The Proinflammatory Mediators C3a and C5a Are Essential for Liver Regeneration

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Abstract

Complement has been implicated in liver repair after toxic injury. Here, we demonstrate that complement components are essential for liver regeneration, and mediate their effect by interacting with key signaling networks that promote hepatocyte proliferation. C3- or C5-deficient mice exhibited high mortality, parenchymal damage, and impaired liver regeneration after partial hepatectomy. Mice with dual C3 and C5 deficiency had a more exacerbated phenotype that was reversed by combined C3a and C5a reconstitution. Interception of C5a receptor signaling resulted in suppression of IL-6/TNFα induction and lack of C3 and C5a receptor stimulation attenuated nuclear factor–κB/STAT-3 activation after hepatectomy. These data indicate that C3a and C5a, two potent inflammatory mediators of the innate immune response, contribute essentially to the early priming stages of hepatocyte regeneration.

Key words: complement • anaphylatoxins • NF-κB • STAT-3 • IL-6

Introduction

Growing evidence suggests that complement proteins not only serve as mediators of innate immune defense against foreign pathogens but can also modulate diverse developmental processes, such as cell survival, growth, and differentiation in various tissues (1, 2). In this respect, complement has recently been implicated as a mediator of lens and limb regeneration in lower vertebrates (3, 4).

In mammals, the liver constitutes a well-established paradigm of tissue regeneration, which can occur after toxic exposure, viral injury, or surgical resection. Terminally differentiated liver cells respond to these perturbations by shedding their quiescent phenotype and by undergoing several cycles of cell division to regenerate lost parenchymal liver mass (5). Liver regeneration is a well-orchestrated and tightly regulated process that proceeds through distinct stages, including priming of hepatocytes, cell cycle progression, proliferation, and cessation of regeneration (6).

The signaling pathways underlying the early priming phase of liver regeneration are thought to be triggered by the synergistic effect of a wide array of stimuli, including cytokines (7), prostaglandins (8), hormones (9), reactive oxygen species (10), and lipopolysaccharides (11, 12) released into the portal circulation. Within this signaling network, IL-6 and TNFα (7), as well as their downstream transcription factors STAT-3 and nuclear factor (NF)–κB (13), have been identified as crucial regulators of the regenerative process. However, to this date, the potential interaction of these cytokine-driven pathways in the liver with cellular or humoral components of the innate immune response, as well as the molecular mechanisms by which such a "crosstalk" might affect the early stages of hepatocyte regeneration, have not been addressed.

In support of this concept, recent works have revealed novel modulatory roles of complement in the hepatic microenvironment. C5a has been shown to costimulate prostaglandin and cytokine secretion from Kupffer cells, and to modulate glucose release and acute-phase gene expression in hepatocytes, in a C5a receptor (C5aR)–dependent fashion (14). Likewise, C3a can mediate metabolic functions in cultured liver macrophages (15) and ex vivo–perfused livers (16). These findings, together with our earlier observation

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Abbreviations used in this paper: BrdU, 5-bromo-2′-deoxyuridine; C5aR, C5a receptor; C5aRa, C5a receptor antagonist; NF, nuclear factor; PHx, partial hepatectomy.

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that $C5^{-/-}$ mice are more susceptible to liver toxic damage (17), formed the conceptual framework of our hypothesis that complement is a critical mediator of liver regeneration.

To define the role of complement in the regenerative process and dissect the molecular interactions of various components with the early signaling networks that promote hepatocyte proliferation, we performed partial hepatectomy (PHx) studies using mice deficient in C3 and C5, two critical components of the complement cascade. Using C3a and C5a in reconstitution studies, we have established that both anaphylatoxins are required for normal liver regeneration and that their stimulatory effect is mediated in an independent and cooperative fashion. Intercepting specific receptor-mediated pathways, we have identified a novel mechanistic association between the complement and cytokine networks in promoting hepatocyte priming and proliferation.

Materials and Methods

Animals. 14–18-wk-old specific pathogen-free mice were used in all experiments. C57BL6-J, B10D2oSn-J ($C5^{-/-}$), and B10D2nSn-J ($C5^{+/+}$) mice were purchased from the Jackson Laboratory. $C3^{-/-}$, $C3^{+/+}$ (both C57BL6), $C3^{+/+}C5^{+/+}$, and $C3^{-/-}C5^{-/-}$ mice were bred in-house. The $C3^{+/+}C5^{+/+}$ and $C3^{-/-}C5^{-/-}$ mice were newly generated by crossbreeding the corresponding C3 and C5 strains. Bacterial colonization or infections that could interfere with the regenerative phenotype of the animals included into the work were ruled out by histological analysis. Lungs, livers (including the resected part of the liver at the time of PHx), kidneys, spleens, duodenums, and hearts were investigated for signs of inflammatory processes. None of these tissue samples showed signs of inflammation or bacterial colonization. The presence of helicobacter species in the livers of mice from our colony was excluded by randomly analyzing the livers of three $C3^{-/-}C5^{-/-}$ mice by PCR, which gave negative results. The PCR studies were performed by the Missouri University Research Animal Diagnostic Laboratory (Columbia, MO). All animal studies were conducted in compliance with the guidelines of the University of Pennsylvania, according to an animal protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Partial Hepatectomy. Approximately 70% PHx was performed according to the method of Higgins et al. (18). The median and left lateral lobes were removed without injuring the remaining liver tissue. In case of severe morbidity (lethargy, trembling, anorexia, or body temperature loss), animals were killed and regarded as nonsurvivors.

To assess the proliferative response of hepatocytes, mice received 50 mg/kg body weight of 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) i.p. 1 h before tissue harvests. C5aR blockade was achieved by i.p. injection of the cyclic hexapeptide AcF[OpdChaWR] 20 min before surgery (1 mg/kg in PBS) (control: peptide IAVVQDWGHHRAT-$CONH_2$, 1 mg/kg in PBS). Animals were perfused with a 10% buffered formaldehyde solution, in which tissues were also placed for paraffin embedding and sectioning. Alternatively, the perfusion was done with PBS and protein extraction.

Liver Morphology. Liver histology was assessed by light microscopy (model BX 60; Olympus) of hematoxylin- and eosin-stained 5-μm sections in a blinded fashion. The extent of necrosis was semi-quantitatively estimated by assigning a severity score (absent, 0; mild, 1; moderate, 2; pronounced, 3; and severe, 4). This score was used to compare the liver damage after PHx between different strains. To compare parenchymal liver necrosis between $C3^{+/+}C5^{+/+}$, $C3^{-/-}C5^{-/-}$, and anaphylatoxin-reconstituted $C3^{-/-}C5^{-/-}$ mice (see Fig. 3), the quantification was performed blinded with the Scion Image Software, version 4.0.2. (National Institutes of Health) using histological slides.

