

A Novel Role of Complement: Mice Deficient in the Fifth Component of Complement (C5) Exhibit Impaired Liver Regeneration¹

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Components of innate immunity have recently been implicated in the regulation of developmental processes. Most strikingly, complement factors appear to be involved in limb regeneration in certain urodele species. Prompted by these observations and anticipating a conserved role of complement in mammalian regeneration, we have now investigated the involvement of complement component C5 in liver regeneration, using a murine model of CCl₄-induced liver toxicity and mice genetically deficient in C5. C5-deficient mice showed severely defective liver regeneration and persistent parenchymal necrosis after exposure to CCl₄. In addition, these mice showed a marked delay in the re-entry of hepatocytes into the cell cycle (S phase) and diminished mitotic activity, as demonstrated, respectively, by the absence of 5-bromo-2'-deoxyuridine incorporation in hepatocytes, and the rare occurrence of mitoses in the liver parenchyma. Reconstitution of C5-deficient mice with murine C5 or C5a significantly restored hepatocyte regeneration after toxic injury. Furthermore, blockade of the C5a receptor (C5aR) abrogated the ability of hepatocytes to proliferate in response to liver injury, providing a mechanism by which C5 exerts its function, and establishing a critical role for C5aR signaling in the early events leading to hepatocyte proliferation. These results support a novel role for C5 in liver regeneration and strongly implicate the complement system as an important immunoregulatory component of hepatic homeostasis. *The Journal of Immunology*, 2001, 166: 2479–2486.

In mammals, the liver has the remarkable ability to regenerate and restore its anatomic and homeostatic integrity in response to partial hepatectomy, toxic exposure, or viral injury (1–3). Liver parenchymal cells, including hepatocytes, and nonparenchymal cells such as endothelial, stellate, and Kupffer cells, respond to these stimuli by shedding their quiescent phenotype and synchronously entering the S phase of the cell cycle and proliferation (4). The regenerative process is normally completed within 6–7 days after hepatectomy or toxic injury, with restoration of the original liver mass or complete recovery of the damaged tissue. Various hormones (insulin, norepinephrine) (2, 5), growth factors (hepatocyte growth factor, epidermal growth factor, TGF- α) (6–8), and proinflammatory cytokines (IL-6, TNF- α) have been implicated in triggering or regulating this complex phenomenon through activation of their respective signal transduction pathways (9, 10). However, the exact molecular events and mechanisms by which these factors contribute to the regulation of liver regeneration are still poorly understood.

The complement system, a phylogenetically ancient arm of the innate immune response (11), has recently been suggested to play novel roles in regulating noninflammatory processes, including cell proliferation and differentiation during development (12, 13). In this context, it has been recently proposed that complement factors may contribute to the regulation of amphibian limb regeneration, as indicated by the expression of the third component of complement (C3) in the regenerating limb blastema cells of certain urodele species (14). This potential involvement of complement in intricate morphogenetic and developmental processes, including tissue remodeling during limb regeneration and muscle cell differentiation in urodeles (14), provided the basis for an intriguing hypothesis that complement may also have been selected during evolution to participate in the regulation of the regenerative response in higher vertebrates.

To investigate the potential role of complement in mammalian regeneration, we have examined the involvement of complement component C5 in the regenerative response of the liver to injury, using a murine model of CCl₄-induced liver toxicity (15) and a congenic strain of mice genetically deficient in C5 (16).

CCl₄-mediated liver injury is a well-established model for the study of liver regeneration in rodents (17, 18). It stimulates hepatocyte proliferation and results in both functional and structural restoration of the hepatic parenchyma. Toxin-induced liver injury is associated with free-radical mediated lipid peroxidation and fat deposition in the liver (15). It is mainly characterized by acute hepatocellular necrosis caused by alterations in the permeability of cellular, mitochondrial, and lysosomal membranes (19), and it has recently been shown to induce hepatocyte apoptosis (20).

Here we report that C5-deficient mice show impaired liver regeneration after toxic exposure to CCl₄. Moreover, these mice show a significant delay in the entry of their hepatocytes into S phase at 36–48 h after the injury, as indicated by the reduced BrdU

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incorporation into C5-deficient livers. This defect in hepatocyte proliferation is corrected and normal liver regeneration is restored in these mice after reconstitution with murine C5. The mechanism by which C5 contributes to normal regeneration was delineated using recombinant C5a as well as a specific C5a receptor antagonist.

Materials and Methods

Animals

Fourteen- to 16-wk-old female B10D2oSn-J mice (The Jackson Laboratory, Bar Harbor, ME) were used in this study. The B10D2oSn-J strain bears a 2-bp (TA) deletion in an exon near the 5' end of the C5 gene, which results in the expression of a truncated protein that accounts for the C5 protein deficiency (16). This strain has been generated by backcrossing the C5-deficient DBA/2 strain onto a C57BL/10 background. As a control group, age- and gender-matched wild-type mice of the C5-sufficient B10D2nSn-J strain (The Jackson Laboratory) were used in these experiments.

All mice were housed in the animal facility of the University of Pennsylvania, within a barrier, on a 12-h light/dark cycle. Water and rodent diet were provided *ad libitum*. Mice were acclimatized for at least 1 wk before the experiments. Studies were conducted in compliance with the guidelines of the University of Pennsylvania, and all experiments were performed in accordance with the animal protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

CCl₄ injury

Acute CCl₄ liver injury was induced in age- and gender-matched C5^{-/-} and wild-type (C5^{+/+}) mice by the i.p. injection of a single 2- μ l/g dose of a 50% (v/v) solution of CCl₄ (Sigma, St. Louis, MO) in mineral oil. Four animals in each cohort were sacrificed at various times after injury (0, 24, 36, 48, 72, 96 and 120 h). Livers were harvested, fixed overnight in 10% neutral buffered formalin, and processed for paraffin embedding, sectioning, and histological evaluation (hematoxylin-eosin staining).

