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Increased activity of the complement system in the liver of patients with alcoholic hepatitis

Hong Shen^b, Barbara A. French^a, Hui Liu^a, Brittany C. Tillman^a, and Samuel W. French^{a,b}

^aLA BioMed at Harbor-UCLA Medical Center, Torrance, CA 90509, USA

^bDepartment of Pathology, Harbor-UCLA Medical Center, Torrance, CA 90509, USA

Abstract

Inflammation has been suggested as a mechanism underlying the development of alcoholic hepatitis (AH). The activation of the complement system plays an important role in inflammation. Although it has been shown that ethanol-induced activation of the complement system contributes to the pathophysiology of ethanol-induced liver injury in mice, whether ethanol consumption activates the complement system in the human liver has not been investigated. Using antibodies against C1q, C3, and C5, the immunoreactivity of the complement system in patients with AH was examined by immunohistochemistry and quantified by morphometric image analysis. The immunoreactivity intensity of C1q, C3, and C5 in patients with AH was significantly higher than that seen in normal controls. Further, the gene expression of C1q, C3, and C5 was examined using real-time PCR. There were increases in the levels of C1q and C5, but not C3 mRNA in AH. Moreover, the immunoreactivity of C5a receptor (C5aR) also increased in AH. To explore the functional implication of the activation of the complement system in AH, we examined the colocalization of C5aR in Mallory-Denk bodies (MDBs) forming balloon hepatocytes. C5aR was focally overexpressed in the MDB forming cells. Collectively, our study suggests that alcohol consumption increases the activity of the complement system in the liver cells, which contributes to the inflammation-associated pathogenesis of AH.

Keywords

alcoholic hepatitis; complement factors; inflammation; Mallory-Denk bodies

1. Introduction

The liver plays a key role in innate immune defense against pathogens (For review, see Gao et al., 2008). Liver receives blood circulating from the intestines, which is rich in bacterial products, environmental toxins, and food antigens. The predominant innate immune system

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Correspondence: Hong Shen, M.D., Ph.D., Department of Pathology, Harbor-UCLA Medical Center, Torrance, CA 90509, USA, hongshenmail@gmail.com, Phone: (310) 222-2643.

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of liver is comprised of phagocytic cells (such as neutrophils and macrophages), lymphocytic cells (natural killer and natural killer T), and humoral factors (complement factors and interferons). The complement system is a major component of the host innate immune system (for reviews see Gasque, 2004; Lu et al., 2008). The complement system can be activated by three different pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). CP is activated by binding of a specific part of the antibody molecule (Fc) to the C1 component and involves C1q, C1r, C1s, C4, C2, and C3 components. LP recognizes pathogen-specific carbohydrate patterns by binding of the mannan-binding lectin (MBL) or ficolin to common carbohydrate structures on microorganisms. AP is continuously activated at very low levels through the spontaneous hydrolysis ("tickover") of the complement component C3 in plasma. All activation conditions result in the formation of C3 and C5 convertases and subsequent generation of major effector molecules such as C3a, C3b, and C5a. These effectors mediate direct lysis, via membrane attack complex (MAC) formation or immune adherence and phagocytosis of the pathogen (C3b and C4b), or function as chemokines, chemoattractants, and activators of immunocompetent cells (C3a and C5a).

