# A complement-dependent balance between hepatic ischemia/reperfusion injury and liver regeneration in mice

Songqing He,[1,2] Carl Atkinson,[1] Fei Qiao,[1] Katherine Cianflone,[3] Xiaoping Chen,[2] and Stephen Tomlinson[1]

[1]Department of Microbiology and Immunology, Darby Children's Research Institute, Medical University of South Carolina, Charleston, South Carolina, USA. [2]Hepatic Surgery Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People's Republic of China. [3]Centre de Recherche Institut Universitaire de Cardiologie et de Pneumologie de Quebec, Université Laval, Quebec City, Quebec, Canada.

**Massive liver resection and small-for-size liver transplantation pose a therapeutic challenge, due to increased susceptibility of the remnant/graft to ischemia reperfusion injury (IRI) and impaired regeneration. We investigated the dual role of complement in IRI versus regeneration in mice. Complement component 3 (C3) deficiency and complement inhibition with complement receptor 2–complement receptor 1–related protein y (CR2-Crry, an inhibitor of C3 activation) provided protection from hepatic IRI, and while C3 deficiency also impaired liver regeneration following partial hepatectomy (PHx), the effect of CR2-Crry in this context was dose dependent. In a combined model of IRI and PHx, either C3 deficiency or high-dose CR2-Crry resulted in steatosis, severe hepatic injury, and high mortality, whereas low-dose CR2-Crry was protective and actually increased hepatic proliferative responses relative to control mice. Reconstitution experiments revealed an important role for the C3a degradation product acylation-stimulating protein (ASP) in the balance between inflammation/injury versus regeneration. Furthermore, liver regeneration was dependent on the putative ASP receptor, C5L2. Several potential mechanisms of hepatoprotection and recovery were identified in mice treated with low-dose CR2-Crry, including enhanced IL-6 expression and STAT3 activation, reduced hepatic ATP depletion, and attenuated oxidative stress. These data indicate that a threshold of complement activation, involving ASP and C5L2, promotes liver regeneration and suggest a balance between complement-dependent injury and regeneration.**

## Introduction

Liver resection has become an increasingly safe procedure, but certain procedures remain high risk, such as massive liver resection and small-for-size (SFS) liver transplantation (1–3). Massive hepatic resection is the only option for some patients with primary or secondary liver tumors. With regard to SFS transplantation, the use of partial liver grafts has the potential to substantially reduce the donor shortage by allowing the donor organ to be split between 2 recipients. In addition, live donor liver transplantation is emerging as an option for some patients, a procedure requiring liver resection and regeneration in the donor and regeneration in the recipient.

The failure of a partial liver to regenerate is considered a critical contributing factor in postsurgical primary liver dysfunction and liver failure, and minimal viable liver volume required for regeneration, following either massive liver resection or SFS transplantation, is an important concept (1–3). Impaired liver regeneration and liver dysfunction has been strongly linked to the extent of hepatic ischemia/reperfusion injury (IRI), an unavoidable consequence of the surgical procedures, and studies in rodent models have shown that small liver fragments and SFS grafts are more susceptible to IRI (3–7). Although the precise mechanisms responsible for liver dysfunction and failure in small liver remnants and SFS grafts are not well understood, complement appears to play an important role in both IRI and liver regeneration.

Studies using rat models indicate a central role for complement in hepatic IRI. These studies have variously shown that complement inhibition with soluble complement receptor 1 (sCR1) reduces Kupffer cell activation, neutrophil accumulation, and microvascular dysfunction and injury in rat liver (8–11). C1-inhibitor and C5a receptor antagonist have also been shown to be protective in rat models of hepatic IRI (9–11), and a role for the terminal cytolytic membrane attack complex (MAC) is indicated by a study showing that deficiency of complement component 6 (C6) (a component of the MAC) is associated with protection from injury, following hepatic IRI in rats (12).

In addition to its role in hepatic IRI, recent evidence indicates that complement activation is required for normal liver regeneration, following either resection or toxic injury (13–17). Using a murine model of 70% partial hepatectomy (PHx), it was shown that C3- and C5-deficient mice exhibited impaired liver regeneration and high mortality after liver resection and that reconstitution of the complement-deficient mice with C3a or C5a improved the regenerative response (14). Data indicate that the complement activation products C3a and C5a play an important role in the proliferative response and hepatocyte regeneration via an effect on TNF-α and IL-6 expression and the subsequent expression of the transcription factors NF-κB and STAT3 (14). A similar role for complement and for C3a receptor (C3aR) and

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**Conflict of interest:** Stephen Tomlinson is a consultant for Taligen Therapeutics Inc., which is developing complement inhibitors for therapeutic use.

**Nonstandard abbreviations used:** ALT, alanine aminotransferase; ASP, acylation-stimulating protein; C3, complement component 3; C3aR, C3a receptor; C5L2, C5a-like receptor 2; CR2, complement receptor 2; Crry, complement receptor 1–related protein y; GPX1, GSH peroxidase; GSH, glutathione; IRI, I/R injury; I/R, ischemia/reperfusion; MDA, malondialdehyde; MPO, myeloperoxidase; PHx, partial hepatectomy; SFS, small for size.

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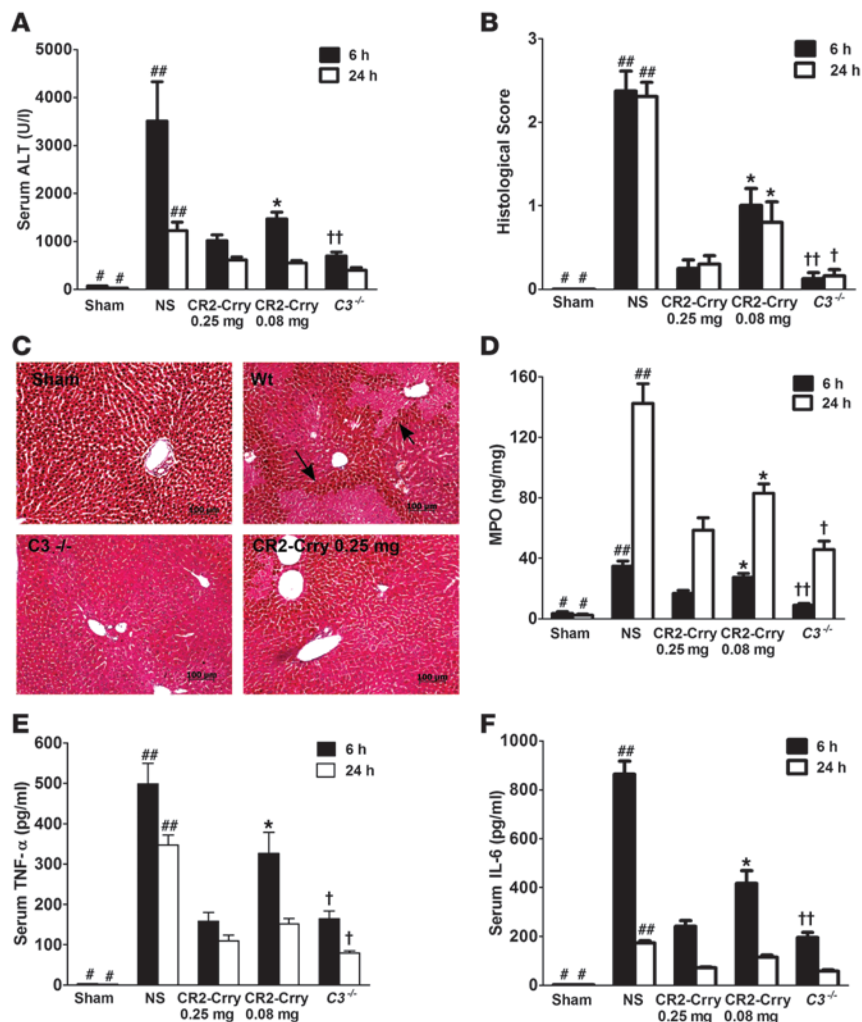


**Figure 1**
Complement deficiency and inhibition protects against hepatic injury and inflammation following I/R. Determinations were performed using liver or serum samples prepared after 30 minutes ischemia and either 6 or 24 hours reperfusion in $C3^{-/-}$ mice or WT mice treated with normal saline (NS) or CR2-Crry (either 0.25 or 0.08-mg dose). (**A**) Serum ALT levels. (**B**) Histological quantification of hepatic necrosis and injury, determined 6 hours after reperfusion, on a scale of 0–4. (**C**) Representative H&E-stained sections 6 hours after reperfusion, with the arrow-outlined area showing widespread hepatic necrosis in WT mice. Original magnification, ×100. (**D**) MPO content in liver samples normalized by total protein content. (**E**) Serum concentration of TNF-α. (**F**) Serum concentration of IL-6. Serum ALT levels, histological scores, liver MPO levels, and serum TNF-α and IL-6 levels were raised significantly in all groups undergoing I/R compared with sham-operated mice. $^{\#}P < 0.01$ versus all IRI groups; $^{\#\#}P < 0.01$ versus other IRI groups, respectively; $^{*}P < 0.05$ versus 0.25 mg CR2-Crry group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus 0.08 mg CR2-Crry group. Results are expressed as mean ± SD; $n = 4$ for all groups.

C5aR signaling in liver regeneration has also been demonstrated in a mouse model of $CCl_4$-induced liver toxicity (13, 15).

Thus, although the studies outlined above indicate that complement inhibition represents a potential therapeutic strategy to protect against hepatic IRI, the important role of complement in liver regeneration would appear to be a contraindication for such a strategy in the context of liver resection and SFS liver transplantation, even though IRI is associated with impaired regeneration. A better understanding of the complement-dependant mechanisms and the relative contribution of complement in IRI versus regeneration as well as the relationship between hepatic IRI and regeneration, may therefore have substantial implications for the development of complement modulatory approaches aimed at improving outcome following massive liver resection or SFS liver transplantation. In the current study, we investigated the role of complement in the relationship between hepatic IRI and liver regeneration using 3 murine models: a warm total hepatic IRI model (similar to the Pringle maneuver), a 70% PHx model, and a combined IRI/PHx model designed to recreate clinical massive liver resection under the Pringle maneuver. In these studies, we used the complement inhibitor CR2–complement component (3b/4b) receptor 1-like (CR2-Crry), a recently described inhibitor of C3 activation that targets to sites of complement activation and provides effective local protection from complement without significant systemic effects (18).