Serum Biochemistry. Serum samples from all mice were collected for measurement of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, and total bilirubin and were stored at −20°C until analysis. All enzymatic assays were performed by Anilytics.

BrdU Immunohistochemistry. Nuclear staining of hepatocytes for BrdU uptake was performed with modifications as described previously (17). The total number of BrdU-labeled hepatocytes was determined by counting positively stained nuclei in 10 high-power fields (×400). Constantly proliferating intestinal crypt epithelium from small intestine served as a positive control for BrdU incorporation and staining.

RNA Isolation and Detection of IL-6 and TNFα mRNA by Semi-quantitative RT-PCR. Total liver RNA was prepared, and cytokine RT-PCRs were performed as described previously (19). Primers used were as follows: IL-6, 5′-primer, 5′-TTCCATCCAGTTGCCTTCTT-3′, 3′-primer, 5′-CAGAATTGCCATTGCACAAC-3′ (size of PCR product, 198 bp); TNFα, 5′-primer, 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′, 3′-primer, 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′ (size of PCR product, 307 bp); and β-actin, 5′-primer, 5′-AGGGTGTGATGGTGGGAATGG-3′, 3′-primer, 5′-AGCCAGAGCAGTAATCTCCTTCTGC-3′ (size of PCR product, 841 bp).

Electrophoretic Mobility Shift Assays and Antibody Supershift Assays. Liver nuclear extracts were prepared and electrophoretic mobility shift assays reactions were performed as described previously (20, 21). For NF-κB binding, the consensus sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′ site (Promega) was used, whereas STAT-3 binding was detected using a gel-purified oligonucleotide from the sis-inducible factor binding element in the c-fos promoter (5′-GATCCTCCAGCATTTCCCGTAAATCCTCCAG-3′). Rabbit reticulocyte lysate with preidentified NF-κB was used to recognize the NF-κB subunit p65 in all assays. Loading control was achieved by detecting E2 binding (5′-GGTTCCAGACCGCGATTGGTGGCTGGA-3′, a gift from J.I. Leu, University of Pennsylvania, Philadelphia, PA; reference 22). For supershift experiments, 2 μg/ml polyclonal rabbit antibodies were used against the p65 subunit on NF-κB (Cat. No. sc109x) and against STAT-3 (Cat. No. sc482x; both from Santa Cruz Biotechnology, Inc.).

Complement Reagents. C3 was purified from human plasma according to a protocol described previously (23). It was applied by a single i.p. injection 20 min before PHx at a dose of 3 mg/mouse. C3a was synthesized chemically according to a protocol described previously (24) with a modification of the cyclization procedure (25, 26). A 6-His-×–tagged murine C5a was expressed in *Escherichia coli* and purified using nickel chelating affinity chromatography (27). Three successive doses of the anaphylatoxins, synthetic mouse C3a, and expressed C5a were also given by i.p. injection at a dose of 15 μg/mouse/injection (one before and two after PHx, 6-h interval). The C5aR antagonist used in this work is a cyclic hexapeptide AcF[OpdChaWR] that was synthetically designed from the COOH terminus of C5a (27). This antagonist has been shown to specifically block C5a-mediated effects in various rodent disease models (28). Peptide synthesis and cyclization were performed as described previously (27). The peptide was purified using preparative reverse phase HPLC. The identity and purity of both

