

C3a and C3b Activation Products of the Third Component of Complement (C3) Are Critical for Normal Liver Recovery after Toxic Injury¹

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Although the complement system has been implicated in liver regeneration after toxic injury and partial hepatectomy, the mechanism or mechanisms through which it participates in these processes remains ill-defined. In this study, we demonstrate that complement activation products (C3a, C3b/iC3b) are generated in the serum of experimental mice after CCl₄ injection and that complement activation is required for normal liver regeneration. Decomplementation by cobra venom factor resulted in impaired entry of hepatocytes into S phase of the cell cycle. In addition, livers from C3-deficient (C3^{-/-}) mice showed similarly impaired proliferation of hepatocytes, along with delayed kinetics of both hepatocyte hyperplasia and removal of injured liver parenchyma. Restoration of hepatocyte proliferative capabilities of C3^{-/-} mice through C3a reconstitution, as well as the impaired regeneration of C3a receptor-deficient mice, demonstrated that C3a promotes liver cell proliferation via the C3a receptor. These findings, together with data showing two waves of complement activation, indicate that C3 activation is a pivotal mechanism for liver regeneration after CCl₄ injury, which fulfills multiple roles; C3a generated early after toxin injection is relevant during the priming of hepatocytes, whereas C3 activation at later times after CCl₄ treatment contributes to the clearance of injured tissue. *The Journal of Immunology*, 2004, 173: 747–754.

Complement is traditionally recognized as part of the innate immune system, defending the host against the invasion of foreign pathogens. However, several recent reports have described novel “unconventional” functions of complement components (1). Those of particular interest and significance include cell survival, growth, and differentiation in various tissues (2, 3). Complement has also been implicated as a mediator of lens and limb regeneration in lower vertebrates (4). Our laboratory has focused considerable effort on defining the role of complement in liver regeneration (5, 6).

Endangered by a multitude of potentially damaging xenobiotics, cellular, and viral agents, the liver has developed the extraordinary ability to regenerate, regulating its own mass and growth. Through a cascade of cytokine and protein signaling, liver cells are induced to reenter the cell cycle from their normally quiescent state. Both parenchymal and nonparenchymal cells proliferate, so that the original liver mass is eventually restored (7). Injection of carbon tetrachloride (CCl₄), leading to necrosis and apoptosis of liver cells and transient hepatic failure, results in liver regeneration. This halogenated alkane, an environmental toxin widely used in the dry-

cleaning industry, has often been applied for the study of hepatic pathophysiology (8–13).

We have recently shown that anaphylatoxin C5a, through an interaction with its receptor, contributes to liver regeneration after toxic injury (5). C5a production is a consequence of the enzymatic cleavage of C5 by the C5 convertase, which can be formed only in the presence of active C3 cleavage products that are generated during complement activation.

C3, the third component of the complement system, has a central role in the complement cascade. The three pathways of complement activation (classical, alternative, and lectin) converge at this key molecule (14). Therefore, C3 deficiency not only eliminates C3-mediated and C3 activation product-mediated functions but also generally prevents the downstream activation of other complement proteins and prevents them from fulfilling their functions. The aim of the study presented was to assess whether complement activation occurs during liver regeneration and whether C3 activation products are involved in the propagation of the regenerative response. To define the role of C3 and C3 activation fragments in the regenerative process, we applied the model of CCl₄-induced liver injury to mice genetically deficient in C3. We set out to determine whether C3 acts indirectly during liver regeneration as an upstream regulator of C5 activation, whether it has a direct role independent of C5 activation, or whether both possibilities exist.

In the present study we show that complement is activated immediately after toxic injury in wild-type mice. In addition, we demonstrate that C3-deficient (C3^{-/-}) mice show impaired liver regeneration after exposure to CCl₄, characterized by a significant delay of hepatocyte entry into S phase and delayed removal of damaged liver parenchyma. Both C3a and the C3a receptor (C3aR) are apparently involved in the regulation of hepatocyte hyperplasia because defective proliferation in C3^{-/-} livers could be corrected by reconstitution with synthetic murine C3a, and C3aR^{-/-} mice

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displayed impaired regeneration. Our studies not only provide new insights into the process of liver regeneration but are also clinically relevant, as complement components may prove to be efficient therapeutics in the treatment of patients exhibiting symptoms of acute liver failure.

Materials and Methods

Animals

C3^{-/-} and C3aR^{-/-} mice used in this study have been previously described (15, 16). The deficient strains were backcrossed nine generations onto a C57BL/6 background, and their wild-type littermates were used as controls. Female mice 14- to 16-wk-old were used for experiments and were housed in an animal facility of the University of Pennsylvania (Philadelphia, PA), within a barrier on a 12-h light-dark cycle. Water and a standard rodent diet were provided ad libitum. Studies were conducted in compliance with the guidelines of the University of Pennsylvania, and all experiments were performed in accordance with an animal protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

CCl₄ injury

Acute CCl₄ injury was induced in age- and gender-matched C3^{-/-}, C3aR^{-/-}, cobra venom factor (CVF)³-treated, and wild-type mice by i.p. injection of a single 2 μl/g dose of a 50% (1/1) solution of CCl₄ (Sigma-Aldrich, St. Louis, MO) in mineral oil. Animals were sacrificed at various time points after injury (3, 24, 36, 48, 72 and 96 h). Livers were harvested, fixed overnight in 10% neutral-buffered formalin, and processed for paraffin embedding, sectioning and histological evaluation (H&E staining), or snap-frozen in liquid nitrogen for immunostaining for C3 deposition.