## Results

*Hepatic IRI.* The role of complement in murine hepatic IRI was investigated using $C3^{-/-}$ mice and in WT mice treated with different doses of the complement inhibitor CR2-Crry (either 0.08 mg or 0.25 mg). Following 30 minutes hepatic ischemia and either 6 hours or 24 hours of reperfusion, survival, liver injury, and local inflammation was assessed. All mice survived for the observed periods after reperfusion. Serum alanine aminotransferase (ALT) levels were determined as a measure of liver function, and ALT levels were raised significantly in all groups undergoing I/R, compared with baseline or with sham-operated mice (Figure 1A). However, ALT levels were significantly higher after reperfusion in WT mice following I/R, compared with $C3^{-/-}$ mice or mice treated with either dose of inhibitor. A 0.08-mg dose of CR2-Crry was less protective than a 0.25-mg dose at 6 hours after reperfusion, although ALT levels were not significantly different at 24 hours after reperfusion. Histological assessment scores of injury were also significantly lower in $C3^{-/-}$ and complement-inhibited mice at both 6 hours and 24 hours after I/R (Figure 1, B and C), with high-dose inhibition providing better protection at both time points of analysis. To assess the effect of complement activation

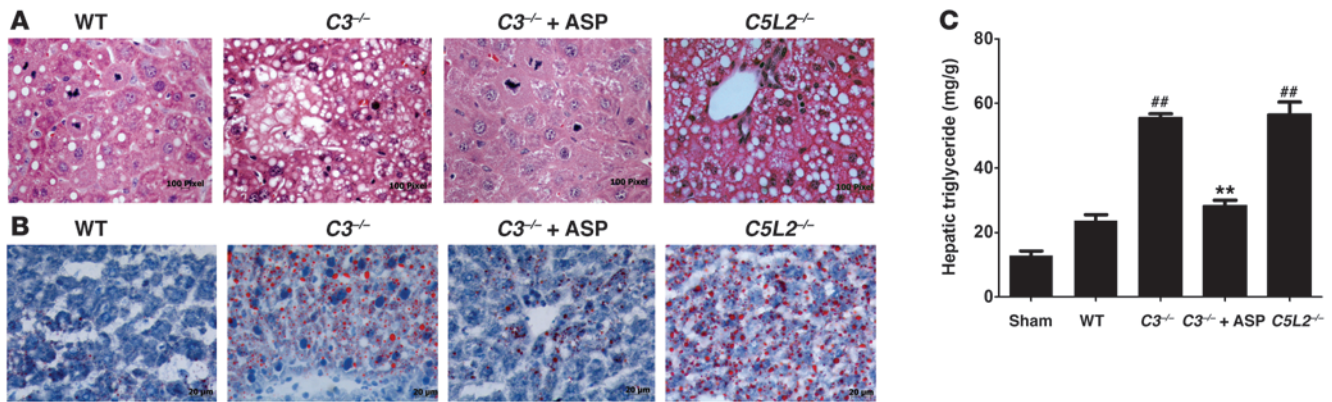


**Figure 2**

Hepatic steatosis in $C3^{-/-}$ and $C5L2^{-/-}$ mice following PHx. All analyses are from liver samples isolated 48 hours after PHx. (**A**) Representative H&E-stained sections showing C3 and C5L2 deficiency is associated with a marked increase in necrosis with hepatic micro- and macrovesicular steatosis, whereas WT mice and $C3^{-/-}$ mice treated with 15 μg ASP exhibit much less apparent steatosis. (**B**) Representative oil red O–stained sections. $C3^{-/-}$ and $C5L2^{-/-}$ mice have increased micro- and macrovesicular steatosis compared with WT mice and $C3^{-/-}$ mice treated with 15 μg ASP. Original magnification in **A** and **B**, ×400. (**C**) Quantification of hepatic triglyceride accumulation. $^{\#\#}P < 0.01$ versus WT groups; $^{**}P < 0.01$ versus $C3^{-/-}$ groups. Results expressed as mean ± SD; $n$ = 4–6.

on neutrophil recruitment, myeloperoxidase (MPO) levels in liver homogenates were determined. Levels of MPO were elevated in all after reperfusion samples compared with baseline and sham-operated controls, but MPO levels were significantly lower in C3-deficient and complement-inhibited mice compared with control WT mice at both 6 hours and 24 hours after reperfusion (Figure 1D). MPO levels were, however, higher at 24 hours compared with those 6 hours after reperfusion in all groups, which did not correlate with reduced ALT and injury scores at 24 hours compared with those 6 hours after reperfusion. Levels of the inflammatory cytokines, TNF-α and IL-6, were also significantly reduced in C3-deficient and complement-inhibited mice compared with WT controls at both time points after reperfusion, with significantly lower levels at 24 hours compared with those 6 hours after reperfusion (Figure 1, E and F). An overall comparison of injury and recovery markers at 6 and 24 hours after I/R indicated that C3 deficiency and high-dose inhibition delays recovery and repair compared with low-dose complement inhibition. For clinical relevance, we used a model of total hepatic ischemia, similar to the Pringle maneuver, a clinical procedure often used in hepatic surgery. Nevertheless, we have shown that complement deficiency and inhibition also protects against IRI in a model of partial hepatic ischemia that does not carry the risk of intestinal venous congestion, a condition that may activate complement and may cause endotoxemia (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38289DS1).

*PHx and liver regeneration*. In broad agreement with previously published data (14), we demonstrated that C3 deficiency results in increased injury following 70% PHx, as measured by increased serum ALT, bilirubin, focal liver necrosis, and mortality. In addition, an impaired regenerative response in $C3^{-/-}$ mice was demonstrated by significantly reduced BrdU incorporation, decreased mitotic index score, and reduced restitution of liver weight (Supplemental Figure 2).

We also observed a significant increase in liver steatosis in $C3^{-/-}$ mice after PHx compared with WT mice, as assessed by histological examination and by triglyceride content. Liver regeneration is associated with transient accumulation of hepatic lipids, and mild macrovesicular steatosis developed in WT mice following PHx. However, C3 deficiency was associated with the development of moderate-to-severe macrovesicular and microvesicular steatosis (Figure 2). It is not known whether there is a mechanistic link between complement, steatosis, and regeneration, but C3a has been shown to play an important role in liver regeneration (14), and its degraded form, C3a-des-Arg (also known as acylation-stimulating protein [ASP]), plays a role in lipid metabolism. ASP increases fat storage in adipocytes through increased triglyceride synthesis and decreased intracellular lipolysis (19), and since mice deficient in C3 (and therefore unable to generate ASP) have delayed triglyceride clearance (20–22), we administered ASP to $C3^{-/-}$ mice following PHx to assess the effect of ASP on liver regeneration and steatosis. Reconstitution of $C3^{-/-}$ mice with a 15-μg dose of recombinant ASP significantly reduced steatosis and hepatic injury, completely restored the proliferative response as measured by BrdU incorporation and restitution of liver weight, and significantly improved survival (Figures 2 and 3). TNF-α and IL-6 are cytokines involved in the priming events of liver regeneration via their effects on NF-κB and STAT3 activation. Confirming previous data (14), C3 deficiency significantly reduced STAT3 activation following PHx (Figure 3F). However, reconstitution of $C3^{-/-}$ mice with 15 μg ASP restored STAT3 activation to WT levels, identifying a putative pathway through which ASP may modulate liver regeneration.

Although not without controversy, the only identified receptor for ASP is C5a-like receptor 2 (C5L2) (23–26), and C5L2 plays an important role in triglyceride synthesis and clearance (25, 26). To investigate a role for C5L2 in liver regeneration and a putative link between ASP and C5L2 in regeneration, we determined the effect of C5L2 deficiency on liver injury and regeneration following PHx. $C5L2^{-/-}$ mice responded to PHx similarly to $C3^{-/-}$ mice, and, compared with WT mice, displayed significantly increased hepatic injury, increased mortality, and impaired liver regeneration (Figure 3). $C5L2^{-/-}$ mice also developed moderate to severe hepatic steatosis following PHx (Figure 2). Also similar to that in $C3^{-/-}$ mice, STAT3 activation was significantly reduced in $C5L2^{-/-}$ mice following PHx compared with WT mice and ASP reconstituted mice. We further determined the effect of ASP administration to $C5L2^{-/-}$ mice following PHx. Treat-

