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...
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 B10D2snJ (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 in 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 harvest. 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 C5\(^{-/-}\)/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 Analytics.

**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 (X400). 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'-TTCATC-CAGTTGCTCTTCTT-3'; 3'-primer, 5'-CAGAATTGCATGTGACAC-3' (size of PCR product, 198 bp); TNFα, 5'-primer, 5'-GGCAAGTCTACTTGTGATCAGTGC-3'; 3'-primer, 5'-CACATTGGAGCTCAGTGAATGCGG-3' (size of PCR product, 307 bp); and B-actin, 5'-primer, 5'-AGGTTGTATGTGTTGGAATGG-3', 3'-primer, 5'-AGCCAGACGATTAATCTCCCTCTGC-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-kB binding, the consensus sequence \(5'-\text{AGTTGAGGGGACTTTCCCAGGC}-3'\) (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 \(5'-\text{GATCCCGAGCATTTCCCAAGCC-3'}\) (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 \(5'-\text{GATCCCGAGCATTTCCCAAGCC-3'}\) (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 \(5'-\text{GATCCCGAGCATTTCCCAAGCC-3'}\) (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 \(5'-\text{GATCCCGAGCATTTCCCAAGCC-3'}\) (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 \(5'-\text{GATCCCGAGCATTTCCCAAGCC-3'}\) (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 \(5'-\text{GATCCCGAGCATTTCCCAAGCC-3'}\) (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. Rabbit reticulocyte lysate with pre-identified NF-kB was used to recognize the NF-kB subunit p65 in all assays. Loading control was achieved by detecting E2 binding (5'-GGTTCCAGACCCGATGTTGCTGGA-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-kB (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.

C5a was synthesized chemically according to a protocol described previously (24) with a modification of the cyclization procedure (25, 26). A 6-His-X–tagged murine C5a was expressed in Escherichia coli and purified using nickel chelating affinity chromatography (27). Three successive doses of the anaphylatoxins, synthetic mouse C5a, and expressed C5a were also given by i.p. injection at a dose of 15 mg/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
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-
The role of complement in liver regeneration.

The mortality 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 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−/−
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 allowed us to test the hypothesis that complement-independent local activation of C3 or C5 (31) would result in cross-inhibition of these responses.

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 regenerative 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).
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−/−C5−/− mice at 44 h after PHx (Fig. 3). 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-
The local release of TNFα in the liver is coupled to the downstream activation of NF-κB and STAT-3. The inclusion of both transcription factors, with the strongest signal detected at 1 h after PHx, was highly specific and localized mainly to the liver, with no induction in other organs (e.g., spleen, lung, and kidney) after PHx and C5aRa treatment (Fig. 4 b). Control peptide-treated animals showed a twofold increase in IL-6 mRNA expression over time. However, C5aRa blockade markedly reduced this induction in IL-6 mRNA expression in the liver within the first 6 h after PHx. The profiles of IL-6 and TNFα mRNA expression in control peptide-treated mice were not significantly different from those in control animals (unpublished data). This finding indicates that 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 C5aR blockade was highly specific and localized mainly to 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-treated mice was most prominently reduced at 2 h after surgery, when compared with control livers (NF-κB 60%–64% 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%–60% and STAT-3 56%–60% 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. The study groups as compared with their controls. Lane 1 represents 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.
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mice are more susceptible to toxic liver damage and display

a delayed regenerative response marked by sustained liver
carcinosis (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 ad-
dress 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 CCl4 toxicity model.

