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Activation of the Complement System in Human Nonalcoholic Fatty Liver Disease

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Activation of the innate immune system plays a major role in nonalcoholic fatty liver disease (NAFLD). The complement system is an important component of innate immunity that recognizes danger signals such as tissue injury. We aimed to determine whether activation of the complement system occurs in NAFLD, to identify initiating pathways, and to assess the relation between complement activation, NAFLD severity, apoptosis, and inflammatory parameters. Liver biopsies of 43 obese subjects with various degrees of NAFLD and of 10 healthy controls were analyzed for deposition of complement factors C1q, mannose-binding lectin (MBL), C4d, activated C3, and membrane attack complex (MAC)-associated C9. Furthermore, hepatic neutrophil infiltration, apoptosis, and pro-inflammatory cytokine expression were quantified. Whereas complement activation was undetectable in the liver of healthy subjects, 74% of the NAFLD patients showed hepatic deposition of activated C3 and C4d. C1q as well as MBL accumulation was found in most activated C3-positive patients. Strikingly, 50% of activated C3-positive patients also displayed MAC-associated C9 deposition. Deposition of complement factors was predominantly seen around hepatocytes with macrovesicular steatosis. Subjects showing accumulation of activated C3 displayed increased numbers of apoptotic cells. Importantly, hepatic neutrophil infiltration as well as interleukin (IL)-8 and IL-6 expression was significantly higher in patients showing activated C3 deposition, whereas patients with C9 deposition additionally had increased IL-1β expression. Moreover, nonalcoholic steatohepatitis (NASH) was more prevalent in patients showing hepatic C9 or activated C3 deposition. *Conclusion:* There is widespread activation of the complement system in NAFLD, which is associated with disease severity. This may have important implications for the pathogenesis and progression of NAFLD given the function of complement factors in clearance of apoptotic cells, hepatic fibrosis, and liver regeneration. (HEPATOLOGY **2009;50:1809-1817.**)

The prevalence of nonalcoholic fatty liver disease (NAFLD) is rapidly increasing.[1] Although once considered a benign condition, a subset of NAFLD patients display a more advanced form of fatty liver disease, so-called nonalcoholic steatohepatitis (NASH). These patients are at risk for progression to fibrosis and cirrhosis, which is associated with increased mortality. Recent studies indicate that inflammatory processes are a driving force behind the progression of NASH.[2-4] As in alcoholic steatohepatitis, the inflammation in NASH is characterized by activation of the innate immune system, with hepatic sequestration of particularly neutrophils.[5]

Neutrophil infiltration into tissues is strongly promoted by C3a and C5a, factors that are generated by intermediate cleavage steps in the complement cascade.[6] Interestingly, activation of the complement system has previously been implicated in the pathogenesis of alcoholic steatohepatitis. For example, ethanol feeding results

Abbreviations: act-C3, activated C3; ASP, acylation-stimulating protein; ELISA, enzyme-linked immunosorbent assay; MAC, membrane attack complex; MBL, mannose-binding lectin; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TNF-α, tumor necrosis factor-alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling.

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in hepatic C3 deposition and increased C3a plasma levels in rodents,[7] whereas C3-deficient mice display attenuated hepatic steatosis after ethanol challenge.[8,9] Moreover, C5-deficient animals have lower serum alanine aminotransferase and reduced hepatic tumor necrosis factor-alpha and interleukin (IL) 6 levels compared with wild-type mice after ethanol feeding.[9] In addition, mice lacking decay-accelerating factor/CD55, a protein that inhibits C3 and C5 activation, display increased hepatic steatosis, serum alanine aminotransferase, and hepatic tumor necrosis factor alpha and IL-6 in response to ethanol feeding.[9]

Complement activation has also been found in other hepatic disorders such as viral hepatitis.[10] Moreover, several components of the complement system are dysregulated in obesity and insulin resistance, which are both associated with NAFLD. For example, C3 plasma levels are increased in obesity and in insulin resistance,[11] and adipose tissue C1q expression is up-regulated in obese insulin-resistant rodents.[12] In addition, hepatic factor D expression is strongly increased in mice in response to a high-fat diet.[13]

Taken together, these observations suggest that the complement system might play an important role in the initiation and perpetuation of the chronic hepatitis associated with steatosis in NAFLD. However, to the best of our knowledge, nothing is known about the involvement of the complement system in the pathogenesis of NASH. We present the first data on the activation and deposition of several complement proteins in the liver of severely obese subjects with NAFLD. Our results indicate that complement activation is associated with excessive fat accumulation, hepatocyte apoptosis, and hepatic neutrophil sequestration and suggest that complement-derived factors contribute to nonalcoholic fatty liver–induced inflammation.

Patients and Methods

Subjects and Biopsies. Forty-three severely obese patients undergoing bariatric surgery and 10 patients undergoing cholecystectomy at the Maastricht University Medical Centre were included in the study. None of the subjects suffered from autoimmune diseases, viral hepatitis, or reported excessive alcohol intake (>20 g/day). The study was approved by the local Ethics Committee and conducted in line with the 1975 Declaration of Helsinki guidelines. All subjects gave written informed consent.