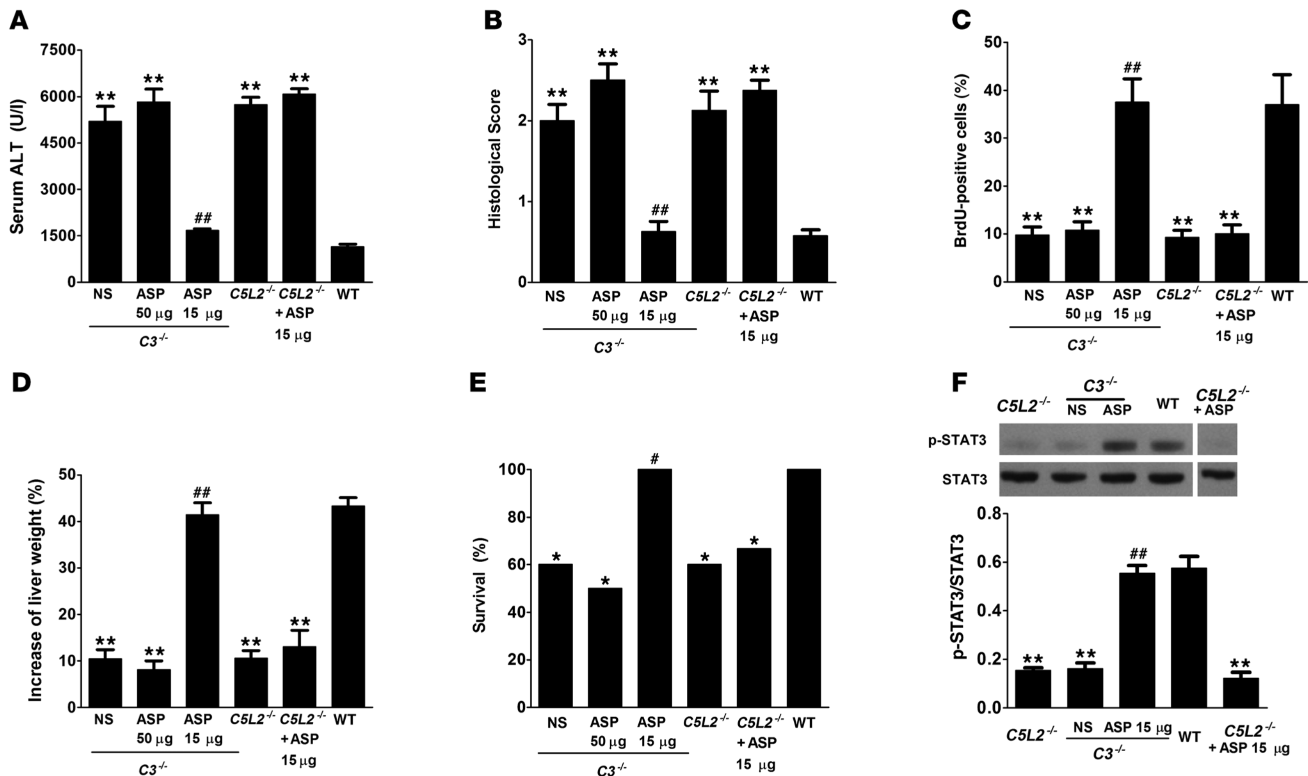


**Figure 3**

Reconstitution of $C3^{-/-}$ mice with ASP following PHx enhances either regeneration or injury depending on dose, and C5L2 (putative ASP receptor) deficiency increases injury and impairs regeneration. A 15-μg or 50-μg dose of ASP was administered to $C3^{-/-}$ mice and a 15-μg dose of ASP was administered to $C5L2^{-/-}$ mice immediately after surgery, and all determinations were made at 48 hours after PHx. (**A**) Serum ALT levels. (**B**) Histological scores. (**C**) Assessment of regeneration by BrdU incorporation. (**D**) Restitution of liver weight. (**E**) Forty-eight-hour survival. (**F**) Western blot assay for phosphorylated form of STAT3 at 3 hours after PHx. Reconstitution of $C3^{-/-}$ mice with low-dose ASP but not high-dose ASP significantly increased 2-day survival. Note that phospho-STAT3 (p-STAT3) levels are strongly reduced in both $C5L2^{-/-}$ and $C3^{-/-}$ mice compared with WT mice. A 15-μg dose of ASP restored activation of STAT3 in $C3^{-/-}$ mice but not $C5L2^{-/-}$ mice. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the $C3^{-/-}$ normal saline group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus the WT group, respectively. For survival study, $n = 10$ for each group; all other studies, $n = 4$–6. Results are expressed as mean ± SD.

ment of $C5L2^{-/-}$ mice with 15 μg ASP following PHx had no effect on any parameter of injury or regeneration in these mice (Figure 3, A–E) and did not effect STAT3 signaling (Figure 3F). Collectively, the data are consistent with the hypothesis that ASP modulates regeneration via a mechanism involving C5L2 modulation of STAT3 activation. Nevertheless, previous studies have indicated a key role for C5a and C5aR signaling in STAT3 activation and liver regeneration (14), and importantly, complement activation was similar in $C5L2^{-/-}$ and WT mice following PHx, as determined by C3 deposition in liver sections (Supplemental Figure 3).

Unexpectedly, reconstitution of $C3^{-/-}$ mice with a high dose of ASP (50 μg) following PHx failed to restore the regenerative response and did not protect against injury (Figure 3). It was not clear why low- versus high-dose ASP had opposing effects in liver regeneration and injury in $C3^{-/-}$ mice following PHx. However, while the complement activation products C3a and C5a have been shown to play a key role in the priming stages of liver regeneration via their effect on TNF-α and IL-6 expression, these cytokines can play dual roles in hepatocyte regeneration and injury, and increased and prolonged expression of these inflammatory cytokines is associated with hepatic injury (27–29). We therefore investigated the effect of high- versus low-dose ASP on TNF-α and IL-6 expression levels and on hepatic neutrophil infiltration (MPO activity) following PHx. At 6 hours after PHx, TNF-α and IL-6 levels were significantly elevated in $C3^{-/-}$ mice treated with either high- or low-dose ASP compared with saline-treated $C3^{-/-}$ mice (Figure 4). However, levels of both cytokines were significantly higher in mice treated with 50 μg ASP compared with those in mice treated with 15 μg ASP. High-dose ASP also correlated with significantly increased neutrophil infiltration after PHx, as determined by MPO activity. Thus, high-dose ASP is associated with a significantly higher inflammatory burden after PHx. We also determined that treatment of WT mice with either a low or high dose of ASP following PHx significantly ($P < 0.05$) increased injury and impaired the proliferative response, with the higher dose of ASP having a more profound effect on injury and regeneration (data not shown). Together, the above data indicate that ASP is a key factor in liver regeneration following PHx, but ASP, at levels higher than normally generated endogenously due to PHx-induced complement activation results, in increased hepatic inflammation and injury and an impaired regenerative response. These data suggest that there is a threshold of complement activation and C3a/ASP production for optimal liver regeneration following PHx.

*Complement inhibition and liver regeneration.* To put the above results in a more clinical context, we investigated the effect of different

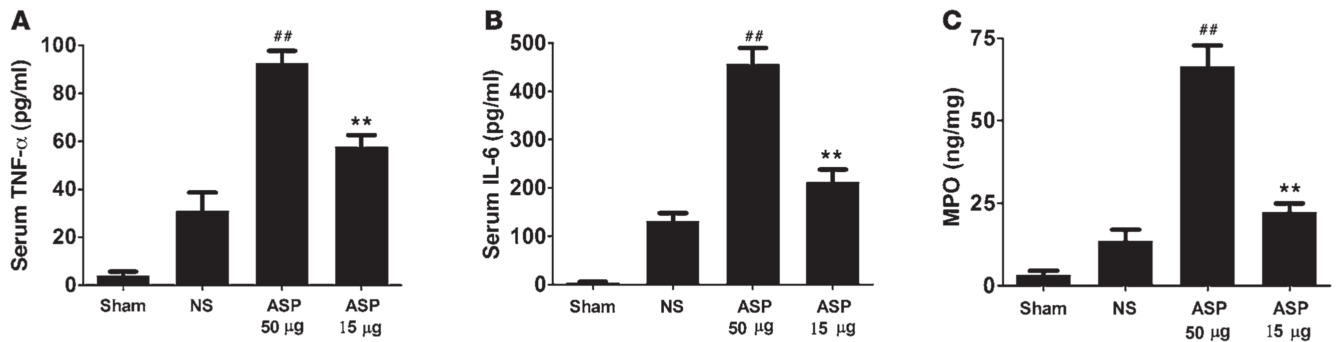


**Figure 4**

Reconstitution of $C3^{-/-}$ mice with high-dose ASP enhances inflammation and injury after PHx. Either a 15-μg or 50-μg dose of ASP was administered to $C3^{-/-}$ mice immediately after surgery, and cytokine and MPO determinations were measured at 6 hours after PHx. (**A**) Serum TNF-α levels. (**B**) Serum IL-6 levels. (**C**) MPO content in liver samples. $^{\#\#}P < 0.01$ versus normal saline group; $^{**}P < 0.01$ versus 50 μg ASP group. Results are expressed as mean ± SD; $n$ = 4–6 for all groups.

doses of a complement inhibitor on liver injury and regeneration following PHx in WT mice. For these studies, we used CR2-Crry at a dose of 0.08 mg or 0.25 mg, the same doses used in the above IRI studies. Similar to the results with $C3^{-/-}$ mice (shown in Supplemental Figure 2), WT mice treated with a 0.25-mg dose of CR2-Crry after PHx showed significantly increased liver injury and impaired proliferative response compared with saline-treated controls (Figure 5). There was also a high mortality of these complement-inhibited mice compared with saline-treated controls (40% versus 0%, respectively, monitored over a 7-day period). As might be expected given the important role for complement activation in liver regeneration, the lower dose of CR2-Crry resulted in less injury and increased BrdU incorporation compared with high-dose CR2-Crry treatment. Unexpectedly, however, low-dose complement inhibition resulted in significantly less hepatic injury and a significantly enhanced proliferative response compared with saline-treated controls (Figure 5). Additional data demonstrated that low-dose CR2-Crry treatment resulted in improved and accelerated regeneration at multiple time points after PHx. By 7 days after PHx, restoration of liver to normal weight was almost complete, and there was no significant difference between mice treated with saline or low-dose CR2-Crry (Supplemental Figure 4, A and B). There was no mortality in mice treated with 0.08 mg of CR2-Crry or saline, but there was some minor injury in control mice following PHx, based on elevated ALT scores and histology scores. There was also a higher morbidity score in control mice compared with low-dose CR2-Crry–treated mice (Supplemental Figure 4C). ALT levels had dropped to normal by 72 hours after PHx (Supplemental Figure 4D). ALT levels were significantly lower at 24 and 48 hours after PHx in low-dose CR2-Crry–treated mice compared with those in saline-treated controls.

Anti-C3d immunofluorescence microscopy of liver sections was used to correlate the effect of the different doses of CR2-Crry with the level of complement activation and liver injury/regeneration. C3d was deposited predominantly on hepatocyte membranes and sinusoidal endothelium within livers isolated from WT mice 48 hours after PHx. C3d was deposited with a greater intensity and was more widely distributed in samples from WT mice compared with samples from mice treated with 0.08 mg CR2-Crry. There was no detectable C3d deposition in samples from mice treated with 0.25 mg CR2-Crry (Figure 6). Together, these data support the concept of a balance between complement-dependent injury and a complement-dependent proliferative response in liver regeneration following PHx. Thus, it is possible that in a clinical setting impaired liver regeneration following resection or SFS transplantation may be a consequence of excessive complement activation and inflammation following I/R.