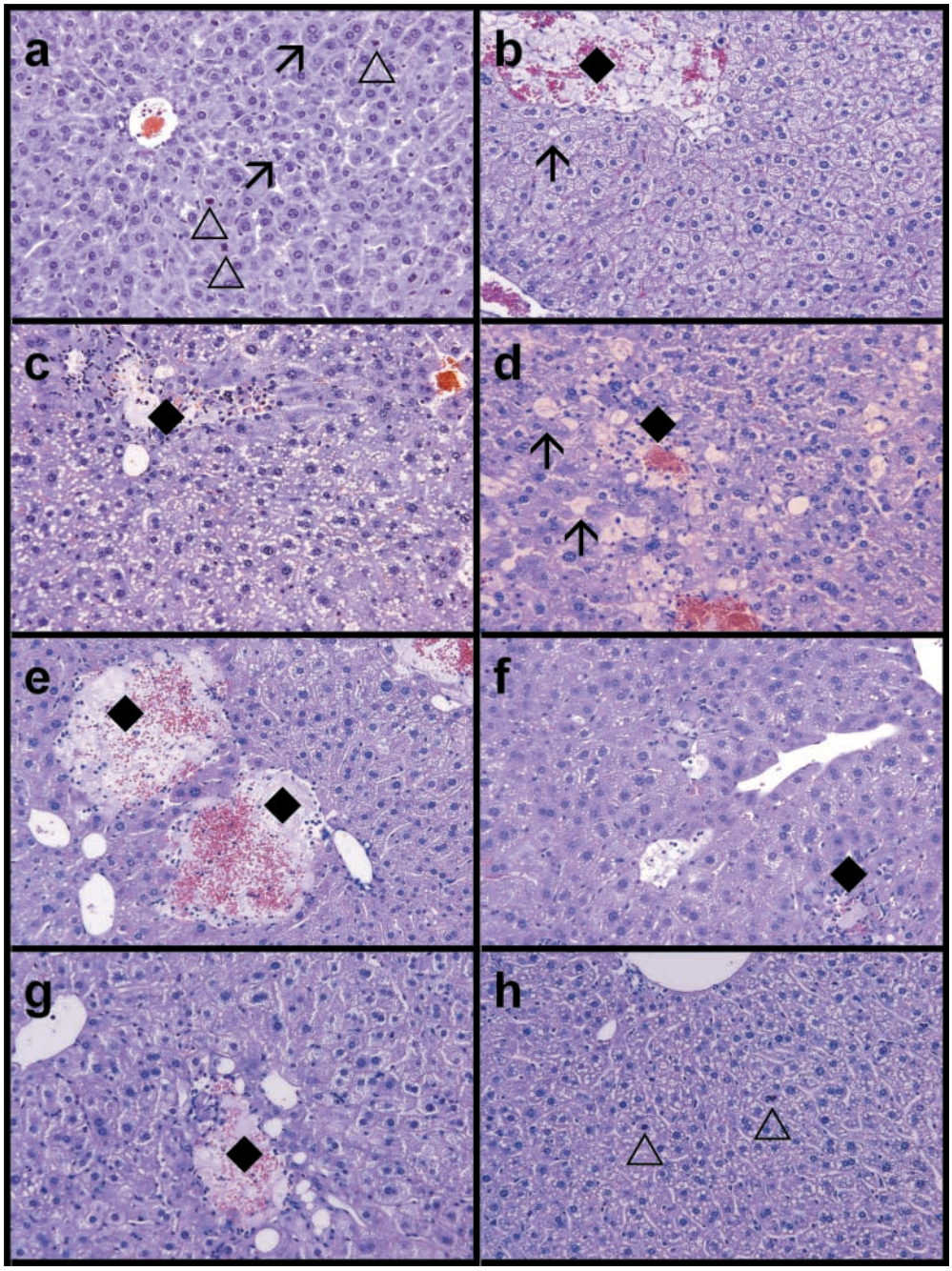


Figure 1. Histological and immunohistochemical findings in $C3^{-/-}$, $C5^{-/-}$, $C3^{-/-}C5^{-/-}$, and $C5^{-/-}$-treated mice and their controls (Hematoxylin and eosin stainings, magnification 200). (a) The liver section of a control animal 44 h after PHx (representative for all control strains used). A vigorous regenerative response with numerous mitotic figures (△) and multinucleated hepatocytes (→) can be seen. $C3^{-/-}$ (b), $C5^{-/-}$ (c), and C5aRa-treated animals (d) 44 h after PHx display areas of parenchymal damage (◆). Hepatocytes adjacent to necrotic areas display ballooning degeneration (↑). (e–h) Representative areas from sections of $C3^{-/-}C5^{-/-}$ livers 44 h after PHx without reconstitution (e), or with reconstitution using C3a (f), C5a (g), or C3a and C5a (h). Severely injured $C3^{-/-}C5^{-/-}$ livers (e) are partially protected from this damage after PHx when they are reconstituted with one of the two anaphylatoxins (f and g). When the combination of both is applied, parenchymal damage is completely prevented and the regenerative capacity recovers (h; mitotic figures, △).

anaphylatoxins was confirmed by mass spectrometry (Maldi-Tof, Tofspec 2E; Micromass). All reagents injected into mice were analyzed for their LPS content using a limulus assay (Pyrochrome) and in all of them it was found to be below 1.5 ng/mg of protein.

Statistical Analysis. Data are expressed as mean ± SE. The Mann-Whitney test or the Chi-Square test was used for the determination of significance.

Results

Liver Damage and Impaired Regeneration in $C3^{-/-}$ Mice after PHx. The three pathways of complement activation (classical, alternative, and lectin) converge at the central complement component C3. Therefore, C3 deficiency not only eliminates C3 activation and cleavage into active C3 fragments but it also largely prevents the downstream activation of C5 and the release of C5-derived activated fragments (29).

This central role of C3 was the basis to study the regenerative phenotype of the corresponding deficient mice ($C3^{-/-}$). In contrast to their wild-type littermates, $C3^{-/-}$ mice displayed an abnormal regenerative response with a markedly compromised clinical postoperative course. The clinical deterioration of the $C3^{-/-}$ mice became evident between 15 and 24 h after PHx and resulted in a high mor-