While the primary source of plasma C1q remains obscure, hepatocytes are the primary cells responsible for biosynthesis of most of complement components found in plasma (Morgan and Gasque, 1997; Nagura et al., 1985; Qin and Gao, 2006). Hepatocytes are also primarily responsible for the biosynthesis of several complement regulator proteins found in plasma, such as factor I, factor H, and the C1 inhibitor (Morgan and Gasque, 1997). Immune cells and endothelial cells, which also biosynthesize complement components, have less contributions compared to hepatocytes (Morgan and Gasque, 1997). Although the complement system provides critical protection against invading organisms, accumulating evidence implicates activation of complements in chronic inflammatory diseases, such as alcoholic liver disease (ALD) (Gao et al., 2011; Pritchard et al., 2007; Qin and Gao, 2006; Wang et al., 2012) and non-alcoholic fatty liver disease (NAFLD) (Rensen et al., 2009). C3 and C5 have been implicated in liver fibrosis and regeneration. Hillebrandt et al. reported that C5 and C5aR play a critical role in the pathogenesis of liver fibrosis (Hillebrandt et al., 2005). C3 and C5 have been demonstrated to be involved in liver regeneration after partial hepatectomy or after toxic injury (Strey et al., 2003). There are increased mortality, parenchymal damage, and impaired liver regeneration after partial hepatectomy in C3- or C5-deficient mice (Strey et al., 2003).

Hepatic C1q and C3 deposition are markedly elevated in mice ALD models (Cohen et al., 2010). Jarvelainen et al. reported that ethanol feeding to rats increased the deposition of complement proteins C3 and C8, but not C1, in the liver (Jarvelainen et al., 2002). Complement C3 has been shown to contribute to ethanol-induced liver steatosis in mice as mice lacking C3 (C3^{-/-}) did not develop hepatic steatosis or increased alanine aminotransferase (ALT) concentrations in response to acute or chronic ethanol exposure (Bykov et al., 2006). In the liver of ethanol-fed mice, ethanol activates CP through C1q binding to apoptotic cells. C1q deletion abolishes ethanol-induced complement activation and the upregulation of TNF α and IL-6 expression in liver and reduces ethanol-induced liver injury (Cohen et al., 2010).

Although these findings indicate that the activation of complement components contributes to inflammatory responses in ALD, the murine findings have not been studied in human. Rensen et al. reported that the complement system is activated in human NAFLD and its activation correlates with disease severity (Rensen et al., 2009). Moreover, despite the obvious ALD relevance of complement system and the wealth of animal data demonstrating its importance in experimental ALD models, little is known about its role in human ALD. There are fragmented data that are based on serum concentration of complements from human. It has been reported that acute alcoholic intoxication does not affect the concentration of total hemolytic complements (Spagnuolo and MacGregor, 1975). The level of circulating complement is increased in chronic alcoholics who withdrew from alcohol consumption after admission (MacGregor et al., 1978). However, the serum concentration of C3 is reduced in alcoholics with cirrhosis (Fox et al., 1971; Petz, 1971). To better understand the role of complements in ALD, our study examined the expression of the complement system in liver biopsies from patients with AH.

2. Materials and Methods

2.1. Biopsies

Human archived formalin-fixed paraffin-embedded (FFPE) liver biopsies from patients who had AH (n = 3–5) were obtained from Harbor UCLA hospital archives. In all the cases MDBs were present in some hepatocytes of liver tissue sections from patients with AH but were not present in the normal control livers (Control; n=3). The biopsy sections were cut 4 μ m thick.

2.2. Immunohistochemical staining

FFPE tissue slides were triple stained for various complements (Rabbit anti-C1q, Bioss, Woburn, MA; Rabbit anti-C3 and Rabbit anti-C5, Abcam, Cambridge, MA; Rabbit anti-C5aR, Novus Biologicals, San Diego, CA) and ubiquitin (Millipore, Temecula CA).

The complements were detected using the second antibody donkey anti-rabbit Alexa Fluor 488 (Jackson Lab, West Grove, PA). Ubiquitin was detected using the second antibody donkey anti-mouse Alexa Fluor 594 (Jackson Lab, West Grove, PA). All slides were stained with the nuclear stain DAPI (Molecular Probes, Eugene, OR). The slides were examined with a Nikon 400 fluorescent microscope. The fluorescence intensity of staining of the protein of interest was measured quantitatively using 40 $\times$ objectives and a standard exposure time of 800 ms using a Nikon 400 fluorescent microscope with three filters (FITC-green, Texas Red, and Tri-Color), and the Nikon morphometric system. The results were displayed as a graph attached to the immunofluorescent photography using a screen snip.

