# Alternative complement pathway component Factor D contributes to efficient clearance of tissue debris following acute CCl₄-induced injury

**Gail A. Cresci, PhD, RD, LD**,
Department of Gastroenterology and Pathobiology Cleveland Clinic

**Daniela Allende, MD**,
Department of Pathology Cleveland Clinic

**Megan R. McMullen, BA**, and
Department of Pathobiology Cleveland Clinic

**Laura E. Nagy, PhD**
Department of Pathobiology and Gastroenterology Cleveland Clinic

## Abstract

Complement, part of the innate immune system, is involved with immune protection against invading pathogens as well as cell survival and tissue regeneration. It is known that complement activation is required for timely hepatocyte recovery following an acute toxic injury, but which pathway of complement activation is involved in response to hepatocyte injury has not been identified. In these studies we utilize mice deficient in *C1qa*, *C4* and *Factor D*, lacking the classical, classical/MBL, and alternative pathways of complement activation, respectively, to identify an essential role for Factor D in the ability of the liver to recover from acute toxic injury. Here we demonstrate that following an acute CCl₄-induced injury, the involvement of the alternative complement pathway is essential for efficient liver recovery.

### Keywords

complement; alternative pathway; factor D; acute liver injury; necrosis

## 1. Introduction

Complement is part of the innate immune system serving conventional roles of defending the host against invading pathogens, and unconventional roles of cell survival, growth and differentiation in various tissues (Reca et al., 2003 and Del Rio-Tsonis, 1998), as well as

tissue homeostasis, with a critical role in clearing apoptotic cells and scavenging of toxic cellular debris (Munoz et al., 2010).

Complement consists of about 30 soluble and membrane bound proteins and functions in cascades of stepwise protease activation. Upon activation, any one of three distinct pathways (classical, mannose-binding lectin (MBL), and alternative) can lead to the cleavage of the complement protein C3; activation can occur either on pathogen/cellular surfaces or in plasma (Qin and Gao, 2006). C1q and MBL are pattern recognition molecules of the classical and lectin pathway, respectively. C1s, C1r, MBL-associated serine proteases (MASP), C2 and C4 also participate in classical and lectin pathway of activation (Degn and Thiel, 2013). The alternative pathway activates C3 spontaneously in combination with Factor B, Factor D (FD) and properdin (Harboe and Mollnes, 2008) and also serves to amplify complement activation via the classical and lectin pathways. C3 cleavage results in the production of the inflammatory anaphylatoxin C3a and the C3b fragment. Binding of C3b to damaged cells acts as an opsonizing agent and facilitates removal of cellular debris from injured tissue (Munoz et al., 2010). As the activation cascade proceeds, the second anaphylatoxin C5a is generated, along with multiple other protein fragments, leading to the formation of the terminal complex, C5b-9n (Nesargikar et al., 2012).

Acute toxic injury to the liver results in the death of hepatocytes by necrosis and apoptosis. In response to injury, the liver undergoes a series of complex, overlapping phases that are precisely timed in order to re-establish tissue homeostasis. These events are classically divided into three phases: inflammation, proliferation and remodeling. Resident macrophages and recruited immune cells coordinate the inflammatory response, activating multiple cytokine and growth factor networks.

There is a growing appreciation that complement is critical to the liver's ability to respond to injury at multiple phases of recovery. Following injury, complement anaphylatoxins (C3a and C5a) are also generated, associated with the activation of other innate immune functions. This dynamic orchestration of multiple innate immune pathways serves to regulate hepatocyte proliferation and liver restoration (Taub, 2004). Complement C3a and C5a also contribute to hepatocyte proliferation (Markiewski et al., 2004). Finally, during the tissue regeneration phase, newly formed fibronectin, cell-adhesion proteins and other basement-membrane proteins are also induced, altering liver architecture (Taub, 2004). Normal liver architecture forms only after normal liver mass is restored. Several mechanisms contribute to tissue remodeling and repair after injury, including the serine protease system and matrix metalloproteinases (Nagase, 1999 and Bezerra, 2001). Since complement is involved in the clearance of necrotic debris by cellular scavengers and remodeling of extracellular matrix components in other injury models (Trouw et al., 2008), it is also likely that complement activation contributes to the clearance of debris during hepatic response to injury, so that a suitable scaffold for newly formed cells can develop.

Exposure of animals to carbon tetrachloride ($CCl_4$) is a commonly used model system to study acute toxic injury to hepatocytes. In response to $CCl_4$, C3 is activated biphasically, peaking at 3 and 36 hr; this activation is closely coupled with activation of a network of cytokine signaling (Markiewski, et al., 2004). The early phase of complement activation

corresponds with the "priming phase" of hepatocytes after injury (Taub, 2004). The second wave of activation likely facilitates the removal of damaged liver tissue, with C3b acting as an opsonin, flagging damaged tissue for removal by phagocytes (Gullstrand et al., 2009). Reconstitution of C3−/− mice with C3a (Markiewski, et al., 2004) and C5−/− mice with C5a (Mastellos et al., 2001) restores hepatocyte proliferation at 48 hr following CCl$_4$-induced injury.

While it is clear that complement activation is required for timely hepatocyte recovery following an acute toxic injury (Markiewski, et al., 2004), the pathway of complement activation involved in response to hepatocyte injury has not been identified. Here we utilized mice deficient in *C1qa*, *C4* and *Factor D*, lacking the classical, classical/MBL, and alternative pathways of complement activation, respectively, to identify an essential role for Factor D in the ability of the liver to recover from acute toxic injury. Interestingly, while Factor D-dependent complement activation was dispensable for an adequate proliferative response, FD-deficient mice were unable to adequately clear injured tissue after a toxic injury. Our results provide new insights into the mechanism and importance of timely clearance of damaged cells after acute toxic injury and suggest that interventions leading to local activation of the alternative pathway of complement activation may enhance early clearance of damaged cells, thus avoiding persistent local inflammation and further damage.

## 2. Materials and Methods

### 2.1 Materials

Carbon tetrachloride and olive oil were purchased from Sigma-Aldrich (St. Louis, MO). All primers for real-time PCR were synthesized by Integrated DNA Technologies (Coralville, IA). Primary antibodies were purchased from the following sources: proliferating cell nuclear antigen (PCNA, clone PC10), (Chemicon/Millipore,Danvers, MA); C3b/iC3b/C3c (C3b) (Hycult Biotechnology, Uden, The Netherlands); Ki67 (Abcam, Cambridge, MA); phospho(Tyr$^{705}$)-STAT3 (Cell Signaling Technology, Danvers, MA); HSC70 (Santa Cruz Biotechnology, Inc, Dallas, TX); cytochrome P450 2E1 (CYP2E1 Research Diagnostics, Inc., Flanders, NJ).

### 2.2 Animals

Ten to 12 week old female C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). A breeding pair for *FD−/−* (Xu et al., 2001) was provided by Dr. Gregory Stahl, Harvard University, Boston; and *C1qa−/−* (Botto et al., 1998) and *C4−/−* (Koller & Smithies, 1992; Fischer et al., 1996) breeding pairs were provided by Dr. Michael Carroll at Center for Blood Research, Harvard University, Boston. Breeding pairs were used to establish breeding colonies at the Cleveland Clinic.