Liver wedge biopsies were obtained during surgery. One part was embedded in Tissue-Tek O.C.T. (Sakura Europe; Zoeterwoude, the Netherlands) and snap-frozen in 2-methylbutane, a second part was snap-frozen in liquid nitrogen, the remaining part was fixed in 4% formalin and embedded in paraffin.

Immunohistochemistry. Immunohistochemical staining was performed as previously described.[4] Sections were incubated with monoclonal antibodies recognizing activated C3 (act-C3); in other words, C3 neo-epitopes on iC3b and C3c (clone bH6; Hycult biotechnology [Hbt], Uden, the Netherlands), C4d (clone 12D11; Hbt), C1q (Quidel, San Diego, CA), MBL (clone 3E7; Hbt), C9 neoantigen in membrane attack complex (MAC) (clone aE11; Hbt), chicken immunoglobulin Y (isotype control; clone 7C2; Hbt), or human neutrophil peptide 1-3 (clone D21; Hbt) (all at 10 μg/mL).

For immunofluorescent staining of act-C3 and MBL, Alexa Fluor 594–conjugated goat anti-mouse immunoglobulin G_{2a} and Alexa Fluor 488–conjugated goat anti-mouse immunoglobulin G_1 were applied (Invitrogen Molecular Probes, Eugene, OR, 4 μg/mL). Nuclei were stained with 4′,6-diamino-2-phenyl-indol.

Apoptosis. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL) method (*in situ* cell death detection kit, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Scoring of Stainings and NASH Severity. Complement staining patterns and distributions were evaluated by two pathologists experienced in the fields of liver and complement-related pathological conditions. The degree of concordance between the pathologists was greater than 95% for all stainings. Complement deposition was scored semiquantitatively on a 0-3 point scale, but because of the limited number of patients, categories 1 through 3 were analyzed as one group positive for complement deposition. Patients in the cholecystectomy group did not show fatty liver disease. In the severely obese patient group, 30 patients (70%) had NASH, whereas 13 patients (30%) showed bland steatosis without significant inflammation (Table 1). NASH severity was assessed according to the Brunt classification.[14] Eleven subjects had grade 1, 12 subjects had grade 2, and seven subjects were classified as grade 3 NASH. The steatosis group comprised patients showing greater than 5% steatotic hepatocytes without inflammation as observed after hematoxylin-eosin staining. Neutrophil numbers and apoptotic cell numbers were quantified by photographing four to six randomly selected parts of human neutrophil peptide 1-3–stained or TUNEL-stained sections at 200× magnification. Pictures were analyzed with Qwin software (Leica Microsystems, Cambridge, UK), allowing automated detection of stained cells. Positive cell number was recorded next to the extent of steatosis as defined by liver parenchyma area

Table 1. Subject Characteristics

Characteristic	Healthy	Steatosis	NASH	ANOVA
Number of patients	10	13	30	
Sex (male:female)	1:9	3:10	10:20	$P = 0.24$
Age (years)	42.2 ± 2.2	42.4 ± 3.9	43.8 ± 1.7	$P = 0.67$
ALT (IU)	23.2 ± 4.2	26.1 ± 5.2	34.2 ± 5.3	$P = 0.59$
AST (IU)	20.4 ± 3.2	24.0 ± 4.6	30.5 ± 3.0	$P = 0.18$
Body mass index (kg/m^2)	29.4 ± 1.4	45.9 ± 1.7*	48.3 ± 1.6*	$P < 0.001$

*$P < 0.001$ versus healthy.

showing no hematoxylin staining (excluding vascular lumen). Furthermore, mean steatotic hepatocyte size was calculated by dividing the steatotic area by the number of steatotic hepatocytes.

Quantitative Polymerase Chain Reaction. RNA isolation and quantitative PCR was performed as previously described.[4] Relative expression was assessed in duplicate by the delta Cycle threshold method after normalization for beta-actin and beta2-microglobulin expression. Sequences of PCR primers are listed in Table 2.

MBL2 Genotyping. Fasting venous blood samples were collected as previously described.[4] Plasma and buffy coat were stored at −80°C. DNA extraction from buffy coat was performed using the Maxwell16 System Blood DNA Purification Kit (Promega, Madison, WI). Genotypes were determined using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) as previously described.[15]

MBL and Activated C3 Enzyme-Linked Immunosorbent Assay. Plasma MBL was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Hbt). Plasma act-C3 was determined using an in-house developed ELISA. ELISA plates were coated with 2 μg/mL antibody bH6 against act-C3. Samples were 500× diluted and incubated for 1 hour. Biotinylated antibody against C3d (Clone 3, Hbt) was applied at 0.5 μg/mL. Horseradish peroxidase–conjugated streptavidin-biotin and tetramethylbenzidine were used for detection. The standard consisted of serum activated with 0.4 mg/mL zymosan A. Plasma samples were analyzed in the same run. The intra-assay coefficient of variance was less than 10%.