2.3. RNA isolation

For RNA isolation of FFPE tissue sections, paraffin was first removed from tissue samples when human liver biopsies were assayed. The paraffin-embedded tissue sections were mounted on a glass slide and dried at 60 $^{\circ}$ C for 30 min. The slides were then submerged in xylene at room temperature for 1 h changing the xylene once after 30 min. The samples were hydrated by washing progressively for 2 min in 100%, 70%, and 50% ethanol, and

then pure RNase-free water before air-drying the samples on the slides for approximately 10 min. RNA isolation was processed using the Pinpoint™ Slide RNA isolation System II (ZYMO, Irvine, CA) by adding Pinpoint™ Solution directly to a small region of the tissue section to allow the solution to dry completely at the room temperature. The embedded tissue was then removed from the slide using a sterile blade or scalpel to scrape tissues from the slides followed by transferring the tissues to a micro-centrifuge tube for subsequent proteinase K digestion. The RNA was extracted and purified according to the manufacturer's protocol. DNA-free RNA can be obtained with subsequent DNase I treatment following the manufacturer's recommended protocol (ZYMO, Irvine, CA). The process described above was also followed for mice frozen liver tissues, except for the deparaffinization step. The quality and yield of the resulting total RNAs were assessed with an absorbance reading at 260 nm (A260) using a spectrophotometer (Thermo, Waltham, MA) by loading 1 µl of the extracted RNA.

2.4. Quantitative real-time PCR analysis

Synthesis of first-strand cDNAs was performed with the above mentioned total RNA (250 ng), and random hexamer primers using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, San Diego, CA) following the instruction. Real-time PCR was performed using the Fast SYBR Green Master Mix on a StepOnePlus™ Real-time PCR System (Applied Biosystems) with a primer concentration of 200 nM. Primer sequences and the related gene Accession Number are listed in Table 1. Reaction conditions consisted of 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Human α -tubulin and mice β -actin were used as controls to normalize the starting quantity of RNA. Quantitative values were obtained from the threshold PCR cycle number (CT) at which point the increase in signal associated with an exponential growth for PCR product starts to be detected. The target mRNA abundance in each sample was normalized to its endogenous control level and the relative mRNA expression levels were analyzed using the 2^{-CT} method. Reaction of each sample was performed in triplicate.

2.5. Statistical analysis

Statistical significance was determined using the t-test. *p* values less than 0.05 were considered statistically significant. All data were presented as the mean $\pm$ S.E.M.

3. Results

3.1. Immunoreactivity of C1q was elevated in patients with AH

Given that the complement system is activated in animal models of ALD, we analyzed whether these observations translate to human AH. To that end, liver biopsies stemming from AH with or without liver fibrosis were included. In the normal control livers, no alcoholic, viral hepatitis, or diabetes was involved.

The possible activation of complement was analyzed by immunohistochemical methods using PPFE liver tissue sections from patients with AH and normal control subjects. Livers from controls showed only minimal or no staining for C1q (Fig. 1A). Increased

immunoreactivity of C1q was found in patients with AH (Fig. 1A). Remarkably, the most intense staining of C1q was found closely associated with the plasma membrane (Fig. 1A). C1q immunoreactivity was also found in Kupffer cells and lymphocytes. The immunoreactivity of C1q of liver cells was quantified and compared between AH and control livers by immunofluorescence intensity (see Methods). The immunoreactivity intensity of C1q in patients with AH was 267% of that in control subjects ($P < 0.05$) (Fig. 1B).

3.2. Immunoreactivity of C3 was elevated in patients with AH

As all complement pathways converge on the central component C3, we next examined activation of C3 in the livers of patients with AH and controls. Increased immunoreactivity of C3 was found in patients with AH (Fig. 2A). The immunoreactivity intensity of C3 in patients with AH was 206% of that in control subjects ($P < 0.05$) (Fig. 2B).