### 2.3 Carbon tetrachloride administration and sample collection

CCl$_4$ was prediluted 1:3 in olive oil before administration. Mice received a single dose at 1μl/g body weight of CCl$_4$ administered by intraperitoneal injection using 100 μl Hamilton syringes fitted with 26G 5/8 inch needles. Mice were anesthetized and blood was drawn from the posterior vena cava precisely at 2, 18, 24, 48, and 72 hours (h) after CCl$_4$. Plasma

was separated from whole blood by centrifugation. After euthanasia, livers were removed, weighed, and portions were fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburg, PA), frozen in optimal cutting temperature (OCT) medium (Takura Finetek USA, Torrance, CA), stored in RNAlater (Ambion, Austin, TX) or snap frozen in liquid nitrogen for further analysis.

## 2.4 Liver histology and plasma alanine aminotransferase (ALT) activities

For histological analysis, formalin-fixed tissues were paraffin-embedded, sectioned (5 μm) and stained with hematoxylin and eosin. Slides were coded prior to examination and reviewed by a liver pathologist blind to the treatment groups. Presence of acidophil bodies (none, few and many), identification and distribution of necrosis (no necrosis, single necrotic cells in zone 3, clusters of necrotic cells in zone 3, diffuse necrosis in zone 3 and zone 3 necrosis extending to zone 2) and neutrophilic inflammation (no neutrophilic inflammation, <2/HPF foci of neutrophilic infiltrate, 2–4 foci/HPF and >4 foci/HPF) were recorded. Images of the histological sections were captured using an Olympus BX41 microscope and digital camera. Plasma samples were assayed for alanine aminotransferase (ALT) activity using Sekisui Chemical Co Ltd enzymatic assay kits (Secaucus, NJ) according to the manufacturer's instructions.

## 2.5 Real-time qPCR

Total RNA was isolated from liver and reverse transcribed as previously described (McMullen et al., 2005). Real-time PCR amplification was performed using gene-specific primers: interleukin-6 (IL-6) FWD: TAGTCCTTCCTACCCCAATTTCC; REV: TTGGTCCTTAGCCACTCCTTC; CD68 FWD: GGACCCACAACTGTCACTCAT REV: AAGCCCCACTTTAGCTTTACC; 18S FWD: ACGGAAGGGCACCACCAGGA REV: CACCACCACCCACGGAATCG; as previously described (Barnes M et al., 2013). Relative amount of target mRNA was determined using comparative threshold (Ct) method by normalizing target mRNA Ct values to those of 18S. Statistics were performed on DCt values.

## 2.6 Immunohistochemistry

C3b/iC3b/C3c and Ki67 immunohistochemical analysis was performed in frozen liver sections as previously described (Roychowdhury et al., 2009). Images were semi-quantified using Image Pro software (Media Cybernetics, Inc, Bethesda, MD).

## 2.7 Liver homogenate preparation, electrophoresis, and immunoblotting

Liver homogenates were prepared and immunoblotting performed, as previously described (Pritchard et al., 2007).

## 2.8 Statistical Analysis

Values reported are mean ± standard error of mean. Data were analyzed by general linear models procedure (SAS Institute, Cary, IN). Data were log transformed if needed to obtain a normal distribution. Follow-up comparisons were made by least square means testing.

Values in graphs with different alphabetical superscripts are significantly different from one another ($p < 0.05$).

## 3. Results

### 3.1 C3b/iC3b/C3c Deposition is Reduced with the Absence of Factor D

Activation of complement, assessed by C3a in plasma and C3b accumulation, exhibits a biphasic response following an acute $CCl_4$-induced toxic injury (Markiewski et al., 2004). To determine which complement activation pathway responds to acute $CCl_4$ liver injury, C3b/iC3b/C3c deposition in liver parenchyma was assessed in wild-type mice and compared to mice deficient in the classical pathway component C1q, the classical/lectin component C4, or the alternative pathway component Factor D. In the absence of $CCl_4$ exposure, C3b/iC3b/C3c was not detected in WT mice (Figure 1A). In WT mice, C3b/iC3b/C3c accumulated as early as 2 hr after $CCl_4$ in a pattern characteristic of a sinusoidal distribution (Figure 1A). At 24 hr, C3b/iC3b/C3c accumulation increased with a noticeably patchy pattern of staining across the liver parenchyma. These patches of C3b/iC3b/C3c accumulation in the centrilobular area were larger at 48 hr after $CCl_4$ injection, but completely absent by 72 hr. Thus, both the dynamics and pattern of C3b/iC3b/C3c deposition changes over time (Figure 1B). In response to $CCl_4$, mice deficient in C1q and C4 exhibited similar dynamics of C3b/iC3b/C3c accumulation to WT mice. However, mice deficient in Factor D (alternative pathway) did not accumulate C3b/iC3b/C3c at any time point following $CCl_4$ exposure, except for a low level of C3b/iC3b/C3c was detected at 48 h.

### 3.2 Deficiency of Factor D Leads to Exacerbated Liver Injury and Inflammatory Cells

Hepatocyte injury, indicated by release of alanine aminotransferase (ALT) into the circulation, as well as hepatocyte apoptosis and necrosis, following a single injection of $CCl_4$ peaks between 36–48 hr (Markiewski, et al., 2004 and Bezerra et al., 2001). ALT was increased in all genotypes 48 hr after $CCl_4$ exposure (Figure 2A). However, plasma ALT activity was higher in the *FD−/−* at 48 hr compared to the other genotypes (Figure 2A). In a more detailed time-course, plasma ALT activity was higher in *FD−/−* mice both at 24 and 48 hr compared to WT. ALT returned to baseline by 72 hr in both genotypes (Figure 2B). Expression of CYP2E1, required to bioactivate $CCl_4$, was not affected by genotype, indicating that the increased injury in *FD−/−* mice was not due to an altered ability to bioactivate $CCl_4$ (Figure 2C).

Histological analysis of acute liver injury was performed by a liver pathologist. As expected, WT and *FD−/−* mice exhibited multiple indications of liver injury following a single $CCl_4$ injection. However, the number of acidophilic bodies, indicative of apoptotic cells, was higher in *FD−/−* mice at 24, 48 and 72 hr compared to WT mice (Figure 3A). The distribution of acidophilic bodies at 48 hr was more extensive and involved multiple perivenular regions (zone 3) in *FD−/−* mice compared to the few, very focal acidophilc bodies noted in WT mice (Figure 3B).

Morphologic features of necrosis were noted in WT and *FD−/−* mice, but necrosis was more extensive and occurred more frequently in the *FD−/−* mice (Figure 4A). Livers from WT mice 24 hr after CCl$_4$ injection had either no necrosis (75% of mice) or clusters of necrotic cells in zone 3 of the liver (25% of mice) compared to *FD−/−* in which more animals exhibited multiple zone 3 clusters of necrotic cells (60% of mice), diffuse necrosis (20% of mice), as well as a few mice with single cell necrosis in zone 3 (20% of mice). 48 hr after CCl$_4$ injection, necrosis was increased in both WT and *FD−/−* mice, but necrosis was more extensive in *FD−/−* mice, with more mice demonstrating clusters of necrotic cells (29% of mice) (Figure 4B) or diffuse necrosis (43% of mice) in zone 3 of the liver lobule (Figure 4C), as well as necrosis extending from zone 3 to zone 2 (29% of mice) (Figure 4D). While necrosis resolved in 50% of the mice in WT and *FD−/−* by 72 hr, the remaining *FD−/−* mice had more severe necrosis in zone 3 of the liver lobule with continued evidence of clusters of necrotic cells, zone 3 necrosis, as well as necrosis extending from zone 3 to zone 2. Taken together, the histological data indicate elevated cellular debris with presence of damaged tissue persisting in the *FD−/−* relative to WT mice after acute exposure to CCl$_4$.