Statistical Analyses. Results are expressed as mean ± standard error of the mean. The Mann Whitney test or Kruskal-Wallis analysis of variance followed by Dunns multiple comparison test was used to analyze differences in continuous variables between groups. Chi-square analysis was used for noncontinuous variables. Data were analyzed using Prism 4.0 software (Graphpad, San Diego, CA). *P*-values ≤ 0.05 were considered statistically significant.

Results

Deposition of Activated C3 Around Steatotic Hepatocytes in NAFLD. All complement pathways converge on the central component C3. Therefore, we first assessed whether activation of C3 occurred in the liver of severely obese subjects with NAFLD. Activated C3 was identified by immunohistochemistry using an antibody that recognizes C3 neo-epitopes on iC3b and C3c, the cleavage fragments of C3b. Deposition of act-C3 was detected in 74% (n = 32/43) of the severely obese patients. Staining was almost without exception located centrilobularly, and in only few cases periportally. Remarkably, the most intense staining was found closely associated with the plasma membrane of steatotic hepatocytes (Fig. 1A). In line with this observation, the degree of steatosis as well as the mean steatotic hepatocyte size was significantly increased in patients showing act-C3 deposition (Fig. 1B). Interestingly, hepatic act-C3 deposition was more frequently detected in subjects with NASH as compared with subjects showing bland steatosis (Fig. 1C, $P = 0.04$). Plasma levels of act-C3 also tended to increase with NAFLD severity (Fig. 1D, $P = 0.07$). Deposition of act-C3 was not detected in subjects without fatty liver disease (Fig. 1A, C), and was not associated with NASH grade or with male or female sex (Supporting Figs. 1, 2).

Table 2. Primers Used for Quantitative Real-Time Reverse Transcription PCR Analysis

Name	Forward 5′ → 3′	Reverse 5′ → 3′
IL-8	CTGGCCGTGGCTCTCTTG	TTAGCACTCCTTGGCAAAACTG
IL-1β	CTGAGCTCGCCAGTGAAATG	TTTAGGGCCATCAGCTTCAAA
IL-6	TCCAGGAGCCCAGCTATGAA	GAGCAGCCCCAGGGAGAA
β-actin	GCTGTGCTACGTCGCCCTG	GGAGGAGCTGGAAGCAGCC
β2-microglobulin	TCCATCCGACATTGAAGTTG	CGGCAGGCATACTCATCTT

