Published in final edited form as:

Mol Immunol. 2016 July ; 75: 122–132. doi:10.1016/j.molimm.2016.05.006.

Differential contribution of complement receptor C5aR in myeloid and non-myeloid cells in chronic ethanol-induced liver injury in mice

Rebecca L McCullough¹, Megan R McMullen¹, Dola Das¹, Sanjoy Roychowdhury¹, Michael G. Strainic², M. Edward Medof², and Laura E Nagy^{1,3,*}

¹Center for Liver Disease Research, Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio

²Institute of Pathology, Case Western Reserve University, Cleveland, Ohio, USA

³Department of Gastroenterology and Hepatology, Cleveland Clinic, Cleveland, Ohio

Abstract

Background—Complement is implicated in the development of alcoholic liver disease. C3 and C5 contribute to ethanol-induced liver injury; however, the role of C5a receptor (C5aR) on myeloid and non-myeloid cells to progression of injury is not known.

Methods—C57BL/6 (WT), global C5aR^{−/−}, myeloid-specific C5aR^{−/−}, and non-myeloid-specific C5aR^{−/−} mice were fed a Lieber-DeCarli diet (32% kcal EtOH) for 25 days. Cultured hepatocytes were challenged with ethanol, TNF α , and C5a.

Results—Chronic ethanol feeding increased expression of pro-inflammatory mediators in livers of WT mice; this response was completely blunted in C5aR^{−/−} mice. However, C5aR^{−/−} mice were not protected from other measures of hepatocellular damage, including ethanol-induced increases in hepatic triglycerides, plasma alanine aminotransferase and hepatocyte apoptosis. CYP2E1 and 4-hydroxynonenal protein adducts were induced in WT and C5aR^{−/−} mice. Myeloid-specific C5aR^{−/−} mice were protected from ethanol-induced increases in hepatic TNF α , whereas non-myeloid-specific C5aR^{−/−} displayed increased hepatocyte apoptosis and inflammation after chronic ethanol feeding. In cultured hepatocytes, cytotoxicity induced by challenge with ethanol and TNF α was completely eliminated by treatment with C5a in cells from WT, but not C5aR^{−/−} mice. Further, treatment with C5a enhanced activation of pro-survival signal AKT in hepatocytes challenged with ethanol and TNF α .

Conclusion—Taken together, these data reveal a differential role for C5aR during ethanol-induced liver inflammation and injury, with C5aR on myeloid cells contributing to ethanol-

* Address for correspondence: Dr. Laura E. Nagy, Department of Pathobiology, Cleveland Clinic, 9500 Euclid Avenue NE40, Cleveland, Ohio 44195, Telephone: (216) 444-4120, Fax: (216) 444-1493, nagyL3@ccf.org.

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

induced inflammatory cytokine expression, while non-myeloid C5aR protects hepatocytes from death after chronic ethanol feeding.

Keywords

alcoholic liver disease; inflammation; complement; apoptosis; hepatocytes

1. Introduction

The innate immune system plays an integral role in host defense and maintenance of healthy tissue. The imbalance of immune defenses that occurs in many chronic diseases, including alcoholic liver disease (ALD), is detrimental and contributes to disease progression. Activation of cellular components of the immune system, including hepatic macrophages, is important for the response to infection and injury, but has also been implicated in the development of ALD [1]. Complement, a component of the innate and adaptive immune system, is an important patho-physiological contributor to ethanol-induced liver injury and is a potential therapeutic target for the treatment of ALD [2, 3].

Complement has been primarily viewed as a key mediator of microbial defense, opsonization and clearance of cellular debris. A growing body of evidence supports a much more diverse and intricate contribution of complement as not only the bridge between innate and adaptive immune systems, but as an effector of chemotaxis, tissue regeneration, and repair [4]. Complement is activated via three different pathways, the classical, lectin, and alternative pathways; these pathways converge at complement component C3. Subsequent activation and cleavage of complement proteins C3 and C5 results in the production of anaphylatoxins C3a and C5a. Both C3a and C5a are powerful chemoattractants involved in recruiting neutrophils, monocytes, and macrophages to the site of complement activation, facilitating phagocytosis and clearance of cellular debris [4, 5]. C5a is recognized to be the more potent chemokine, signaling via interaction with its cognate G-protein coupled receptor, C5aR [6, 7].