In response to cellular stress and injury, immune cells infiltrate into damaged tissue to assist with its recovery. Liver sections of both genotypes identified the presence of neutrophilic infiltrates predominantly located in zone 3 (Figure 5A). More neutrophilic infiltrates were detected per high power field in *FD−/−* mice than the WT mice 24 hr following CCl$_4$ injection; however, at 48 hr there was no effect of genotype on neutrophilic bodies. Interestingly, by 72 hr, when other markers of liver injury were resolving in both genotypes, the *FD−/−* mice continued to exhibit a higher number of foci of neutrophilic infiltrates (>4 foci/HPF) than the WT mice (Figure 5A). The localization of neutrophilic infiltrates also differed between the WT and *FD−/−* mice at 72 hr; WT mice exhibited scattered foci of neutrophilic inflammation (Figure 5 B) whereas the *FD−/−* mice exhibited numerous and confluent neutrophilic aggregates around the central veins (Figure 5C). Additionally, mRNA expression of CD11b, a marker of activated neutrophils, was elevated in *FD−/−* mice at 48 hr (Figure 5D).

### 3.3 Absence of Factor D Does Not Impair Hepatocyte Proliferation

Delayed or reduced hepatocyte proliferation could be one mechanism for impaired recovery of *FD−/−* mice after CCl$_4$. IL-6/STAT3 signaling is associated with the early priming phase of liver regeneration (Fausto et al., 2006). A single CCl$_4$ injection induced IL-6 mRNA expression in WT and *FD−/−* mice at 2 and 24 hr; this induction was greater in *FD−/−* mice compared to WT at 24 hr (Figure 6A). As with the IL-6 mRNA, phosphorylation of STAT3 was elevated in response to a single CCl$_4$ injection in both genotypes; induction was higher in *FD−/−* mice at 24 hr, corresponding to the time that IL-6 mRNA was also greater in the *FD−/−* mice (Figure 6B).

Consistent with markers of IL-6/STAT3 signaling, measures of hepatocyte proliferation were also induced. WT and *FD−/−* mice mounted a similar proliferative response to CCl$_4$ – induced injury as assessed by proliferating cell nuclear antigen (PCNA) mRNA expression (data not shown) and protein expression via immunoblotting at 24, 48 and 72 hr (Figure 7A). Since liver sections 72 hr following CCl4 induced injury had more neutrophilic infiltrates,

we further analyzed if this correlated with Ki67, a cellular marker for proliferation. Frozen liver sections probed for antigen Ki67 revealed Ki67-positive cells to be similarly distributed throughout the liver lobule between genotypes; no difference in the number of Ki67-positive cells was found between WT and *FD−/−* mice at 72 hr (Figure 7B).

## 4. Discussion

The present study identifies the importance of Factor D, a component in the alternative pathway of complement activation, in facilitating complement opsonization and efficient clearance of damaged tissue in the liver in response to acute toxic injury. WT, *C1qa−/−* and *C4−/−*, but not *FD−/−*, mice rapidly activated complement, evidenced by the accumulation of C3b across the liver parenchyma, in response to exposure to $CCl_4$. The failure to activate complement was associated with increased hepatocyte injury in *FD−/−* mice compared to WT, *C1qa−/−* and *C4−/−* mice. Interestingly, while *FD−/−* were able to mount a normal proliferative response to acute toxic injury, deficiency of *FD* was associated with increased acidophilic bodies, neutrophilic infiltration and a prolonged accumulation of necrotic tissue. Taken together, these data suggest that the alternative pathway, as indicated by the absence of FD, is critical to the appropriate opsonization of damaged tissue by C3b, facilitating the timely clearance of hepatocellular debris. Impaired clearance of this debris in *FD−/−* mice exacerbated and prolonged liver injury.

Genetic deficiencies of key molecules of the complement cascade (C3 and C5) are associated with defective liver regeneration and extensive parenchymal necrosis following toxic liver injury induced by $CCl_4$ in mice (Markiewski et al., 2004 and Mastellos et al., 2001). Carbon tetrachloride, a halogenated alkane used to study hepatic injury, (Rahman and Hodgson, 2000, Yamada and Fausto, 1998, Weber et al., 2003) is activated by the cytochrome system (e.g., CYP2E1) to form the trichloromethyl radical, $CCl_3\cdot$. This radical can react with oxygen to form the trichloromethylperoxy radical $CCl_3OO\cdot$, a highly reactive species known to induce lipid peroxidation, oxidative stress, hepatic necrosis and apoptosis (Weber et al., 2003 and Taub, 2003). Administration of C3a and C5a in these gene deficient mice restores hepatocyte proliferation, indicating that the anaphylatoxins act as early regulators of normal liver recovery after $CCl_4$ induced injury (Markiewski et al., 2004 and Mastellos et al., 2001).

Tissue repair requires not only generation of new cells, but also efficient removal of damaged cells; complement plays an important role in clearance of cellular debris (Trouw et al., 2008). Following injury and complement activation, C3b is released and deposited on necrotic and apoptotic cells, signaling phagocytes to clear damaged tissue (Takizawa et al., 1996 and Mevorach et al., 1998). While Markiewski, et al. (2004) had identified C3a and C5a as key to hepatocyte proliferation after injury, identification of complement activation products involved in clearing damaged tissue has not yet been studied. We hypothesized that deposition of C3b on damaged tissue is a key mediator of clearance of cellular debris and is necessary for timely liver recovery following $CCl_4$-induced injury.

The identification for a critical role for FD in the alternative pathway for complement activation in the response to acute toxic injury is of particular relevance to clearance of

cellular debris. While binding of complement initiation molecules does not typically take place on early apoptotic cells (Trouw et al., 2008); several reports indicate that the alternative pathway plays a role in priming apoptotic cells for safe clearance by phagocytes, being most prominent with late apoptotic/necrotic cells (Mevorach et al., 1998, Tsuji et al., 1994, Gaipel et al., 2001). If apoptotic cells are not rapidly and efficiently removed, they enter the stage of secondary necrosis which can exacerbate inflammatory cellular conditions (Munoz et al., 2010). The present studies indicate that Factor D in the alternative pathway is important for early complement opsonization and plays a significant role in facilitating damaged tissue clearance.

If removal of damaged tissue is inadequate and injury persists, reorganization of the extracellular matrix is impaired. We find the absence of Factor D and lack of C3b/iC3b/C3c deposition following $CCl_4$ -induced injury was associated with more severe and prolonged liver injury. Attracted by multiple signals, neutrophils are amongst the early immune responders to the injury site (Nathan, 2006). Amplifying the local inflammatory environment, neutrophils produce a variety of immunostimulatory effectors involved with tissue repair and regeneration (Chosay et al., 1997). Continuation of tissue injury coincides with inflammation which activates various cell types, including neutrophils. Interestingly, while the WT mice appeared to be recovering from the acute toxic injury at 72 hr, this was associated with resolution of inflammation. In comparison, prolonged tissue injury in *FD−/ −* mice, as indicated by the increased presence of acidophilic bodies and necrosis, was associated with a persistent presence and abundance of neutrophilic infiltrates around the central vein.

The presence of more neutrophils at 24 hr in the *FD−/−* mice was associated with higher mRNA expression of the proinflammatory cytokine IL-6. There is increased evidence of the importance of cytokines, and in particular IL-6/STAT3 signaling, in having critical roles in liver regeneration (Taub, 2003). Our data reveal that deficiency of Factor D did not impair hepatocyte proliferation, as there was no effect of genotype on IL-6/STAT3, as well as proliferative markers, in response to $CCl_4$-induced injury.