Mice deficient in C3 showed severely disrupted liver re-
generation 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 indepen-
dent of downstream C5 activation. The partial recovery of
the regenerative phenotype of C3-/-/C5-/- mice upon re-
constitution with C3a or C5a further underlines the impact
of the anaphylatoxins in the regenerating liver. The com-
bined 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 antago-
nist provided an ideal tool for directly targeting and dis-
secting the role of C5aR stimulation and the involved mo-

cular 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 fol-

low. 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 compen-
sated for by a variety of other signals during the develop-

ment 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
C5 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 on-
set of regeneration (7, 34–36). This finding supported the
hypothesis that C5aR activation is required for the prim-
ing 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 an-
imals 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 comple-
ment 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
clavage of C3 or C5 leads to the generation of the potent anaphylatoxins
C3a and C5a. Our current results support a regulatory role for these an-
aphylatoxins 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/B
(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.
toocyte growth response by activating STAT-3 and NF-kB. 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 provide 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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References

1. Mastellos, D., and J.D. Lambris. 2002. Complement: more than a ‘guard’ against invading pathogens? Trends Immunol. 23:485–491.
2. Reca, R., D. Mastellos, M. Majka, L. Marquez, J. Rajaczak, S. Franchini, A. Godek, M. Honczarenko, L.A. Spruce, A. Janowska-Wieczorek, et al. 2003. Functional receptor for C3a anaphylatoxin is expressed by normal hematopoietic stem/progenitor cells, and C3a enhances their homing-related responses to SDF-1. Blood. 101:3784–3793.
3. Tsonis, K., P.A. Tsonis, I.K. Zarkadis, A.G. Tsagas, and J.D. Lambris. 1998. Expression of the third component of complement, C3, in regenerating limb blastema cells of urodèles. J. Immunol. 161:6819–6824.
4. Kimura, Y., M. Madhavan, M.K. Call, W. Santiago, P.A. Tsonis, J.D. Lambris, and K. Tsonis. 2003. Expression of complement 3 and complement 5 in newt limb and lens regeneration. J. Immunol. 170:2331–2339.
5. Michalopoulos, G.K., and M.C. DeFrances. 1997. Liver regeneration. Science. 276:60–66.
6. Taub, R. 1996. Liver regeneration 4: transcriptional control of liver regeneration. FASEB J. 10:413–427.
7. Diehl, A.M. 2000. Cytokine regulation of liver injury and repair. Immunol. Rev. 174:160–171.
8. Rudnick, D.A., D.H. Perlmuter, and L.J. Muglia. 2001.
Prostaglandins are required for CREB activation and cellular proliferation during liver regeneration. 

9. Fausto, N. 2000. Liver regeneration. J. Hepatol. 32:19–31.

10. Decker, K.F., and M.Y. Obolenskaya. 1995. Cytokines, nitric oxide synthesis and liver regeneration. J. Gastroenterol. Hepatol. 10:512–S17.

11. Cornell, R.P., B.L. Liljequist, and K.F. Bartzial. 1990. Depressed liver regeneration after partial hepatectomy of germ-free, athymic and lipopolysaccharide-resistant mice. Hepatology. 11:916–922.

12. Gao, C., R. Jokert, P. Gondipalli, S.R. Cai, S. Kennedy, M.W. Flye, and K.P. Ponder. 1999. Lipopolysaccharide potentiates the effect of hepatocyte growth factor on hepatocyte replication in rats by augmenting AP-1 activity. Hepatology. 30:1405–1416.

13. Fausto, N. 2001. Liver regeneration. In The Liver, Biology and Pathobiology. M.A. Irwin and J.L. Boyer, editors. Lipincott Williams and Wilkins, New York. 591–610.

14. Mack, C., K. Jungermann, O. Gotze, and H.L. Schieferdecker. 2001. Anaphylatoxin C5a actions in rat liver: synergistic enhancement by C5a of lipopolysaccharide-dependent alpha(2)-macroglobulin gene expression in hepatocytes via IL-6 release from Kupffer cells. J. Immunol. 167:3972–3979.

15. Puschel, G.P., U. Hespeining, M. Oppermann, and P. Dieter. 1993. Increase in prostanoit formation in rat liver macrophages (Kupffer cells) by human anaphylatoxin C3a. Hepatology. 18:1516–1521.