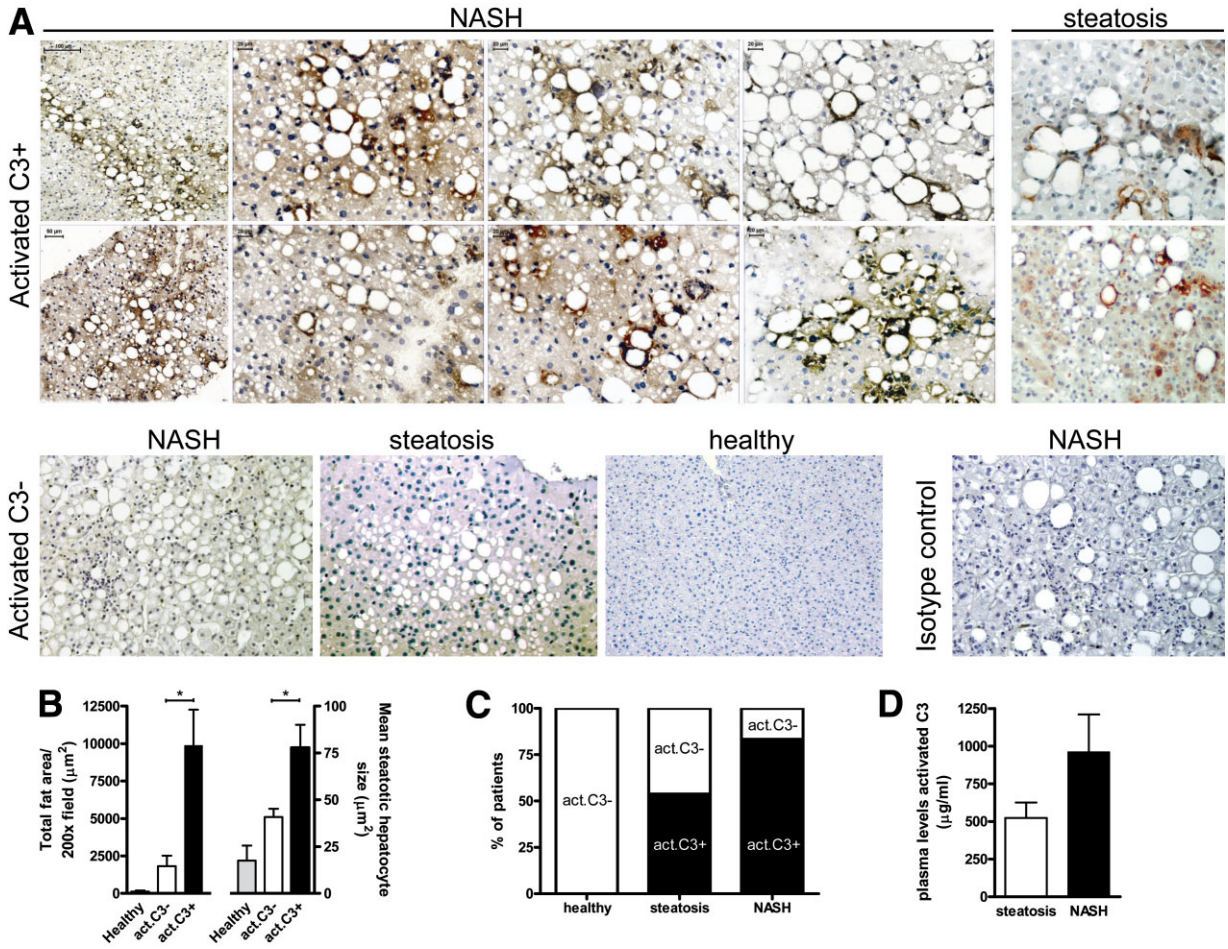

Fig. 1. Deposition of activated C3 in the liver of patients with NAFLD is associated with steatosis and disease severity. (A) Representative images of act-C3 staining of different patients with various degrees of NAFLD, showing that hepatic C3 activation is mostly associated with steatotic hepatocytes (upper panel). Lower panel: Representative images of act-C3–negative stainings and isotype control of a NASH patient. Healthy livers never showed C3 activation. (B) Increased hepatic steatosis in patients showing act-C3 in the liver ($P < 0.01$). In addition, mean steatotic hepatocyte size is bigger in patients with act-C3 deposition ($P = 0.02$). Healthy livers only displayed minimal steatosis. (C) Deposition of act-C3 in the liver is more often observed in patients with NASH as compared with subjects with bland steatosis ($P = 0.04$). (D) Act-C3 plasma levels show a trend to increase in patients with NASH ($P = 0.07$).

Classical and Lectin Pathway Activation in NAFLD. Whereas act-C3 is detectable for only a limited period after deposition,[16] C4d, the stable split product of C4, can be detected for 4 to 8 days after deposition,[17] giving a better indication of complement activation over time. Moreover, because C4 figures in the classical and lectin pathway but not in the alternative complement pathway, C4d detection gives information on the pathways involved in C3 activation. Interestingly, C4d staining was observed in a pattern similar to that of act-C3 in 85% (n = 17/20) of 20 consecutive patients that were found to show act-C3 accumulation (Fig. 2A). Patients without detectable act-C3 never showed C4d deposition. This suggested complement activation in the liver of most of the severely obese subjects through either the classical or the lectin pathway or both.

To further delineate the initiating pathways responsible for activation of C4 and C3, we first analyzed C1q, a protein of the classical pathway initiating complex. C1q was detected in 60% (n = 12/20) of the livers showing act-C3 deposition. Similar to the act-C3 deposition pattern, C1q staining showed the highest intensity around steatotic hepatocytes (Fig. 2B). Likewise, substantial MBL accumulation was observed around steatotic hepatocytes in 55% (n = 11/20) of these patients (Fig. 2C). Weaker cytoplasmic staining of hepatocytes was also detected. All patients showing strong MBL staining also displayed C1q immunoreactivity. Like act-C3, deposition of C4d, C1q, and MBL around steatotic hepatocytes was more frequently detected in NASH (Fig. 2D). Healthy livers did not show C4d deposition, whereas occasionally C1q-positive cells could be identified, as well as