In mouse models of partial hepatectomy and carbon tetrachloride-induced toxicity, complement activation is required for normal liver regeneration [7-10]. Studies employing C3aR^{-/-} and C5aR^{-/-} mice have elucidated the important function for complement in both liver cell proliferation and hepatocyte apoptosis [7]. In models of ALD, C5^{-/-} mice are protected from ethanol-induced elevations of plasma alanine transaminase (ALT) and hepatic cytokine production, while C3^{-/-} were protected from steatosis following chronic ethanol feeding [11, 12]. While these reports illustrate an important contribution of C3 and C5 to ethanol-induced liver injury, our understanding of anaphylatoxins and their role in the progression of ALD is still unclear. To better understand the role of C5a and its cognate receptor C5aR in ALD, we have utilized C5aR^{-/-} mice in a model of chronic ethanol feeding. Using bone marrow transplants, we have identified a differential contribution of C5aR on myeloid and non-myeloid cells following chronic ethanol feeding.

2. Experimental Procedures

2.1 Materials and animals

Female C57BL/6 (WT) mice (8-10 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Lieber-DeCarli high-fat ethanol and control diets were purchased from Dyets (Bethlehem, PA; Cat#710260). C5aR^{-/-} mice on a WT background [13, 14] were bred in-house and were from Dr. M.E. Medof (Case Western Reserve University, Cleveland, OH).

Plasma alanine aminotransferase (ALT) assay kit was purchased from Sekisui Diagnostics (Framingham, MA). Triglyceride assay kits were purchased from Pointe Scientific Inc. (Lincoln Park, MI). AML12 cells were from ATCC and all culture media was from Sigma Aldrich (St. Louis, MO) and media supplements were from ThermoFisher (Grand Island, NY) and Cayman Chemicals (Ann Arbor, MI).

Antibodies were from the following sources: TNF α (Fitzgerald Industries International, Acton, MA; cat# 70R-TR008), CYP2E1 (EMD Millipore, Billerica, MA; cat# AB1252), 4-HNE (Kindly provided by Dr. Dennis Petersen, University of Colorado Anschutz Medical Campus), RIP3 (ABGENT, San Diego, CA; cat# AP7819b), phospho-Ser345-Mixed lineage kinase domain-like (MLKL) (Abcam, Cambridge, MA; ab196436), pAKT (Cell Signaling Phospho Ser473; cat#: 9271) and C5aR/CD88 (Santa Cruz Biotechnology, Dallas, Texas; cat# sc-25774). Alexa fluor-488 conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA). Caspase-generated fragments of cytokeratin-18 were detected using an M30 CytoDEATH staining kit (Roche, Mannheim, Germany; cat#12140322001).

2.2 Mouse Models

2.2.1 Chronic Ethanol Feeding—All procedures using animals were approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Female mice were housed in shoe box cages (two animals per cage) with microisolator lids. Mice were age- and weight-matched, then randomized into ethanol-fed and pair-fed groups. At the beginning of the study, all mice were acclimated to the liquid control diet for two days. Ethanol-fed mice were fed liquid diet *ad libitum*. Control mice received isocalorically-substituted maltose dextrins for ethanol over the entire feeding period. The chronic ethanol feeding model consisted of 1% (vol/vol) ethanol for 2 days followed by 2% ethanol for 2 days, 4% ethanol for 1 week, 5% ethanol for 1 week, and 6% ethanol for a final week (32% of total calories, denoted as 25d/32%). Ethanol and pair-fed mice increased their body mass over the course of the study and consumed an equal amount of diet (Table 1).