BrdU incorporation and immunohistochemistry

5-Bromo-2'-deoxyuridine (BrdU)³ (Sigma) was administered to the mice, 2 h before harvesting of the livers, by i.p. injection, at 50 mg/kg body weight. Paraffin-embedded liver sections were then subjected to immunohistochemical staining using 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. Antigenic sites were made accessible by denaturation of the tissue 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 the primary anti-BrdU Ab (0.2 μ g/ml) for 45 min at 37°C. This step was followed by the addition of a secondary biotinylated horse anti-mouse IgG (7.5 μ g/ml for 30 min at 37°C), and the subsequent incubation with the avidin-biotin-peroxidase complex (ABC reagent; Vector Laboratories). BrdU reactivity in the tissue sections was detected with the addition of diaminobenzidine. BrdU-positive hepatocytes were identified by their large, round, dark brown-stained nuclei under high-power magnification.

Sections were counterstained with hematoxylin (Gill's formulation) to localize nonreplicating hepatocytes (with blue-stained nuclei).

Biochemical evaluation of liver injury-serum toxicity markers

Blood was collected by cardiac puncture of metophane-anesthetized mice at various times after CCl₄ injury. After clotting, serum was obtained by centrifugation at 14,000 rpm for 5 min, and stored in -70°C until analysis. The extent of liver toxic injury was determined by measuring the degree of elevation of alanine aminotransferase (ALT), aspartate aminotransferase, and total bilirubin in the serum of CCl₄-treated mice. All enzymatic assays were performed by Anilytics (Gaithersburg, MD).

Purification of murine C5

Murine C5 was purified from normal mouse serum by a modification of a method previously described (21). Mouse serum (50 ml) (obtained from Cocalico Biologicals, Reamstown, PA) was precipitated with 4% polyethylene glycol (PEG) at 4°C for 30 min in the presence of 20 mM EDTA, 10

mM benzamidine, and 1 mM PMSF. The supernatant collected after centrifugation was further precipitated with 10% PEG. The resulting precipitate after the centrifugation of this mixture (4–10% PEG precipitate) was resuspended in 20 mM NaH₂PO₄ buffer, pH 7.4, loaded onto a DEAE 40 HR (6.5 \times 5.0 cm) anion exchange chromatography column (Millipore, Bedford, MA) and eluted with a linear salt gradient (0–500 mM NaCl). C5-containing fractions were identified by SDS-PAGE, pooled, and dialyzed overnight against PBS, pH 7.4, at 4°C. This C5 pool was subsequently passed through an affinity chromatography column that was prepared by covalently coupling a mouse mAb raised against murine C5 (clone BB5.1) (22) to a cyanogen bromide-activated Sepharose matrix (Pharmacia, Piscataway, NJ). After extensive washing of the column with PBS, the bound protein was eluted with 2 M KBr. The fractions containing C5, which were >99% pure as judged by SDS-PAGE, were immediately pooled and dialyzed overnight against PBS, pH 7.4. This affinity-purified C5 preparation was assayed in a rabbit erythrocyte lysis assay for hemolytic activity mediated through the alternative pathway of complement activation.

Reconstitution of C5^{-/-} mice with murine C5

B10D2oSn-J (C5^{-/-}) mice were injected i.p. with 150 μ g of affinity-purified, hemolytically active, murine C5 in a solution of PBS, 20 min before the administration of CCl₄.

The mice were injected with an amount of protein sufficient to yield a reconstituted serum concentration of C5 identical with that of wild-type animals (75–80 μ g/ml).

In vivo C5aR blockade using a peptide antagonist

The cyclic hexapeptide AcF[OpdChaWR] was used in this study as a specific C5aR antagonist. Peptide synthesis and cyclization were performed as previously described (23). The peptide was purified using preparative reversed-phase HPLC, and eluted fractions were characterized by mass spectrometry (matrix-assisted laser desorption ionization). For the C5aR blockade study, a cohort of C5-sufficient (B10D2nSn-J) mice received i.v. three successive doses (1 mg/kg body weight, in PBS) of antagonist at 6 h-intervals after CCl₄ injury. Mice treated with PBS alone served as a control cohort in this experiment. Both groups were treated i.p. with CCl₄, and BrdU incorporation and immunohistochemistry were performed as described above.

Results

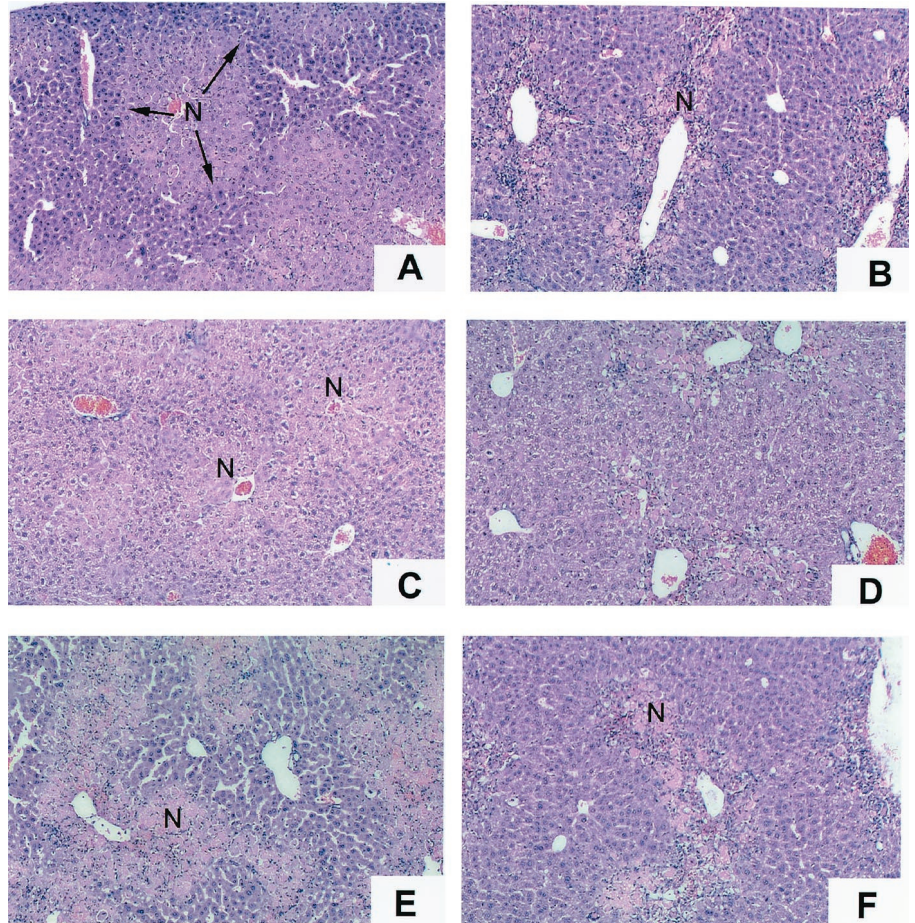
Impaired liver regeneration in C5^{-/-} mice after CCl₄ toxic injury