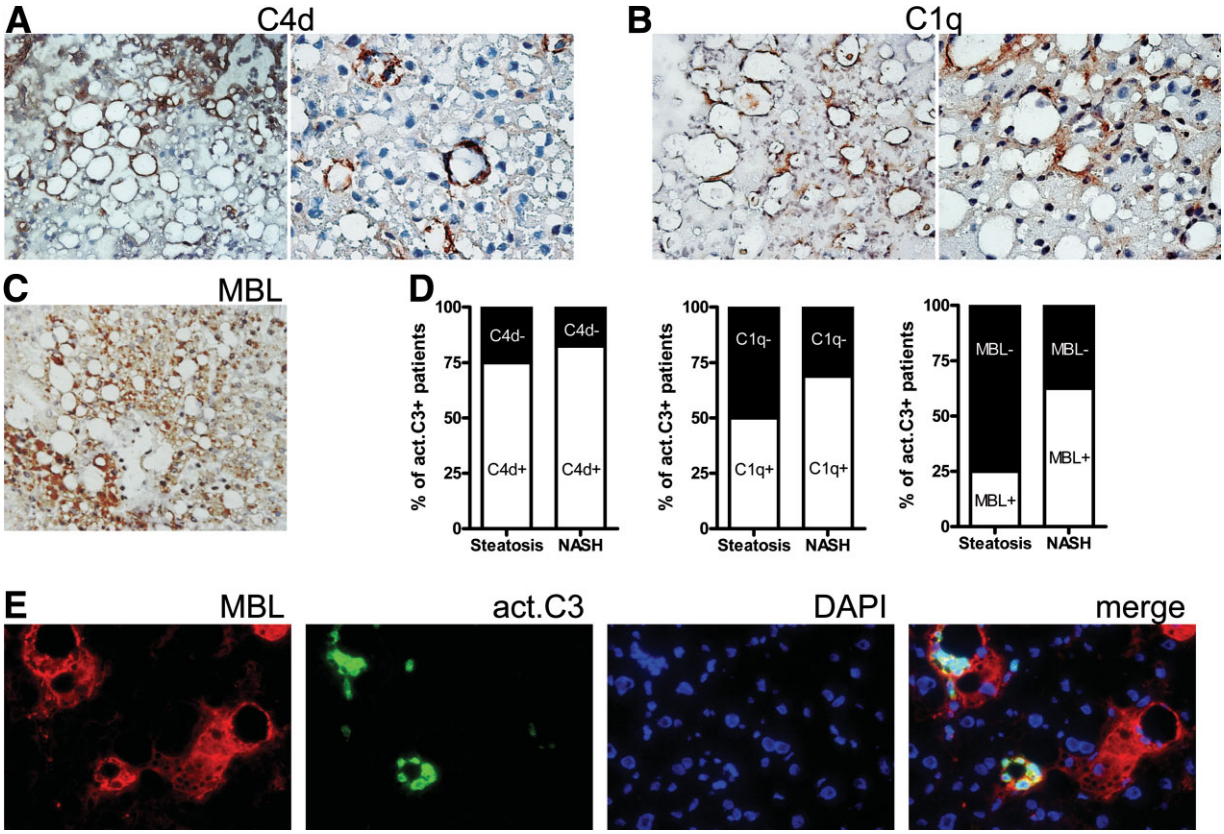

Fig. 2. Lectin and classical complement pathway activation in NAFLD. (A) Representative images of C4d staining of the liver of two subjects with NASH (100×/400× magnification). The C4d staining pattern mirrors the distribution of act-C3, showing association with steatotic hepatocytes. (B) Representative images of C1q staining of liver sections of two subjects with NAFLD (100×/400× magnification), showing staining around steatotic hepatocytes. (C) Intense staining for MBL around steatotic hepatocytes in NAFLD (representative image, original magnification 100×). Weak cytoplasmic staining of hepatocytes without steatosis is probably attributable to endogenous MBL synthesis. (D) Hepatic accumulation of C4d, C1q, and MBL is more frequently detected in act-C3–positive subjects with NASH. (E) Immunofluorescent double staining of act-C3 (green) and MBL (red) showing occasional co-localization (merge) (original magnification, 400×).

a uniform MBL staining in hepatocytes (Supporting Fig. 3). Furthermore, MBL and act-C3 occasionally co-localized, as shown by immunofluorescent double staining (Fig. 2E), suggesting that MBL accumulation may be directly coupled to C3 activation.

Complement Activation in NAFLD Is Associated with Apoptosis. Because the complement system is involved in the recognition and clearance of apoptotic cells,[18] we next investigated whether complement activation was associated with the degree of hepatocyte apoptosis as determined by the TUNEL assay. Interestingly, the number of apoptotic hepatocytes was significantly higher in patients showing act-C3 deposition as compared with patients without such deposition (Fig. 3, $P < 0.01$), suggesting that accumulation of complement products may be related to an increased number of apoptotic events.

Activation of the Terminal Complement Pathway in NAFLD. To investigate whether C3 activation ultimately resulted in activation of the terminal complement pathway, we studied the distribution of the MAC in the liver of patients with act-C3 deposition. We took advantage of the fact that MAC assembly is associated with conformational changes of C9 causing exposure of neoepitopes that are absent from native C9, which is mainly produced by hepatocytes. Remarkably, 50% (n = 10/20) of the patients with act-C3 deposition also exhibited MAC-associated C9. Similar to act-C3, MAC-associated C9 deposition was detected around steatotic hepatocytes (Fig. 4A). As expected, hepatic MAC assembly was associated with more steatosis as well as bigger steatotic hepatocyte size (Fig. 4B). In addition, MAC-associated C9 was more frequently observed in patients with NASH as compared with patients with bland steatosis (Fig. 4C). Subjects without NAFLD never displayed MAC-associated C9 deposition (Supporting Fig. 3).

Complement Activation in NAFLD Is Associated with Hepatic Neutrophil Infiltration and Pro-inflammatory Cytokine and Chemokine Expression. Activation of C3 and C5 results in formation of the C3a and C5a peptides, which exert strong chemotactic activity to-