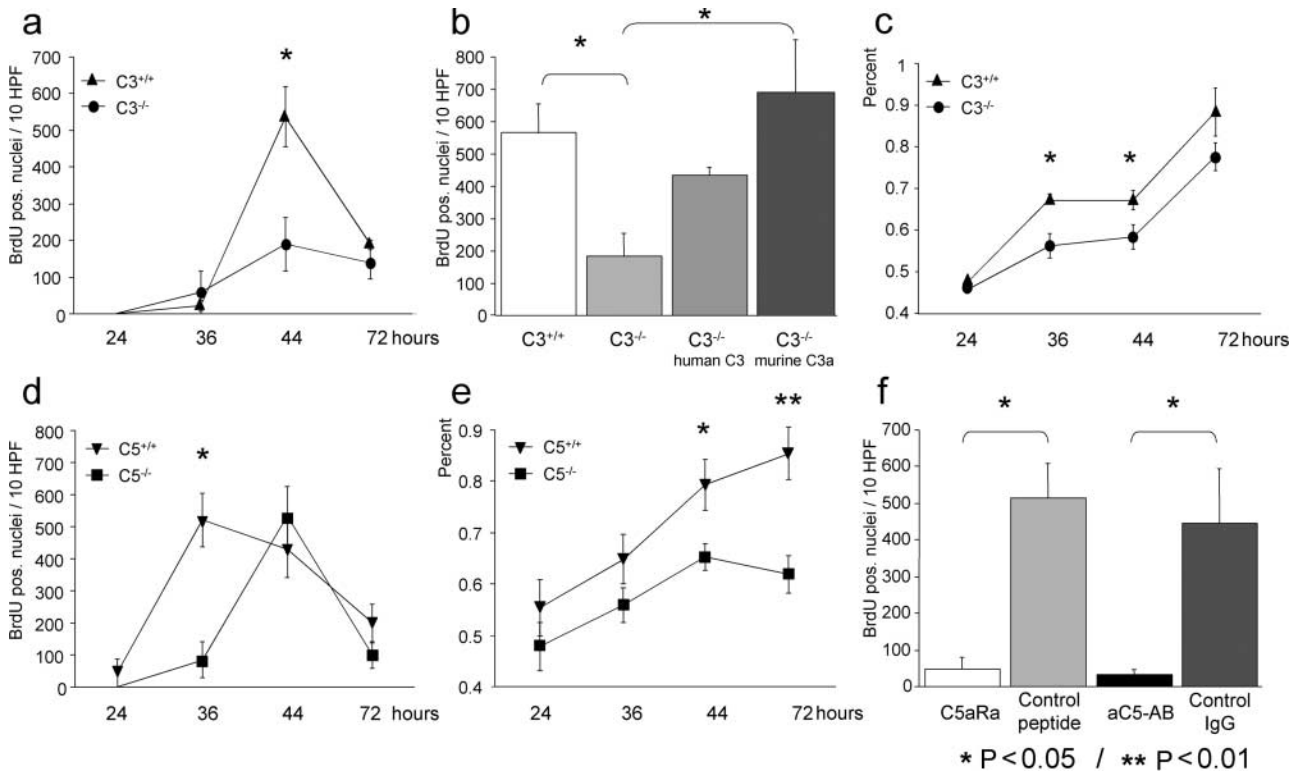


Figure 2. Defective hepatocyte S-phase entry after PHx in C3- and C5-deficient mice or after C5aR blockade. Graphs in a, b, d, and f depict numbers of BrdU-labeled hepatocytes (total number of labeled hepatocytes in 10 high-power fields at various times after PHx). (a) BrdU incorporation levels in $C3^{-/-}$ ($n = 9$) mice over time are clearly reduced and show a significant difference compared with control ($n = 8$) at 44 h after PHx ($P < 0.05$). (b) Reconstitution with either human C3 ($n = 3$) or C3a ($n = 3$) is capable of restoring BrdU incorporation to nearly wild-type levels at 44 h after PHx. (c) The low number of BrdU-positive nuclei in $C3^{-/-}$ mice is paralleled by a delayed gain in liver weight, as measured by the percentage of original liver weight before PHx at harvest time. (d) Numbers of BrdU-labeled hepatocytes in $C5^{-/-}$ and $C5^{+/+}$ mice at various times after PHx reveal a delayed peak of BrdU incorporation. (e) This delay was associated with slower liver weight recovery after PHx. (f) The number of BrdU-positive nuclei is significantly reduced at 44 h after PHx after C5aRa treatment ($P < 0.01$; BrdU incorporation levels in C5aRa-treated mice [$n = 10$], white bar; BrdU uptake in control peptide-treated mice [$n = 3$], lightly shaded bar). The same pattern is observed for animals after anti-C5 antibody treatment ($n = 3$, black bar) when compared with control IgG treatment ($n = 3$, darkly shaded bar; $P < 0.05$).

tality of 40% (21:52 mice) as compared with 15% (3:20) for their wild-type littermates ($P < 0.05$). This clinical impairment was associated with disruption of the lobular architecture of the liver and the presence of randomly distributed necrotic areas throughout the parenchyma. Ballooning degeneration was evident in the cytoplasm of hepatocytes adjacent to the necrotic areas (Fig. 1 b). This prominent presence of necrosis and hepatocyte degeneration in $C3^{-/-}$ mice correlated with lower BrdU incorporation at 44 h after hepatectomy and with reduced liver weight recovery in the surviving $C3^{-/-}$ mice (Fig. 2, a and c).

Biochemical analysis of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin serum levels at 44 h after PHx in $C3^{-/-}$ mice revealed higher aminotransferase values in the $C3^{-/-}$ mice as compared with their controls. These elevated levels of hepatic enzymes confirmed the observation of a higher incidence of liver failure and a more pronounced level of liver damage in the $C3^{-/-}$ cohort (unpublished data).

Complement Component C5 Is Required for Normal Liver Regeneration. C5, the initial component participating in the assembly of the membrane attack complex, is activated through cleavage by the classical or alternative pathway C5 convertase, a protein complex containing activated C3 molecules (C3b). Complement activation occurring during liver regeneration would lead to cleavage and activation of native C5. Having shown that C3 was required for a normal regenerative response, we investigated whether C5, the downstream activation target of C3, might be essential for liver regeneration too.

Similar to $C3^{-/-}$ mice, $C5^{-/-}$ animals exhibited an abnormal regenerative response and increased mortality after hepatectomy as follows: 23% (9:30) for $C5^{-/-}$ mice as compared with 4% (1:23) for $C5^{+/+}$ mice ($P < 0.05$). However, the livers of the surviving $C5^{-/-}$ mice showed less parenchymal damage than those of $C3^{-/-}$ mice (Fig. 1 c), and the rate of BrdU incorporation into $C5^{-/-}$ hepatocytes over time revealed a delayed rather than severely diminished DNA synthetic response after PHx when compared with that of wild-type littermates (Fig. 2 d). It should be noted that this delayed proliferative response of $C5^{-/-}$ livers had a significant impact on the recovery of liver weight (Fig. 2 e). As in the case of $C3^{-/-}$ mice, analysis of aminotransferase and total bilirubin serum levels in $C5^{-/-}$