The coagulation cascade is another pathway critical to the clearance of tissue debris in response to toxic liver injury, coordinating timely proteolysis of matrix elements and clearance of tissue debris (Bezerra et al., 2001). In response to $CCl_4$, expression of mRNA for the plasminogen activators, tPA and uPA, was strong in both *FD−/−* and WT mice, with *FD−/−* exhibiting an even more robust induction (data not shown). Thus, under the conditions of our study, activation of the coagulation pathway was insufficient to maintain timely clearance of cellular debris, suggesting that clearance is dependent on the activation of both the coagulation cascade and alternative pathway driven complement-dependent opsonization.

## 5. Conclusion

In summary, complement plays a critical role following an acute toxic liver injury not only in assuring an adequate proliferative response is generated, but also in marking of cellular debris for safe and efficient removal by phagocytes in order for timely tissue repair to

proceed. Here we demonstrate the important role of the alternative complement pathway in facilitating opsonization of damaged tissue for a timely reparative process following an acute toxic liver injury induced by $CCl_4$. Early marking of damaged cells for efficient clearance is important to minimize accumulation of necrotic and secondary necrotic cells that could exacerbate and prolong the inflammatory and immune response.

## Acknowledgements

## References

Barnes MA, McMullen MR, Roychowdhury S, et al. Macrophage migration inhibitory factor contributes to ethanol-induced liver injury by mediating cell injury, steatohepatitis, and steatosis. Hepatology. 2013; 57:1980. [PubMed: 23174952]

Bezerra JA, Currier AR, Melin-Aldana H, et al. Plasminogen activators direct reorganization of the liver lobule after acute injury. 2001; 158:921.

Botto M, Dell'Agnola C, Bygrave AE, et al. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nature Genet. 1998; 19:56. [PubMed: 9590289]

Chosay JG, Essani NA, Dunn CJ, et al. Neutrophil margination and extravasation in sinusoids and venules of liver during endotoxin-induced injury. Am J Physiol. 1997; 272:G1195. [PubMed: 9176230]

Degn SE, Thiel S. Humoral pattern recognition and the complement system. Scand. J. Immunol. 2013; 78:181. [PubMed: 23672641]

Del Rio-Tsonis K, Tsonis PAA, Zarkadis IK, et al. Expression of the third component of complement, C3, in regenerating limb blastema cells of urodeles. J. Immunol. 1998; 161:6819. [PubMed: 9862713]

Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology. 2006; 43:S45. [PubMed: 16447274]

Fischer MB, Ma M, Goerg S, et al. Regulation of the B cell response to T-dependent antigens by classical pathway complement. 1996; 157:549.

Gaipl US, Kuenkele S, Voll RE, et al. Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death. Cell Death Differ. 2001; 8:327. [PubMed: 11550084]

Gullstrand B, Martensson Ul, Sturfelt g, et al. Complement classical pathway components are all important in clearance of apoptotic and secondary necrotic cells. Clin Experimental Immunology. 2009; 156:303.

Harboe M, Mollnes TE. The alternative complement pathway revisited. J.Cell. Mol. Med. 2008; 12:1074. [PubMed: 18419792]

Koller BH, Smithies O. Altering genes in animals by gene targeting. Annu Rev Immunol. 1992; 10:705. [PubMed: 1591000]

Markiewski MM, Mastellos D, Tudoran R, et al. C3a and C3b/iC3b/C3c activation products of the third component of complement (C3) are critical for normal liver recovery after toxic injury. J Immunology. 2004; 173:747. [PubMed: 15240660]

Mastellos D, Papadimitriou JC, Franchini S, et al. A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration. J Immunol. 2001; 166:2479. [PubMed: 11160308]

McMullen MR, Pritchard MT, Wang Q, et al. Early growth response-1 transcription factor is essential for ethanol-induced fatty liver injury in mice. Gastroenterology. 2005; 128:2066. [PubMed: 15940638]

Mevorach DJ, Mascarenhas O, Gershov K, et al. Complement-dependent clearance of apoptotic cells by human macrophages. J Exp Med. 1998; 188:2313. [PubMed: 9858517]

Muñoz LE, Lauber K, Schiller M, et al. The role of defective clearance of apoptotic cells in systemic autoimmunity. Nat. Rev. Rheumatol. 2010; 6:280. [PubMed: 20431553]

Nagase H, Woessner J. Matrix metalloproteinases. J. Biol Chem. 1999; 1999:21491. [PubMed: 10419448]

Nathan C. Neutrophils and immunity: challenges and opportunities. Nature Reviews Immunology. 2006; 6:173.

Nesargikar PN, Spiller B, Chavez R. The complement system: history, pathways, cascade and inhibitors. Eur. J. Microbiol. Immunol. (Bp). 2012; 2:103. [PubMed: 24672678]

Pritchard MT, Roychowdhury S, McMullen MR, et al. Early growth response-1 contributes to galactosamine/lipopolysaccharide-induced acute liver injury in mice. Am J Physiol Gastrointest Liver Physiol. 2007; 293:G1124. [PubMed: 17916644]

Qin X, Gao B. The complement system in liver disease. Cellular & Molecular Immunity. 2006; 3:333.

Rahman TM, Hodgson HJ. Animal models of acute hepatic failure. Int. J. Exp. Pathol. 2000; 81:145. [PubMed: 10762442]

Reca R, Mastellos D, Jajka M, et al. Functional receptor for C3a anaphylatoxin is expressed by normal hematopoietic stem/progenitor cells, and C3a enhances their homing-related responses to SDF-1. Blood. 2003; 101:3784. [PubMed: 12511407]

Roychowdhury S, McMullen MR, Pritchard MT, et al. An early complement-dependent and TLR-4-independent phase in the pathogenesis of ethanol-induced liver injury in mice. Hepatology. 2009; 49:1326. [PubMed: 19133650]

Takizawa F, Tsuji S, Nagasawa S. Enhancement of macrophage phagocytosis upon iC3b/iC3b/C3c deposition on apoptotic cells. FEBS Lett. 1996; 397:269. [PubMed: 8955361]

Taub R. Hepatoprotection via the IL-6/STAT3 pathway. J Clin Investigation. 2003; 112:978.

Taub R. Liver regeneration: from myth to mechanism. Nature Reviews. 2004; 5:836.

Tegla CA, Cudrici C, Patel S, et al. Membrane attack by complement: the assembly and biology of terminal complement complexes. Immunol. Res. 2011; 51:45. [PubMed: 21850539]

Trouw LA, Bengtsson A, Gelderman K, et al. C4b-binding protein and factor H compensate for the loss of membrane bound complement regulators to protect apoptotic cells against complement attack. J. Biol. Chem. 2007; 282:28540. [PubMed: 17699521]

Tsuji S, Kaji K, Nagasawa S. Activation of the alternative pathway of human complement by apoptotic human umbilical vein endothelial cells. J Biochem (Tokyo). 1994; 116:794. [PubMed: 7883753]

Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit. Rev. Toxicol. 2003; 33:105. [PubMed: 12708612]

Xu V, Ma M, Ippolito GC, et al. Complement activation in Factor D-deficient mice. Proc National Acad Sci. 2001; 98:14577.

