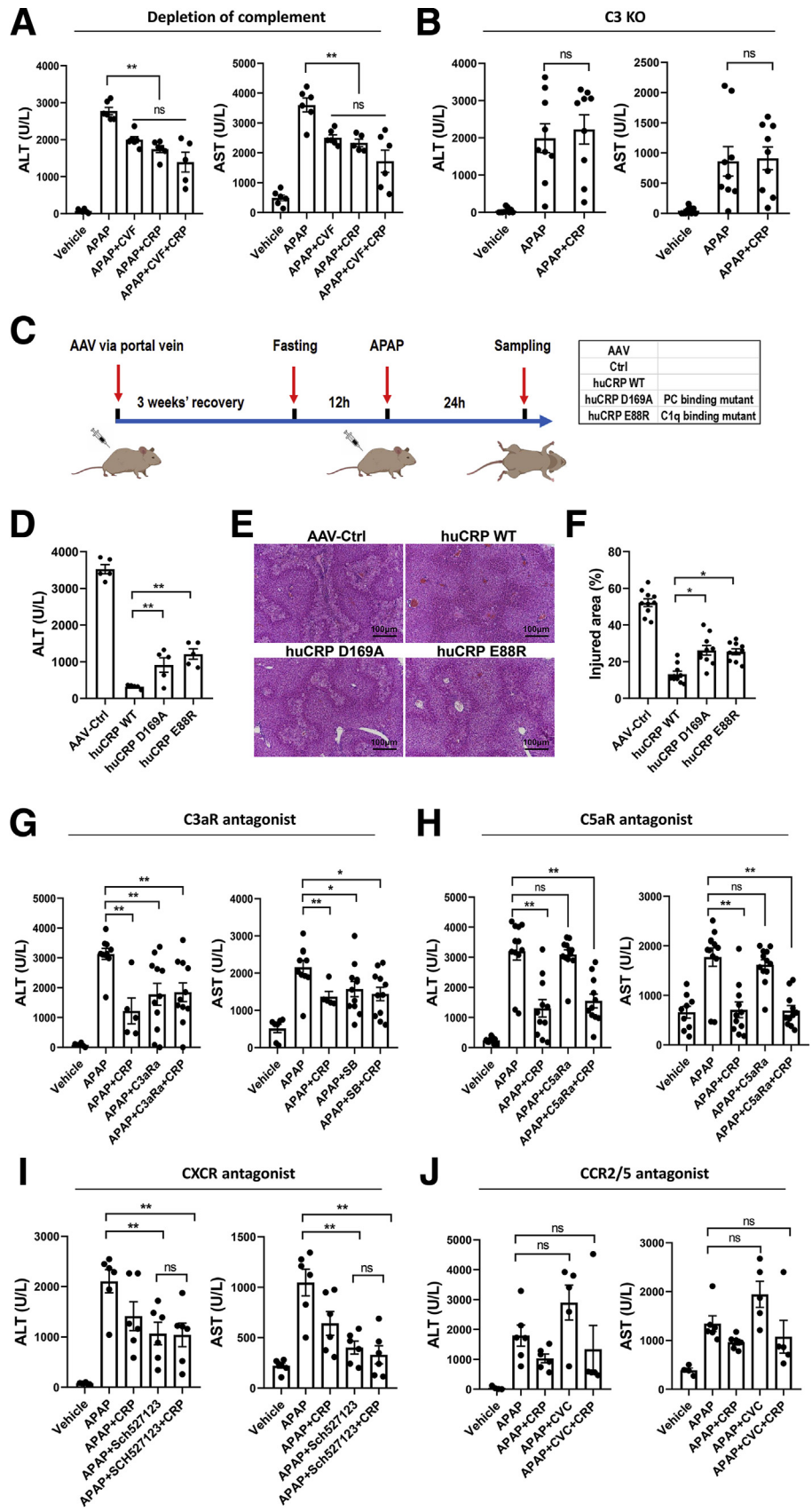


Figure 10. CRP inhibits C3a-mediated and neutrophil-dependent amplification of inflammation in the late phase of AILI. HuCRP were administered 2 hours post-AILI induction (A) in female WT mice pretreated with CVF (7.5 U/mice, twice) (n = 5–6) or (B) in C3 KO mice (n = 9). Both pre-depletion of complement with CVF and C3 KO abrogated the protection of huCRP. (C) Control adeno-associated virus (AAV) or AAVs expressing huCRP WT, D169A mutant (defective in PC binding), or E88R mutant (defective in C1q binding) were injected into female CRP KO mice via portal vein. After recovery for 3 weeks, 350 mg/kg APAP were intraperitoneally injected into these mice. (D) Circulating levels of ALT, (E) hematoxylin and eosin staining of liver tissues, and (F) quantification of injured areas (n = 5–10) were then determined. The protection by hepatic expression of huCRP were compromised by mutations impairing its complement regulatory activities. huCRP were administered 2 hours post-AILI induction in female WT mice treated with (G) SB 290157 (C3aR antagonist), (H) PMX 53 (C5aR antagonist), (I) SCH 527123 (CXCR1/2 inhibitor), or (J) cenicriviroc (CCR2/CCR5 inhibitor). The C3aR antagonist and CXCR1/2 inhibitor abrogated the protection of huCRP, whereas the C5aR antagonist and CCR2/5 inhibitor showed little effect. Data are presented as mean ± SEM, *P < .05, **P < .01, 1-way analysis of variance with Tukey post hoc. Data in Figure 10D, 10F, 10I, and 10J are obtained from 1 individual experiment. Data in Figure 10A, 10B, 10G, and 10H are pooled from 2 to 3 repetitive experiments.