ELISA for measuring C3b/iC3b/C3c

The reactivity of mAb 2/11 with a neoantigenic site on the mouse C3 activation fragments C3b/iC3b/C3c (17) allowed us to design a sandwich ELISA that could directly detect C3 activation products in the presence of native C3. Blood was collected from the tails of wild-type mice with 0.02 M EDTA at various times after CCl₄ or mineral oil injection. Plasma was separated from blood cells by centrifugation at 1000 × g for 15 min at 4°C and stored at -70°C until analysis. Microtiter plates (Immunoplate Maxi-sorp; Nunc Immunoplate, Roskilde, Denmark) were coated with a rabbit anti-rat IgG Fc Ab (2.3 μg/ml dilution in PBS, pH 7.4, ICN Pharmaceuticals, Bryan, OH) for 2 h. Nonspecific binding to the wells was prevented by incubation with blocking buffer (1% BSA in PBS) for 1 h. The supernatant of a hybridoma cell culture producing mAb 2/11 was diluted 1/50 in blocking buffer, added to the wells, and incubated for 1 h. Serial dilutions of mouse plasma (1/50 starting dilution in blocking buffer) were added and incubated for 1 h. Subsequently, a HRP-conjugated polyclonal goat anti-mouse C3 Ab (3.2 μg/ml in blocking buffer, ICN Pharmaceuticals) was added and incubated for 1 h. Bound Ab was detected by the addition of substrate solution (0.05% ABTS; Roche Diagnostic, Indianapolis, IN) and 0.03% (H₂O₂ in 0.1 M sodium citrate buffer, pH 4.2).

OD was measured in an ELISA reader at 405 nm. All incubations were conducted at room temperature, and the wells were washed with PBS containing Tween 20 (0.05%) between incubation steps. For quantification purposes, CVF-activated wild-type mouse plasma was used in all experiments as a standard for 100% complement activation, with a starting dilution of 1/500 in blocking buffer.

Liver morphology

Liver histology was assessed by light microscopy (Olympus B X60) of H&E-stained 5-μm sections in a blinded fashion. The extent of parenchymal damage was quantitatively estimated with Scion Image software (version 4.0.2; National Institutes of Health, Bethesda, MD) using histological slides under ×40 magnification.

Biochemical evaluation of liver injury in serum

Blood was collected by cardiac puncture of isoflurane-anesthetized mice at various times after CCl₄ injury. After clotting, serum was obtained and stored until analysis as previously described for plasma. The extent of injury was determined by measuring the degree of elevation of alanine aminotransferase, aspartate aminotransferase, and total bilirubin in the se-

rum of CCl₄-treated mice. All enzymatic assays were performed by Anilytics (Gaithersburg, MD).

BrdU incorporation and immunostaining

To detect S phase in proliferating hepatocytes, 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) was administered to mice by i.p. injection of a dose of 50 mg/kg body weight 2 h before livers were harvested. Paraffin-embedded liver sections were then stained with a mouse anti-BrdU mAb (Boehringer Mannheim, Indianapolis, IN) and an avidin-biotin-peroxidase conjugate (Vectastain ABC kit; Vector Laboratories, Burlingame, CA): In brief, paraffin-embedded liver sections were deparaffinized and then rehydrated through a series of alcohol solutions. The tissue sections were denatured in 10 mM citric acid, pH 6.0, and endogenous peroxidase activity was quenched by incubating the slides in a mixture of methanol and H₂O₂. After successive blocking steps in avidin, biotin, and 4% horse serum/PBS, the tissue was incubated with primary anti-BrdU Ab (0.2 μg/ml) for 45 min at 37°C, then with secondary biotinylated horse anti-mouse IgG (7.5 μg/ml) for 30 min at 37°C, followed by the avidin-biotin-peroxidase conjugate. BrdU reactivity in the tissue sections was detected with diaminobenzidine (DAB, Vector Laboratories). BrdU-positive hepatocytes were identified by their round, dark-stained nuclei under high-power magnification. Sections were counterstained with hematoxylin (Gill's formulation) to localize nonreplicating hepatocytes (blue-stained nuclei). BrdU-positive nuclei were quantified by counting 10 fields per slide at ×400 magnification (10 high-powered fields).

Immunostaining to assess C3 deposition

Frozen sections 5-μm thick, taken from livers of C3-sufficient mice harvested at various time points after CCl₄ or mineral oil injections, were incubated overnight at 4°C with polyclonal anti-mouse C3b/iC3b/C3c Ab (1/1000 dilution) generated in rabbit using purified mouse C3c. Preimmune rabbit IgG was used as an isotype control. Additionally, to exclude the possibility that complement components are adsorbed from serum in a nonspecific manner by damaged tissue (18), additional immunostaining was performed using a monoclonal rat anti-C3a (3/11) and anti-C3b/iC3b (2/11, 3/26) Abs as previously described (17). Biotinylated goat anti-rabbit or rabbit anti-rat IgG (7.5 μg/ml) was then applied to slides for 30 min at 37°C, followed by an avidin-biotin-peroxidase conjugate. Immunoreactivity in the tissue sections was detected with DAB (Vector Laboratories). Livers from CCl₄-treated C3^{-/-} mice served as additional negative controls.

Detection of apoptosis in liver sections

The presence of apoptosis in liver tissue was assessed by two different assays to ensure detection of both early and late phases of cell death. Paraffin sections were stained for the presence of apoptotic cells by TUNEL assay (late phase) using ApopTag Plus Peroxidase In Situ Apoptosis Detection kit (Intergen Company, Purchase, NY). Additionally, immunohistochemistry was performed with the use of the mouse mAb M30 CytoDEATH (Roche Diagnostics, Mannheim, Germany), which recognizes the specific caspase 3 cleavage site on cytokeratin18 (early phase), diluted 1/250. The protocols for these assays were previously described (19, 20).

CVF depletion of complement

To achieve virtually complete depletion of serum complement, CVF (50 μg/animal) was injected i.p. into wild-type mice (21, 22). To minimize possible acute effects of decaplementation, this treatment was administered in two boluses. The first bolus (half the dose) was given 24 h before the actual experiments; the second half-dose was injected 2 h later.