If C5 is an essential component of normal liver regeneration, mice deficient in this complement protein should exhibit an abnormal regenerative response to toxic liver injury. Indeed, when we treated C5-deficient mice and their wild-type (C5^{+/+}) counterparts with a single dose of CCl₄ and subsequently sacrificed the mice at various times after the challenge, we found that the livers of C5^{-/-} mice displayed extensive necrosis and a diffuse pattern of degeneration that extended throughout the hepatic parenchyma and persisted until 72 h after the injury (Fig. 1). In contrast, wild-type mice displayed a localized and almost exclusively centrilobular pattern of necrosis at 48 h, with the tissue manifesting definitive signs of recovery and regeneration at 72 h after the injury. Of particular note was the significant degree of fat deposition throughout the parenchyma of the C5^{-/-} livers at 48 and 72 h after injury, a feature that was almost absent from the CCl₄-treated C5^{+/+} mice and indicated that the effect of the hepatotoxin was more pronounced in the C5^{-/-} animals (Fig. 2, D and F).

As opposed to the diffuse pattern of injury found in C5^{-/-} livers, the centrilobularly focused lesional zones of the C5^{+/+} livers were sharply demarcated from the surrounding, viable liver parenchyma. The C5^{+/+} liver parenchyma exhibited marked basophilic staining and prominent hepatocyte karyomegaly (nuclear enlargement) at 48 h after the injury, indicating a normal regenerative response. However, the C5^{-/-} liver parenchyma showed widespread fat deposition and a generalized eosinophilic staining indicative of ongoing cell necrosis in the tissue, and no signs of karyomegaly were evident even at 72 h after the injury. The brisk regenerative response of the C5^{+/+} mice was followed by a

³ Abbreviations used in this paper: BrdU, 5-bromo-2'-deoxyuridine; ALT, alanine aminotransferase; PEG, polyethylene glycol.

FIGURE 1. Defective hepatocyte regeneration and extensive parenchymal necrosis in $C5^{-/-}$ mice after CCl_4 injury. Low-power photomicrographs of hematoxylin/eosin-stained liver sections from CCl_4 -treated wild-type, $C5$ -deficient, and $C5$ -reconstituted mice. *A* and *B*, $C5^{+/+}$ livers at 48 and 72 h after injury, respectively. *C* and *D*, $C5^{-/-}$ livers at 48 and 72 h after injury, respectively. *E* and *F*, $C5^{-/-}$ livers at 48 and 72 h after injury and reconstitution with $C5$, respectively. $C5^{-/-}$ livers show a pattern of extensive and diffuse injury throughout the tissue (*C–D*) with a lack of demarcation between the necrotic centrilobular areas and the surrounding parenchyma, whereas $C5^{+/+}$ livers display a localized pattern of centrilobular necrosis as indicated by the arrows in *A*. Widespread parenchymal necrosis (indicated by the prominent eosinophilic staining of hepatocytes) persists in $C5^{-/-}$ livers until 72 h after injury, as shown in *D*. Marked shrinkage of necrotic zones and vigorous hepatocyte regeneration is observed in $C5^{+/+}$ and $C5$ -reconstituted livers at 72 h (*B–F*). N, Centrilobular areas of necrotic tissue. Magnification, $\times 10$.



marked shrinkage of the centrilobular necrotic zones at 72 h after the challenge with CCl_4 , and by a corresponding expansion of the regenerating tissue into the previously damaged areas (Fig. 1).

Another marked difference between the $C5^{-/-}$ and the wild-type livers was the presence of increased parenchymal apoptosis in the wild-type mice during the early stages following CCl_4 exposure (36–48 h), as demonstrated by the presence of numerous cells with condensed (pyknotic) nuclei and apoptotic bodies in the centrilobular areas of hematoxylin/eosin-stained liver sections (Fig. 2, *A* and *C*). The $C5^{-/-}$ livers appeared to have a significantly lower rate of apoptosis during the first 48 h, whereas extensive cell necrosis due to severe toxicity was prominent throughout the tissue (Fig. 2, *B* and *D*). However, after 72 h, increased apoptosis was also detected in the $C5^{-/-}$ livers, comparable to that observed at earlier stages in the wild-type mice.

To evaluate the extent of toxic injury induced to the liver, in terms of plasma transaminase activity and hepatic enzyme release, we assayed sera collected from $C5^{+/+}$ and $C5^{-/-}$ mice at various times after injury for ALT activity (Fig. 3*B*). A comparable increase in the levels of serum ALT was observed in both cohorts at 48 and 72 h after CCl_4 administration, indicating that the toxic effect was similar in both groups and therefore excluding the possibility that the $C5^{-/-}$ livers may have shown defective hepatocyte regeneration and a decreased proliferative response due to a greater protection from toxic damage.