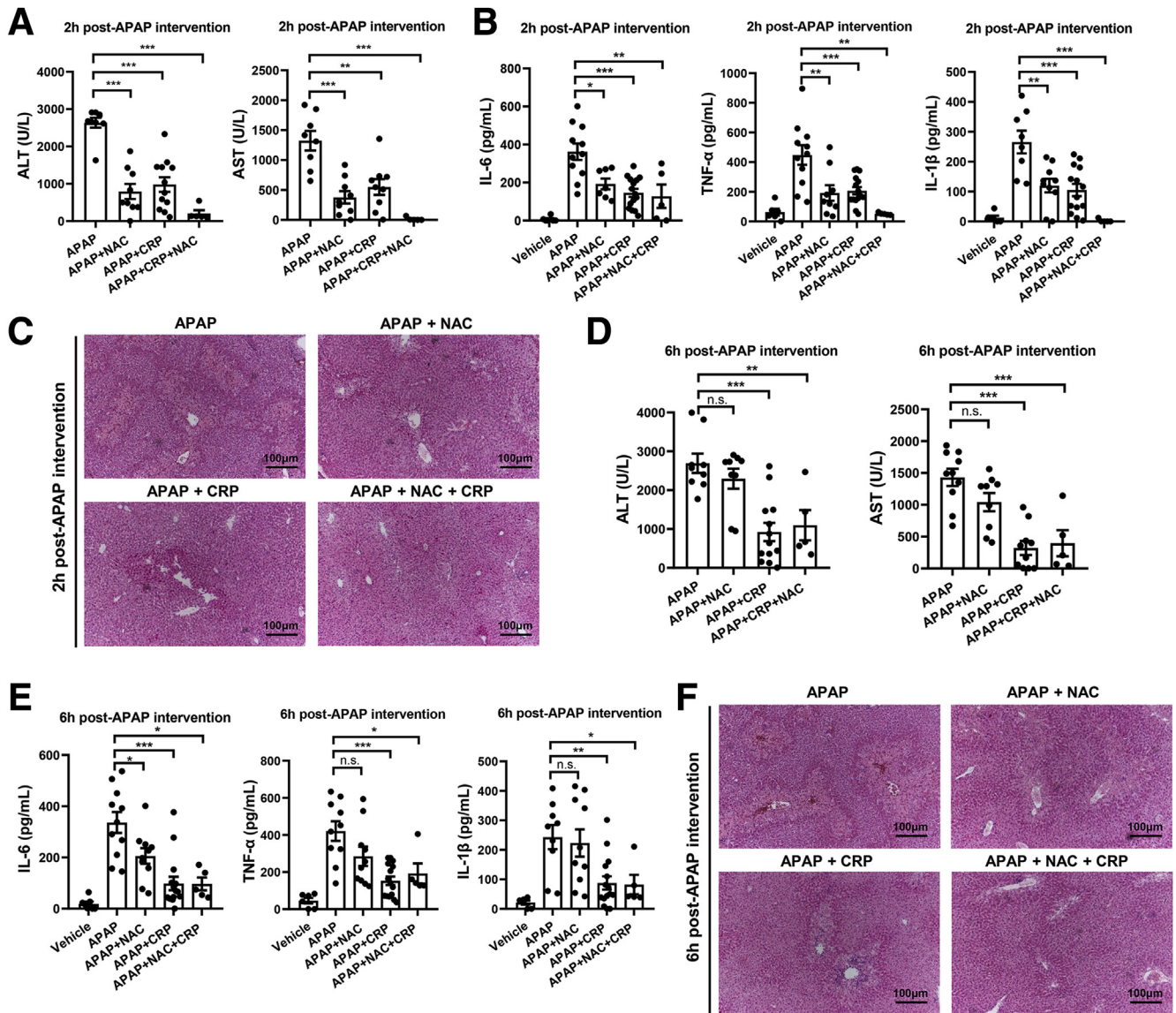


Figure 11. CRP treatment attenuates tissue injury in AILI with a prolonged therapeutic time window. A total of 350 mg/kg APAP was intraperitoneally injected into female WT mice for 24 hours. 2.5 mg/kg CRP, 500 mg/kg NAC, or their combination were intraperitoneal administrated at 2 or 6 hours post-APAP injection. (A, D) Circulating levels of ALT and AST ($n = 5-12$), (B, E) circulating levels of IL-6, TNF- α , and IL-1 β ($n = 5-16$), and (C, F) hematoxylin and eosin staining of liver tissues were then determined. CRP treatment reduced the severity of AILI both at 2 and 6 hours post-APAP injection, whereas NAC treatment was effective only at 2 hours post-APAP injection. Data are presented as mean \pm SEM, * $P < .05$, ** $P < .01$, *** $P < .001$, 1-way analysis of variance with Tukey post hoc. Data in Figure 11A, 11B, 11D, and 11E are pooled from 3 repetitive experiments.

number: C0060; lot number:20181212) for 10 minutes. The sections were then examined by a Nikon A1R/Ti2-E confocal microscopy (Tokyo, Japan) in DAPI-Fluoromount-G clear mounting media (SouthernBiotech, Birmingham, AL; catalog number: 0100-20; lot number: C3619-PI19B). To visualize cell death, cryosections of mouse liver tissues were stained with a TUNEL kit (Beyotime Biotechnology; catalog number: C1088; lot number: 071018180920).

Cell Activation

Mouse primary hepatocytes⁴⁴ and marrow-derived macrophages⁴⁵ were isolated as described. Human

hepatocellular carcinoma cell line Hep3B were obtained from cell bank of Chinese Academy of Sciences (Shanghai, China). Cells were treated with APAP (5 mM), CRP (20 μ g/mL), or lipopolysaccharide (100 ng/mL) for 24 hours. Total RNA was extracted with RNAiso Plus (Takara, Shiga, Japan; catalog number: 9109; lot number: AKA3402, AKA5802). cDNA was synthesized from 2 μ g total RNA using PrimeScript RT Master Mix system (Takara; catalog number: RR036A; lot number: AK4102, AK4403). Gene expression was determined with q-PCR using RealStar Green Power Mixture (Genestar, Beijing, China; catalog number: A311; lot number: 7AB01) in a StepOne Plus real-time PCR system (Thermo Fisher Scientific). The gene expression levels were