Synthesis of murine C3a

Mouse C3a was synthesized on an Applied Biosystems (Foster City, CA) 433A peptide synthesizer using standard Fmoc protocols (23, 24). Synthesis was conducted on a preloaded Fmoc-Arg(Pmc) p-hydroxymethylphenoxymethyl polystyrene (HMP) resin (Applied Biosystems) with *N*-α-9-fluorenylmethoxycarbonyl-protected amino acids (Novabiochem, San Diego, CA), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (Applied Biosystems) coupling, and 20% piperidine (Applied Biosystems) in *N*-methylpyrrolidone (Fisher Scientific, Fair Lawn, NJ) deprotection. The protein was cleaved from the resin with trifluoroacetic acid (TFA; Applied Biosystems)/phenol (Fisher Scientific)/thionisole (Sigma-Aldrich)/1,2-ethanedithiol (Fluka, Geneva, Switzerland)/H₂O (82.5/5/5/2.5/5) as previously described (25). After cleavage, the protein was washed with ether (Sigma-Aldrich), then extracted with 0.1% TFA in 50% acetonitrile (Fisher Scientific) and lyophilized. The crude

³ Abbreviations used in this paper: CVF, cobra venom factor; TFA, trifluoroacetic acid; DAB, diaminobenzidine; BrdU, 5-bromo-2'-deoxyuridine.

product was purified by reverse phase HPLC on a C-18 column (Vydac, Hesperia, CA) using a 30–70% acetonitrile gradient containing 0.1% TFA. The purity was assessed by reverse phase HPLC, and the mass was confirmed by MALDI on a Micromass Tofspec 2E (Beverly, MA) (26).

The purified product was folded by subjecting it to air oxidation (27, 28). It was solubilized in a small volume of 0.1% TFA and slowly added to 0.1 M ammonium bicarbonate, pH 8.5, with stirring, to a final concentration of 0.1 M. The solution was gently bubbled with O₂ at 4°C for 5 days. Folding progress was monitored by reverse phase HPLC, with no change being noted after 3 days. The folded protein was aliquotted and lyophilized. An aliquot was solubilized in H₂O and dialyzed against PBS, pH 7.4.

Protein purity was assessed by HPLC and SDS-PAGE, and the mass was verified by MALDI. The presence of free thiols was assessed using pHMB (29). Chemotactic activity of synthetic C3a was the same as expressed protein. Synthetic C3a was analyzed for its LPS content using a *Limulus* assay (Pyrochrome), and LPS levels were found to be below 1.5 ng/mg protein.

Reconstitution of C3^{-/-} mice with synthetic murine C3a

C3^{-/-} mice were injected i.p. with three successive doses of synthetic murine C3a 20 min before and at two 6-h intervals after CCl₄ administration (15 μg/mouse/injection).

Statistical analysis

Data are expressed as means ± SE. The Mann-Whitney *U* test or the χ² test was used for the determination of significance. At least four animals per cohort per time point were used for experiments and data analysis.

Results

C3 is activated after CCl₄ toxic injury

Quantification by ELISA of C3 cleavage products in mouse plasma revealed two waves of complement activation after CCl₄ injury (Fig. 1). An increase in the level of active C3 fragments was observed as early as 1 h after CCl₄ injection, and the first peak was reached 2 h later. A more prominent wave of complement activation was seen between 24 and 48 h after CCl₄ injection. The amount of circulating C3a at the highest peak of complement activation was estimated to be ~2.5 μg, taking into consideration that the estimated total C3 concentration is 0.5 mg/ml in adult mouse serum. Plasma samples from animals treated with mineral oil alone showed no increase in the level of C3 cleavage products when compared with baseline levels. The differences in the level of complement activation between various time points after toxic injury, as well as the differences between CCl₄- and mineral oil-treated animals, were statistically significant (Fig. 1, *p* < 0.05). To rule out the possibility that CCl₄ directly activates C3 on its own,

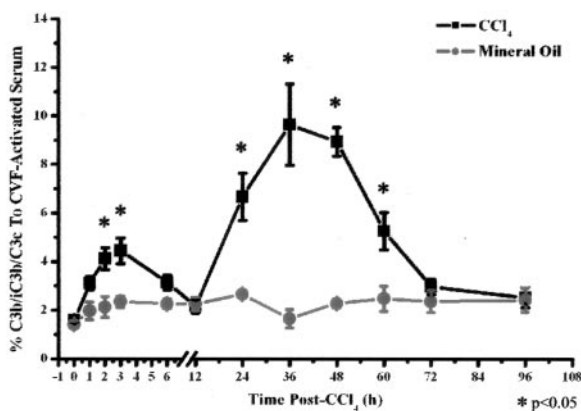


FIGURE 1. Amount of C3b/iC3b/C3c detected in the plasma of wild-type mice at various time points after CCl₄ injection. Asterisks represent the time points at which differences between animals treated with mineral oil and CCl₄ were statistically significant.

we performed experiments in which plasma and whole blood were incubated with various doses of CCl₄ for 30 min at 37°C. We observed no C3 activation by CCl₄ at the concentrations used in this experiment (data not shown).

CVF treatment abolishes the regenerative capability of hepatocytes after toxic injury by CCl₄

CVF, together with Bb, forms a very stable convertase and completely activates C3, thus depleting C3 in the injected animal 24 to 72 h after treatment (21, 22). If the post-CCl₄ injection complement activation previously described plays a role during liver regeneration, then animals with depleted complement proteins should exhibit impaired regeneration. Indeed, CVF-treated wild-type mice showed significantly decreased levels of BrdU incorporation into hepatocyte nuclei 48 h after toxic injury when compared with wild-type controls with normal levels of complement proteins (Fig. 2, column 1 vs 4, *p* < 0.01).

The DNA synthetic response in C3^{-/-} livers is blunted after CCl₄-induced injury

Liver regeneration after CCl₄ injection in C3^{-/-} mice was examined to confirm the results obtained using CVF-treated animals and to further dissect the contribution of C3 to the regenerative response after toxic liver injury. In contrast to their wild-type littermates, C3^{-/-} mice displayed an abnormal regenerative response, illustrated by a lower number of BrdU-positive hepatocyte nuclei at 36 and 48 h after CCl₄-induced injury, along with a delayed peak of hepatocyte proliferation (Fig. 2, column 2 vs 4, and Fig. 3, *p* < 0.01). However, no differences in survival were observed between C3^{-/-} mice and wild-type littermates despite significant impairment of hepatocytes proliferation in C3^{-/-} mice.