At the termination of the study, mice were anesthetized, blood was taken into non-heparinized syringes from the posterior vena cava, and livers excised. Blood was transferred into EDTA-containing Microtainer tubes (BD Biosciences, Franklin Lakes, NJ) for isolation of plasma. Plasma was stored at -80°C until further analysis. Portions of the liver were either fixed in formalin or frozen in optimal cutting temperature (OCT) compound (Sakura Finetek U.S.A. Inc., Torrance, CA) for histology, frozen in RNAlater (Qiagen, Valencia, CA), or flash frozen in liquid nitrogen and stored at -80°C .

2.2.2 Bone Marrow Transplants: Generation of Myeloid-C5aR^{-/-} and Non-Myeloid-C5aR^{-/-} mice—Bone marrow chimeras were created by lethally irradiating 5 week old female WT and C5aR^{-/-} and DKO mice with 600 RAD two times, two hours apart. Donor marrow was isolated by flushing the femur and tibia bones of 10 week old mice with sterile phosphate buffered saline, and lysing red blood cells with AKC lysis buffer. Donor BMCs were injected (10×10^6 cells) via the tail vein to irradiated recipients. All chimeras were kept in sterile cages with radiated food and autoclaved water. 5 weeks post-transplant, chimeras were dosed with a single injection of clodronate-containing liposomes (Encapsula NanoSciences, Nashville, TN SKU # 8909) to deplete resident macrophages in the liver as previously described [15]; Seven days post-clodronate, chimeras started the chronic ethanol feeding paradigm. Adoptive transfer of recipient marrow into donors was confirmed in isolated peripheral blood monocytic cells (PBMC). RNA was isolated from PBMCs using a blood RNA isolation kit (Ambion Life Technologies, Grand Island, NY) per the manufacturer's instructions. Complementary DNA was reverse transcribed and standard PCR was performed using primers specific for WT and C5aR^{-/-} alleles (WT Forward (oIMR7178) 5'-GGTCTCTCCCCAGCATCATA-3'; C5aR Mutant Forward (oIMR7179) 5'-GCCAGAGGCCACTTGTGTAG-3'; Common Reverse (oIMR7415) 5'-GGCAACGTAGCCAAGAAAAA-3'.

2.2.3 Primary Hepatocyte Isolation—Hepatocytes from WT and C5aR^{-/-} mice were isolated using methods previously described [16, 17]. Hepatocytes were isolated from livers perfused via the portal vein with modified Hank's solution (free of Ca²⁺ and Mg²⁺) containing 1 mM EGTA and 10 mM HEPES and then with 0.05% type I collagenase in Williams' E medium at a flow rate of 6 mL/min. A cell suspension was formed by gentle disruption of the collagenase-treated livers in Williams' E medium containing 10% fetal bovine serum. Cells were washed twice with Williams' E and dead/damaged hepatocytes were separated out with a 30% Percoll gradient. Purified cells were counted, plated on collagen I coated 24-well plates (0.063×10^6 /mL) and subsequently utilized for cytotoxicity experiments.

2.3 Culture and treatment of AML12 cells

AML12 mouse hepatocytes were obtained from ATCC and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Sigma Aldrich) with 1X insulin, transferrin and selenium (Gibco), 1X dexamethasone (Cayman Chemical), 10% fetal bovine serum and 1% penicillin streptomycin. Cells were subcultured at a density of 0.044×10^6 cells/cm² in a 24-well plate, 24 hrs later cells were treated with 50mM EtOH [18]. Following 24hr culture with ethanol, cells were then treated with 10 ng recombinant mouse C5a (R&D Systems, Minneapolis, MN) and/or 50 ng recombinant rat TNF α (Invitrogen, Carlsbad, CA) and 50 mM EtOH in serum-free DMEM/F12 +1% penicillin streptomycin for 24 hrs. Cytotoxicity assays were performed per the manufacturer's instructions. Lysates were prepared and used for Western blot analysis.

2.4 Immunocytochemistry

Following treatment, AML12 cells were fixed in freshly-prepared 4% paraformaldehyde. Slides were quenched with 25 mM glycine in PBS and blocked for 1 hour in 2% bovine

serum albumin, 5% fish gelatin, and 0.02% saponin as previously described [19]. Slides were incubated overnight with primary antibody at 4°C in blocking buffer. Following three washes in PBS, slides were then incubated with Alexa-fluor 488 secondary antibody (1:300) for 1 hr in blocking buffer. After three washes in PBS, slides were mounted with Vectashield mounting medium and at least four representative images were captured on an upright confocal microscope (Leica Microsystems, Buffalo Grove, IL). Negligible nonspecific staining was assessed by incubating cells without primary antibody (data not shown).