*Complement inhibition in a combined model of IRI and PHx.* Since hepatic I/R results in a significant level of complement activation and complement-dependent injury (refer to Figure 1), we investigated the effect of complement inhibition in a model that incorporates both IRI and 70% PHx, a model mimicking the procedure used for massive liver resection under the Pringle maneuver. WT or $C3^{-/-}$ mice were subjected to 30-minute hepatic ischemia, during which time 70% PHx was performed. WT mice were treated with either 0.08 mg CR2-Crry or 0.25 mg CR2-Crry immediately after surgery. Only 20% of $C3^{-/-}$ mice survived for 48 hours following surgery, compared with 90% survival of WT mice (Figure 7A). Compared with WT mice, surviving $C3^{-/-}$ mice had significantly increased hepatic injury and an impaired proliferative response (Figure 7, B–E). Of note, WT mice that underwent the combined surgery had a worse outcome in terms of hepatic injury and hepatocyte proliferation than WT mice that underwent 70% PHx alone (refer to Figure 3). Treatment of WT mice with 0.25 mg CR2-Crry, a dose that was highly protective against IRI, also resulted in a significantly poorer outcome in the combined model, with increased hepatic injury, decreased BrdU incorporation, and lower liver weights at 48 hours after surgery compared with control animals (Figure 7). In contrast, low-dose CR2-Crry treatment resulted in no mortality and a significantly improved outcome in terms of hepatic injury and liver regeneration when compared with all other groups, including, importantly, WT control. The level of hepatic injury correlated with neutrophil infiltration, as measured by MPO activity (Figure 7F).

We also investigated the effect of complement inhibition on TNF-α and IL-6 levels. At 6 hours after reperfusion in this combined model, serum TNF-α levels positively correlated with hepatic injury. Serum IL-6 levels, on the other hand, were negatively correlated with injury, with significantly higher IL-6 levels seen in mice treated with 0.08 mg CR2-Crry compared with all other groups (Figure 8, A and B). This is consistent with the important role for IL-6 in the regenerative response, and although TNF-α levels were lower in 0.08 mg CR2-Crry–treated mice compared with other test groups, this level was still significantly elevated compared

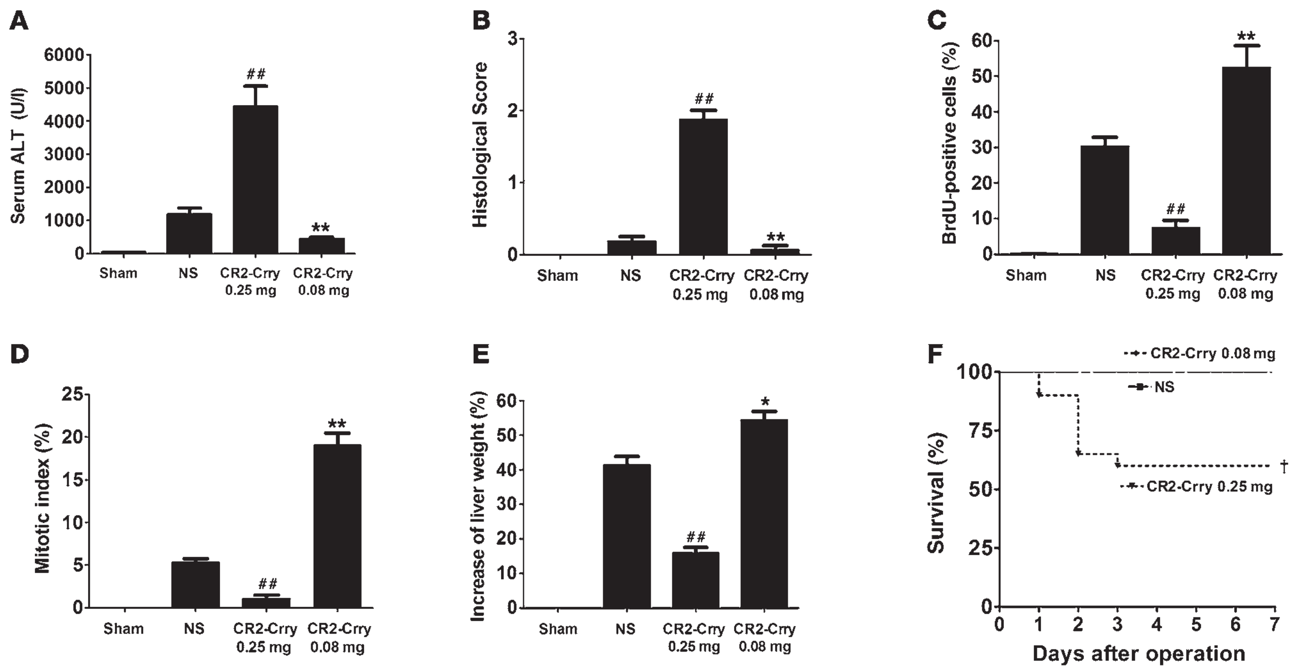


**Figure 5**

Opposing effects of high- and low-dose complement inhibition on hepatic injury and regeneration following PHx. WT mice were treated with normal saline or CR2-Crry at a dose of either 0.25 mg or 0.08 mg immediately after surgery. All determinations were made at 48 hours after PHx. (**A**) Serum ALT levels. (**B**) Histological quantification of hepatic necrosis and injury determined on a scale of 0–4. (**C**) Assessment of regeneration by BrdU incorporation. (**D**) Mitotic index evaluated by calculating the percentage of hepatocytes undergoing mitosis in H&E-stained sections. (**E**) Restitution of liver weight expressed as percentage of regenerated liver mass relative to total liver weight. (**F**) Accumulative survival rate, 7 days after 70% PHx. ##*P* < 0.01 versus normal saline group; ***P* < 0.01 versus all other PHx groups; **P* < 0.05 versus normal saline group. Results expressed as mean ± SD; *n* = 6 for all groups. †*P* < 0.01 compared with normal saline and CR2-Crry 0.08 mg treatment groups; *n* = 20.

with sham-operated mice. Interestingly, at 48 hours after reperfusion, the situation for IL-6 was reversed, with serum IL-6 levels in low-dose CR2-Crry–treated mice being significantly lower than in *C3*$^{-/-}$ mice or mice treated with high-dose CR2-Crry. Serum TNF-α levels remained significantly lower in low-dose CR2-Crry–treated mice compared with all other groups (Figure 8, C and D). TNF-α and IL-6 are considered important for the priming phase of the regenerative response, and hepatic expression of these cytokines peaks at around 1–2 hours and 3–6 hours after PHx, respectively. We therefore also determined hepatic levels of TNF-α and IL-6 at 3 hours after IRI and PHx. Compared with WT mice, C3 deficiency and high-dose complement inhibition resulted in significantly reduced levels of TNF-α and IL-6 in the liver (Figure 8, E and F). In contrast, low-dose CR2-Crry treatment correlated with significantly increased hepatic levels of both cytokines relative to all other groups, including WT. Thus, low-dose complement inhibition and enhanced liver regeneration is associated with increased early hepatic production of these cytokines and with diminished systemic levels of the inflammatory cytokines by 48 hours after PHx, compared with all other groups.

Effect of complement deficiency and complement inhibition on signaling pathways, ATP levels, and oxidative injury following IRI and PHx. Additional studies were performed to further elucidate potential mechanisms of hepatoprotection and regeneration in CR2-Crry–treated mice. In addition to regulating the activation of STAT3, IL-6 also activates the PI3K/Akt survival pathway, a pathway that has been shown to play an important role in the early regenerative response following PHx and that regulates progression of the $G_1$ phase during regeneration (30). We therefore determined whether the high levels of early IL-6 expression associated with low-dose complement inhibition correlated with increased STAT3 and Akt activation. Phosphorylation of STAT3 and Akt after IRI and PHx was determined in livers isolated from complement-deficient and complement-inhibited mice. C3 deficiency and high-dose complement inhibition markedly reduced STAT3 activation following IRI and PHx, compared with that in saline-treated mice and mice treated with low-dose complement inhibition (Figure 9A). Further, there was an increase in STAT3 activation in low-dose CR2-Crry–treated mice compared with saline controls at both 3 and 6 hours after IRI and PHx. Low-dose CR2-Crry treatment was also associated with an increase in Akt phosphorylation at 6 hours after IRI and PHx (Figure 9A).

Mitochondrial dysfunction and oxidative injury occurs in the liver after I/R and also after massive resection. Also, cellular ATP stores have been shown to play an important role in liver regeneration, by supplying energy and regulating posttranscriptional activation of cyclin D1/cyclin-dependent kinase (cdk) complexes (31–33). To investigate whether the effect of complement inhibition on hepatoprotection and liver regeneration was associated with hepatic ATP levels, ATP concentrations were measured in liver samples from all groups at various time points after IRI and PHx. There was a marked reduction in hepatic ATP in all groups at 6 hours after IRI and PHx (Figure 9B). However, whereas ATP levels remained low in *C3*$^{-/-}$ mice and mice treated with high-dose CR2-

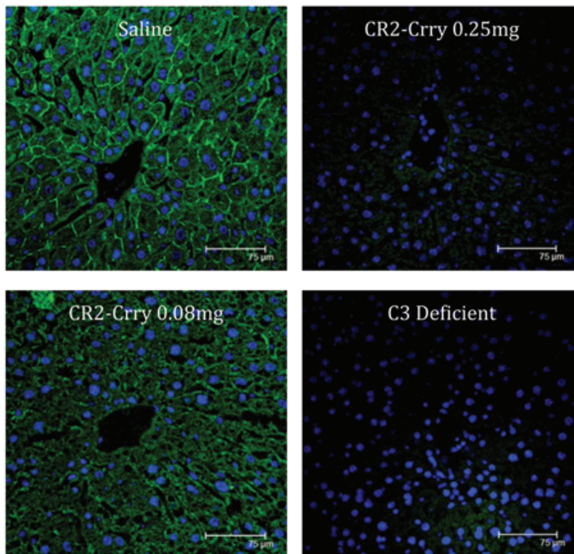


**Figure 6**
Treatment of mice with CR2-Crry following PHx results in a dose-dependent decrease in hepatic C3d deposition. WT mice were treated with normal saline or CR2-Crry at a dose of 0.25 mg or 0.08 mg immediately after surgery. *C3*$^{-/-}$ mice received no treatment. At 48 hours after PHx, livers were removed and sections were analyzed for C3d deposition by immunofluorescence microscopy. Complement deposition was localized to the central lobular areas and was associated with hepatocyte and sinusoidal endothelial cells in WT mice. C3 deposition was reduced in mice treated with 0.08 mg CR2-Crry and was absent in mice treated with 0.25 mg CR2-Crry and in *C3*$^{-/-}$ mice. Representative images, $n = 3$. Images are representative of 3 experiments. Scale bars: 75 μm.