Reconstitution of C3^{-/-} mice with murine C3a restores liver regeneration

We found that C3 deficiency resulted in impaired liver regeneration, characterized by decreased and delayed DNA synthesis in hepatocytes. To identify which cleavage products of C3 are crucial for liver regeneration, we performed reconstitution experiments with synthetic murine C3a. The amount of C3a injected into C3^{-/-} mice (3 × 15 μg) is sufficient to reconstitute the level of

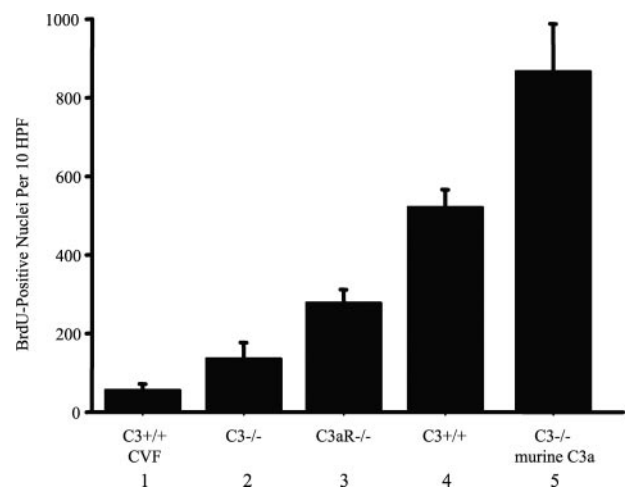


FIGURE 2. BrdU incorporation into hepatocytes 48 h after CCl₄ injection. BrdU was injected into wild-type mice treated with CVF (column 1), C3-deficient mice (column 2), C3aR-deficient mice (column 3), wild-type mice without CVF treatment (column 4), and C3-deficient mice reconstituted with synthetic murine C3a (column 5). HPF, high-power field.

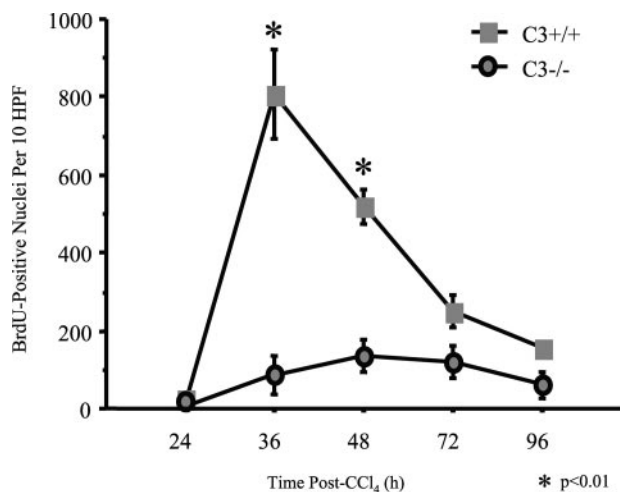


FIGURE 3. BrdU incorporation in C3-deficient animals vs wild-type controls at various time points after CCl₄ challenge. Asterisks represent the time points at which differences between wild-type and C3-deficient animals were statistically significant. HPF, high-power field.

C3a in these mice to the level generated in wild-type animals after CCl₄ injection. Reconstitution of C3-deficient animals with synthetic murine C3a completely restored the wild-type regenerative phenotype. DNA synthesis in C3a-reconstituted animals 48 h after toxic injury not only reached wild-type levels but showed a trend toward surpassing the values typical for these controls (Fig. 2, column 4 vs 5). However, these differences were not statistically significant. These data support the hypothesis that impaired liver regeneration after CCl₄-induced injury in C3^{-/-} mice is not a secondary phenomenon but is indeed specifically related to the lack of C3 in knockout animals. Moreover, they imply that hepatocyte regeneration is connected to C3a generated in serum through C3 cleavage immediately after CCl₄-induced injury. This response to CCl₄ suggests an important role for the C3a anaphylatoxin in hepatocyte priming because initial complement activation occurs soon after CCl₄ injection (Fig. 1).

C3aR is required for effective hepatocyte entry into S phase of the cell cycle after CCl₄ challenge

The observation that reconstitution with synthetic murine C3a completely restored the proliferative response in regenerating hepatocytes of C3^{-/-} mice led us to study whether the C3a-C3aR interaction was important in this context, or whether C3a exerted its function in a C3aR-independent manner. Therefore, we examined liver regeneration after CCl₄ injection in C3aR^{-/-} mice. BrdU incorporation measured 48 h after challenge was significantly lower in C3aR-deficient animals than in their wild-type littermates (Fig. 2, column 3 vs 4, $p < 0.01$). These results demonstrate that C3aR signaling after C3a stimulation is essential for normal liver regeneration and identify C3a-C3aR cross-talk as an important factor for hepatocyte proliferation after toxic injury. However, the level of DNA synthesis in C3aR-deficient animals was slightly higher than in C3^{-/-} mice, indicating a possible role for other C3-mediated processes.

Clearance of damaged liver tissue is delayed in C3^{-/-} mice after CCl₄ treatment

H&E-stained histological sections from both C3^{-/-} mice and their wild-type littermates revealed a centrilobular pattern of injury after