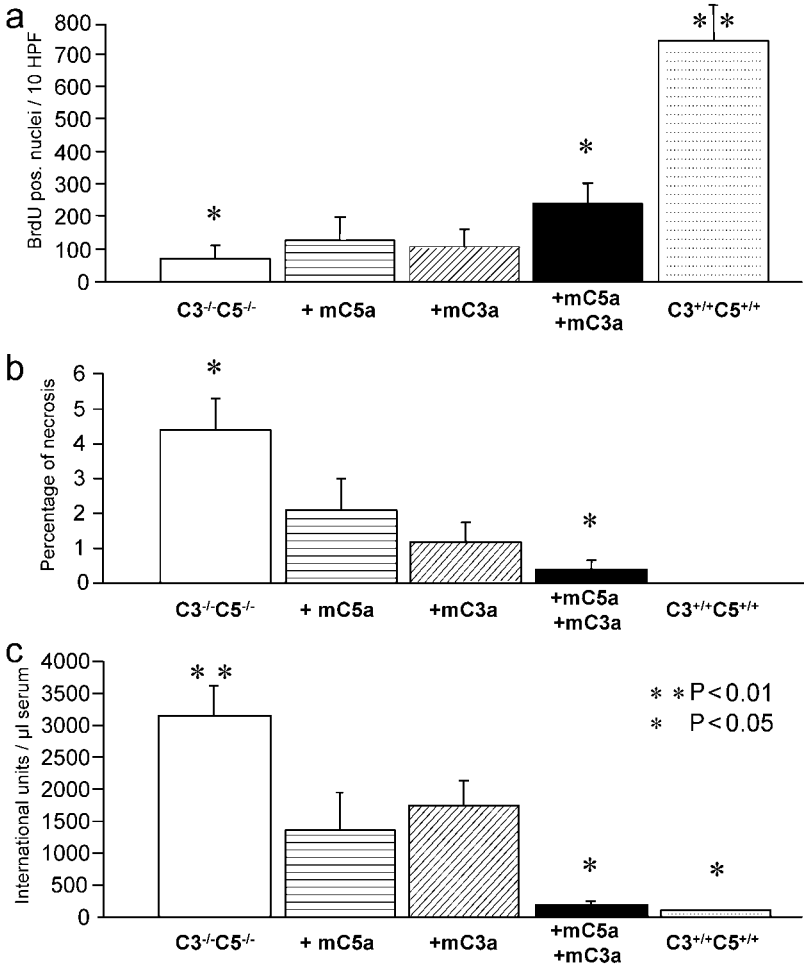


Figure 3. S-phase entry by hepatocytes after PHx is abrogated by combined C3 and C5 deficiency and reversed by anaphylatoxin reconstitution. (a) BrdU incorporation into nuclei of hepatocytes from $C3^{-/-}C5^{-/-}$ mice is minimal after PHx ($n = 16$, white bar). Reconstitution with C5a ($n = 7$) or C3a ($n = 4$) alone (diagonally striped bar) or with the combination of C3a and C5a ($n = 5$; shaded bar) reverses the proliferative response partially, as measured by the number of BrdU-positive nuclei. The reconstitution does not reach the proliferative response of $C3^{+/+}C5^{+/+}$ ($n = 3$, dotted bar) mice. (b) The same pattern can be detected for liver damage at 44 h after PHx. The percentage of parenchymal liver necrosis (quantified with "Scion Image" software; Materials and Methods) is highest in $C3^{-/-}C5^{-/-}$ livers without reconstitution (white bar). Single reconstitution with either anaphylatoxin reduces the extent of necrosis slightly (diagonally striped bar). Simultaneous reconstitution with C3a and C5a (black bar) reveals a significant additive protective effect in terms of the extent of necrosis ($P < 0.01$). (c) The serum levels of the aspartate amino transferase (I.U./ml) mirror and confirm the amount of parenchymal damage measured by histology (b). Combined C3a and C5a reconstitution (black bar) significantly decreases serum transaminase levels when compared with untreated $C3^{-/-}C5^{-/-}$ animals (white bar).

mice at 44 h after PHx confirmed the higher incidence of liver failure and more pronounced liver damage in this group (unpublished data).

To further substantiate our finding that C5 is required for normal liver regeneration, we also assessed the regenerative response of wild-type mice treated with a monoclonal antibody (BB5.1) that inhibits the cleavage of C5 to C5b and C5a (30). Mice treated with a single preoperative dose of this anti-C5 antibody showed impaired liver regeneration and diminished BrdU incorporation into hepatocytes at 44 h after PHx (Fig. 2 f). This finding indicates that C5 activation contributes to the regulation of liver regeneration after PHx.

Combined C3/C5 Deficiency Induces a More Severe Regenerative Defect than Single C3 or C5 Deficiency after PHx. Given that the absence of both C3 and C5 caused defective regeneration after PHx, it was important to establish whether C3 merely serves as an intermediate activator of C5 or whether both components mediate distinct functions; this was explored, for example, by activating different pathways via their anaphylatoxins C3a and C5a and their downstream receptor-mediated interactions. To evaluate both the independent and combined effects of C3 and C5 and their anaphylatoxins to liver regeneration, we crossbred both deficient strains and generated a C3/C5 double-deficient strain. These $C3^{-/-}C5^{-/-}$ mice provided an animal model for delineating the independent effect of isolated components (C3, C5) and also a platform for performing reconstitution experiments with each of their respective anaphylatoxins. Furthermore, choosing to assess the regenerative response of the double-deficient mice alleviated our concern that complement-independent local activation of C3 or C5 (31) would result in cross-interference or compensatory effects in the single-deficient strains. $C3^{-/-}C5^{-/-}$ mice showed no histological sign of liver regeneration (mitotic figures, cellular hypertrophy) at 44 h after hepatectomy. Parenchymal necrosis and hepatocyte degeneration in $C3^{-/-}C5^{-/-}$ livers were more pronounced (Fig. 1 e) as well as higher levels of aminotransferases (not depicted) than in the single-deficient strains. Similarly, BrdU incorporation into hepatocytes was extremely low in $C3^{-/-}C5^{-/-}$ livers (Fig. 3 a). No mortality among the $C3^{-/-}C5^{-/-}$ animals was observed within the first 44 h of the observation period. However, all $C3^{-/-}C5^{-/-}$ displayed clinical signs of liver failure with reduced physical activity and dark yellow urine, which predicted that survival would have been compromised if these mice had been allowed to stay beyond the 44 h time point.

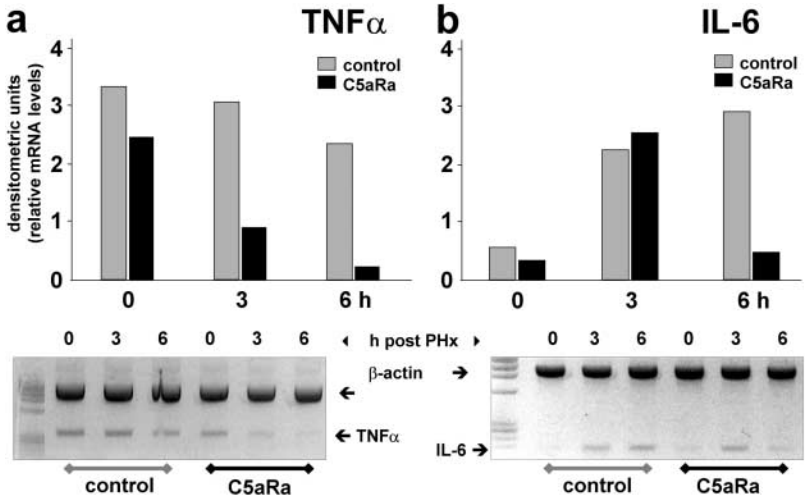


Figure 4. C5aRa blockade decreases TNFa (a) and IL-6 (b) mRNA synthesis in whole liver mRNA extracts during the early phase after PHx. (a) Black bars show the levels of TNFα mRNA synthesis in livers of C5aRa-treated animals in densitometric arbitrary units at 0, 3, and 6 h. Gray bars show the corresponding values for control peptide-treated animals. The quantification was performed on the bases of relative intensities of the corresponding bands in the displayed agarose gels. Normalization was done according to β-actin mRNA intensity in each sample to compensate for loading differences. Control peptide-treated animals showed a constant decrease in TNFα mRNA synthesis over time after PHx. In C5aRa-treated animals, this decrease was more pronounced than in control livers at 3 and 6 h after PHx. (b) Black bars show the relative levels of IL-6 mRNA synthesis in livers of C5aRa-treated animals at 0, 3, and 6 h. Gray bars show the corresponding values for control peptide-treated animals (quantification was performed as described in A). Control peptide-treated animals show a constant approximate fivefold increase in IL-6 mRNA synthesis over time after PHx. C5aRa treatment causes an abrogation of this increase in IL-6 mRNA expression. (mRNA samples from three separate experiments were pooled for each time point).