Yamada Y, Fausto N. Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1 but not type 2 tumor necrosis factor receptor. Am. J. Pathol. 1998; 152:1577. [PubMed: 9626061]

**Highlights**

- We examine which complement activation pathway is involved for timely liver recovery following an acute toxic injury

- We utilized mice deficient in *C1qa*, *C4* and *Factor D*, lacking the classical, classical/MBL, and alternative pathways of complement activation

- Factor D-deficient mice had worse liver injury compared to other genotypes

- Factor D-deficient mice were unable to adequately clear injured tissue

- We demonstrate the importance of the alternative complement pathway in facilitating opsonization of damaged tissue for a timely reparative process following an acute toxic liver injury
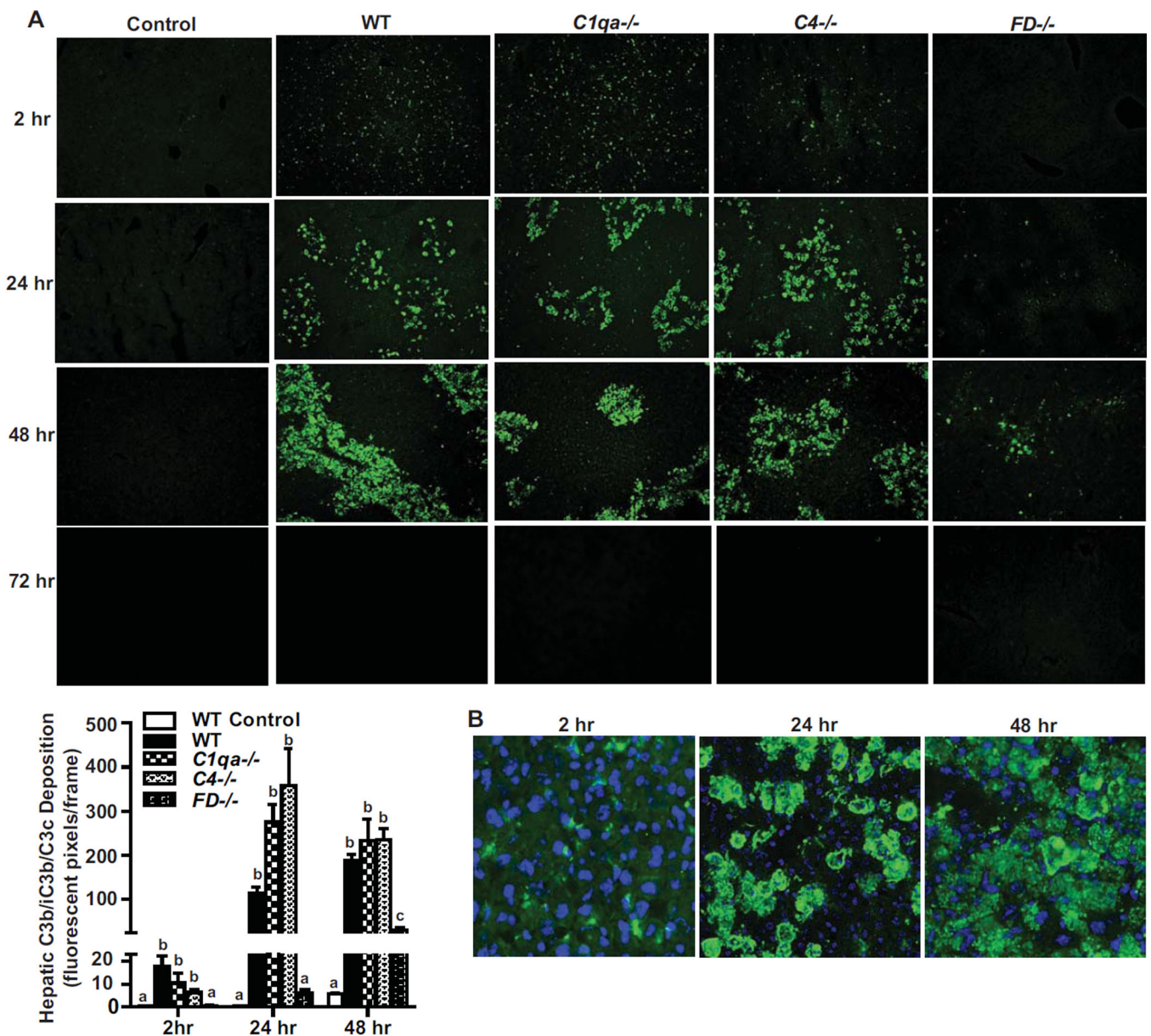
**Figure 1. Factor D is required for C3b/iC3b/C3c deposition in liver following acute exposure to CCl₄**