3.3. Immunoreactivity of C5 was elevated in patients with AH

We next examined the activation of C5 in the livers of patients with AH and controls. Increased immunoreactivity of C5 was found in patients with AH (Fig. 3A). The immunoreactivity intensity of C5 in patients with AH was 234% of that in control subjects ($P < 0.05$) (Fig. 3B).

3.4. The expression of C1q and C5, but not C3, mRNA was elevated in patients with AH

We examined the expression of C1q, C3, and C5 mRNA in livers using real-time PCR. The levels of C1q and C5 mRNA increased significantly in patients with AH as compared to control subjects ($P < 0.01$) (Fig. 4A and C). There was no significant difference in the levels of C3 mRNA between AH and controls (Fig. 4B).

3.5. Increased immunoreactivity of C5aR in patients with AH

Most of the biological functions of C5a are mediated through C5a receptor (C5aR), a specific G protein-coupled receptor (Riedemann et al., 2003). We next examined the immunoreactivity of C5aR in livers of patients with AH and controls. Increased immunoreactivity of C5aR was found in patients with AH (Fig. 5A). The immunoreactivity intensity of C5aR in patients with AH was 490% of that in control subjects ($P < 0.05$) (Fig. 5B).

3.6. Increased immunoreactivity of C5aR in MDBs forming cells in patients with AH

MDBs are large eosinophilic hepatocellular cytoplasmic protein aggregates which are characteristic hallmarks of alcoholic steatohepatitis but they also occur in a variety of other liver diseases such as non-alcoholic steatohepatitis, Wilson's disease, chronic cholestasis, or hepatocellular carcinoma, etc. (Strnad et al., 2013; Zatloukal et al., 2007). Given the critical role of innate immune response in alcohol-induced steatohepatitis (Gao et al., 2011), we further examined the colocalization of C5aR and MDBs in patients with AH. The liver sections from patients with AH were stained with antibodies against C5aR and Ubiquitin (Fig. 6A) and the fluorescence intensity of the immunostaining of C5aR in MDB forming cells and non-MDB forming cells was measured (Fig. 6B). C5aR was focally overexpressed

by the MDB forming hepatocytes in the AH biopsies (Fig. 6A and B). The fluorescence intensity of the immunostaining of C5aR in MDB forming cells was 141% of that in non-MDB forming cells ($P < 0.01$) (Fig. 6B).

4. Discussion

It has been demonstrated that inflammation plays a critical role in the pathogenesis of ALD (Gao et al., 2008; Gao et al., 2011; Wang et al., 2012). Inflammation, which originates in response to a variety of stress conditions, can be either beneficial or detrimental to the liver. Mild hepatic inflammation has been shown to protect hepatocytes from injury and to promote tissue repairing and the re-establishment of homeostasis (Gasque, 2004). However, excessive or chronic hepatic inflammation may exacerbate the liver injury. The complement system, which bridges innate and adaptive immunity, plays beneficial roles in host defense, wound healing, and response to tissue injury (Gasque, 2004). Although it has been shown that ethanol-induced activation of the complement system contributes to the pathophysiology of ethanol-induced liver injury in mice, whether ethanol consumption activates the complement system in human liver is unknown. In the present study, we compared the immunoreactivity and gene expression of complement factors C1q, C3, and C5 in patients with AH and in normal controls. We also examined the immunoreactivity of C5aR and the correlation between C5aR immunoreactivity and MDB in AH. To our knowledge, our study is the first investigation of the immunoreactivity and gene expression of complement factors in human AH tissue. Our data showed an increase in C1q, C3, C5, C5aR immunoreactivity intensity in patients with AH compared to normal controls. We also showed that increased gene expression of C1q and C5, but not C3, in patients with AH. Moreover, C5aR was focally overexpressed by the MDB forming cells. Our data are generally in line with the previous observations that activation of complement system contributes to ALD.