Crry, ATP stores recovered to near preoperation levels by 48 hours after IRI and PHx in mice treated with low-dose CR2-Crry.

The production of reactive oxygen species and lipid peroxidation is considered a major mechanism of hepatic injury following I/R and extreme liver resection. The effect of complement inhibition on oxidative injury to the liver following IRI and PHx was examined by measuring levels of hepatic glutathione (GSH), GSH peroxidase (GPX1), and malondialdehyde (MDA). There were reduced levels of GSH (antioxidant) and increased levels of MDA (index of lipid peroxidation) in saline-treated animals after IRI and PHx, which is indicative of oxidative stress (Figure 10, A and B). Levels of the free radical scavenger GPX1 were also reduced in saline-treated animals following IRI and PHx (Figure 10C). In contrast, treatment of mice with 0.08 mg CR2-Crry after IRI and PHx protected against oxidative stress as indicated by significantly increased levels of GSH and GPX1 and decreased levels of MDA.

*IL-6 blockade and complement inhibition following IRI and PHx*. Finally, since low-dose complement inhibition increased IL-6 levels after IRI and PHx, and since IL-6 signaling is essential for the priming phase of liver regeneration, we sought to clarify whether there was a link between the hepatoprotective and pro-regenerative effect of low-dose complement inhibition and IL-6 expression. IL-6 block-

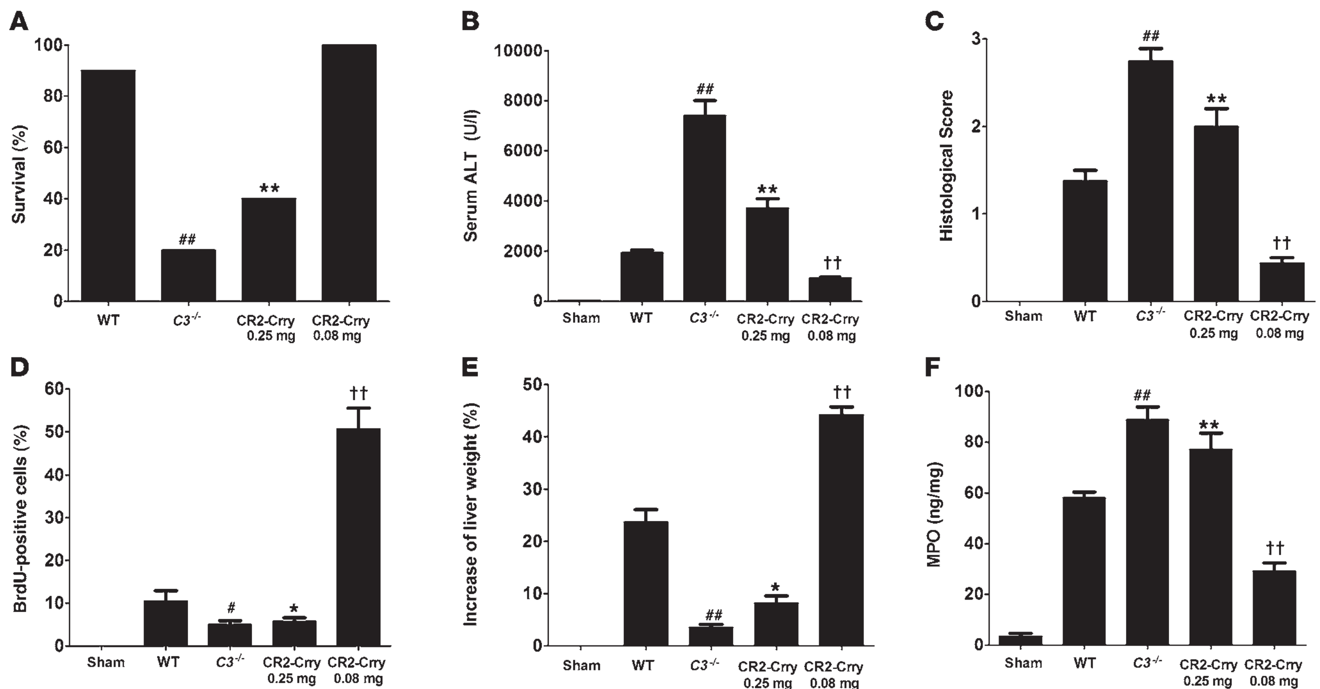


**Figure 7**
Opposing effects of high- and low-dose complement inhibition on hepatic injury and regeneration in a model incorporating both IRI and PHx. Mice were treated with normal saline or CR2-Crry at a dose of either 0.25 mg or 0.08 mg immediately after surgery. *C3*$^{-/-}$ mice received no treatment. All determinations made 48 hours after I/R and PHx. (**A**) Mouse survival. (**B**) Serum ALT levels. (**C**) Histological quantification of hepatic necrosis and injury determined on a scale of 0–4. (**D**) Assessment of regeneration by BrdU incorporation. (**E**) Restitution of liver weight. (**F**) MPO content in liver samples. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus WT group; $^{**}P < 0.01$ versus WT group (similar to WT normal saline group); $^{\dagger\dagger}P < 0.01$ versus all other groups; $^{*}P < 0.05$, $^{**}P < 0.01$ versus WT group. Results are expressed as mean ± SD; $n = 6$–$10$.

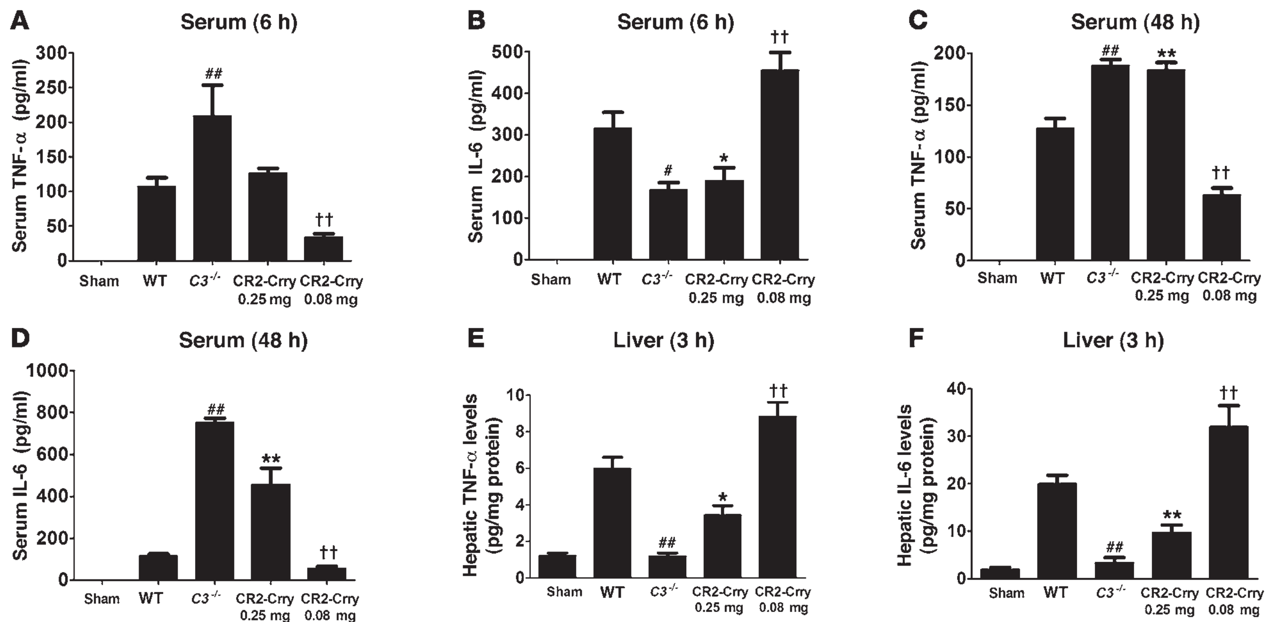


**Figure 8**

Effect of C3 deficiency and complement inhibition on hepatic and serum levels of TNF-α and IL-6 following IRI and PHx. Mice were treated with normal saline or CR2-Crry at a dose of either 0.25 mg or 0.08 mg immediately after surgery. *C3*$^{-/-}$ mice received no treatment. (**A**) Serum TNF-α levels 6 hours after PHx. (**B**) Serum IL-6 levels 6 hours after PHx. (**C**) Serum TNF-α levels 48 hours after PHx. (**D**) Serum IL-6 levels 48 hours after PHx. (**E**) Hepatic TNF-α levels 3 hours after PHx. (**F**) Hepatic IL-6 levels 3 hours after PHx. Low-dose CR2-Crry treatment was associated with high hepatic levels of IL-6 and TNF-α early after PHx relative to other groups and lower relative serum cytokine levels by 48 hours after PHx. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus WT group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus WT group (similar to WT normal saline group); $^{\dagger\dagger}P < 0.01$ versus CR2-Crry 0.25 mg and *C3*$^{-/-}$ groups. Results are expressed as mean ± SD; $n = 6$ for all groups.

ade by administration of anti–IL-6 antibodies, together with CR2-Crry treatment, reduced hepatic levels of IL-6 by about 65% at 3 hours after IRI and PHx and reduced serum levels of IL-6 by about 50% at 6 hours after IRI and PHx (Figure 11, A and B). Further, IL-6 blockade significantly reduced levels of phosphorylated STAT3 following IRI and PHx, indicating a direct relationship between increased levels of IL-6 and STAT3 activation (Figure 11C). IL-6 blockade resulted in significantly increased liver injury (serum ALT) and a significantly impaired regenerative response (BrdU incorporation) in mice subjected to IRI and PHx and treated with low-dose CR2-Crry (Figure 11, D and E). In addition, only 4 of 10 mice receiving anti–IL-6 antibody and CR2-Crry survived for more than 48 hours after IRI and PHx (data not shown). Thus, the protective effect of low-dose complement inhibition following IRI and PHx was lost when complement inhibitor treatment was combined with IL-6 blockade. Taken together, these results suggest that the hepatoprotective effect of low-dose (but not high-dose) complement inhibition is due to the role of complement in IL-6 expression and subsequent priming of the regenerative response.