Defective hepatocyte proliferation in $C5^{-/-}$ mice: blunted DNA synthetic response in $C5^{-/-}$ livers after CCl_4 injury

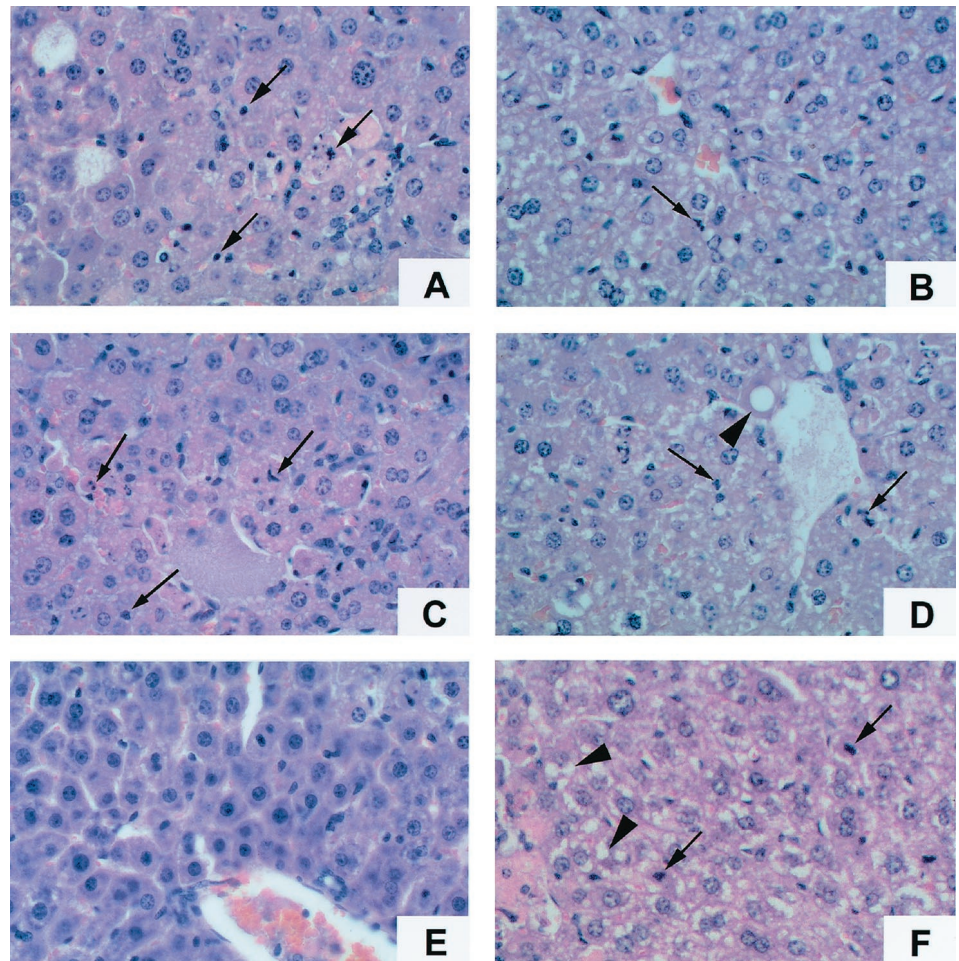
BrdU incorporation detected by immunohistochemistry (24) was used to determine whether the absence of $C5$ causes abnormalities

in the entry of $C5^{-/-}$ hepatocytes into the S phase (DNA synthesis) of the cell cycle, thus hindering the regenerative response of the liver after toxic injury. When a single dose (50 mg/kg) of BrdU was administered i.p. to CCl_4 -treated $C5^{+/+}$ and $C5^{-/-}$ mice, 2 h before the livers were harvested, the level of BrdU incorporation in $C5^{-/-}$ livers was markedly lower than that in wild-type livers, at both 36 and 48 h after injury (Fig. 3*A*). Almost no reactivity was detected in $C5^{-/-}$ hepatocytes, and the very low overall staining in these livers was attributed to replicating nonparenchymal cells (Fig. 4). In contrast, the $C5^{+/+}$ livers exhibited a normal pattern of BrdU reactivity, with incorporation into replicating hepatocytes peaking at 36 h after injury and remaining at elevated levels by 48 h (Fig. 3*A*). BrdU incorporation in $C5^{+/+}$ livers decreased to basal levels at 96 h after injury, whereas in $C5^{-/-}$ livers, a delayed peak was detected at 72 h after CCl_4 administration, corresponding to only $\sim 30\%$ of the wild-type peak BrdU incorporation at 36 h. These results clearly demonstrate the inability of $C5^{-/-}$ mice to promote hepatocyte entry into S phase, a prerequisite for normal liver regeneration in response to toxic injury.

Diminished mitotic activity in $C5^{-/-}$ mice after CCl_4 treatment

To evaluate the regenerative response in terms of actual mitotic activity and hepatocyte proliferation, mitotic figures were identified and counted under a light microscope in hematoxylin/eosin-stained liver sections of $C5^{-/-}$ and $C5^{+/+}$ mice at 48 and 72 h after CCl_4 -induced injury. No mitoses were detected in $C5^{-/-}$ livers at 48 h after injury, in contrast to the numerous mitoses found throughout the liver parenchyma of $C5^{+/+}$ mice at the same time (Fig. 5). The sluggish regenerative response and delayed tissue repair exhibited by the $C5^{-/-}$ mice was further substantiated