In a group of eight $C3^{-/-}C5^{-/-}$ mice that were allowed to survive until 96 h after PHx, three animals died (unpublished data). Five animals showed recovery of their liver function accompanied by increasing liver weight 96 h after PHx and after transient clinical signs of liver dysfunction (icteric urine, low physical activity). These observations suggest that $C3^{-/-}C5^{-/-}$ mice eventually regenerate once they have survived a period of liver dysfunction that is probably due to their delayed onset of regeneration. Together, these results strongly indicate that C3 and C5 act cooperatively in inducing hepatocyte proliferation and that they also have distinct functions required to initiate the regenerative response.

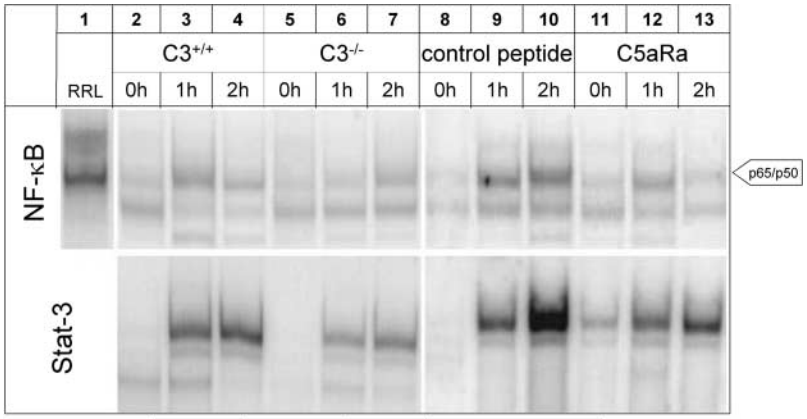
C3a and C5a Exert a Cooperative Effect during Liver Regeneration. Reconstitution of $C3^{-/-}$ mice with human C3 or murine C3a reversed liver damage and significantly restored hepatocyte proliferation (Fig. 2 b) In light of this finding, we subsequently reconstituted $C3^{-/-}C5^{-/-}$ mice with synthetic C3a and recombinant C5a to assess whether these cleavage products of C3 and C5, respectively, mediate independent effects during liver regeneration.

Single reconstitutions with C3a (Fig. 1 f) or C5a (Fig. 1 g) decreased parenchymal damage and increased hepatocyte BrdU incorporation (Fig. 3 a). The combined application of both anaphylatoxins had an additive effect in terms of protection from liver failure and tissue damage (Fig. 1 h and Fig. 3 b) as well as in inducing hepatocyte proliferation. However, the reconstitution with both anaphylatoxins was not able to completely restore the proliferative response to that of wild-type animals (Fig. 3 a), which may be due to the rapid inactivation of the injected anaphylatoxins by carboxypeptidases (32).

The cooperative effect of C3a and C5a in preventing liver failure and promoting hepatocyte proliferation was also reflected by the serum aminotransferase profile of the C3a/C5a-reconstituted $C3^{-/-}5^{-/-}$ mice at 44 h after PHx (Fig. 3 c). These results strongly suggest that both C3 and C5 contribute to normal liver regeneration and that this is achieved by the concerted action of both anaphylatoxins C3a and C5a.

C5aR Blockade Disrupts Liver Regeneration after PHx. To further dissect the mechanism by which complement anaphylatoxins promote the mitogenic priming of hepatocytes after PHx, we focused on the action of C5a and assessed whether C5aR–CD88-mediated interactions are involved in the early priming stage of liver regeneration. For this purpose, mice were treated with a specific C5a receptor antagonist (C5aRa; references 28, 33) before PHx. This small peptide antagonist of C5aR exhibits highly inhibitory properties for C5a-mediated functions and has been used previously and characterized in several animal models (28). C5aRa treatment almost completely abrogated liver regeneration, with a high mortality of 37% (6:16), as compared with 0% (0:3 mice) for their peptide-treated controls ($P < 0.05$) at 44 h after surgery. Hepatocyte DNA synthesis was almost completely inhibited (Fig. 2 f), and extensive parenchymal damage was evident at 44 h after PHx (Fig. 1 d). The total number of control animals used in these studies was 12. This number includes control peptide-treated and nontreated animals because we found no differences between the two control groups. In Fig. 2 f, only the results from the control peptide-treated animals are included. The detrimental effect of C5aR blockade on liver regeneration indicated that the anaphylatoxin C5a exerts its stimulatory effect on hepatocytes via C5aR-mediated interactions.

Intrahepatic TNFα and IL-6 Release after PHx Requires C5aR Stimulation. To determine whether the proliferative effect mediated by C5aR activation involves modulation of cytokine responses in the liver, we investigated the influence of C5aR blockade on the intrahepatic expression of TNFα and IL-6 immediately after PHx. Both of these cytokines are among the earliest priming factors that have been implicated in liver regeneration after loss of parenchy-



	C3-/-		C5aRA	
	1 h	2 h	1 h	2 h
NF-κB	54%	11%	13%	60%
Stat-3	56%	18%	19%	37%

Figure 5. C3 deficiency and C5aRa blockade diminishes NF-κB and STAT-3 activation in nuclear liver extracts after PHx. The top depicts NF-κB activation, and the bottom shows STAT-3 activation in both study groups as compared with their controls. Lane 1 represents NF-κB in rabbit reticulocyte lysates (RRL). WT (lanes 3 and 4) and control peptide-treated (lanes 9 and 10) mice show induction of both transcription factors, with the strongest signal detected at 1 or 2 h after PHx. C3 deficiency (lanes 6 and 7) and C5aRa treatment (lanes 12 and 13) diminishes the induction of both factors after PHx, with the most prominent difference at 1 h in $C3^{-/-}$ mice (NF-κB 54%- and STAT-3 56%-less activation than in control) and 2 h after C5aRa treatment (NF-κB 60%- and STAT-3 37%-less activation than in control). The table summarizes the reduction of activation of both transcription factors in $C3^{-/-}$ and C5aRa-treated mice when compared with control (% values). The densitometric values from gels of two separate PHx experiments were averaged (Materials and Methods). Lanes 2, 5, 8, and 11 represent the baseline activation of both transcription factors in each group without surgery.

mal liver mass (34). Control peptide-treated animals displayed a constant induction of TNFα mRNA expression over time, as determined by semi-quantitative RT-PCR. In contrast, C5aRa-treated animals showed a decline in TNFα mRNA levels at 3 and 6 h (Fig. 4 a).