## Discussion

Failure of the liver to regenerate following massive liver resection or SFS liver transplantation often leads to liver dysfunction. A component of these surgical procedures is a period of ischemia and subsequent reperfusion that is injurious to the liver and which is thought to have a deleterious effect on regeneration. Here, we investigated the dual role of complement in hepatic IRI and liver regeneration.

The pathogenic mechanisms involved in IRI are complex and multifaceted, but it is clear that activation of complement is a key initiating event. An important role for complement in hepatic IRI is fairly well documented in rat models (see Introduction), and in the first set of studies reported here, we show that complement deficiency and complement inhibition is protective in a mouse model. In a second set of studies, we confirmed and extended previous studies that demonstrated an important role for complement in liver regeneration, and we also observed that a significant increase in hepatosteatosis was associated with C3 deficiency. Since C3a has been shown to play an important role in liver regeneration (14), and since its degraded form, ASP (C3ades-Arg), plays a role in lipid metabolism, we investigated the effect of ASP reconstitution in *C3*$^{-/-}$ mice following PHx. Previous studies have shown that the hepatic proliferative response is restored in *C3*$^{-/-}$ mice reconstituted with multiple doses of C3a (14) and that liver regeneration is impaired in *C3aR*$^{-/-}$ mice (13). However, ASP does not bind to C3aR. Although controversial, the only identified receptor for ASP is C5L2 (23–26, 34, 35), a receptor that also binds C3a (and C5a/C5ades-Arg) and that plays an important role in triglyceride synthesis and clearance (25, 26). We found that administration of 15 μg ASP to *C3*$^{-/-}$ mice after PHx significantly ($P < 0.05$) reduced hepatosteatosis, protected against injury, restored BrdU incorporation to the level seen in WT mice, and reversed the decrease in STAT3 phosphorylation seen in *C3*$^{-/-}$ mice. These data indicate that the involvement of complement in the proliferative response can be independent of C3aR signaling and indicate a key role for ASP in hepatoprotection and liver regeneration following PHx. In view of previous data showing that *C3aR*$^{-/-}$ mice have an impaired regenerative response, there may be different and intercepting roles for complement in hepatoprotection and regeneration induced following PHx. One possibility is that since ASP plays an important role in lipid metabolism, steatosis observed

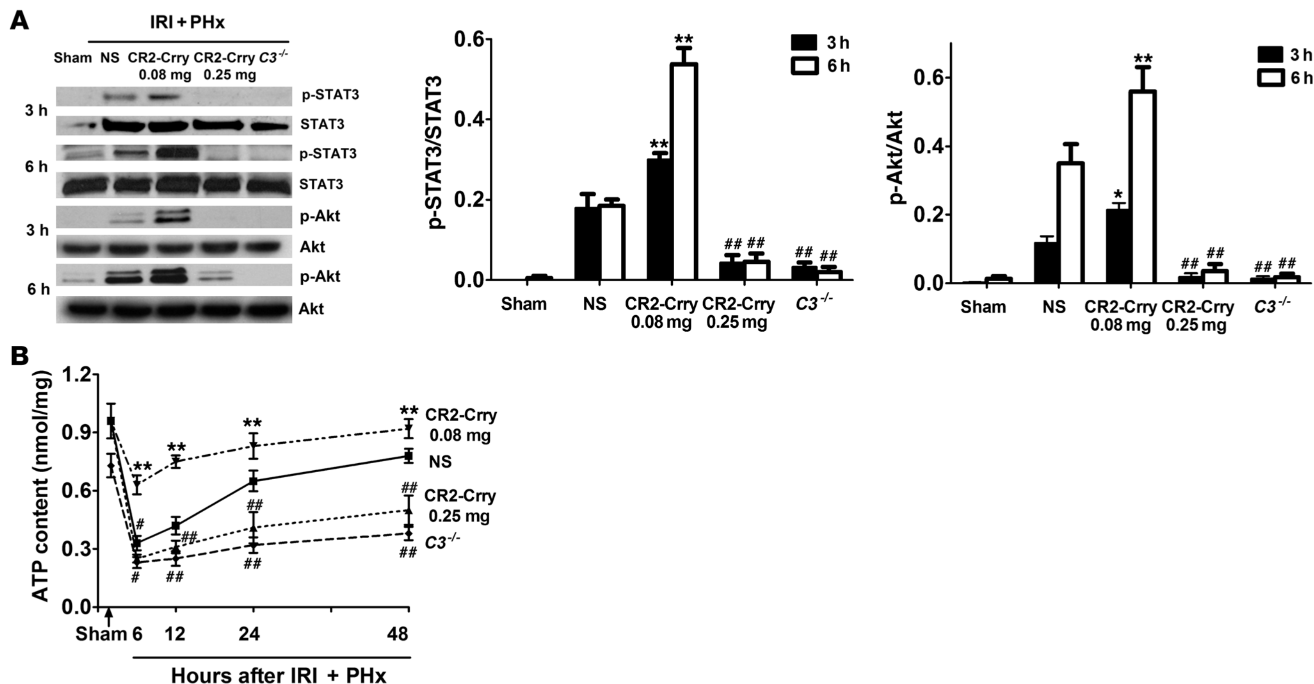


**Figure 9**

Opposing effects of high- and low-dose complement inhibition on STAT3 and Akt activation and on hepatic ATP levels following IRI and PHx. Mice were treated with normal saline or CR2-Crry at a dose of either 0.25 mg or 0.08 mg immediately after surgery. $C3^{-/-}$ mice received no treatment. (**A**) Western blot analysis of STAT3 and Akt phosphorylation using liver samples taken 3 hours and 6 hours after IRI and PHx. Low-dose complement inhibition with CR2-Crry was associated with increased STAT3 and Akt activation. In contrast, expression of phospho-STAT3 and phospho-Akt (p-Akt) was significantly reduced in mice treated with high-dose CR2-Crry and in $C3^{-/-}$ mice. (**B**) ATP content in liver tissue samples taken different time points after IRI and PHx. Low-dose complement inhibition with CR2-Crry was associated with less ATP depletion and higher overall ATP levels compared with all other groups. *$P < 0.05$ versus normal saline group; **$P < 0.01$ versus all other IRI and PHx groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the normal saline group, respectively. $n$ = 4–6 for all groups. Results are expressed as mean ± SD.

in $C3^{-/-}$ mice following PHx may be due to an inhibition of productive triglyceride lipolysis and fatty acid release, resulting in an insufficient energy supply for regeneration. The putative receptor for ASP, C5L2, also plays an important role in lipid metabolism, and we show here that C5L2 deficiency also results in steatosis and impaired regeneration following PHx. Based on current and previous data (reviewed in refs. 36, 37), with the controversy over ASP/C5L2 interaction notwithstanding, one potential mechanism for the development of steatosis in $C3^{-/-}$ and $C5L2^{-/-}$ mice following PHx is that in the absence of ASP/C5L2 signaling, there is a decreased rate of triglyceride synthesis in adipose tissue. This may lead to increased fatty acid delivery to the liver and, due to delayed clearance of triglycerides in the liver, the development of steatosis. The finding that administration of 15 μg ASP to $C5L2^{-/-}$ mice after PHx had no effect on hepatic injury, regeneration, or STAT3 activation is consistent with the hypothesis that ASP modulates regeneration via a mechanism involving its interaction with C5L2. Nevertheless, we demonstrated complement activation in $C5L2^{-/-}$ mice following PHx, indicating endogenous production of ASP, and further research is needed to better understand the role of ASP and C5L2 in liver regeneration. Also in need of clarification are the relative and interacting roles of C5aR and C5L2, since an important role for C5a/C5aR in liver regeneration has been documented (14).

An unexpected finding was that reconstitution of $C3^{-/-}$ mice with a high dose of ASP (50 μg) following PHx failed to restore liver regeneration and induced severe injury. Moreover, WT mice treated with either a low or high dose of ASP exhibited a significant increase in liver injury with impaired regeneration compared with untreated WT animals ($P < 0.05$, data not shown). While previous and current data together indicate a role for both C3a and ASP in liver regeneration, both peptides have proinflammatory properties, although removal of the C-terminal Arg from C3a inactivates certain activities (26, 38). The significant increases seen in liver MPO activity and in serum levels of TNF-α and IL-6 associated with high-dose ASP treatment in $C3^{-/-}$ mice testifies to the proinflammatory properties of ASP and suggests that there is a threshold of complement activation and C3a/ASP production for optimal liver regeneration following PHx. Indeed, while TNF-α and IL-6 play important roles in liver regeneration, there appear to be dual roles for these cytokines in injury versus hepatoprotection and regeneration (27, 39). Although IL-6–dependent processes are associated mainly with protective responses, TNF-α expression is clearly associated with inflammation and injury, and these cytokines can modulate the expression of each other. For example, IL-6 plays a key role in initiating the proliferative response and IL-6 expression appears to be dependent on NF-κB activation that is induced by TNF-α (40). NF-κB activation is also dependent on other factors that influence inhibitory IkBα, and TNF-α may initiate apoptotic pathways in the absence of NF-κB activation (41). In other studies, IL-6 has been shown to be protective in a model of warm IRI via a mechanism that appears to involve the downregulation of TNF-α (42), and liver injury in various models has been shown to be associated with increased expression of both TNF-α and IL-6.