Apoptosis of hepatocytes is a major characteristic of ALD (Higuchi et al., 1996; Oliva et al., 2009). Our current study shows increased C1q immunoreactivity and gene expression in patients with AH. C1q is activated in the classic pathway and the classic pathway is also involved in apoptosis. The relationship between activation of the complement system and apoptosis has been well established. C1q can bind to apoptosis-associated signals on the surface of apoptotic cells and mark them for clearance by phagocytes. As C1q can induce TNF- α protein and receptor expression and up-regulate C3 in macrophages (Bajtay et al., 2000), C1q may also directly increase the expression of inflammatory cytokines in Kupffer cells. Our data show that chronic alcohol exposure increased C1q immunoreactivity in hepatocytes. This is consistent with the observation that chronic ethanol exposure, but not short-term ethanol exposure, increases apoptosis of hepatocytes and C1q is required for chronic ethanol-induced hepatocyte apoptosis (Cohen et al., 2010) and implicates a possible role of the complement system in the apoptosis of hepatocytes in AH.

We found that, in addition to C1q, immunoreactivity of C3 and C5 also increased in patients with AH. C3a and C5a anaphylatoxins are cytokine-like polypeptides generated during complement system activation and released at the inflammatory site (Gasque, 2004). Hepatic C3 deposition is markedly elevated in mice ALD models (Cohen et al 2010). C3 and C8

increase in rat livers after ethanol feeding (Jarvelainen et al., 2002). In a human study, it has been reported recently, that increased circulating levels of activated C3 (C3a) is associated with liver fat content and hepatocellular injury, at least in subjects who consume considerable amounts of alcohol daily (Wlazlo et al., 2013). C3 and C5 have been shown to make differential contributions to the pathophysiologic progression of alcohol-induced liver steatosis in mice (Pritchard et al., 2007). C3 contributes to the development of hepatic steatosis, whereas C5 acts primarily to mediate increased proinflammatory cytokine production and injury to hepatocytes (Pritchard et al., 2007). The increased deposition of C3 and C5 has been linked to ethanol-induced expression of proinflammatory cytokines including tumor necrosis factor alpha (TNF- α), interleukin-6, and interferon-gamma in mice liver (Pritchard et al., 2007; Roychowdhury et al., 2009). Mice lacking the C5 (C5 $^{-/-}$ mice) but not C3 (C3 $^{-/-}$ mice) were completely protected from chronic (4-week) ethanol feeding-induced increases of inflammatory cytokines (Pritchard et al., 2007). On the other hand, the short-term (4-day) ethanol feeding-induced increase in hepatic TNF- α expression coincided with the deposition of C3 in the liver while TNF- α induction was lacking in C3 $^{-/-}$ mice (Roychowdhury et al., 2009). In addition, the absence of Toll-like receptor 4 (TLR-4) had no effect on the early, ethanol-induced increase in either C3b or TNF- α (Roychowdhury et al., 2009). It has been shown that the MyD88-dependent TLR4/NF κ B pathway was upregulated in hepatocytes where MDBs formed, via the NF κ B-CXCR4/7 pathway (Liu et al., 2014). Ethanol-induced expression of proinflammatory cytokines occurs prior to the increased plasma alanine aminotransferase activities and the induction of cytochrome or oxidative stress (Menon et al., 2001). The possibility that increased immunoreactivity of C3 and C5 in liver of patients with AH is an early event needs to be further investigated.