2.5 Plasma ALT measurements and liver triglycerides

Plasma samples were analyzed for alanine aminotransferase (ALT) via enzymatic assay according to the manufacturer's instructions. Flash frozen liver samples were used to quantify triglyceride accumulation using the Triglyceride Reagent Kit.

2.6 MTT Assay

AML12 cells and primary mouse hepatocytes were assayed for viability using the MTT assay (Sigma Aldrich, St. Louis, MO). Absorbance values were normalized to cell count to determine percent cytotoxicity.

2.7 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from liver stored in RNAlater using RNeasy Mini kits per the manufacturer's instructions (Qiagen, Germantown, MD). 4 µg of liver RNA was reverse transcribed and analyzed with PowerSYBR qRT-PCR kits (ThermoFisher, Grand Island, NY) on an Mx3000p analyzer (Stratagene, La Jolla, CA). Relative messenger RNA (mRNA) expression was determined using gene-specific primers: Tumor necrosis factor alpha (TNFα): 5'-CCCTCACACTCAGATCATCTTCT-3' and 5'-GCTACGACGTGGGCTACAG-3'; monocyte chemoattractant protein-1 (MCP-1): 5'-AGGTCCTGTGTCATGCTTCTG-3' and 5'-TCTGGACCCATTCCTTCTTG-3'; interleukin-6 (IL-6): 5'-TAGTCCTTCCTACCCCAATTTCC-3' and 5'-TTGGTCCTTAGCCACTCCTTC-3'; 18S: 5'-ACGGAAGGGCACCACCAGGA-3' and 5'-CACCACCACCCACGGAATCG-3'. Statistical analyses were performed on the Ct values (average Ct of gene of interest – average Ct of 18S) [20].

2.8 Immunohistochemistry and Immunofluorescence

Paraffin embedded liver sections were deparaffinized and stained with antibodies against M30, a caspase-dependent cleavage product of cytokeratin 18 [21], phospho-MLKL, receptor interacting kinase 3 (RIP3)[22], TNFα [23], CYP2E1 and 4-HNE [24]. Slides were coded and at least 3 images were acquired per tissue section and semi-quantification of positive staining was performed using ImagePro Plus software. No specific immunostaining was seen in sections incubated with PBS rather than the primary antibody (data not shown).

2.9 Western blot Analysis

Protein concentrations were measured in lysates from AML12 cells with the DC protein assay kit from Bio-Rad (Hercules, CA). Proteins were separated by SDS-PAGE for Western blot analysis. GAPDH was used as a loading control. Western blot analysis was performed

using enhanced chemiluminescence for signal detection. Signal intensities were quantified by densitometry using Image J software (NIH, Bethesda, MD).

2.10 Statistical Analysis

All values are reported as means $\pm$ SEM (For global C5aR^{-/-} studies n=8 for pair-fed and n=8-10 for ethanol-fed mice; in BMT studies, WT $\rightarrow$ WT n=4 for pair-fed and 7 for ethanol-fed, C5aR $\rightarrow$ WT n=10 for pair-fed and 13 for ethanol-fed, WT $\rightarrow$ C5aR n=7 for pair-fed and 10 for ethanol-fed). Analysis of variance was performed using the general linear models procedure and data were tested for normality using the Shapiro-Wilk test (SAS; Carey, NC). Data were log transformed if necessary to obtain a normal distribution. Follow-up comparisons were made by least square means testing.