FIGURE 2. Characteristic features of hepatocellular necrosis and injury in $C5^{-/-}$ mice. High-power photomicrographs of hematoxylin/eosin-stained liver sections from $C5^{+/+}$ and $C5^{-/-}$ mice at various times after CCl_4 injury. *A*, *C*, and *E*, $C5^{+/+}$ livers at 36, 48, and 72 h after injury, respectively. *B*, *D*, and *F*, $C5^{-/-}$ livers at 36, 48, and 72 h after injury, respectively. Marked apoptotic activity is observed in $C5^{+/+}$ livers at 36 h, as shown in *A* (arrows indicate apoptotic hepatocytes). Less apoptosis is observed in $C5^{-/-}$ livers at the same time point (*B*, arrow indicates apoptotic cell). Marked shrinkage of necrotic areas and prominent regenerative activity is observed in $C5^{+/+}$ livers at 48 h (*C*, arrows indicate apoptotic hepatocytes). Considerable fat deposition (large arrowhead) and apoptosis (small arrows) is observed in $C5^{-/-}$ livers at 48 h after injury (*D*). Marked basophilic staining of hepatocytes and karyomegaly (increased nuclear size) are prominent in $C5^{+/+}$ livers at 72 h (*E*), indicating a brisk regenerative response to injury. All of these features are absent from $C5^{-/-}$ livers at 72 h after injury (*F*). In contrast, $C5^{-/-}$ livers show persisting fat accumulation (large arrowheads), eosinophilic staining indicative of hepatocyte necrosis, and increased apoptosis (small arrows) throughout the parenchyma.



by the rare occurrence of mitotic hepatocytes, even at 72 h after the administration of CCl_4 . These observations are consistent with the results of the BrdU incorporation time-course experiments and strongly indicate that $C5^{-/-}$ mice are incapable of mounting a normal regenerative response to toxic liver injury induced by CCl_4 . Furthermore, these results suggest that C5 may play a critical role in priming normally quiescent hepatocytes to re-enter the cell cycle and proliferate in response to toxic injury, by providing essential signaling cues for the G_1 -S phase transition in the cell cycle.

Reconstitution of $C5^{-/-}$ mice with murine C5 restores liver regeneration

If defective regeneration and abnormal hepatocyte proliferation after CCl_4 injury is the direct consequence of C5 deficiency and not a secondary defect in the $C5^{-/-}$ mice, then it should be possible to correct this defect and restore the regenerative response by reconstituting $C5^{-/-}$ mice with C5. Therefore, we treated $C5^{-/-}$ mice with a single dose of purified, hemolytically active murine C5, 20 min before CCl_4 administration, and then monitored these mice for hepatocyte BrdU incorporation and corresponding mitotic activity. Reconstitution of $C5^{-/-}$ mice with hemolytically active C5 restored BrdU incorporation in hepatocytes, at 48 and 72 h after injury, to nearly 80% of the peak wild-type levels at 36 h (Fig. 6). Furthermore, the mitotic indices of the C5-reconstituted mice were similar to those of the $C5^{+/+}$ mice, with the number of mitotic figures (% mitosis) rising to 70% of wild-type levels at 48 h after injury (Fig. 5). Histologic analysis indicated that the livers of the C5-reconstituted mice displayed an injury pattern during the first 48 h that closely resembled that of the wild-type mice (Fig. 1). The

areas of necrotic tissue were localized around the central veins (centrilobular necrosis) and were easily distinguished from the surrounding parenchyma, in which considerable basophilic staining and hepatocyte karyomegaly were evident. At 72 h after exposure to CCl_4 the $C5$ -reconstituted livers manifested extensive tissue repair, with the regenerating tissue replacing the previously necrotic areas. These histologic findings were consistent with the results of the BrdU immunohistochemistry and together provide considerable evidence to support a role for C5 as an essential component in normal liver regeneration after toxic injury.

Restoration of the regenerative response in $C5^{-/-}$ mice after treatment with C5a

There are a number of mechanisms by which C5 could contribute to normal liver regeneration. It would be reasonable to propose that its function is mediated by one or more of its activation products (C5a, C5b, C5b-9). Therefore, to dissect the mechanism by which C5 mediates its effect during liver regeneration, we assessed whether C5a, an activation product of C5, could promote normal liver regeneration and effectively restore the wild-type ($+/+$) phenotype when administered to $C5^{-/-}$ mice that had been treated with CCl_4 . Indeed, when $C5^{-/-}$ mice were injected with three successive doses ($3.5 \mu\text{g}/\text{animal}$) of human, recombinant C5a, at 6-h intervals after the exposure to CCl_4 , hepatocyte BrdU incorporation was restored to 70% of the peak wild-type levels at 48 h after injury (Fig. 6). However, no effect on hepatocyte DNA synthesis or proliferation was observed when C5a was administered to a cohort of $C5^{-/-}$ mice that had not been exposed to CCl_4 , strongly indicating that C5a is specifically involved in the normal