CCl₄ treatment. Livers harvested 24 h after CCl₄ injection showed more eosinophilic staining of centrilobular regions compared with uninjured livers, and no apoptotic or necrotic damage. The morphological features of apoptosis and necrosis were not seen until 36 h after CCl₄ injection (Fig. 4). This observation was confirmed by TUNEL assay and immunostaining with the Ab M-30 (Fig. 5 and Fig. 6). Both methods for apoptosis detection revealed few apoptotic cells throughout liver sections from C3^{+/+} and C3^{-/-} mice 24 h after CCl₄ injection. Moreover, these cells were not localized in centrilobular regions, indicating that their presence was not related to CCl₄ toxicity. Only isolated TUNEL-positive nuclei were seen around central veins 36 h after toxic injury in both strains (Fig. 5, B and F), despite obvious morphological features of tissue injury seen in H&E slides that indicated severe damage of liver parenchyma. Compared with TUNEL staining, significantly more cells were stained with the M-30 Ab in both C3^{+/+} and C3^{-/-} livers at the 36 h time point (Fig. 6). Some of the apoptotic cells detected by this assay were already engulfed by cells with typical macrophage morphology (elongated, vesicular, kidney-shaped nuclei) (Fig. 6). Livers harvested 48 h after CCl₄ injection showed numerous apoptotic cells using both apoptosis detection assays, and at this time point almost all liver cells showing features of injury in H&E slides were also positive in the TUNEL assay (Fig. 5, C and G) and in immunostaining using the M-30 Ab (data not shown). The extent of overall injury at various time points was primarily assessed semiquantitatively in a blinded fashion based on H&E staining. Quantification was performed using Scion Image Software. The magnitude of tissue damage was expressed as a percentage of the total area in the histological sections.

We did not observe any significant differences in the extent of injury between C3^{-/-} mice and their wild-type littermates at 24, 36, or 48 h after CCl₄ injection (Fig. 7). This lack of differences was corroborated by similar levels of aminotransferases and bilirubin in C3^{-/-} and wild-type mice at 36 h (data not shown), the time of maximum tissue damage after CCl₄ injection as indicated by histological analysis (Fig. 4, B and F). However, H&E-stained sections from wild-type animals, but not from C3-deficient mice, showed histological signs of regression of damaged tissue (a sharp demarcation of necrotic and apoptotic areas from surrounding parenchyma, with mononuclear cell infiltration) at 48 h (Fig. 4, C and G). Histological sections revealed striking differences in the amount of damaged tissue 72 h after CCl₄ injection (Fig. 4, D and H, and Fig. 7). Injured tissue was hardly visible in wild-type animals at this time compared with C3^{-/-} mice. In centrilobular regions of 72 h C3^{+/+}, but not C3^{-/-} livers, single clusters of deeply eosinophilic "ghost" cells were still present, and in some areas the damaged parenchyma was already replaced by an inflammatory infiltrate. In contrast, the extent of injury in 72 h C3^{-/-} livers was similar to that seen in C3^{-/-} animals sacrificed at 48 h, whereas it was significantly higher than in 72 h wild-type controls (Fig. 4 and Fig. 7, $p < 0.05$). The extent of tissue injury for both cohorts 72 h after CCl₄ correlated with the number of apoptotic cells as visualized by apoptosis stainings (Fig. 5). C3^{+/+} livers showed scattered islands composed of few apoptotic cells localized mainly around central veins (Fig. 5D), whereas C3^{-/-} livers still presented a centrilobular pattern of injury with numerous apoptotic cells (Fig. 5H). In these 72 h C3^{-/-} livers some of the apoptotic cells had already undergone disintegration and fragmentation, and therefore less "intact" TUNEL-positive nuclei were seen when compared with C3^{-/-} livers 48 h after CCl₄ injection (Fig. 5, G and H). However, the overall extent of tissue damage, measured as a percentage of injured liver parenchyma, was similar in C3^{-/-} mice 48 and 72 h after CCl₄ challenge (Fig. 7) These

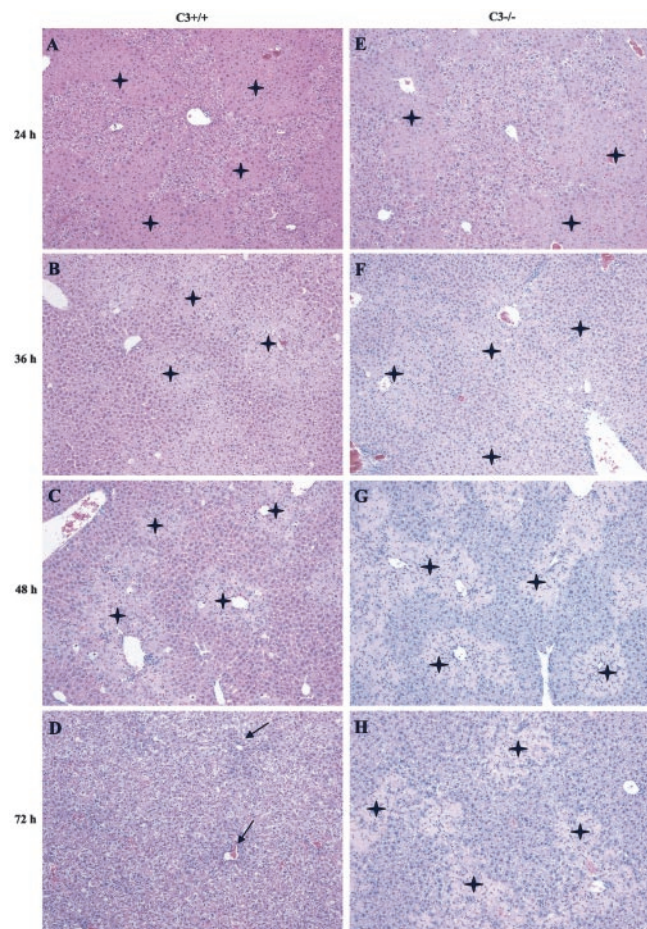


FIGURE 4. Histological H&E-stained liver sections from $C3^{+/+}$ and $C3^{-/-}$ mice. Liver sections from wild-type controls (A–D) and $C3$ -deficient animals (E–H) at various time points (A and E, 24 h; B and F, 36 h; C and G, 48 h; D and H, 72 h) after CCl_4 injection are shown. Asterisks and arrows indicate damaged liver parenchyma. Magnification, $\times 40$.

results suggest a defect in the removal of necrotic and apoptotic cells that is related to $C3$ deficiency, possibly as a result of the inability of $C3^{-/-}$ mice to engage macrophage CR3 receptors with $C3b/iC3b$ on apoptotic cells.