3. RESULTS and DISCUSSION

Absence of C5aR prevented ethanol-induced inflammation

During the progression of ethanol-induced liver injury, cellular components of the innate immune system, including Kupffer cells, become activated, secrete pro-inflammatory mediators and contribute to liver injury [1]. Complement also contributes to alcohol-induced liver injury in mice [11, 12, 25, 26]. Complement activation is observed in mouse models of ALD; deposition of C1q and C3b/iC3b/C3c can be detected in the liver [25, 27] and serum levels of C3a are elevated [11]. Serum concentrations of C5a [28] and the expression of C5aR in the liver [29] are also elevated in patients with alcoholic hepatitis. Previous reports have focused on understanding the involvement of initiator complement components, including C1q [25], as well as effector components, including C3, C5, and regulator CD55/DAF [11, 12] in ethanol-induced liver injury. The current report defines the contribution of C5aR to chronic ethanol-induced liver injury in mice.

Given the known pro-inflammatory actions of C5a, we hypothesized that C5aR^{-/-} mice should be protected from ethanol-induced expression of pro-inflammatory mediators and, as a consequence of reduced inflammation, also be protected from ethanol-induced hepatocyte injury. TNF α is a key pro-inflammatory cytokine that induces significant liver injury during ethanol exposure [30, 31]. The expression of TNF α in liver sections was evaluated by immunohistochemistry (Figure 1A) and quantified (Figure 1B) in WT and C5aR^{-/-} mice. In pair-fed WT and C5aR^{-/-} mice, TNF α expression was low and localized near the portal vein. Ethanol feeding to WT mice increased TNF α expression throughout the lobule, with punctate expression along the sinusoids as well as disperse expression near the central lobular zone in cells histologically appearing to be hepatocytes. In contrast, C5aR^{-/-} mice were completely protected from ethanol-induced increases in hepatic TNF α (Figure 1A/B). We have previously reported that TNF α expression in response to ethanol feeding co-localizes predominantly with F4/80, a marker of resident macrophages in the liver [26]. Consistent with increased expression of immunoreactive TNF α , expression of TNF α , IL-6 and MCP-1 mRNA was increased in liver of WT mice after chronic ethanol feeding, compared to pair-fed controls. Expression of these mRNA was blunted in C5aR^{-/-} mice (Figure 1C).

Despite reduced pro-inflammatory cytokines, C5aR^{-/-} mice still have elevated injury

Sustained inflammatory responses in the liver contribute to hepatocellular injury and death, reflected in the elevation in plasma ALT activity [2]. Plasma ALT activity was increased in WT mice after ethanol feeding compared to pair-fed controls (Figure 2A). Unexpectedly, despite the reduction in inflammatory mediators in the C5aR^{-/-} mice, ALT activity was still elevated after ethanol feeding in C5aR^{-/-} mice compared to pair-fed controls (Figure 2A). In addition, ethanol feeding elevated hepatic triglycerides in both C5aR^{-/-} and WT mice (Figure 2B). A well-known consequence of ethanol metabolism by cytochrome P4502E1 (CYP2E1) is increased generation of ROS and lipid peroxidation [32]. Ethanol feeding induced CYP2E1 expression in WT and C5aR^{-/-} mice (Figure 2C); expression was particularly strong throughout zone 3 of the hepatic lobule (Figure 2D). 4-hydroxynonenal (4-HNE) protein adducts, an indicator of oxidative stress, were elevated in livers of ethanol-fed WT and C5aR^{-/-} mice (Figure 2E).

C5aR^{-/-} mice were not protected from markers of cell death

Cell death by both apoptosis and necrosis/necroptosis is a hallmark of alcoholic liver disease. Ethanol-induced increases in the expression of death receptor ligands, in particular TNF α , are implicated in hepatocellular cell death [2, 22, 31]. Increased hepatocellular death can itself also contribute to further inflammatory responses, setting off a cycle of sustained liver injury and inflammation [2]. Since TNF α was not increased in response to chronic ethanol feeding in C5aR^{-/-} mice (Figure 1), we would expect that chronic ethanol-induced cell death would also be ameliorated in C5aR^{-/-} mice. However, unexpectedly, ethanol feeding increased the accumulation of M30, a caspase-dependent cleavage product of CK18 that serves as a hepatocyte-specific measure of apoptosis, in both WT and C5aR^{-/-} mice (Figure 3A/D).