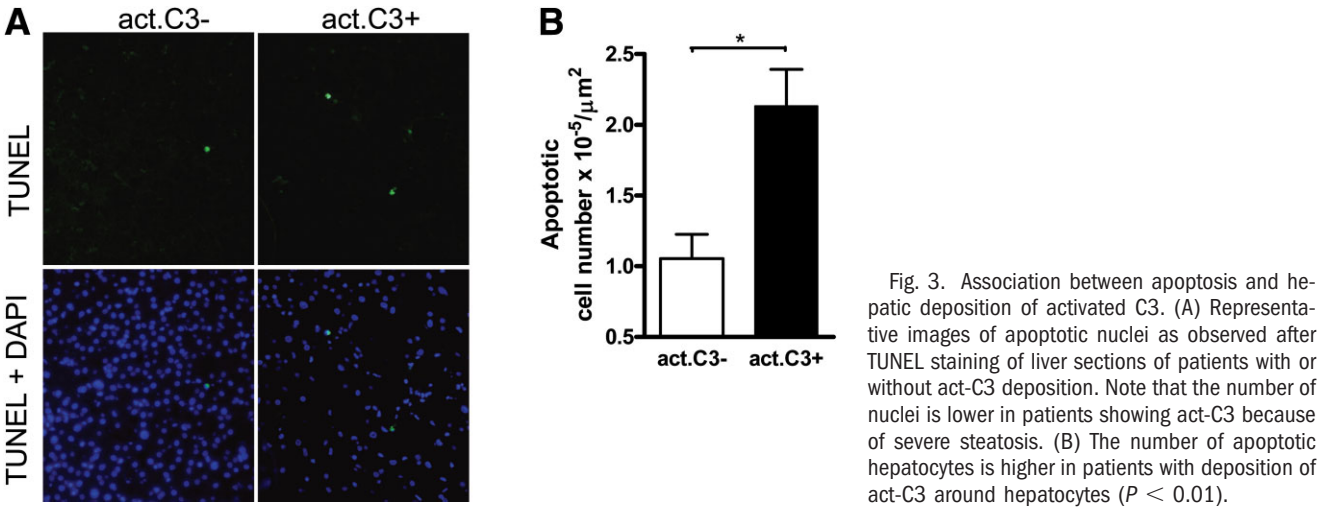

Fig. 3. Association between apoptosis and hepatic deposition of activated C3. (A) Representative images of apoptotic nuclei as observed after TUNEL staining of liver sections of patients with or without act-C3 deposition. Note that the number of nuclei is lower in patients showing act-C3 because of severe steatosis. (B) The number of apoptotic hepatocytes is higher in patients with deposition of act-C3 around hepatocytes ($P < 0.01$).

ward neutrophils. MAC assembly can lead to the release of cellular products that are powerful stimuli of leukocyte attraction as well. Therefore, we next assessed whether activation of C3 and assembly of the MAC translated into increased hepatic neutrophil sequestration. Indeed, the number of human neutrophil peptide 1-3–positive neutrophils was increased in patients with hepatic act-C3 deposition (Fig. 5A, $P < 0.01$). Patients with both act-C3 and MAC-associated C9 deposition showed even higher hepatic neutrophil numbers as compared with patients with act-C3 but without MAC assembly (Fig. 5A, $P = 0.03$). Moreover, hepatic messenger RNA expression of IL-8 and IL-6 was elevated in patients with act-C3 deposition (Fig. 5B, $P < 0.01$ and $P = 0.03$, respectively). Subjects with both act-C3 and C9 deposition showed, additionally, higher expression of IL-1β (Fig. 5C, $P = 0.02$).

Discussion

In the current study, we show that steatotic hepatocytes are frequently associated with activated complement factors. Complement activation was accompanied by hepatic neutrophil sequestration, suggesting that chemotactic factors generated during complement activation contribute to the infiltration of the liver by neutrophils in NAFLD. Importantly, we report full activation of the complement system up to the assembly of cytotoxic