**A**. Hepatic C3b/iC3b/C3c (C3b) deposition in mice 2, 24, 48 and 72 hr after a single injection of CCl₄ in WT, *C1qa−/−, C4−/− and FD−/−* mice. WT control mice received injection of olive oil. Immunoreactive C3b/iC3b/C3c was visualized by immunohistochemistry in frozen liver sections. Images are shown at 20× magnification. Total number of fluorescent pixels per 20× field was determined using Image Pro software. Values with different alphabetical superscripts were significantly different from each other, p<.05. **B**. Representative images of localization of immunoreactive C3b/iC3b/C3c deposition in frozen liver sections of WT mice 2, 24 and 48 hr following treatment with a single injection of CCl₄ are shown. Magnification 40×.
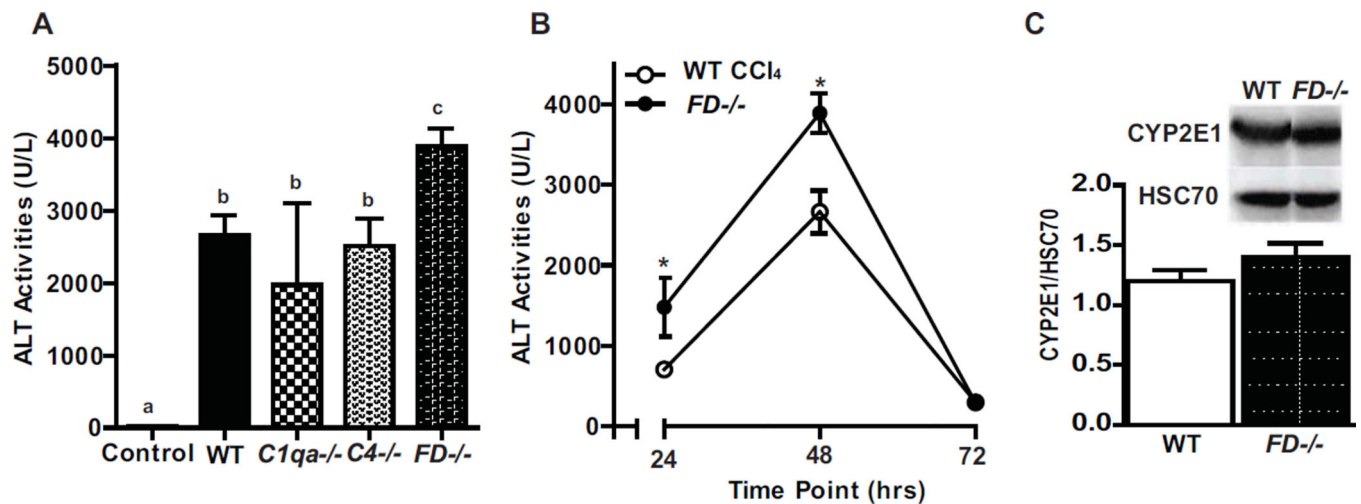
**Figure 2. Factor D deficient mice have exacerbated CCl₄-induced liver ALT activity**
WT, *C1qa−/−, C4−/− and FD−/−* mice were exposed to a single injection of CCl₄ and plasma ALT was measured at **A**. 48 hr and **B**. WT and *FD−/−* mice ALT levels at 24, 48 and 72 hr. N=5–8 mice per group. **C**. Immunoblots of total hepatic protein from control WT and *FD−/−* mice were performed to determine baseline expression of cytochrome P450 2E1 (CYP2E1). Quantification of band intensity measured by densitometry after normalization to HSC70 is shown in the bar graphs. n = 4 mice per group. Values represent mean ± standard error of mean. Values with different alphabetical superscripts or points represented by an (*) were significantly different from each other, p<.05.
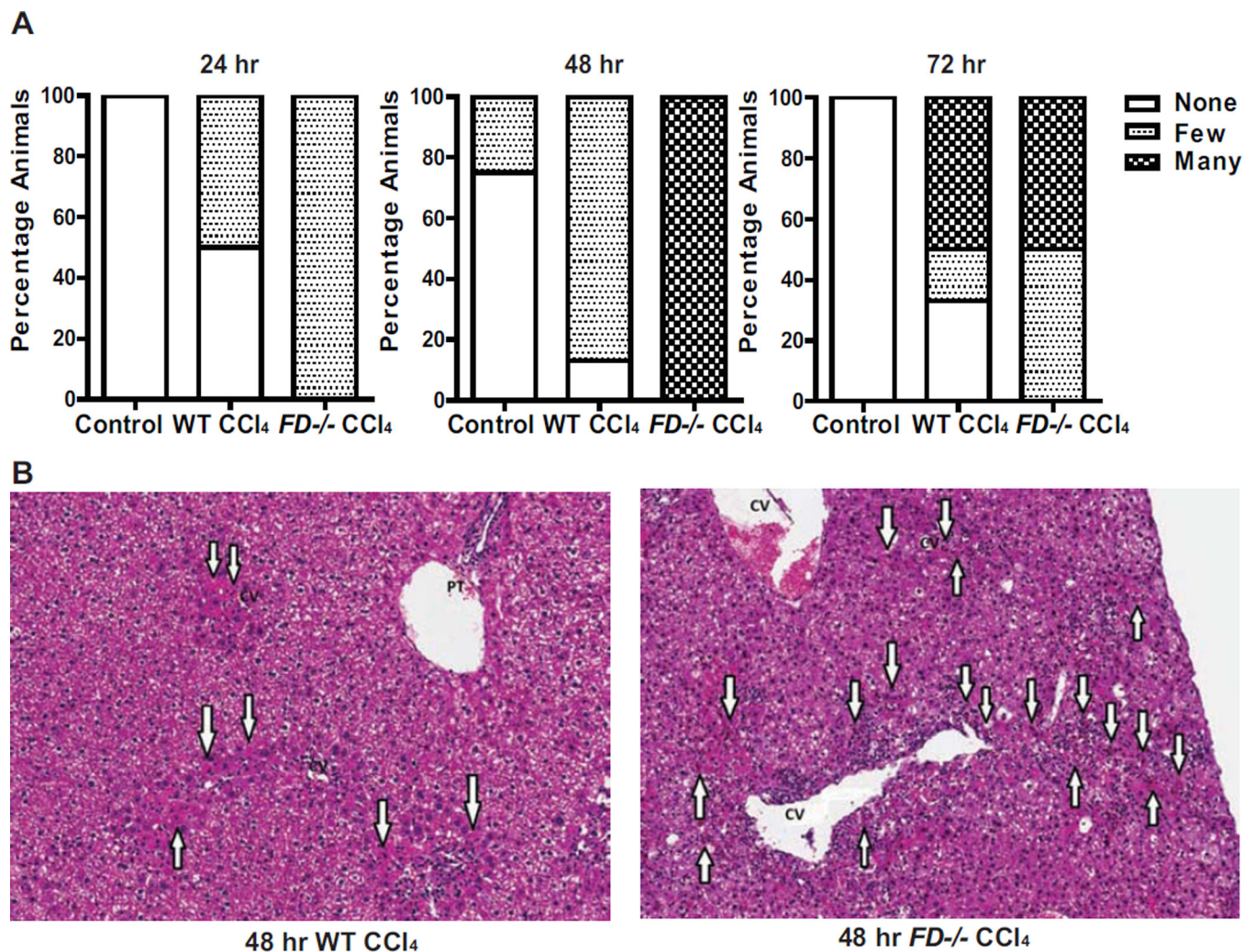
**Figure 3. Increased and persistent presence of acidophilic bodies in the liver of *FD−/−* mice following CCl₄-induced injury**

**A**. Evaluation of the quantity of acidophilic bodies evaluated in Hematoxylin and eosin-stained histologic sections of livers from WT and *FD−/−* mice at 24, 48 and 72 hr following a single injection of $CCl_4$ are shown. WT controls received a single injection of olive oil. Bars represent percentage of mice in each group exhibiting the designated quantity of acidophilic bodies. 24 hr: Control (n=4); WT CCl₄ (n=4); FD CCl₄ (n=5); 48 hr: Control (n=4); WT CCl₄ (n=8); FD CCl₄ (n=7); 72 hr: Control (n=4); WT CCl₄ (n=6); FD CCl₄ (n=6). **B**. Representative H & E histological sections of livers from WT and *FD−/−* mice 48 hr after a single injection of $CCl_4$ are shown. A greater number of acidophil bodies was noted in the *FD−/−* cases. (arrows indicate the acidophilic bodies) (H&E stain, 100×).
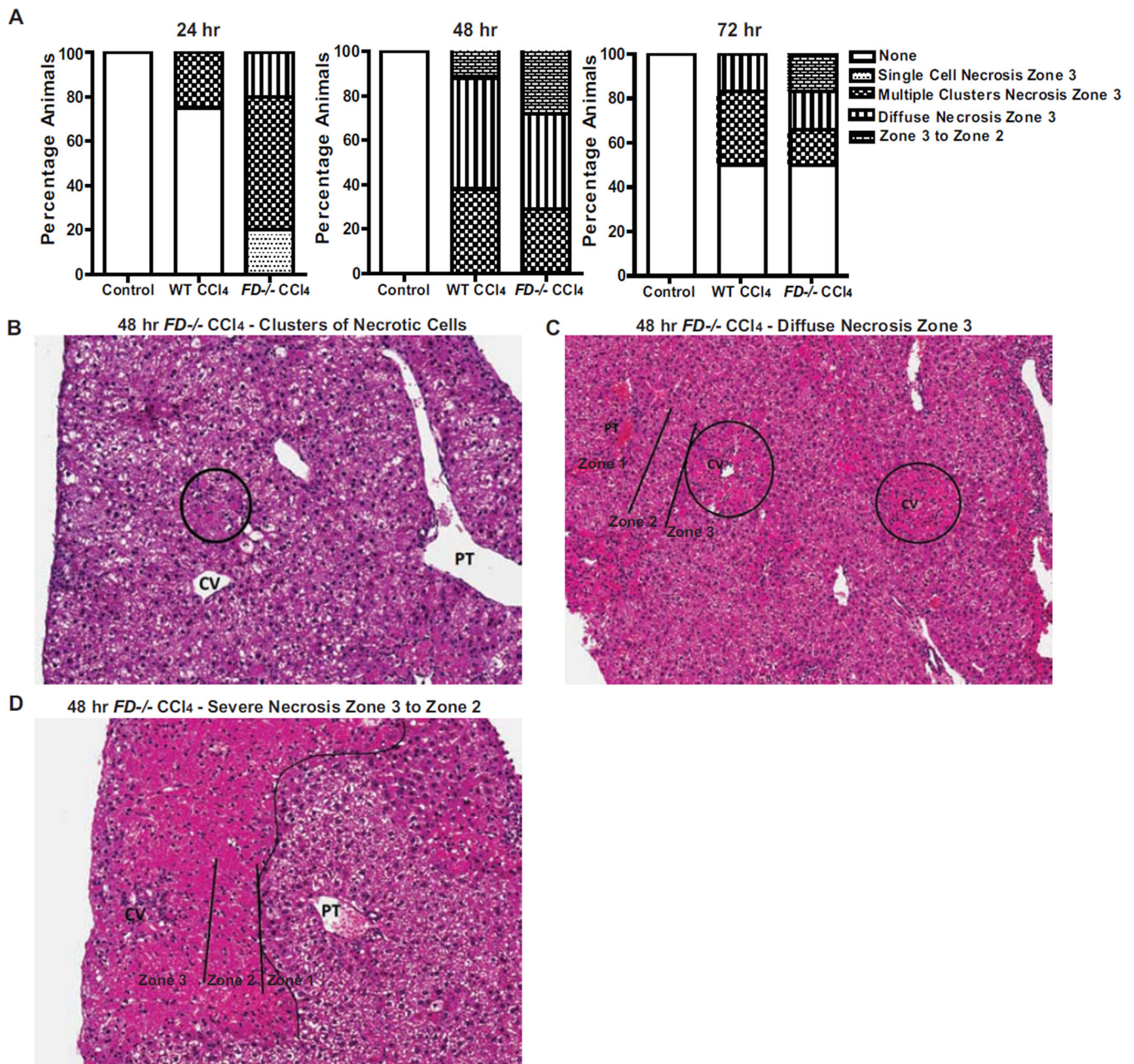
**Figure 4.** *FD−/−* **mouse livers exhibit more severe necrosis following CCl₄ exposure**
**A**. Identification of the presence of cellular necrosis evaluated from histological Hematoxylin and eosin (H&E)-stained sections of livers from WT and *FD−/−* mice at 24, 48 and 72 hr following a single injection of CCl₄ are shown. Bars represent percentage of mice in each group exhibiting the pattern of cellular necrosis described. The number of mice at each time point are the same as those indicated in Figure 3. **B–D**. Representative histological sections of livers from *FD−/−* mice exhibiting the predominant patterns of necrosis at 48 hr after a single injection of CCl₄ are shown (CV: central vein; PT: portal tract): **B**. Clusters of necrotic cells in the lobules (H&E stain, 200×) **C**. Diffuse zone 3 necrosis involving the centrilobular region (also called zone 3) (H&E stain, 100×) **D**. More