C3 is deposited in damaged liver parenchyma

To assess whether $C3$ is deposited in liver parenchyma as a consequence of complement activation after CCl_4 injection, immunostaining was performed using polyclonal and mAbs on frozen liver sections harvested at times when maximum levels of complement activation occurred. Wild-type mouse livers harvested 3 h after CCl_4 injection showed $C3$ deposition limited to only a few small spots throughout liver sections (Fig. 8A). At 24 h, livers showed a patchy pattern of staining with numerous small areas of $C3$ deposition seen in liver parenchyma (Fig. 8B). At 36 and 48 h after CCl_4 injection, deposited $C3$ was detected mainly in areas of tissue injury (centrilobular regions), but not in viable parenchyma (Fig. 8, C and D), and the amount of $C3$ deposited within the tissue correlated with the extent of injury. None of the $C3^{-/-}$ livers used as negative controls showed positive immunoreactivity. Similarly $C3$ deposition was not detected in liver parenchyma of mineral oil-treated mice (data not shown). In addition, no positive staining was detectable with the use of isotype controls or anti- $C3a$ Ab, suggesting that the reactivity seen with anti- $C3b/iC3b/C3c$ Abs was not the result of nonspecific adsorption of serum proteins by damaged tissue.

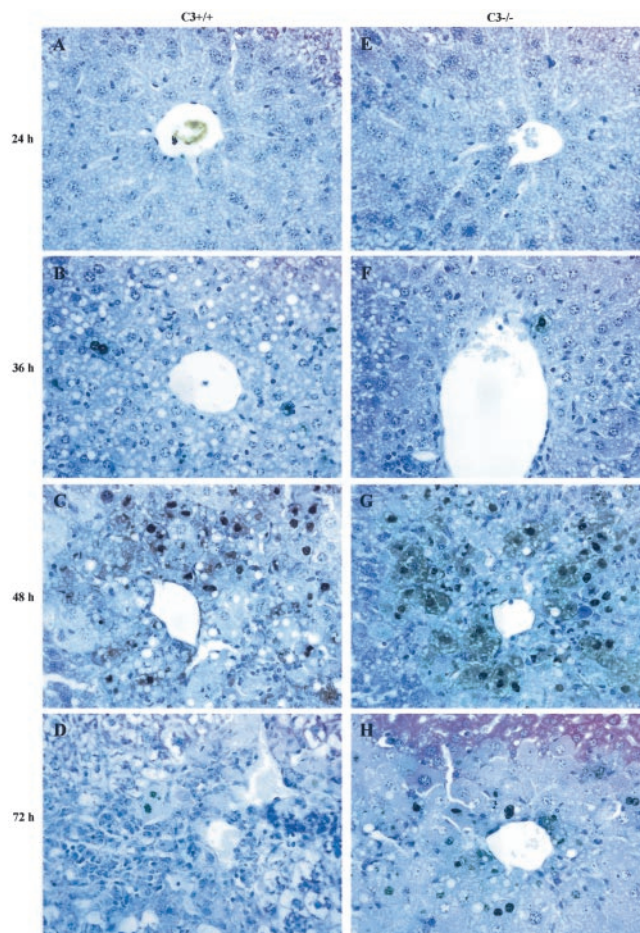


FIGURE 5. TUNEL staining showing apoptosis in liver sections from $C3^{+/+}$ and $C3^{-/-}$ mice. Liver sections from wild-type controls (A–D) and $C3$ -deficient animals (E–H) at various time points (A and E, 24 h; B and F, 36 h; C and G, 48 h; D and H, 72 h) after CCl_4 injection are shown. Magnification, $\times 400$.

Discussion

Complement activation is a phenomenon that occurs in response to various inflammatory stimuli (30, 31). This well-regulated system, composed of over 30 different proteins, is also involved in the pathogenesis of numerous inflammatory and autoimmune diseases (32, 33). However, reports addressing functions of complement other than its involvement in inflammation are still relatively rare (1). CCl_4 -induced liver injury, which is a well-established model for the study of liver regeneration in rodents, can be considered a process that triggers an inflammatory reaction, but this reaction occurs as a late response to damaged liver parenchyma (34). In this model we have shown that activation of $C3$ occurs in two waves (with peaks at 3 and 36 h after CCl_4 injection) during liver regeneration after toxic injury and is involved in the regulation of this process. Depletion of complement proteins with CVF resulted in a blunted DNA synthetic response in hepatocytes of wild-type mice 48 h after toxic exposure. Mice deficient in $C3$, the central component of the complement activation cascade, showed impaired liver regeneration after toxic injury, characterized by significantly decreased BrdU incorporation at various time points, a delayed peak of hepatocyte proliferation and an inability to remove damaged liver parenchyma. Reconstitution of $C3^{-/-}$ mice with $C3a$ completely restored the wild-type phenotype for hepatocyte DNA synthesis. $C3aR^{-/-}$ mice also exhibited impaired liver regeneration after CCl_4 injury, indicating that $C3a$ exerts its function through the $C3aR$.

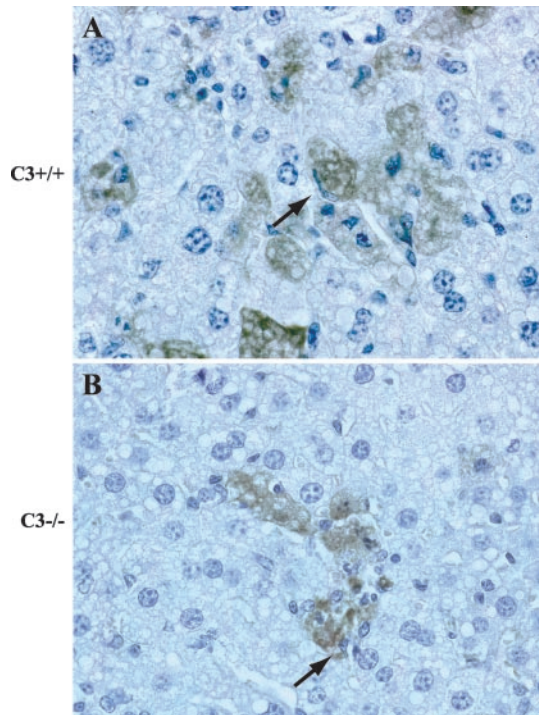


FIGURE 6. Cytokeratin-18 staining for apoptosis in 36 h post-CCl₄ liver sections from C3^{+/+} (A) and C3^{-/-} (B) mice. Magnification, $\times 600$. Arrows indicate macrophages engulfing apoptotic cells.