Necroptosis, a caspase-independent form of programmed cell death, also contributes to ethanol-induced liver injury [22, 33]. Because TNF α is an important activator of the necroptotic pathway [22,33], we also expected for necroptosis to be reduced in C5aR^{-/-} mice compared to WT, since they expressed less TNF α in liver after chronic ethanol exposure. Ethanol feeding increased the expression of receptor interacting protein kinase 3 (RIP3), an important effector protein in necroptosis, in the centralobular zone in WT mice; this induction was similar in C5aR^{-/-} mice (Figure 3B/D). Mixed lineage kinase domain-like (MLKL) is a down-stream target of RIP3 in mediating necroptotic cell death [34, 35]. Chronic ethanol feeding increased the phosphorylation of MLKL in WT and C5aR^{-/-} mice compared to pair-fed controls (Figure 3C/D). Therefore, despite the reduction in expression of the death receptor ligand TNF α , C5aR^{-/-} mice were still susceptible to chronic ethanol-induced hepatocellular death by both apoptotic and necroptotic pathways.

Taken together, these data suggest a complex role for C5aR in the pathogenesis of ethanol-induced injury since the absence of C5aR can prevent increased inflammatory responses in the liver, but does not protect from increased oxidative stress and hepatocellular injury. This dichotomy between expression of inflammatory mediators and other measures of injury could be the result of multiple factors, such as an imbalance between anti- and pro-inflammatory mediators or a potential cell-specific role for C5aR in the progression of

chronic ethanol-induced liver injury. Here we have focused on determining whether there are cell-specific contributions of C5aR to the hepatic response to chronic ethanol because of evidence in the literature that C5aR has differential functions in other systems. While in many cell types, C5aR signaling is pro-inflammatory [3,6], C5a can also activate cell protective signaling pathways. For example, C5aR regulates T cell proliferation and differentiation [36], as well as delays apoptosis of neutrophils via activating cell survival pathways [37, 38].

Since ethanol-induced hepatocyte injury was sustained in C5aR^{-/-} mice, despite a decrease in inflammatory responses, we hypothesized that C5aR might protect hepatocytes from ethanol-induced injury. In healthy animals, C5aR expression on hepatocytes is relatively low, however, elevated cellular stress induces C5aR expression on hepatocytes, activating regenerative and proliferative cell pathways in mice [39]. IL-6 is considered to have a primary role in inducing C5aR expression on hepatocytes [40]. Immunohistochemical analysis has also revealed an increase in the expression of C5aR in the liver of patients with alcoholic hepatitis [29].

Myeloid specific C5aR^{-/-} mice were protected from ethanol-induced hepatic inflammation

If C5aR has cell specific effects in the hepatic response to liver injury, then these differences could be revealed by distinguishing the differential roles for C5aR in cells of myeloid and nonmyeloid lineage during ethanol-induced liver injury. To test this hypothesis, myeloid specific C5aR^{-/-} (C5aR^{-/-}→WT) and non-myeloid specific C5aR^{-/-} (WT→C5aR^{-/-}) chimeric mice were generated. WT chimeras (WT→WT) were generated as a control. Adoptive transfer of donor marrow was confirmed in the PBMCs of recipient mice (Fig 4D). During chronic ethanol feeding, ethanol consumption was similar for all chimeras; however, ethanol-fed WT→WT and WT→C5aR^{-/-} chimeric mice gained less weight compared to their pair-fed controls (Table 2).