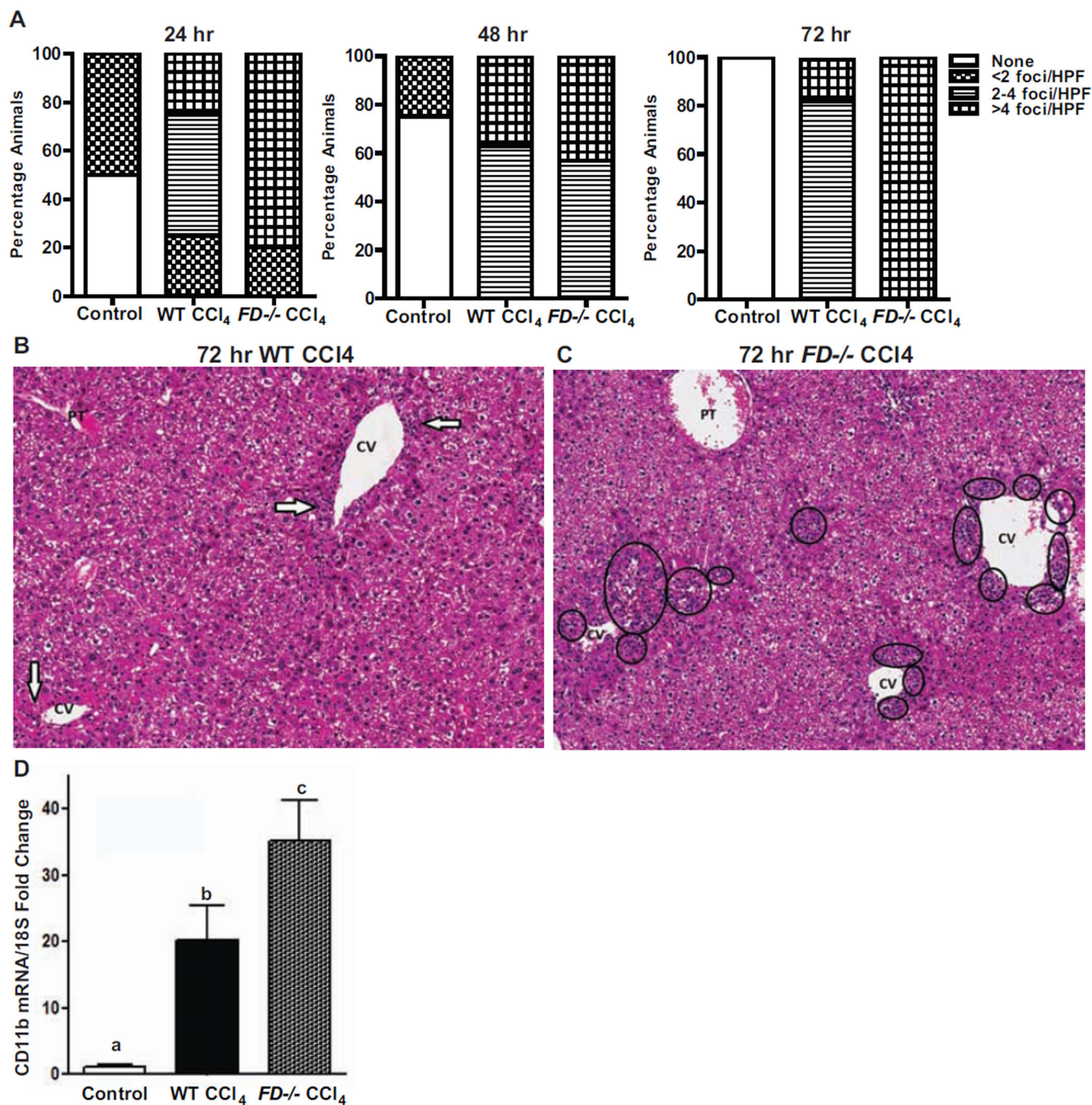extensive necrosis not only involving the zone 3, but also extending to zone 2 (H&E stain, 200×).