16. Puschel, G.P., M. Oppermann, F. Neuschafer-Rube, O. Gotze, and K. Jungermann. 1991. Differential effects of human anaphylatoxin C3a on glucose output and flow in rat liver during orthograde and retrograde perfusion: the portal scavenger cell hypothesis. Biochem. Biophys. Res. Commun. 176:1218–1226.

17. Mastellos, D., J.C. Papadimitriou, S. Franchini, P.A. Tsonis, and J.D. Lambris. 2001. A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration. J. Immunol. 166:2479–2486.

18. Higgins, G.M., and R.M. Anderson. 1931. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch. Pathol. 12:186–202.

19. Aldeguer, X., F. Debonera, A. Shaked, A.M. Krasinkas, A.E. Gelman, X. Que, G.A. Zamir, S. Hiyozasu, K.K. Kovalovich, R. Taub, and K.M. Olthoff. 2002. Interleukin-6 from Kupffer cells by human anaphylatoxin C3a on glucose output and flow in rat liver following partial surgical removal. Hepatology. 31:149–159.

20. Cressman, D.E., R.H. Diamond, and R. Taub. 1995. Rapid activation of the Stat3 transcription complex in liver regeneration. Hepatology. 21:1443–1449.

21. Kovalovich, K., R.A. DeAngelis, W. Li, E.E. Furth, G. Ciliberto, and R. Taub. 2000. Increased toxin-induced liver injury and fibrosis in interleukin-6-deficient mice. Hepatology. 31:149–159.

22. Crissey, M.A., J.I. Leu, R.A. De Angelis, L.E. Greenbaum, L.M. Scearce, K. Kovalovich, and R. Taub. 1999. Liver-specific and proliferation-induced deoxyribonuclease I hyper-sensitive sites in the mouse insulin-like growth factor binding protein-1 gene. Hepatology. 30:1187–1197.

23. Sahu, A., A.M. Soulika, D. Morikis, L. Spruce, W.T. Moore, and J.D. Lambris. 2000. Binding kinetics, structure-activity relationship, and biotransformation of the complement inhibitor compstatin. J. Immunol. 165:2491–2499.

24. Chino, N., S. Kubo, Y. Nishiuchi, S. Kumagaye, K.Y. Kumagaye, M. Takai, T. Kimura, and S. Sakakibara. 1988. Synthesis of porcine C5a anaphylatoxin by the solution procedure and confirmation of the reported structure. Biochem. Biophys. Res. Commun. 151:1285–1292.

25. Fields, G.B., and R.L. Noble. 1990. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int. J. Pept. Protein Res. 35:161–214.

26. Sahu, A., D. Morikis, and J.D. Lambris. 2003. Compstatin, a peptide inhibitor of complement, exhibits species-specific binding to complement component C3. Mol. Immunol. 39:557–566.

27. Finch, A.M., A.K. Wong, N.J. Paczkowski, S.K. Wadi, D.J. Craik, D.P. Fairlie, and S.M. Taylor. 1999. Low-molecular weight peptidic and cyclic antagonists of the receptor for the complement factor C5a. J. Med. Chem. 42:1965–1974.

28. Morikis, D., and J.D. Lambris. 2001. Structural aspects and design of low-molecular-mass complement inhibitors. Biochem. Soc. Trans. 30:1026–1036.

29. Sahu, A., and J.D. Lambris. 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. ImmunoL Rev. 180:35–48.

30. Frei, Y., J.D. Lambris, and B. Stockinger. 1987. Generation of a monoclonal antibody to mouse C5 application in an ELISA assay for detection of anti-C5 antibodies. Mol. Cell. Probes. 1:141–149.

31. Huber-Lang, M., E.M. Younkin, J.V. Sarna, N. Riedemann, S.R. McGuire, K.T. Lu, R. Kunkel, J.G. Younger, F.S. Zetoune, and P.A. Ward. 2002. Generation of C5a by phagocytic cells. Ann. J. Pathol. 161:1849–1859.

32. Campbell, W.D., E. Lazoura, N. Okada, and H. Okada. 2002. Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. Microbiol. Immunol. 46:131–134.