If C5a is acting in a pro-inflammatory manner on myeloid cells, then myeloid-specific C5aR^{-/-} chimeras should be protected from ethanol-mediated cytokine and chemokine production. In WT→WT chimeric mice, hepatic TNFα was elevated in ethanol-fed mice throughout the parenchyma (Figure 4A/B), similar to the response of non-transplanted mice (Figure 1A). Ethanol-fed C5aR^{-/-}→WT chimeras were protected from increases in TNFα; however, WT→C5aR^{-/-} chimeras had substantially higher levels of TNFα, with a greater intensity near the centralobular zone (Figure 4A/B). Pair-fed WT→C5aR^{-/-} chimeric mice also displayed modestly elevated numbers of TNFα positive cells compared to other pair-fed groups. In WT→WT, but not C5aR^{-/-}→WT, chimeras, ethanol feeding elevated TNFα and MCP-1 mRNA (Figure 4C), similar to non-transplanted mice (Figure 1C). Further, ethanol feeding strongly increased TNFα and MCP-1 mRNA in WT→C5aR^{-/-} mice (Figure 4C). Taken together, these data are consistent with a role of C5aR on myeloid cells in the generation of inflammatory cytokines/chemokines in response to chronic ethanol feeding. It is also interesting to note that the increase in hepatocyte death in the WT→C5aR^{-/-} chimeras (see below) may also contribute, along with the activation of myeloid cells, to the greater elevation in TNFα and MCP-1 mRNA in these chimeras (Figure 4C), as hepatocyte

death can in turn contribute to increased inflammatory responses due to the release of danger signals from injured and dying cells [2].

WT→C5aR^{-/-} chimeric mice had elevated injury and hepatocyte apoptosis after chronic ethanol feeding

In C5aR global knockout mice, there was a divergence between inflammation and liver injury in response to chronic ethanol feeding. If C5aR protects from hepatocyte injury, WT→C5aR^{-/-} chimeras, lacking C5aR on hepatocytes, should have elevated hepatocyte injury after chronic ethanol feeding. In WT→WT chimeras, ethanol increased plasma ALT (Figure 5A). C5aR^{-/-}→WT mice had lower increases in ALT compared to WT→WT chimeras after chronic ethanol feeding. Interestingly, ethanol-fed WT→C5aR^{-/-} chimeras had an even greater elevation in ALT compared to WT→WT chimeras. Hepatic triglycerides were elevated to an equivalent degree in all three chimeric groups after ethanol feeding compared to pair-fed controls (Figure 5B). M30 positive cells were elevated in WT→WT and C5aR^{-/-}→WT chimeras and even further increased in WT→C5aR^{-/-} chimeras (Figure 5C).

Taken together, these data suggest that C5aR signaling on non-myeloid cells protects from hepatocyte injury caused by ethanol feeding. Of interest, hepatocyte apoptosis in chimeric mice lacking C5aR^{-/-} in myeloid cells was similar to that of WT→WT chimeras, despite some reduction in inflammatory cytokines. Continued hepatocellular injury in this context is likely due to the contribution of additional mediators, including oxidative stress and/or increases in LPS [43], that also lead to hepatocyte injury during chronic ethanol feeding.

Activation of C5aR prevented TNF α -induced cell death in hepatocytes cultured in ethanol

Data from the *in vivo* bone marrow transplant studies suggested that C5aR protected hepatocytes from injury in response to chronic ethanol. We made use of hepatocyte cell cultures to confirm that C5a could directly protect hepatocytes from TNF α -induced cytotoxicity in the context of ethanol exposure. C5aR expression was increased in AML12 cells cultured with 50 mM ethanol for 24 h (Figure 6A). Consistent with this response in cultured hepatocytes, the expression of C5aR mRNA in the liver of WT mice was higher in ethanol-fed mice compared to pair-fed controls (Figure 6B).

To further investigate the potential protective role of C5aR in hepatocytes, AML12 hepatocytes (Figure 6C) and primary mouse hepatocytes (Figure 6D) were cultured in ethanol and then challenged with TNF α and/or C5a. Healthy hepatocytes are resistant to TNF α -induced toxicity; indeed, TNF α supports cellular homeostasis and regeneration in healthy hepatocytes [41]. In contrast, exposure to ethanol shifts the response of hepatocytes to TNF α from cell protective signaling to pathways of cell death [18, 41, 42]. Culture of AML12 cells with 50 mM ethanol increased cytotoxicity; this cytotoxicity was further increased when cells were challenged with TNF α (Figure 6C). While treatment with C5a had no effect on cytotoxicity induced by ethanol alone, it completely protected cells from cytotoxicity induced by the combination of ethanol and TNF α (Figure 6C). In order to confirm a role for C5aR in this protective function of C5a, primary hepatocytes were

isolated from WT and C5aR^{-/-} mice and cultured in 50 mM ethanol for 24h (Figure 6D). Ethanol alone and in combination with TNF α increased cytotoxicity in primary hepatocytes from both genotypes (Figure 6D). Treatment with C5a protected hepatocytes from WT, but not C5aR-deficient, mice from ethanol/TNF α -induced cytotoxicity (Figure 6D).