Furthermore, the effect of C5aRa on IL-6 synthesis in the liver was even more pronounced (Fig. 4 b). Control peptide-treated animals showed a gradual approximate twofold increase in IL-6 mRNA level over time. However, C5aR blockade markedly reduced this induction in IL-6 mRNA within the first 6 h after PHx. The profiles of IL-6 and TNFα mRNA expression in other organs (e.g., spleen, lung, and kidney) after PHx and C5aRa treatment were not significantly different from those in control animals (unpublished data). This finding indicates that the effect of the C5aRa was highly specific and localized mainly in the liver and did not affect cytokine release from remote organs. Together, these results demonstrate that C5a exerts its effect during liver regeneration by mediating the induction of proinflammatory cytokines that, in turn, stimulate hepatocytes to reenter the cell cycle.

C5aR Engagement Recruits NF-κB and STAT-3–dependent Pathways in the Regenerating Liver. The local release of TNFα and IL-6 and the subsequent stimulation of their receptors in the liver is coupled to the downstream activation of the latent transcription factors NF-κB and STAT-3 (6). Both transcription factors have been shown to largely determine the regenerative capacity of hepatocytes in the early stages after PHx (20). Therefore, the decreased mRNA levels of IL-6 and TNFα observed in C5aRa-treated animals after PHx could be associated with decreased activation of STAT-3 and NF-κB transcription complexes. To test this hypothesis, the DNA binding activity of these complexes was assessed in nuclear extracts prepared from C5aRa-treated livers after PHx (Fig. 5, lanes 8–13). NF-κB– and STAT-3–binding activity in C5aRa livers was most prominently reduced at 2 h after surgery, when compared with control livers (NF-κB 60%- and STAT-3 37%-less activation than in control). This pronounced reduction of transcription factor activation upon blockade of the C5aR explains in part the severely impaired regenerative phenotype of the antagonist-treated mice and identifies a potential molecular pathway by which C5a and its receptor C5aR modulate the early growth response of the liver after surgical resection.

Complement Component C3 Is Required for Normal STAT-3/NF-κB Activation during Liver Regeneration. In C3-deficient mice, we observed markedly reduced binding activity of both transcription factors (NF-κB and STAT-3) in liver nuclear extracts at 1 h after PHx (NF-κB 54%- and STAT-3 56%-less activation than in control; Fig. 5, lanes 2–7). Although the pattern of the diminished transcription factor activation in $C3^{-/-}$ mice was different from that in C5aRa-treated animals (a prominent difference was observed at 2 h after PHx in $C3^{-/-}$ livers), this finding suggests that both C3 and C5a mediate their stimulatory effect during liver regeneration by interacting with similar downstream signaling effectors.

Discussion

To date, the influence of innate immunity components on the molecular pathways that regulate liver regeneration has not been addressed. Here, we report that both complement components C3 and C5 contribute to liver regeneration after PHx and that this effect is mainly achieved through the concerted action of their anaphylatoxins. C3 or C5 deficiency led to diminished liver regeneration, accompanied by transient or fatal liver failure after PHx. Combined deficiency of both components exacerbated the regenerative defect of the liver. The recovery of DNA synthesis in regenerating hepatocytes after simultaneous reconstitution of $C3^{-/-}C5^{-/-}$ mice with C3a and C5a clearly indicated that both anaphylatoxins are required to initiate

hepatocyte proliferation. We demonstrated that C3 and C5 engage in the early growth response of regenerating hepatocytes by mediating the activation of priming signals (STAT-3 and NF-κB), which are essential for the initiation of the regenerative response (6). Additionally, C5aR blockade after PHx revealed that C5aR stimulation is required for normal induction of the proinflammatory cytokines IL-6 and TNFα, two factors that have been identified as crucial regulators of the regenerative capacity of hepatocytes. An essential role of complement in liver regeneration is also supported by our recent paper showing that $C5^{-/-}$ mice are more susceptible to toxic liver damage and display a delayed regenerative response marked by sustained liver necrosis (17).

However, a clear distinction should be drawn between these two models of liver regeneration. Acute liver toxicity induces vigorous parenchymal regeneration but also evokes a major inflammatory response at the site of tissue damage. The partial hepatectomy model has now allowed us to address the direct effect of complement on the regenerative potential of hepatocytes without interference from a more generalized inflammatory parenchymal insult, such as that associated with the CCl_4 toxicity model.

Mice deficient in C3 showed severely disrupted liver regeneration after PHx. Reconstitution of $C3^{-/-}$ mice with human C3 restored their regenerative capacity, indicating that C3 mediates a specific effect during liver regeneration. The recovery of DNA synthesis in hepatocytes after C3a administration to $C3^{-/-}$ mice suggested that C3 activation could have an effect on regenerating hepatocytes independent of downstream C5 activation. The partial recovery of the regenerative phenotype of $C3^{-/-}C5^{-/-}$ mice upon reconstitution with C3a or C5a further underlines the impact of the anaphylatoxins in the regenerating liver. The combined reconstitution of $C3^{-/-}C5^{-/-}$ animals with C3a and C5a resulted in a significant increase of DNA synthesis in hepatocytes and complete protection from liver damage. These findings support the hypothesis that C3a and C5a do not only have independent effects on liver regeneration but they also act in concert to promote hepatocyte proliferation after PHx. However, it has to be noted that combined C3a and C5a reconstitution did not restore DNA synthesis to wild type levels. This gives room to speculate on the need for additional complement activation products other than the anaphylatoxins.

The in vivo administration of a specific C5aR antagonist provided an ideal tool for directly targeting and dissecting the role of C5aR stimulation and the involved molecular pathways during liver regeneration. In comparison with $C5^{-/-}$ animals, C5aRa treatment displayed a more severe impairment of liver regeneration after PHx. This observation can be explained in a number of ways as follows. The process of liver regeneration relies on multiple redundant pathways that ensure its control when single components within this regulating network malfunction. The lack of a C5a signal in $C5^{-/-}$ mice might be compensated for by a variety of other signals during the development of the deficient organism. In the case of short-term C5aR blockade, a compensatory mechanism cannot be initiated. Therefore, this blockade can unmask the actual contribution of C5a to the regulation of liver regeneration. The fact that $C3^{-/-}C5^{-/-}$ animals apparently cannot compensate for their regenerative defect could suggest that C3 and its activation products might indeed be the source for the compensatory capability in $C5^{-/-}$ mice. C5aRa treatment induced a severe regenerative defect after PHx, which was associated with decreased levels of IL-6 and TNFα mRNA, two cytokines that are crucial for the onset of regeneration (7, 34–36). This finding supported the hypothesis that C5aR activation is required for the priming of hepatocyte regeneration. The changes in cytokine mRNA synthesis, the decreased NF-κB activation, and the low STAT-3 activation in livers of C5aRa-treated animals provide an explanation for the observed absence of DNA replication in livers of this study group. TNFα has been described as the main mediator of NF-κB activation in hepatocytes and nonparenchymal liver cells. In turn, NF-κB targets the IL-6 gene, leading to IL-6 synthesis (37). Defective activation of both STAT-3 and NF-κB was also observed in $C3^{-/-}$ mice after PHx. Thus, C3 may either serve as an upstream mediator of C5aR activation or exert an C5a-independent effect on the early hepa-