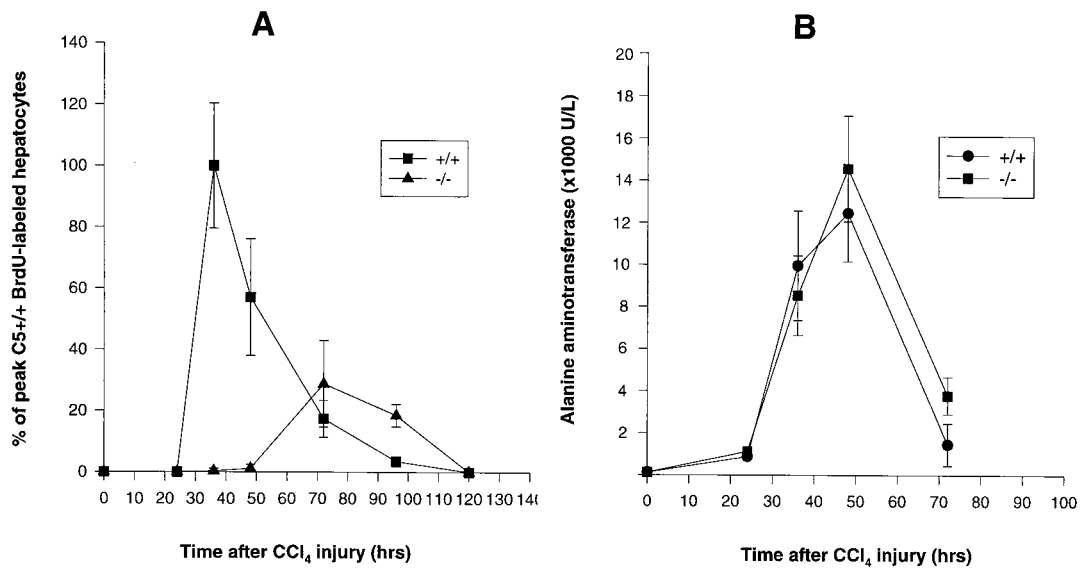


FIGURE 3. Blunted DNA synthetic response in C5^{-/-} hepatocytes after CCl₄ injury. *A*, BrdU incorporation profile of C5^{+/+} and C5^{-/-} mice at various times after CCl₄ treatment. C5^{+/+} and C5^{-/-} mice were treated with CCl₄ and injected with BrdU 2 h before sacrifice and liver harvest. Livers were harvested at the indicated times, fixed, sectioned, and stained with an anti-BrdU mAb. BrdU incorporation in hepatocytes was quantitated by counting the number of BrdU-positive cells (dark brown nuclei) per high-power field in 10 randomly selected fields. At least three mice from each group were used for each time point. The mean value for each time point was then plotted as a percentage of the mean number of BrdU-labeled cells at the peak time of BrdU incorporation in C5^{+/+} mice (36 h). SDs are shown for each time point. *B*, ALT activity in serum collected from C5^{+/+} and C5^{-/-} mice at various times after CCl₄ injury. SD is indicated for each time point.

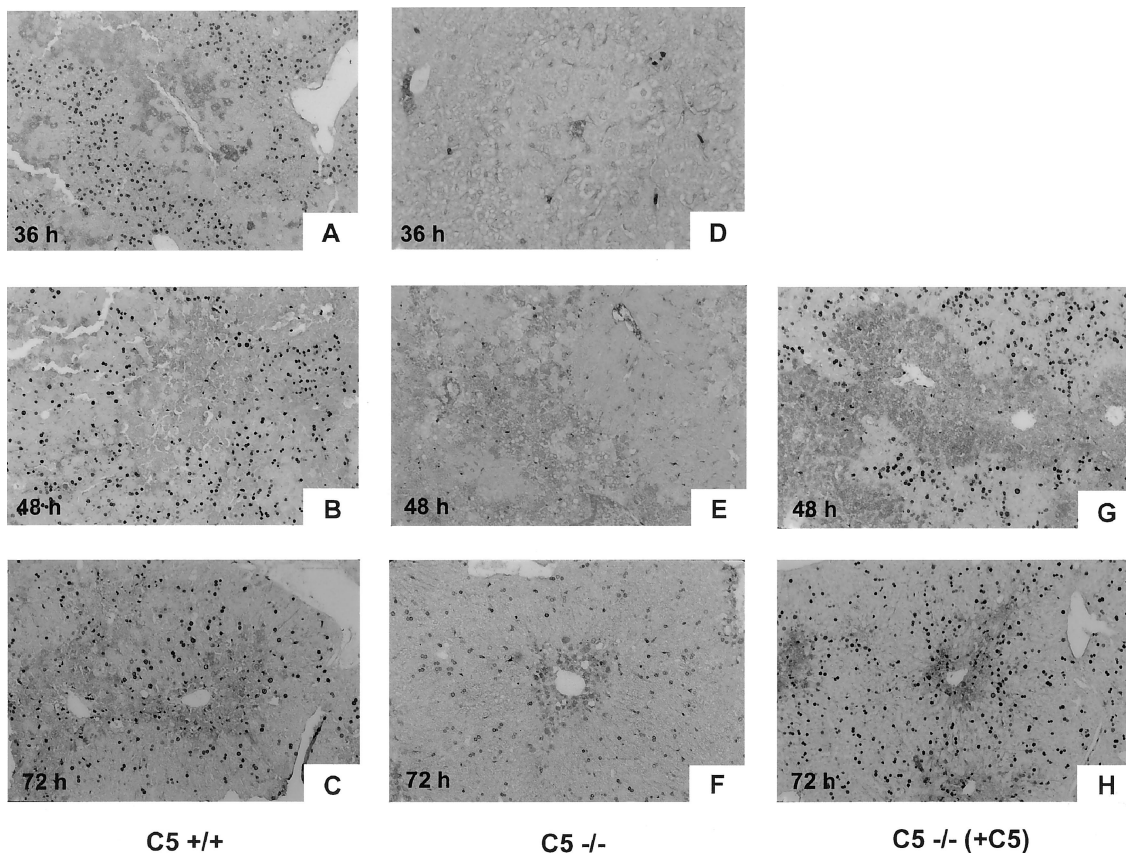


FIGURE 4. Diminished BrdU incorporation in C5^{-/-} mice after CCl₄ injury and restoration of hepatocyte proliferation after reconstitution with C5. Low-power photomicrographs of BrdU-stained liver sections from C5^{+/+} (*A-C*), C5^{-/-} (*D-F*), and C5^{-/-} (+C5) mice (*G* and *H*) at various times after CCl₄ treatment. Indicated times are: (*A*) 36 h, (*B*) 48 h, and (*C*) 72 h, for C5^{+/+} sections; (*D*) 36 h (*E*) 48 h, and (*F*) 72 h, for C5^{-/-} sections; and (*G*) 48 h and (*H*) 72 h after CCl₄ injury, for C5^{-/-} (+C5) sections. Round, dark nuclei indicate positively stained hepatocytes. Slides from C5^{+/+} and C5^{-/-} mice were processed and stained simultaneously. Magnification, $\times 20$.