The first wave of complement activation and the generation of active C3 cleavage products (C3a and C3b) occurs early after CCl₄ injection. This phase of liver regeneration is referred to as the

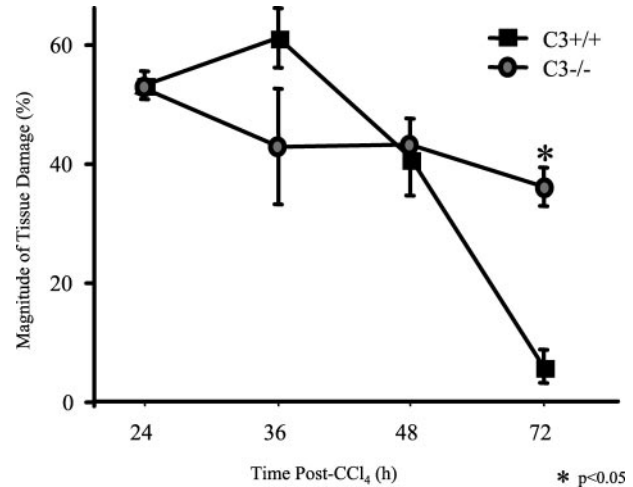


FIGURE 7. Liver tissue damage measured in histological slides in C3-deficient and wild-type mice at various time points after CCl₄ injection. The differences between the two groups of animals at 72 h after CCl₄ injection were statistically significant.

priming of liver cells (35, 36). Therefore, considering the successful reconstitution of C3^{-/-} mice with C3a, we suggest that complement activation and the generation of C3a participate in the regulation of the priming phase of liver regeneration. C3b is also involved, as it is deposited in liver parenchyma early after CCl₄ injury, and contributes to the formation of the C5 convertase, leading to C5 cleavage and C5a production. The importance of C5a for normal liver regeneration after CCl₄ injury has been demonstrated by our laboratory in a previous study (5). In addition, both C3a and

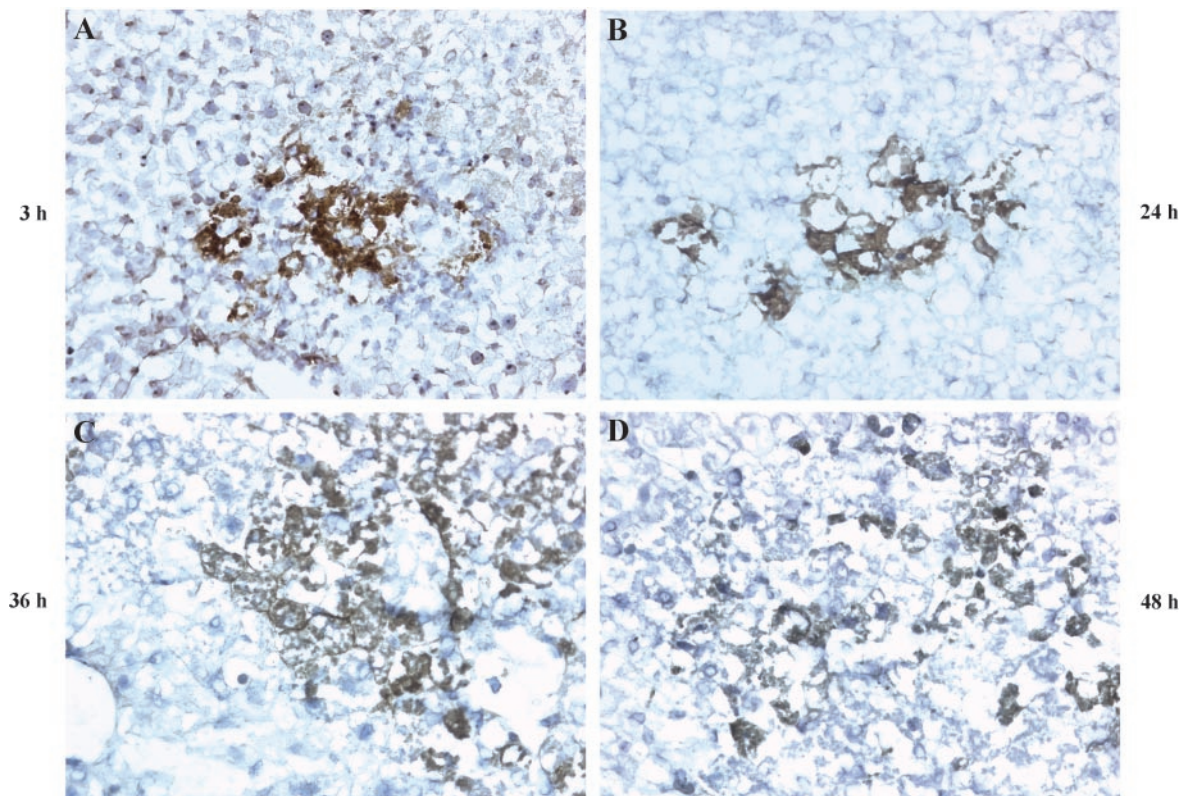


FIGURE 8. C3 deposition detected with polyclonal anti-C3b/iC3b/C3c Ab in frozen liver sections from C3^{+/+} mice after CCl₄ treatment. Time points shown are 3 h (A), 24 h (B), 36 h (C) and 48 h (D). Magnification, $\times 400$.

C5a have been shown to be involved in hepatocyte priming during liver regeneration after partial hepatectomy (6).