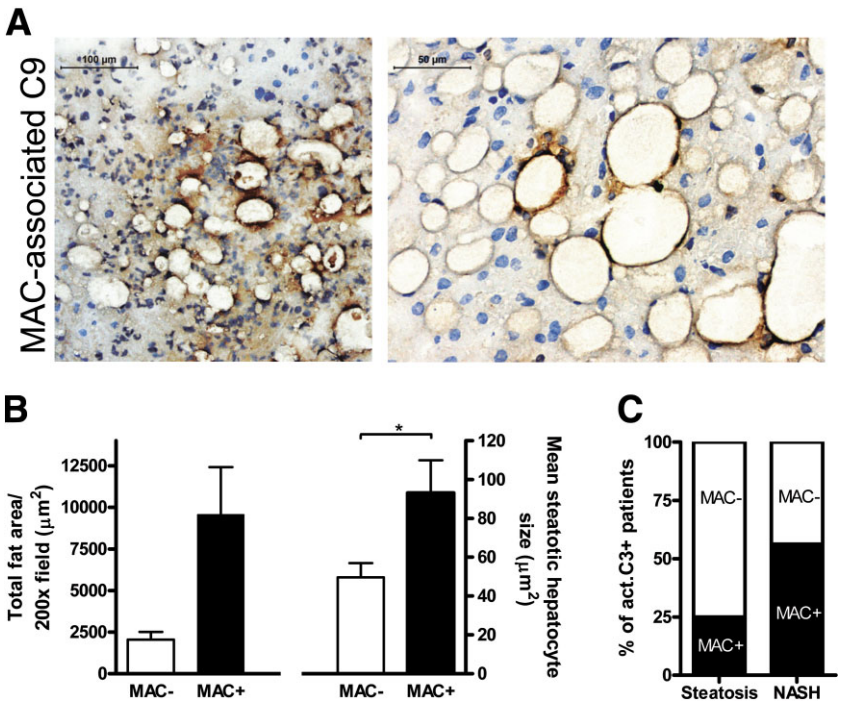

Fig. 4. Membrane attack complex formation in NAFLD is associated with steatosis and disease severity. (A) Representative images of MAC-associated C9 neo-epitope staining, revealing activation of the terminal complement pathway around steatotic hepatocytes. (B) The degree of hepatic steatosis as well as the mean steatotic hepatocyte size is increased in patients showing assembly of MAC ($P = 0.05$ and $P = 0.02$, respectively). (C) Assembly of MAC in the liver is more often observed in patients with NASH as compared with subjects with bland steatosis ($P = 0.02$).

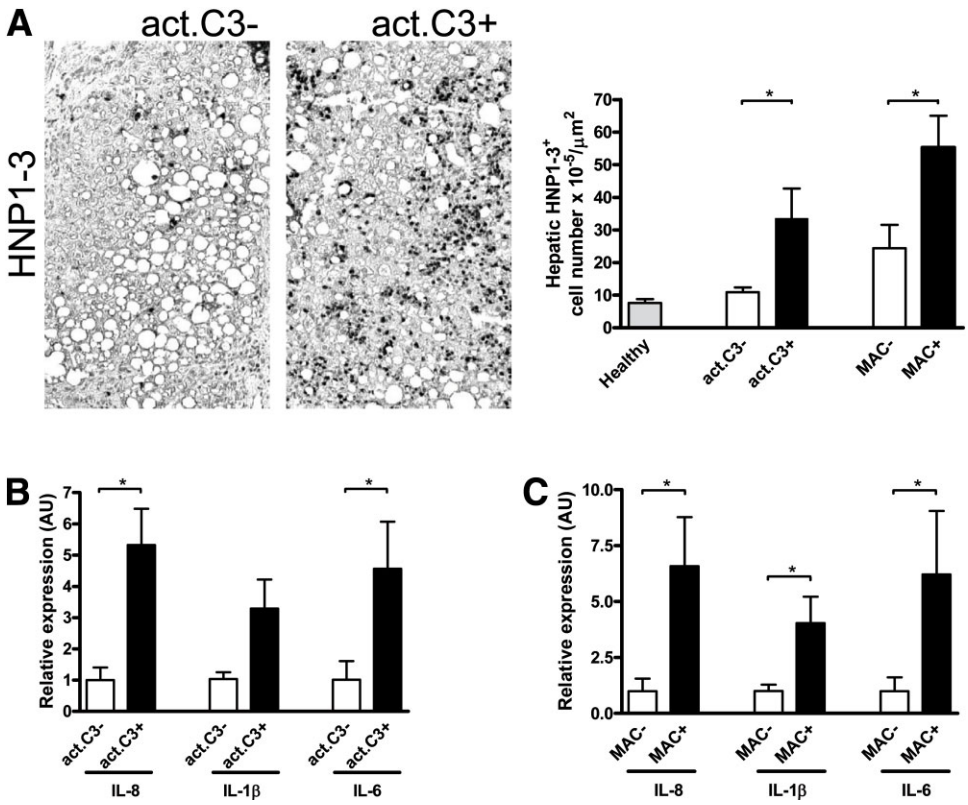

Fig. 5. Complement activation in NAFLD is associated with hepatic neutrophil influx and expression of pro-inflammatory chemokines and cytokines. (A) Left: Representative human neutrophil peptide 1-3 stainings of patients with and without act-C3 deposition. Right: Higher hepatic neutrophil numbers in patients showing act-C3 ($P < 0.01$) or MAC assembly ($P = 0.03$). (B) Hepatic gene expression of IL-8 and IL-6 is higher in patients with act-C3 in the liver ($P < 0.01$, $P = 0.03$, respectively), whereas expression of IL-1β is elevated to a lesser extent ($P = 0.09$). (C) IL-8, IL-6, and IL-1β gene expression is up-regulated in the liver of patients with MAC assembly around steatotic hepatocytes ($P = 0.03$, $P < 0.01$, $P = 0.02$, respectively).

MAC, indicating that there are strong and persistent stimuli of complement activation in NAFLD. Thus, complement activation may be a significant factor in the pathogenesis of NAFLD and its associated complications.

Comparison of healthy controls and NAFLD patients indicates that hepatic complement activation is specifically found in NAFLD. However, the subjects without NAFLD in our study were not obese. Therefore, we cannot rule out that activation of the complement system in the liver is related to obesity as such. Nevertheless, patients with NASH displayed more complement activation than patients with only steatosis, indicating that complement may contribute to NAFLD progression.