neutrophilic foci at 48 hr after a single injection of $CCl_4$ are shown (H&E stain, 200×) (CV: central vein; PT: portal tract). **B**. Arrows indicate single foci with scattered neutrophils and rare associated lymphocytes noted in the centrilobular region in WT mice. **C**. Circles indicate the numerous zone 3 clusters of neutrophils seen in the *FD−/−* mice **D**. mRNA expression of CD11b was detected in mouse liver 48 hr after a single injection of $CCl_4$. Values represent means ± SEM. Values with different alphabetical superscripts were significantly different from each other, p<.05.
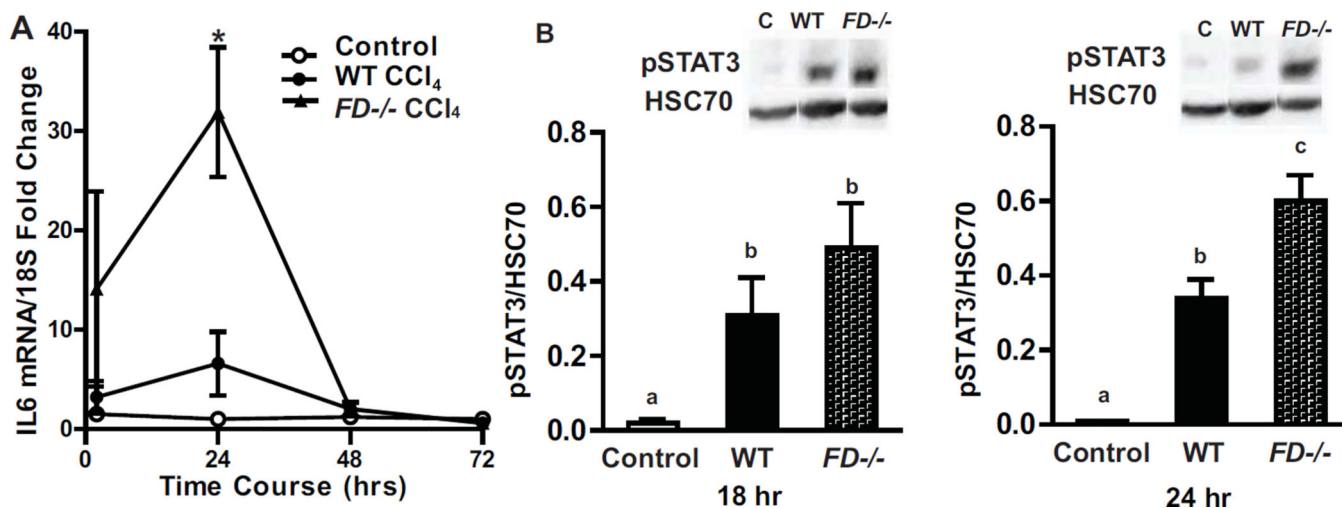
**Figure 6. IL-6 and pSTAT3 were increased in *FD−/−* mice 24 hr after CCl₄ exposure**
Expression of IL-6 mRNA and phosphorylated STAT3 was detected in mouse livers at after a single injection of CCl4. **A**. IL-6 mRNA was detected in WT and *FD−/−* mice 2, 24, 48 and 72 hr following a CCl4 challenge. **B**. Liver lysates of total hepatic protein were prepared and levels of phosphorylated (Tyr$^{705}$)-STAT3 was detected 18 and 24 hr following a single injection of CCl4. Quantification of band intensity measured by densitometry after normalization to HSC70 are shown in the bar graphs. n = 4–6 mice per group. Bars are means ± SEM. Values with different alphabetical superscripts or points represented by an (*) are significantly different from each other, p<.05.
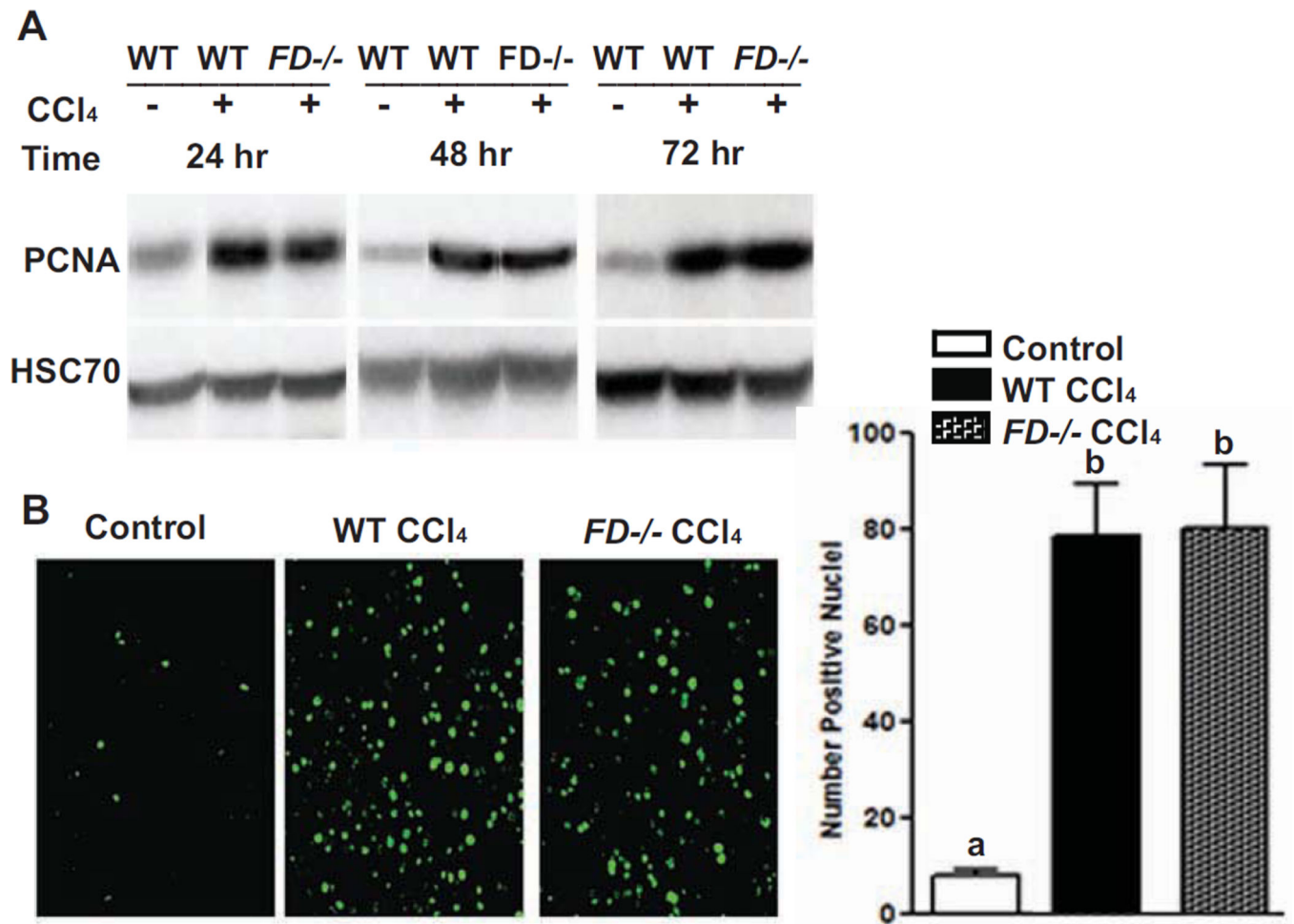
**Figure 7. Absence of Factor D does not impair hepatocyte proliferation following CCl₄-induced toxic injury**

WT and *FD−/−* mice were provided with a single injection of CCl₄. **A**. Immunoreactive PCNA (proliferating cell nuclear antigen) protein in liver lysates was assessed by Western blot analysis 24, 48 and 72 hr after injection. Quantification of band intensity measured by densitometry after normalization to HSC70 showed no difference between genotypes (data not shown). **B**. Ki67 was visualized by immunohistochemistry in frozen liver sections 72 hr following injury. Total number of fluorescent pixels per 10× field was determined using Image Pro software. Images are representative of n = 4–6 mice per group. Bars are means ± SEM. Values with different alphabetical superscripts are significantly different from each other, p<.05.