normalized to that of GAPDH. The primer sequences used were: human IL-6 (forward: 5'-ACTCACCTCTTCA-GAACGAATTG-3'; reverse: 5'-CCATCTTTGGAAGGTTTCAGGTTG-3'); human TNF- α (forward: 5'-GAGGCCAAGCCCTGGTATG-3'; reverse: 5'-CGGGCCGATTGATCTCAGC-3'); human IL-1 β (forward: 5'-ATGATGGCTTATTACAGTGGCAA-3'; reverse: 5'-GTCGGAGATTTCGTAGCTGGA-3'); human IL-10 (forward: 5'-GACTTTAAGGGTTACCTGGGTTG-3'; reverse: 5'-TCA-CATGCGCCTTGATGTCTG-3'); human GAPDH (forward: 5'-CTGGGCTACTGAGCACC-3'; reverse: 5'-AAGTGGTCGTT-GAGGGCAATG-3'). Primer sequences of mouse genes were described previously.

Complement Binding and Activation

The interactions of CRP with complement were examined by enzyme-linked immunosorbent assay and flow cytometry. In enzyme-linked immunosorbent assay assays, 20 $\mu\text{g}/\text{mL}$ PC-KLH (Santa Cruz Biotechnology, Dallas, TX; catalog number: sc-396490; lot number: J0915) or CRP was immobilized onto microtiter wells in coating buffer (0.05 M NaHCO_3 - Na_2CO_3 , pH 9.6) or in TBS-Ca (10 mM Tris, 140 mM NaCl, 2 mM CaCl_2 , pH 7.4) to mimic the membrane-bound, activated conformation.³³ After overnight immobilization at 4°C, microtiter wells were washed and blocked with 1% BSA in TBS-Ca for 1 hour at 37°C. Mouse or rat sera (10% for C1q and CFH binding or 1% for C3 activation in 1% BSA, PBS, 0.15 mM CaCl_2 , 0.5 mM MgSO_4) without or with 5 mM EGTA or EDTA were then added. After incubation for 1 hour (for C1q and CFH binding) or 0.5 hour (for C3 activation), microtiter wells were washed and further incubated with anti-C1q (Abcam; catalog number: ab182451; lot number: GR3266536-2; 1:2000), anti-CFH (Abcam; catalog number: ab8842; lot number: GR324556-4; 1:1000), anti-C3b (Hycult Biotech; catalog number: HM1605; lot number: 24277M0518-A; 1:500), or anti-C3d (R&D Systems, Minneapolis, MN; catalog number: AF2655; lot number: VFL0518101; 0.5 $\mu\text{g}/\text{mL}$) antibodies. The deposition of these complement components was then detected with horseradish peroxidase-conjugated secondary antibodies followed by development with tetramethyl benzidine.

In flow cytometry assays, mouse primary hepatocytes or Hep3B cells were treated with or without APAP (20 or 6 mM) \pm huCRP (20 or 50 $\mu\text{g}/\text{mL}$) in serum-free media for 24 hours. Cells were then incubated with 10% normal human sera (ZKKA Biotechnology, Beijing, China; catalog number: ZK911250) for 1.5 hours at 37°C. FITC-labeled anti-C1q (Abcam; catalog number: ab4223; lot number: GR3235926-4; 1:100), APC-labeled anti-C3b (BioLegend; catalog number: 846106; lot number: B264941; 1:80), or anti-C5b-9 (Abcam; catalog number: ab55811; lot number: GR3210984-1; 1:1000) and a Alexa Fluor 488-labeled secondary antibody (Abcam; catalog number: ab150077; lot number: GR3203087-1; 1:1000) were then added for 15 min at 4°C. Following washing, cells were resuspended in precooled PBS and examined with a A00-1-1102 flow cytometer (Beckman Coulter, Atlanta, GA).

Statistical Analysis

Data are presented as mean \pm SEM. Statistical analysis was performed by 2-tailed Student's *t* test, 1-way analysis of variance with Tukey post hoc or Kolmogorov-Smirnov test as appropriate. Differences were considered significant at values of $P < .05$.

All authors had access to the study data and had reviewed and approved the final manuscript.

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