Our present study shows robust increases in gene expression of C1q and C5, but not C3, in patients with AH. These are the first data documenting mRNA levels of C1q, C3, and C5 in AH. The finding that levels of hepatic C1q and C5 mRNA increased in patients with AH is consistent with the immunohistochemistry data from previous study in animal models of ALD (Bykov et al., 2006; Cohen et al., 2010; Jarvelainen et al., 2002; Pritchard et al., 2007), a microarray study using high-fat diet with alcohol in mice (Bykov et al., 2007), and our present study (Fig. 1 and 3). While our immunohistochemical analysis used quantification of immunoreactivity of complement factors in hepatocytes and our gene expression data reflect changes in whole hepatic tissue including various cell types (e.g. hepatocytes, Kupffer cells, lymphocytes, stellate cells, etc.), the observation that mRNA levels of C1q and C5 increased in AH suggests that alcohol exposure induce *de novo* synthesis of C1q and C5 in liver. It is surprising that there was no change of C3 mRNA levels in patients with AH when compared to controls. A microarray study showed that high-fat diet with alcohol intake slightly increased hepatic synthesis of C3 in mice when compared to high-fat diet with carbohydrates (Bykov et al., 2007). The immunohistochemistry data from the previous study in animal models of ALD and our present study (Fig. 2) demonstrate that ethanol exposure increases C3 immunoreactivity. The elevated C3 immunoreactivity in AH may be caused by the regulation of C3 on protein levels via regulating translation or the cleavage of C3. It has been shown that deficiency in decay-accelerating factor (CD55/DAF), a complement regulatory protein that terminates the complement cascade at the level of C3 and C5

cleavage, exacerbated C3-associated alcohol-induced liver steatosis in mice liver (Pritchard et al., 2007). It is also possible that the elevated C3 immunoreactivity in AH is due to sequestering and deposition of C3 from the circulation, as a response to cellular damage by alcohol (Qin and Gao, 2006).

Our observation that C5aR immunoreactivity increased in patients with AH is consistent with the previous finding that C5aR was not present in hepatocytes of normal rat liver but can be induced after lipopolysaccharide challenge, which triggers inflammation (Daveau et al., 2004; Koleva et al., 2002). C5aR contributes to a variety of functions in the liver including stimulation of glucose release, induction of acute phase protein synthesis, and stimulation of hepatocyte proliferation (Buchner et al., 1995; Daveau et al., 2004; Koleva et al., 2002). Recently, Hillebrandt et al. demonstrated C5 and C5aR play a critical role in the pathogenesis of liver fibrosis (Hillebrandt et al., 2005).

Importantly, there was an increase in C5aR immunoreactivity in the MDB forming hepatocytes compared to that in non-MDB forming hepatocytes. MDB formation occurs in 80% of AH liver biopsy specimens (French, 1981). The ballooning of hepatocytes and MDBs are major features of ongoing inflammation in AH (French et al., 2010). We recently showed that MyD88-dependent TLR4/NF κ B pathway was upregulated in hepatocytes where MDBs formed (Liu et al., 2014). The crosstalk between TLRs and C5aR in AH is intriguing. Deficiency in TLR-4 did not affect ethanol exposure-induced expression of C3b or TNF- α in mice model of ALD (Pritchard et al., 2007). On the other hand, TLR activation enhances the C5a-induced pro-inflammatory responses (Raby et al., 2011). Deciphering the interaction between TLRs and the complement system will provide insights into the mechanisms underlying AH and new therapeutic avenues to control inflammation. Based on the report that C5aR antagonist attenuated liver fibrosis in mice (Hillebrandt et al., 2005), C5aR could be a drug target in AH treatment.

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Abbreviations

ALD	alcoholic liver disease
AH	alcoholic hepatitis
AP	the alternative pathway
C1q	complement component 1 q
C3	complement component 3
C5	complement component 5
CP	the classical pathway
FFPE	formalin-fixed, paraffin-embedded
HCC	hepatocellular carcinoma

LP	the lectin pathway
MDBs	Mallory–Denk bodies
NAFLD	non-alcoholic fatty liver disease

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Highlights

- This is the first study of the expression of the complement system in liver biopsies from patients with alcoholic hepatitis (AH).
- The immunoreactivity of C1q, C3, and C5 increased in AH.
- Levels of C1q and C5, but not C3, mRNA increased in AH.
- Immunoreactivity of C5a receptor increased in AH.
- C5a immunoreactivity focally overexpressed in the Mallory-Denk body forming cells.