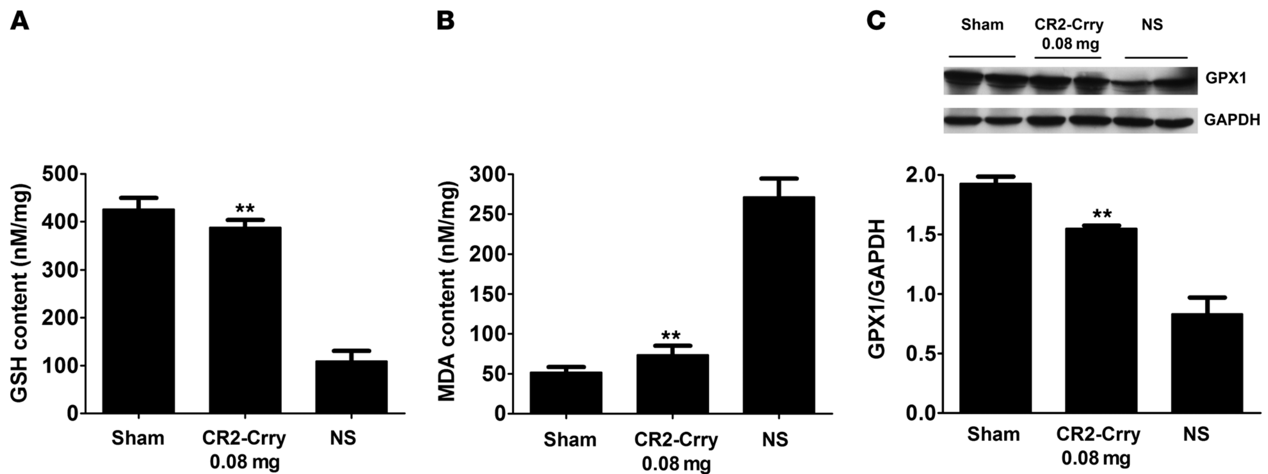


**Figure 10**
Low-dose CR2-Crry treatment after IRI and PHx decreases levels of markers for oxidative stress. (**A**) GSH and (**B**) MDA content 6 hours after IRI and PHx as determined spectrophotometrically and expressed as nM/mg protein in liver samples. (**C**) Western blot analysis of GPX1, demonstrating that low-dose CR2-Crry treatment prevents relative decease in GPX1 levels after IRI and PHx. Western blot data quantitated by image analysis of autoradiograms. Results are expressed as mean ± SD. $^{**}P < 0.01$ versus normal saline group; $n = 4$.

In a more clinically relevant setting of complement inhibition, we found that a 0.25-mg dose of CR2-Crry administered immediately after PHx resulted in severe liver injury and impaired regeneration, similar to that seen in $C3^{-/-}$ mice. Surprisingly, however, low-dose CR2-Crry treatment following PHx resulted in an improved outcome relative to control saline treatment, with CR2-Crry–treated mice displaying significantly less hepatic injury and a significantly enhanced proliferative response. Taken together, the above data indicate that there is a threshold of complement activation and C3a/ASP production for optimal liver regeneration following PHx, above which increased levels of C3a/ASP (and indeed other complement activation products) tip the balance toward injury and impairment of regeneration. This balance will likely be influenced by the varying roles of complement in hepatic metabolism, inflammation, and regeneration. Although there is no direct significant ischemic or inflammatory insult to remnant liver after 70% PHx, there are substantial hemodynamic changes, with an increase in portal blood and a very likely decrease in oxygen pressure that could trigger a hypoxic response, leading to complement activation. We observed increased ALT levels and increased morbidity in control mice, compared with mice treated with low-dose CR2-Crry early after PHx, indicating some liver injury. This is consistent with the hypothesis that PHx alone results in a level of complement activation that is slightly in excess of that required for optimal regeneration and that low-dose complement inhibition tips the injury/regeneration balance toward regeneration. In a clinical setting of massive liver resection or SFS transplantation, hepatic I/R is unavoidable and results in complement activation and inflammation, and several studies have highlighted the deleterious effect of IRI on liver regeneration. Thus, we further investigated the role of complement in the balance between liver injury and regeneration in a model incorporating both IRI and PHx.

Although C3 deficiency protected against hepatic IRI, the combination of IRI and PHx in $C3^{-/-}$ mice resulted in significant liver damage compared with WT mice, as well as a failure of the liver to regenerate. The result was similar in mice treated with 0.25 mg CR2-Crry. Low-dose CR2-Crry treatment, however, significantly improved outcome in terms of liver injury and regeneration, not only when compared with $C3^{-/-}$ and high-dose CR2-Crry–treated mice but also compared with WT mice. We propose that while hepatic I/R results in complement activation, incomplete blockade of the complement pathway provides a level of protection from IRI, while allowing generation of C3a (and C5a) at levels sufficient to promote regeneration.

To further address the protective mechanisms of low-dose complement inhibition following IRI and PHx, we determined the effect of CR2-Crry on neutrophil infiltration, cytokine levels, activation of transcription factors, hepatic ATP levels, and oxidative injury. Hepatic MPO activity following IRI and PHx correlated with injury and was significantly lower in mice treated with 0.08 mg CR2-Crry compared with all other test groups. Neutrophil infiltration has been shown to play a vital role in hepatic IRI and impairment of liver regeneration, and C3a and C5a can mediate this process.

Hepatic levels of TNF-α and IL-6 were significantly higher at 3 hours after IRI and PHx in the low-dose CR2-Crry–treated group, and early expression of these cytokines is important for priming the regenerative response. At later time points, however, cytokine expression increased in $C3^{-/-}$ mice and in high-dose CR2-Crry–treated mice relative to low-dose CR2-Crry mice, which together with MPO data is indicative of increased and ongoing inflammation. IL-6–induced activation of STAT3 plays a key role in cell-cycle progression in liver regeneration, and elevated early expression of IL-6 in low-dose CR2-Crry–treated mice correlated with increased STAT3 activation. Low-dose CR2-Crry also resulted in enhanced activation of Akt, a transcription factor involved in the phosphorylation of downstream targets that control cell growth and survival. Lipid peroxidation is considered a major mechanism of tissue damage following I/R, and the measurement of various markers of oxidative injury following IRI and PHx revealed a significant attenuation of oxidative stress in mice treated with low-dose CR2-Crry compared with other groups. An increased capacity to protect against lipid peroxidation and membrane injury may also contribute to the improved maintenance of ATP levels in mice treated with low-dose CR2-Crry. These results identify several potential mechanisms by which low-dose complement inhibition improves regeneration following IRI and PHx, including induction of IL-6,

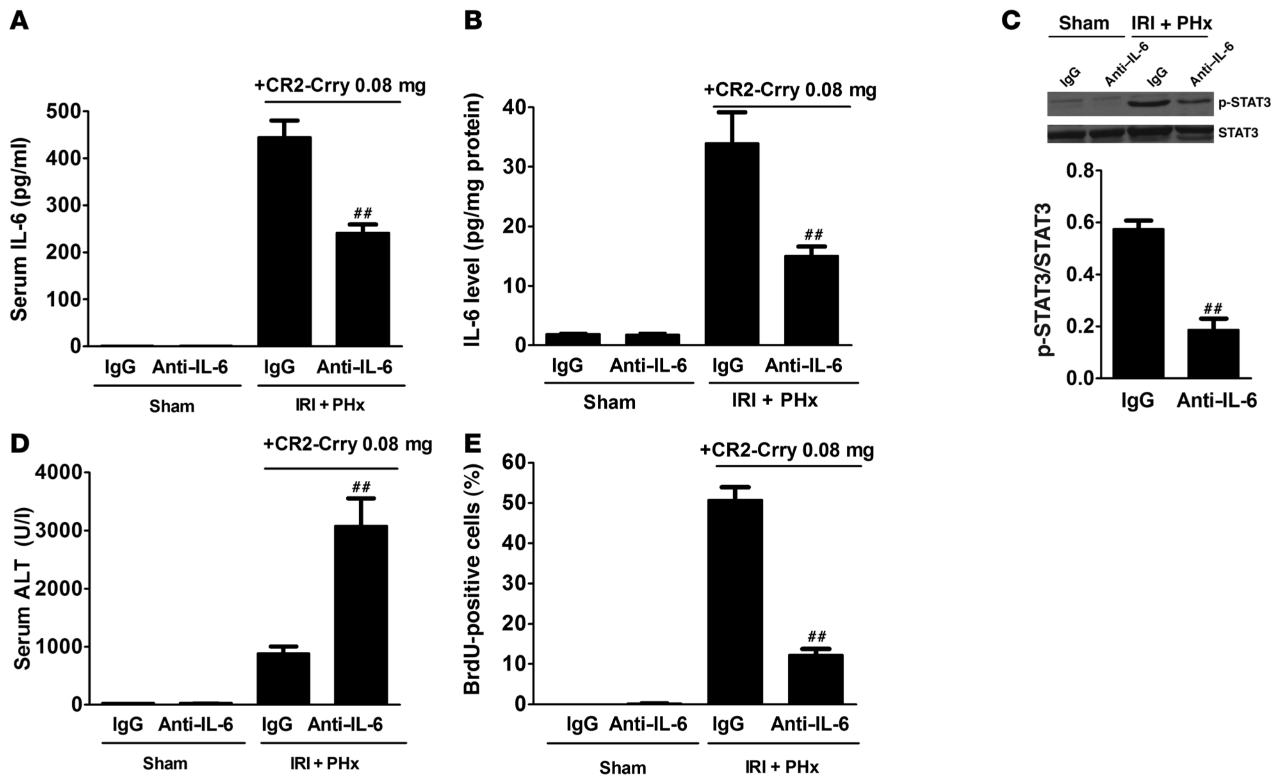


**Figure 11**
IL-6 blockade reverses protective effect of low-dose complement inhibition following IRI and PHx. Mice were treated with 0.08 mg CR2-Crry and either anti-mouse IL-6 antibody or control IgG immediately after IRI and PHx. (**A**) Serum IL-6 levels at 6 hours after PHx. (**B**) Hepatic IL-6 levels at 3 hours after PHx. (**C**) Western blot analysis of phosphorylated form of STAT3 at 3 hours after PHx, together with densitometric quantification. Phospho-STAT3 levels were strongly reduced in mice treated with anti–IL-6 antibodies. (**D**) Serum ALT levels at 48 hours after PHx. (**E**) BrdU incorporation at 48 hours after PHx. Results are expressed as the mean ± SD; $n = 4$. ##$P < 0.01$ versus the IgG control IRI and PHx group.

enhancement of STAT3 and Akt activation, reduction in hepatic ATP depletion, and attenuation of oxidative injuries.