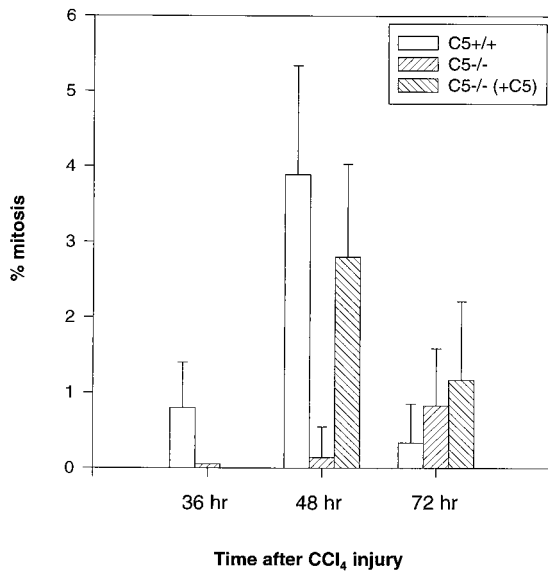


FIGURE 5. Decreased mitotic activity in C5^{-/-} mice after CCl₄ injury. Mitotic figures were counted in liver sections of C5^{+/+}, C5^{-/-}, and C5-reconstituted mice, at the indicated times after CCl₄ exposure. Mean values were plotted as the percentage of mitotic cells among the total number of hepatocytes present in five high-power fields. SDs were calculated on the basis of at least three mice per time point.

regenerative response of the liver. These findings implicate complement activation as a mechanism that may contribute to normal liver regeneration, because C5a is generated upon complement activation and subsequent cleavage of C5 (25). Furthermore, they support a novel role for the C5a receptor (C5aR) that is expressed in the liver (26), suggesting that C5aR-mediated signaling is a critical component of the regenerative response to liver injury.

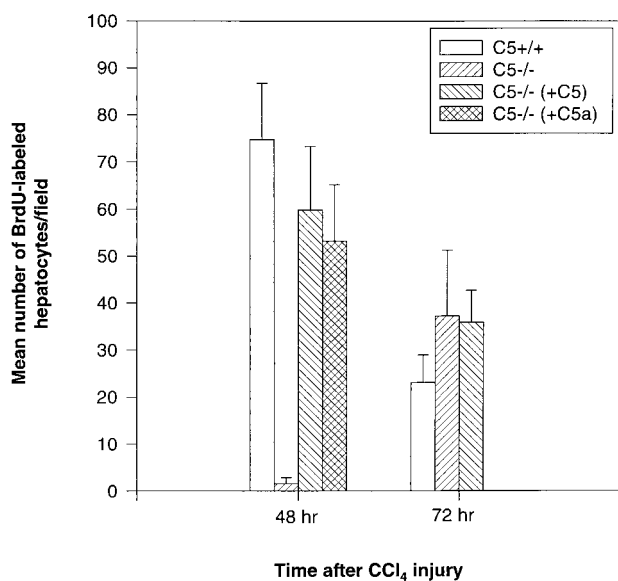


FIGURE 6. Restoration of hepatocyte BrdU incorporation in C5^{-/-} mice at 48 and 72 h after CCl₄ injury by reconstitution with murine C5 or human C5a (48 h). BrdU-positive hepatocytes were counted in liver sections from C5^{+/+}, C5^{-/-}, C5^{-/-} (+C5), and C5^{-/-} (+C5a) mice at the indicated times after injury, and values were plotted as the mean number of BrdU-labeled hepatocytes identified in 10 randomly selected high-power fields (magnification, $\times 40$). SDs are shown for each group of mice (at least three mice from each group were evaluated per time point).

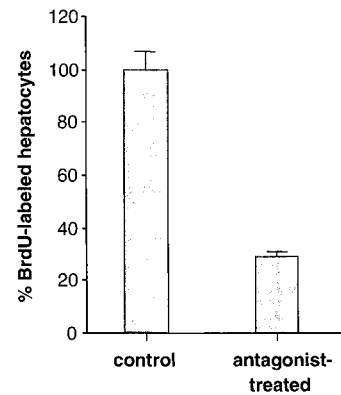


FIGURE 7. Effect of C5aR blockade on hepatocyte proliferation. C5aR signaling is required for cell cycle progression (S phase entry) in hepatocytes. C5^{+/+} mice (B10D2nSn-J) were treated with the C5aR antagonist (right bar), or with PBS alone (control, left bar). Toxic liver injury was induced in both cohorts by CCl₄ administration. Following BrdU incorporation, livers were harvested at 48 h after injury and sections were stained with an anti-BrdU mAb. Data were plotted as the percentage of BrdU-positive hepatocytes identified in 10 randomly selected high-power fields. Three mice were evaluated from each cohort.

C5aR stimulation is required for cell cycle progression in regenerating hepatocytes

C5a may have pleiotropic effects on regenerating hepatocytes. A requirement for C5a activity during liver regeneration was clearly demonstrated by reconstituting C5-deficient mice with C5a and restoring their regenerative phenotype. To determine whether this effect is exerted through stimulation of the C5a receptor expressed in the liver, we performed *in vivo* inhibition studies using a specific C5a receptor (C5aR) antagonist derived synthetically from the COOH terminus of C5a (23). This potent antagonist is a small cyclic peptide that exhibits a C5a inhibitory activity for human leukocytes in the low nanomolar range, and it has been shown to specifically block C5a-mediated effects in various rodent models of disease (27, 28). When we treated C5-sufficient mice with this antagonist at various times after toxic liver injury, BrdU incorporation into hepatocytes was found to be significantly blunted, indicating a defective proliferative response to injury that was marked by the inability of hepatocytes to re-enter the cell cycle (S phase) and proliferate (Fig. 7). The livers of these mice showed persistent necrotic damage at 72 h after CCl₄ administration, which was comparable to the extent of necrosis observed in C5-deficient mice at the same time point. These results clearly demonstrate that C5aR signaling after C5a stimulation is essential for liver regeneration and identify the C5a receptor as a novel regulatory checkpoint for cell cycle progression in regenerating hepatocytes.