Based on these previous findings and the results presented in this report, we propose that both anaphylatoxins generated during complement activation act as early regulators of normal liver regeneration after toxic injury. C3a injected alone into C3^{-/-} mice significantly increased hepatocyte proliferation when compared with nontreated C3^{-/-} mice. In this experimental environment, C3^{-/-} mice reconstituted with C3a are not able to generate C3b, and consequently cannot form the C5 convertase that is responsible for C5 cleavage. However, the proliferation of hepatocytes in these mice, even in the absence of C5 convertase, returned to wild-type levels. Therefore, the effect of C3a reconstitution on the regenerative response in C3^{-/-} mice raises the question of how animals lacking the ability to form a C5 convertase (C3b3bBb or C4b2b3b) can successfully restore proliferation of liver cells if C5a is an essential priming factor. A recent report has shown that C5a can be generated by activated phagocytic cells independently of a complement convertase (37). This may allow the production of C5a in CCl₄-treated C3^{-/-} mice, especially considering that liver macrophages are rapidly activated by TNF- α , released as a response to stimuli initiating liver regeneration (9). Another possible explanation is that the lack of C5a in C3^{-/-} mice might be compensated for by a variety of other signals generated by the deficient strain to maintain a proper homeostatic response to various factors.

The overlap in anaphylatoxin functions during the regulation of liver regeneration after partial hepatectomy (6) and CCl₄ injury is not surprising. C3a and C5a share several activities in various pathophysiological situations. For example, both C3a and C5a are responsible for increased endothelial permeability in small blood vessel and have the potential to induce smooth muscle contraction (38). Both also have the ability to trigger oxidative bursts in various leukocytes (38). The growing list of activities common to these anaphylatoxins is long and is discussed in detail in review articles (39). However, in contrast to these shared activities, C3a and C5a may also have quite opposite effects on cellular functions, and their influence on the course of certain disease processes may be significantly different. A classical example of these diverse activities is seen in airway hyperresponsiveness in asthma (39).

Thus, it seems reasonable that both C3a and C5a share similar actions in the regulation of liver regeneration in both models mentioned earlier in this report. However, more diffuse patterns of tissue injury observed in C5^{-/-} mice after CCl₄ injection may suggest additional cytoprotective functions of C5a besides the regulation of hepatocyte proliferation (5). This supposition may be additionally supported by findings that C5a protects neurons from exogenous toxin-induced degeneration and apoptosis (40).

C3^{-/-} mice, apart from the obvious defect in hepatocyte proliferation, demonstrated a significant delay in the removal of damaged liver parenchyma. These observations are in concordance with reports showing that C3 cleavage products (C3b, iC3b) facilitate phagocytosis of apoptotic and necrotic cells, as well as apoptotic bodies, by macrophages (41). We have shown in this study that C3b/i3Cb is deposited in damaged liver parenchyma, and we have confirmed that apoptosis is involved in cell death after CCl₄ acute injury.

Tissue phagocytes express CR1 and CR3 on their surface, and these receptors interact with C3b/iC3b deposited on apoptotic and necrotic cells (42). Removal of damaged tissue occurred more slowly in C3^{-/-} mice lacking C3b/iC3b, as shown in our study by quantification of damaged tissue at 72 h post-CCl₄ injury. At this time point, C3^{-/-} mice still presented morphological features and molecular markers of extensive apoptosis and necrosis, while at

the same time damaged tissue was rarely seen in their wild-type counterparts, despite similar initial levels of injury in both strains. The involvement of C3b and iC3b in the elimination of damaged liver parenchyma may offer a plausible explanation for the second peak of complement activation in wild-type mice 36 h after CCl₄ injection. During this second wave of complement activation, which was even more prominent than the first, high amounts of C3b and iC3b were generated and deposited in damaged liver parenchyma, as shown by immunostaining. Another aspect to be considered is that several reports have shown apoptotic cells to activate complement (43, 44). Our conclusion is that tissue injury induces the second wave of C3 activation. This wave peaked 36 h after CCl₄ treatment, which correlated with the presence of maximum tissue damage and the results of immunostaining that showed massive C3 deposition 36 h after the beginning of the experiment. Moreover, complement activation occurred as long as apoptotic and necrotic cells were present in the liver. It began 24 h after CCl₄ injection, when only slight morphological features of injury were present, and lasted until injury was nearly resolved at 72 h.

In summary, we postulate that complement activation after CCl₄-induced liver injury and generation of C3a and C5a, as well as C3b/iC3b, is an important homeostatic mechanism in the course of liver regeneration. C3a and C5a generated during the first few hours after toxic exposure are involved in the priming of liver cells for regeneration. C3b/iC3b, generated in large amounts later in the course of toxic injury, facilitate the removal of damaged liver tissue.

There is little literature in which hepatic disease and complement activation have been addressed. A report dating to 1994 shows increased C3d levels in patients with acute and subacute hepatic failure caused by viral infection (45). In contrast, patients with viral hepatitis, without signs of liver failure, did not have elevated C3d levels. In TNF- α -galactosamine-induced lethal hepatitis, levels of total complement that produces 50% lysis in hemolytic activity (CH₅₀ and APCH₅₀) were decreased, suggesting strong activation of both the classical and alternative pathways (46). C3 deposition on cells was also seen, but complement did not influence the outcome. A report addressing the role of complement activation in chronic ethanol-induced liver injury has described complement activation as a harmful process contributing to the development of liver damage (47). These seemingly contradictory reports not only reflect the dual role of complement, its function as a "double-edged sword," (30, 48) but they also demand a rigorous differentiation between different models: acute vs chronic liver injury, virally vs toxically induced hepatitis and transient vs lethal liver failure.

Our work presented in this report identifies complement activation and generation of C3 cleavage fragments as a pivotal mechanism by which liver regeneration is initiated. New insight is provided into the role of complement in acute toxic liver injury, furthering our understanding of the functions of complement proteins. However, the mechanism by which complement is activated requires further investigation, especially at early time points after toxic injury. It seems possible that autoantibodies circulating in mouse plasma are able to recognize molecules expressed in liver as a consequence of toxic injury, and may participate in the removal of damaged liver parenchyma (49). This hypothesis is currently under investigation in our laboratory.

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