Several factors could induce complement activation in response to hepatic steatosis. First, lipopolysaccharide, which is a strong complement-activating factor,[19] is known to promote the development of NASH.[20] Hence, it is possible that accumulation of bacterial products in the liver contributes to the deposition of complement factors. However, we presented evidence that initiation of complement activation may be related to apoptosis. The occurrence of hepatocyte apoptosis in NASH has been well documented.[21] Both C1q and MBL have been implicated in the recognition and clearance of apoptotic cells.[18] C1q may directly bind apoptotic blebs to enable phagocytosis as well as recognize immunoglobulin M antibodies or C-reactive protein bound to apoptotic cells, causing activation of the classical complement pathway.[18,22] MBL can also recognize apoptotic cells and mediate their phagocytosis by macrophages.[18] C3 deposition within damaged liver parenchyma has been shown to promote clearance of apoptotic tissue as well.[23] In the absence of rapid clearance, apoptotic cells undergo secondary necrosis, causing leakage of pro-inflammatory intracellular contents into the interstitium. Thus, activation of complement on steatotic hepatocytes as observed in our study may initially serve to prevent the development of an excessive inflammatory response that causes additional tissue injury.

Replacement of dead hepatocytes occurs by replication of adjacent hepatocytes, a process impaired by steatosis.[24] The steatotic hepatocyte-associated deposition of complement factors as observed in our study may have implications in the context of liver regeneration, because complement signaling is known to be required for this process.[25] For example, liver regeneration is impaired in C3-deficient and C5-deficient mice through downstream signaling events dependent on C5.[23,26] Thus, deposition of complement factors around steatotic hepatocytes may prime adjacent hepatocytes for replication. However, we did not observe a clear increase in the number of dividing hepatocytes in association with complement deposition.

Next to the beneficial effects of complement with respect to clearance of apoptotic cells and promotion of liver

regeneration, complement activation also can result in tissue injury. This may occur directly by formation of cytolytic MAC, or indirectly by promoting the recruitment and certain functional properties of inflammatory cells. The assembly of MAC in a significant proportion of the patients implies that both C3a and C5a are generated in the fatty liver. These peptides are very potent chemoattractants for neutrophils.[27] In line with this, we observed increased neutrophil infiltration in patients with act-C3 and MAC deposition around steatotic hepatocytes. Importantly, several neutrophil characteristics that give them a high potential of causing tissue injury are affected by complement factors. For example, C1q and C5a stimulate the respiratory burst,[28] thereby causing formation of aggressive oxygen radicals. C5a also promotes the release of granules containing myeloperoxidase, which further contributes to tissue injury.[29] In addition, C5a inhibits neutrophil apoptosis, thereby enhancing neutrophil survival.[30] Moreover, C3a and C5a stimulate tumor necrosis factor alpha and IL-6 secretion by Kupffer cells,[26] and C5a induces IL-8 and IL-1β in various cell types,[27] which may explain the increased expression of these cytokines in patients with complement activation in our study. Interestingly, C5 also has been shown to be involved in liver fibrogenesis,[31] suggesting a possible role of complement activation in advanced stages of NASH.

On top of their effects on inflammation, complement-derived factors also may affect the development of steatosis. Acylation-stimulating protein (ASP) is a C3 derivative that is involved in adipocyte lipid metabolism by stimulating triglyceride synthesis.[32] Interestingly, plasma ASP levels are increased in NAFLD.[33] Although this was considered to be related to "ASP resistance" of adipose tissue of NAFLD patients, it is tempting to speculate that elevated ASP in NAFLD may result from increased hepatic C3 activation as described in our study. It is also conceivable that, analogous to its function in adipocytes, ASP may promote triglyceride accumulation in hepatocytes, thereby creating a vicious cycle in which complement activation promotes steatosis, which in turn increases complement activation.

Our results suggest that both the classical and the lectin pathway of complement activation may be important in the context of NAFLD, although the observed MBL accumulation could also be caused by increased MBL synthesis by the liver. Moreover, we cannot rule out that there is additionally alternative pathway activation. In fact, it is likely that the alternative pathway is responsible for the act-C3 deposition that was observed in the patients without C4d staining. Definitive proof would require detection of the factor B-derived Bb fragment, which is challenging because of its short half-life (M. Daha, personal communication).

The lectin pathway has previously been implicated in other metabolic diseases. For example, MBL deficiency is associated with the development of diabetic vascular complications,[34] and serum MBL concentrations are decreased in obesity and insulin resistance.[35] However, neither *MBL2* genotype distribution nor MBL plasma levels appeared to be related to accumulation of MBL around steatotic hepatocytes or NAFLD severity in our study (see Supporting Figs. 4-7 and Supporting Tables 1 and 2), although the study group was small.

In summary, we have shown significant complement activation in the liver of the majority of subjects with NAFLD. Complement activation was associated with increased apoptosis, excessive hepatocyte steatosis and neutrophil influx, and increased with disease severity. This may have important implications for the pathogenesis and progression of NAFLD given the function of complement factors in hepatic inflammation, fibrosis, clearance of apoptotic cells, and liver regeneration. The putative involvement of the complement cascade in the progression of NAFLD as suggested by our study opens up new opportunities for treatment, because complement inhibitors are already in clinical use.[36]

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