Discussion

In this study, we have shown that C5 is an essential component in liver regeneration following toxic injury. Mice deficient in this complement protein were unable to mount a normal regenerative response after toxic exposure to CCl₄. This was demonstrated by the delayed entry of C5^{-/-} hepatocytes into the cell cycle, the severely compromised mitotic activity detected in C5^{-/-} livers, as well as the extensive parenchymal necrosis and fat deposition in the deficient mice that persisted until 96 h after the injury (data not shown). Reconstitution of the C5^{-/-} mice with murine C5 resulted in a significant recovery of their regenerative phenotype, as shown by the restored hepatocyte DNA synthetic response and mitotic activity at 48 h after the toxic challenge. It should be noted that the

C5^{-/-} mice showed signs of acute necrosis and persisting degeneration until 4 days after the exposure to CCl₄, a time at which the wild-type animals had completely recovered from injury. Both wild-type and deficient mice eventually regenerated their livers in a course of 6–7 days. This observation is in agreement with the results of previous studies that have used TNF- α receptor I and IL-6-deficient mice in a partial hepatectomy model of liver regeneration (9, 10). Although these studies have shown a requirement for these components of the cytokine network in the early stages of liver regeneration, they point to a functional redundancy of their signaling pathways later on in the process. The observation that both C5-deficient and wild-type mice eventually recover from injury further stresses the concept that the regenerating liver recruits several interdependent molecular pathways that act cooperatively, and in a compensating manner, to ensure its complete recovery from injury. Moreover, it suggests that C5 is one among essential factors that mediate liver regeneration, and that it probably exerts its function in an early stage during this process.

C5, a serum protein that is an integral component of the complement activation cascade, generates two distinct products upon proteolytic cleavage: C5b, which participates in the assembly of the C5b-9 complex (the membrane attack complex, or MAC) that induces the lysis of complement-targeted cells (25); and the anaphylatoxic fragment C5a, which has a potent chemoattractive effect on various myeloid cells, stimulating the migration of neutrophils, eosinophils, basophils, and monocytes and causing the degranulation of mast cells (29). The pleiotropic effects of C5a on a wide array of tissues are mainly mediated by stimulation of its G-protein coupled receptor C5aR (30).

The ability of C5a to restore the DNA synthetic response and proliferation of hepatocytes in C5^{-/-} mice indicates that this anaphylatoxin may play an important role in normal liver regeneration and that stimulation of its receptor C5aR may trigger signal transduction events that prime quiescent hepatocytes to re-enter the cell cycle. To address this plausible mechanism by which C5 may contribute to liver regeneration and establish that C5aR signaling is an essential component of the pathway leading to mitogenic priming of hepatocytes, we performed in vivo blockade studies using a specific C5aR antagonist. Mice treated with the C5aR antagonist exhibited significantly blunted hepatocyte proliferation after toxic injury, which was marked by a defective S phase transition profile, clearly demonstrating that stimulation of the C5a receptor is required for cell cycle progression in hepatocytes and normal liver regeneration after toxic injury.

Recent studies in a human neuroblastoma cell line have suggested that a fragment of C5a participates in apoptotic signal transduction pathways through binding to the neuronal C5a receptor nC5aR (31). From another perspective, it has been reported that C5a may protect neurons from excitotoxin-induced degeneration and apoptosis, and this neuroprotective function may be mediated by binding of C5a to its neuronal receptor (32). These observations suggest that C5 may contribute to the regulation of the regenerative response of the liver through its activation product, C5a, and that signaling through the C5a receptor, which is expressed in human liver parenchymal cells (26, 33), and in rat liver nonparenchymal cells (34), may elicit a growth factor-like response after liver injury, stimulating hepatocyte DNA synthesis and proliferation. Our data from the C5a reconstitution studies demonstrate that C5a is indeed a critical intermediate in this process. The results of the inhibition studies identify the C5a receptor (C5aR) as a novel regulatory element driving hepatocyte proliferation and therefore provide a mechanism by which C5 and its activation fragment C5a exert a mitogenic effect on hepatocytes.

Binding of C5a to its receptor, found primarily in Kupffer cells (liver macrophages) and hepatic stellate cells (34), could result either in direct stimulation of cell proliferation or in activation of apoptotic pathways leading to the clearance of superfluous or irreversibly damaged cells during the course of regeneration. In this context, it should be noted that we observed very little apoptosis in the C5^{-/-} liver parenchyma within the first 48 h after CCl₄ treatment, a feature that could be attributed to the absence of a possible apoptotic stimulus associated with C5 or one of its activation fragments. A similar concept implicating apoptosis as a possible regulatory response of the liver to acute injury was recently discussed in a study involving IL-6-deficient mice (35). Moreover, the significant restoration of hepatocyte proliferation in C5^{-/-} mice, after reconstitution with human C5a, suggests that C5a may also have a direct effect on stimulating cell proliferation during liver regeneration through binding to its receptor C5aR. In support of this plausible mechanism, a recent study has demonstrated that C5a can exert a direct effect on hepatocytes through the inducible expression of C5aR on their surface after treatment with IL-6 (36). A possible induction of C5aR expression in hepatocytes during liver regeneration cannot be excluded because it has been shown that cytokine levels in portal circulation, including those of IL-6, are rapidly elevated after partial hepatectomy or toxic liver injury as part of the hepatic acute-phase response (1).

Another way in which C5 could contribute to the regulation of liver regeneration is by mediating, as an upstream element, the release from nonparenchymal cells (bearing C5a receptors) of proinflammatory cytokines (IL-6, TNF- α) that have been shown to be essential for normal liver growth and regeneration.

Our finding that intact C5a receptor signaling is essential for hepatocyte proliferation in a murine model of liver regeneration can be further explored with the availability of mice that bear targeted disruption of the C5aR gene (37).

In conclusion, the results of this study show that complement, diverging from its established role as an innate defense mechanism against infection, appears to play a novel, integrated role in the maintenance of hepatic homeostasis and the physiological response of the liver to toxic injury.

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