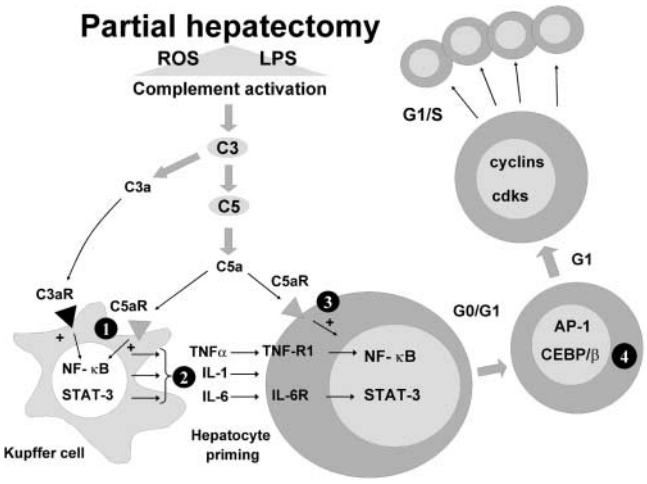


Figure 6. Summary of the proposed mechanisms by which complement activation products modulate the priming of hepatocytes after PHx. PHx causes among other factors the release of reactive oxygen species (ROS) and LPS. These factors can trigger complement activation either locally, in the portal circulation, or systemically. After complement activation cleavage of C3 or C5 leads to the generation of the potent anaphylatoxins C3a and C5a. Our current results support a regulatory role for these anaphylatoxins in liver regeneration. Their respective G-protein coupled receptors C3aR and C5aR are activated on Kupffer cells (1) and thereby enhance the release of TNFα and IL-6 (2). These cytokines activate NF-κB and STAT-3 in hepatocytes via the corresponding receptors, and, thus, initiate the transcription of immediate early genes. The final transition into G1 phase and the transcription of cell cycle genes is supported by the activation of several transcription factors including AP-1 and CEBP/β (4). Alternatively, the release of IL-6 might induce C5aR activation on hepatocytes after PHx (3). This direct effect of C5a on hepatocytes would further promote their priming.

tocyte growth response by activating STAT-3 and NF-κB. Hepatocyte DNA replication and the transcription of a broad range of acute phase genes is known to be the result of the synergistic effect of various transcription factors (e.g., STAT-3, NF-κB, C/EBPβ, and AP1) (38, 39). NF-κB activation has also been reported as a response to stimulation of C5aR on mononuclear cells (40) as well as C3aR and C5aR stimulation on HeLa cells (41). These observations open the possibility for a direct, cytokine-independent effect of C3a and C5a on transcription factor activation in liver cells. In this respect, the $C3^{-/-}C5^{-/-}$ animals together with the $C3^{-/-}$ and $C5^{-/-}$ animals will help to dissect the influence of the individual anaphylatoxins on transcription factor activation during the early events of liver regeneration.

Several lines of evidence support our finding that both anaphylatoxins contribute to the early signaling and transcriptional network driving hepatocyte proliferation. It has been reported that release of IL-6 from Kupffer cells can be triggered by C5a in concert with LPS, thereby mediating the expression of acute-phase genes in cultured hepatocytes (14). Furthermore, LPS is considered one of the earliest priming signals for liver regeneration (11). In light of these findings, a synergistic interaction of C5aR and LPS-mediated pathways in nonparenchymal liver cells seems feasible, which could modulate early responses of hepatocytes during liver regeneration. Hepatocytes could also serve as direct targets for C5a-dependent activation during liver regeneration. It was recently shown that C5aR expression can be stimulated de novo in hepatocytes after administration of LPS or IL-6 in rats (42). Therefore, it is reasonable to hypothesize that IL-6 or LPS release might affect the responsiveness of various liver cell types to C5a stimulation, by causing an increase in C5aR expression during liver regeneration.

In comparison to the functions of C5a, there is also evidence to indicate that C3a modulates cell metabolism and intracellular signaling. C3a was shown to modulate prostaglandin synthesis in cultured Kupffer cells (15) and to change IL-6, TNFα, and IL-1β production in peripheral blood mononuclear cells (43–45). It was also reported that C3a is capable of activating a signal transduction pathway in endothelial cells that is distinct from C5a-dependent mechanisms (46). These findings further support the concept of a cooperative role of the two anaphylatoxins, which might be mediated through activation of distinct signaling pathways in target liver cells.

Moreover, the recent identification of a second C5aR molecule that is able to bind with high affinity not only C5a but also C3a and C3a desArg (47) adds more complexity to the interpretation of our data and provides greater conceptual flexibility as to how these two anaphylatoxins can interact and signal through their receptors in the liver. The fascinating possibility that these ligands might physically share the same receptor but trigger divergent signaling pathways, or bind to different isoforms of the same receptor, and thereby activate distinct pathways in liver cells during regeneration, cannot be excluded and warrants further investigation.

In conclusion, we present our working hypothesis as to how the early mechanisms of liver regeneration that we have discussed interact with the complement system, including the role of the C5aR (Fig. 6). Future works are needed to confirm the direct effect of anaphylatoxin-dependent signaling on hepatocytes during liver regeneration. We cannot exclude that complement activation products other than C5a and C3a (such as C3b, C4a, C5adesArg, and sublytic concentrations of C5b-9 [the membrane attack complex]) might also play a role in hepatocyte priming.

Liver regeneration is critical for the successful outcome of liver resections and is especially important in the context of living donor liver transplantations. Our results provides new insights into the mechanisms regulating liver regeneration by identifying complement components as early priming factors of hepatocytes. This finding offers a new perspective in the search for possible therapeutic interventions to enhance liver regeneration.

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