33. Paczkowski, N.J., A.M. Finch, J.B. Whitmore, A.J. Short, A.K. Wong, P.N. Monk, S.A. Cain, D.P. Fairlie, and S.M. Taylor. 1999. Pharmacological characterization of antagonists of the C5a receptor. Br. J. Pharmacol. 128:1461–1466.

34. Cressman, D.E., L.E. Greenbaum, R.A. DeAngelis, G. Ciliberto, E.E. Furth, V. Poli, and R. Taub. 1996. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science. 274:1379–1383.

35. Trautwein, C., T. Rakemann, M. Niehoff, S. Rose-John, and M.P. Manns. 1996. Acute-phase response factor, increased binding, and target gene transcription during liver regeneration. Gastroenterology. 110:1854–1862.

36. Yamada, Y., E.M. Webber, I. Kirillova, J.J. Peschon, and N. Fausto. 1998. Analysis of liver regeneration in mice lacking type 1 or type 2 tumor necrosis factor receptor: requirement for type 1 but not type 2 receptor. Hepatology. 28:959–970.

37. Kirillova, I., M. Chaison, and N. Fausto. 1999. Tumor necrosis factor induces DNA replication in hepatic cells through nuclear factor kappA activation. Cell Growth Different 10:819–828.

38. Hsu, J.C., R. Bravo, and R. Taub. 1992. Interactions among LRF, JunB, c-Jun, and c-Fos define a regulatory program in the G1 phase of liver regeneration. Mol. Cell. Biol. 12: 4654–4665.

39. Greenbaum, L.E., W. Li, D.E. Cressman, Y. Peng, G. Ciliberto, V. Poli, and R. Taub. 1998. CCAAT enhancer-binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. J. Clin. Invest. 102:996–1007.

40. Hsu, M.H., M. Wang, D.D. Browning, N. Mukaida, and
R.D. Ye. 1999. NF-kappaB activation is required for C5a-induced interleukin-8 gene expression in mononuclear cells. *Blood.* 93:3241–3249.

41. Yang, M., H. Sang, A. Rahman, D. Wu, A.B. Malik, and R.D. Ye. 2001. G alpha 16 couples chemoattractant receptors to NF-kappa B activation. *J. Immunol.* 166:6885–6892.

42. Schieferdecker, H.L., G. Schlaf, M. Koleva, O. Gotze, and K. Jungermann. 2000. Induction of functional anaphylatoxin C5a receptors on hepatocytes by in vivo treatment of rats with IL-6. *J. Immunol.* 164:5453–5458.

43. Takabayashi, T., E. Vannier, J.F. Burke, R.G. Tompkins, J.A. Gelfand, and B.D. Clark. 1998. Both C3a and C3a (desArg) regulate interleukin-6 synthesis in human peripheral blood mononuclear cells. *J. Infect. Dis.* 177:1622–1628.

44. Takabayashi, T., E. Vannier, B.D. Clark, N.H. Margolis, C.A. Dinarello, J.F. Burke, and J.A. Gelfand. 1996. A new biologic role for C3a and C3a desArg: regulation of TNF-alpha and IL-1 beta synthesis. *J. Immunol.* 156:3455–3460.

45. Fischer, W.H., M.A. Jagels, and T.E. Hugh. 1999. Regulation of IL-6 synthesis in human peripheral blood mononuclear cells by C3a and C3a (desArg). *J. Immunol.* 162:453–459.

46. Schraufstatter, I.U., K. Trieu, L. Sikora, P. Sriramaraao, and R. DiScipio. 2002. Complement c3a and c5a induce different signal transduction cascades in endothelial cells. *J. Immunol.* 169:2102–2110.

47. Kalant, D., S.A. Cain, M. Maslowska, A.D. Sniderman, K. Cianflone, and P.N. Monk. 2003. The chemoattractant receptor-like protein C5L2 binds the C3a des-Arg77/acylation-stimulating protein. *J. Biol. Chem.* 278:11123–11129.