Fig. 1. Increased immunoreactivity of C1q in the livers of patients with AH

A, The liver sections from patients with AH and controls were stained with an antibody against C1q (top picture). The liver sections from patients with AH stained with greater intensity for C1q compared to the controls. The bottom picture shows the fluorescence intensity measurement of the immunostaining of C1q. A representative screen snip was obtained from morphometric screen. The fluorescence intensity was traced along the yellow line in the top panel and shown as a green tracer in the bottom picture. B, Quantification of fluorescence intensity of the immunostaining of C1q. Data represent mean values $\pm$ S.E.M. * $p < 0.05$.

Fig. 2. Increased immunoreactivity of C3 in the livers of patients with AH

A, The liver sections from patients with AH and controls were stained with an antibody against C3 (top picture). The liver sections from patients with AH stained with greater intensity for C3 compared to the controls. The bottom picture shows the fluorescence intensity measurement of the immunostaining of C3. A representative screen snip was obtained from morphometric screen. The fluorescence intensity was traced along the yellow line in the top panel and shown as a green tracer in the bottom picture. B, Quantification of fluorescence intensity of the immunostaining of C3. Data represent mean values $\pm$ S.E.M. * $p < 0.05$.

Fig. 3. Increased immunoreactivity of C5 in the livers of patients with AH

A, The liver sections from patients with AH and controls were stained with an antibody against C5 (top picture). The liver sections from patients with AH stained with greater intensity for C5 compared to the controls. The bottom picture shows the fluorescence intensity measurement of the immunostaining of C5. A representative screen snip was obtained from morphometric screen. The fluorescence intensity was traced along the yellow line in the top panel and shown as a green tracer in the bottom picture. B, Quantification of fluorescence intensity of the immunostaining of C5. Data represent mean values $\pm$ S.E.M. * $p < 0.05$.

Fig. 4. Gene expression levels of C1q, C3, and C5 in the livers of patients with AH and controls
Gene expression levels of C1q (A), C3 (B), and C5 are measured by real-time PCR. Data represent mean values $\pm$ S.E.M. * $p < 0.01$.

Fig. 5. Increased immunoreactivity of C5aR in the livers of patients with AH

A, The liver sections from patients with AH and controls were stained with an antibody against C5aR (top picture). The liver sections from patients with AH stained with greater intensity for C5aR compared to the controls. The bottom picture shows the fluorescence intensity measurement of the immunostaining of C5aR. A comprehensive screen snip was obtained from morphometric screen. The fluorescence intensity was traced along the yellow line in the top panel and shown as a green tracer in the bottom picture. B, Quantification of fluorescence intensity of the immunostaining of C5aR. Data represent mean values $\pm$ S.E.M. * $p < 0.05$.

Fig. 6. Increased immunoreactivity of C5aR in MDB forming cells in the livers of patients with AH

A, The liver sections from patients with AH were stained with antibodies against C5aR (middle of the picture) and Ubiquitin (left of the picture). Arrow indicates the C5aR immunoreactivity that was colocalized with MDBs. B, Quantification of fluorescence intensity of the immunostaining of C5aR in MDB forming cells and non-MDB forming cells. Data represent mean values $\pm$ S.E.M. * $p < 0.01$.

Table 1

Sequences of the forward and reverse primers of C1q, C3, and C5 for real-time PCR.

Gene name (species)	Accession number	Sequences of primer
C1q (Human)	NM_015991	Forward primer: 5'-CATCACCAACCAGGAAGAAC-3' Reverse primer: 5'-TGTGGTGGTGTACAGAAG-3'
C3 (Human)	NM_000064	Forward primer: 5'-CCTGGCTCCACAGTTCTTA-3' Reverse primer: 5'-AGTGGAGAAGACCTGCTGTG-3'
C5 (Human)	NM_001735	Forward primer: 5'-ATTGGGAAGGCTACACATGA-3' Reverse primer: 5'-TGCCTTGACAGTATCAGCAA-3'