In conclusion, this study provides additional information on the role of complement in hepatic IRI and regeneration and provides data supporting a complement-dependent balance in the link between IRI and impaired regeneration. These findings shed new light on the pathogenic mechanisms involved in the failure of liver regeneration and suggest that an approach of modulated complement inhibition represents a potential therapeutic strategy for patients undergoing massive liver resection or SFS liver transplantation.

## Methods

*Animal studies*. *C3*$^{-/-}$ mice and WT controls were obtained from The Jackson Laboratory. *C5L2*$^{-/+}$ heterozygous mice were provided by Regeneron Pharmaceuticals Inc., courtesy of Joseph Sorrentino (Tarrytown, New York, USA), and *C5L2*$^{-/-}$ and WT littermates determined by PCR genotyping. All procedures were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee, in accordance with the National Research Council 1996 guide for the care and use of laboratory animals. All mice were on C57BL/6 background and were used when 8–10 weeks old and weighing between 22.5–25 g. Mice were fed a pellet diet with water ad libitum and kept on a 12-hour-light/12-hour-dark cycle. For all procedures, mice were anesthetized with a intraperitoneal injection of 0.05 ml/10 g body weight of a "ketamine cocktail", consisting of ketamine (13 mg/ml), xylazine (2.6 mg/ml), and acepromazine (0.15 mg/ml) in sterile normal saline. Animals were subjected to 1 of the following 3 different procedures. (a) For procedures involving hepatic IRI, mice were subjected to total warm hepatic I/R, as previously described (43). The portal vein and hepatic artery were occluded for 30 minutes with a microaneurysm clamp to induce hepatic ischemia, followed by a 6-hour period of reperfusion. For some experiments, we also used a model of partial warm hepatic IRI (see Supplemental Figure 1). Mice were subjected to occlusion of the left lateral and median lobes of the liver, by applying an atraumatic microvascular clamp to the vascular pedicle (44). After 90 minutes of partial warm ischemia, the clamp was removed, initiating hepatic reperfusion. In all IRI studies, CR2-Crry or normal saline was administered i.p. immediately after ischemia. Mice were sacrificed at predetermined time points after reperfusion for serum and liver sampling. (b) For procedures involving 70% PHx, surgery was performed as previously described (45, 46), with resection of the median and left lateral liver lobes. (c) For procedures involving combined I/R and PHx, a model was developed incorporating both of the above hepatic I/R and PHx procedures. The portal vein and hepatic artery were occluded for 30 minutes, and during the ischemic period, 70% PHx was performed. Following surgeries, mice were sacrificed, and livers and blood samples were collected at different time points after operation. Blood was also collected from the vena cava for serum preparation at the time of sacrifice and at 6 hours in the PHx and IRI and PHx models. Harvested livers were weighted to assess regeneration, and portions of liver tissue were either fixed in 10% neutralized formalin for histological evaluation or were snap frozen in liquid nitrogen and maintained at –80°C until homogenization for various biochemical assays. In therapeutic protocols with complement inhibition, CR2-Crry or normal

saline was administered i.p. immediately after surgery. CR2-Crry was administered at a dose of 0.25 mg, based on effective protection in previous studies of intestinal and cerebral IRI (47, 48), and at one-third the dose, 0.08 mg. CR2-Crry was prepared as previously described (47). Based on a previous study done in rats (49), IL-6 blockade was accomplished using goat anti-mouse IL-6 antibody (R&D Systems) injected i.p. at 200 μg/kg body weight. The IL-6 antibody or normal goat IgG (control) was injected i.p. immediately after surgery and just prior to CR2-Crry administration.

*ASP/C3ades-Arg reconstitution*. Recombinant human ASP/C3ades-Arg was prepared and purified by a modification of the original procedure (50), using a His-tag at the amino-terminal, with initial purification on a Ni-Sepharose column (Amersham Biosciences), followed by HPLC. No denaturing agents were used at any step in the purification to avoid ASP inactivation. ASP was administered at a dose of either 15 μg or 50 μg per mouse in 200 μl saline, by i.p. injection immediately after PHx. Endotoxin levels in the ASP preparation were analyzed by the Limulus Amebocyte Lysate assay (E-Toxate kit; Sigma-Aldrich) and 25 μg/μl ASP (100 times higher concentration than used) tested negative for endotoxin. Mouse ASP and human ASP are not identical, but it has been shown that human ASP interacts with mouse C5L2, activates mouse cells, and enhances postprandial triglyceride clearance in WT and $C3^{-/-}$ mouse models (26).

*Microscopy*. For histological examination, tissue blocks were placed in 10% buffered formaldehyde solution for 48 hours before being embedded in paraffin. Liver histology was assessed by light microscopy (Olympus BH -2) of H&E-stained 4-μm sections in a blinded fashion. Ten random fields on each slide were assessed for necrosis by standard morphologic criteria (loss of architecture, vacuolization, karyolysis, increased eosinophilia), and the extent of necrosis was semiquantitatively estimated by assigning a severity score on a scale of 0–4 as previously described (51) (absent, 0; mild, 1; moderate, 2; severe, 3; and total necrotic destruction of the liver, 4). The score was used to compare the liver damage after I/R and/or PHx between different study groups. Steatosis was assessed by oil red O staining as previously described (52). C3 deposition in liver samples was determined by immunofluorescence using anti-mouse FITC-conjugated C3d antibody (Dako Cytomation) as previously described (18).

*Biochemical and immunological assays*. Serum levels of ALT and total bilirubin were determined using analytical kits from Sigma-Aldrich according to manufacturer's instructions. Serum levels of TNF-α and IL-6 were measured by ELISA using kits from eBiosciences. For measurement of hepatic TNF-α and IL-6 levels, frozen liver samples were homogenized in extraction buffer (50 mmol/l Tris, pH 7.2, 150 mmol/l NaCl, Triton X-100, and a protease inhibitor cocktail). The homogenate was centrifuged at 10,000 *g* and 4°C for 8 minutes, and TNF-α and IL-6 levels in supernatants were measured by ELISA using a kit from eBiosciences. For quantitative assessment of neutrophil infiltration into the liver parenchyma, liver MPO content was assessed, using the Hbt mouse MPO ELISA Kit from Hycult Biotechnology according to manufacturer's instructions. Liver samples were prepared, and hepatic triglyceride content of samples was determined using a triglyceride test kit as described by the manufacturer (Stanbio Laboratory). The levels of GSH and MDA in liver samples were determined spectrophotometrically by commercially available kits (Bioxytech GSH and MDA-586 kits; OXIS International Inc.), as reported previously (43). The calculated concentrations of lipid peroxidation products were normalized by protein concentration and expressed as nmol/mg protein.

*Assessment of liver regeneration*. Three independent markers for hepatic regeneration were used. Reconstitution of liver weight was expressed as percentage of regenerated liver mass relative to total liver weight and was calculated as described previously (53). For assessment of hepatic proliferation, BrdU was injected (50 mg/kg i.p.) 2 hours prior to harvesting of liver. BrdU incorporation in liver sections was determined by immunohistochemical staining, as described previously (54). Positive and negative cells were counted in 10 randomly selected fields by light microscopy using a ×40 objective lens. Constantly proliferating intestinal crypt epithelium served as a positive control for BrdU incorporation and staining. The mitotic index was determined in H&E sections using previously reported criteria for mitosis as follows: complete absence of cell membrane, slight eosinophilic staining of nucleus, nuclear spindle matrix formation, absence of a nucleolus, and slight increase in cell size (55). The mitotic index was expressed as the rate of positive cells per 1,000 hepatocytes/high-power field. All analyses were performed with the operator blinded to the experimental groups.

*Assessment of morbidity*. Clinical scores of morbidity were assessed 48 hours after PHx, as previously described (56). Each mouse was graded from 0 to 3 (normal, 0; slight effect, 1; moderate effect, 2; severe effect, 3) for posture, coat, and activity, and scores combined to a final score on a scale from 0 to 9.

*Measurement of liver ATP content*. Approximately 50 mg frozen liver tissue was homogenized in 500 μl ice-cold tissue lysis buffer (Sigma-Aldrich) with a protease inhibitor cocktail (Pierce). The homogenates were centrifuged at 10,000 *g* for 8 minutes at 4°C, and ATP in supernatants was extracted using 1.5% trichloroacetic acid. Supernatants were then diluted in 1:150 Tris acetate buffer (pH 7.85), and 100 μl reconstituted luciferin-luciferase solution was added to a 100 μl diluted sample (Enliten, Promega), and luciferase activity was immediately evaluated luminometrically. ATP content in the samples was determined by comparison to a concurrent standard curve. Protein concentration was also determined, and the calculated concentrations of ATP content were normalized by protein concentration and expressed as mmol/mg protein.

*Western blot analysis of GPX1 and STAT3 and Akt activation*. Liver samples were homogenized on ice in lysis buffer (Sigma-Aldrich), containing protease inhibitor cocktail (Pierce). Homogenates were sonicated and centrifuged at 10,000 *g* at 4°C to remove cellular debris. Protein concentrations were determined. Samples containing equal amounts of protein in equal volumes of sample buffer were separated in a 4%–15% Tris-HCl polyacrylamide gel and transferred to PVDF membrane (Bio-Rad). Nonspecific binding sites were blocked with Tris-buffered saline, containing 5% nonfat dry milk, for 1 hour at room temperature. Membranes were then incubated with antibodies to GPX1, Akt, phospho-Akt, STAT3, phospho-STAT3 (Cell Signaling Technology), or GAPDH (Santa Cruz Biotechnology Inc.) in Tris-buffered saline with 0.1% Tween 20. Membranes were washed and incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were detected via enhanced chemiluminescence.

*Statistics*. Data are expressed as mean ± SD. Significant differences between groups were determined by ANOVA, with a Bonferroni correction for continuous variable and multiple groups. Two-tailed Student's *t* test was used for the comparison of a normally distributed continuous variable between 2 groups. For the survival studies, Kaplan-Meier log-rank analysis was performed. *P* values of less than 0.05 were considered statistically significant.


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Address correspondence to: Stephen Tomlinson, Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425, USA. Phone: (843) 792-1450; Fax: (843) 792-2464; E-mail: tomlinss@musc.edu.

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