In other cell types, C5a protects from cytotoxicity/apoptosis via activation of the PI3K-Akt [36, 37] signaling pathways. When AML12 cells were cultured with ethanol and then challenged with TNF α , treatment with C5a increased the phosphorylation of Akt, a downstream mediator of the PI3K pathway (Figure 6E). Taken together, these data demonstrate that activation of C5aR protects hepatocytes from cytotoxicity induced by exposure to ethanol/TNF α , likely via similar cytoprotective signaling pathways that are activated by C5aR in other cell types, including CD4⁺ T-cells and neutrophils [36, 37].

4. Conclusion

In summary, the data presented here reveal a complex role for C5aR during ethanol-induced inflammation and hepatocyte injury in mice. Utilizing global C5aR^{-/-} mice and bone marrow chimeric mice, in conjunction with studies in cultured hepatocytes, we identified a protective role for C5aR on hepatocytes against cell death induced by ethanol/TNF α . These data add ethanol-exposed hepatocytes to the growing list of cell types for which C5a stimulates pro-survival/anti-apoptotic signals [36,37,38]. Thus, our data suggest that while C5aR on myeloid cells contributes to enhanced production of inflammatory mediators during chronic ethanol exposure, C5aR on hepatocytes likely counters the injurious effects of inflammatory signals on hepatocyte homeostasis.

It is also important to comment on the clinical implications of these data. ALD remains a serious socioeconomic burden and its prevalence is only increasing [44]. Thus, the need for therapeutic treatments remains high, despite the large effort directed toward this common goal. Because of the differential role of C5aR on cells of myeloid and non-myeloid origin in the liver, it will be an important consideration during in the development of therapeutics to not only dampen the pro-inflammatory function of C5aR on myeloid cells, but also maintain and/or enhance the protective actions of C5aR on hepatocytes.

Acknowledgements

This work was supported in part by NIH grants P20 AA017837, P50 AA024333, U01 AA020821 and R37 AA011876 to LEN; NIH grant R21AA020941 to SR; R01HL109561 and R01AR067182 to MEM; T32 DK00731935 to RLM; and contributions from the Case Western Reserve University/Cleveland Clinic CTSA UL1RR024989.

Abbreviations

4-HNE	4-hydroxy-2-nonenal
ALD	alcoholic liver disease
ALT	alanine aminotransferase
C5aR	C5a Receptor

CCl₄	carbon tetrachloride
CK18	cytokeratin18
CYP2E1	cytochrome P450 2E1
IL-6	interleukin-6
MCP-1	macrophage chemoattractant protein-1
mRNA	messenger RNA
MLKL	mixed lineage kinase domain-like
RIP	receptor-interacting protein kinase
ROS	reactive oxygen species
TG	triglyceride
TNFα	tumor necrosis factor- α

Reference List

- [1]. Dixon LJ, Barnes M, Tang H, Pritchard MT, Nagy LE. Kupffer cells in the liver, *Comprehensive Physiology*. 2013; 3:785–797. [PubMed: 23720329]
- [2]. Gao B, Seki E, Brenner DA, Friedman S, Cohen JI, Nagy L, Szabo G, Zakhari S. Innate immunity in alcoholic liver disease, *American journal of physiology. Gastrointestinal and liver physiology*. 2011; 300:G516–525. [PubMed: 21252049]
- [3]. Ricklin D, Lambris JD. Complement in immune and inflammatory disorders: therapeutic interventions. *Journal of immunology*. 2013; 190:3839–3847.
- [4]. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nature immunology*. 2010; 11:785–797. [PubMed: 20720586]
- [5]. van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. *Cellular microbiology*. 2007; 9:2095–2102. [PubMed: 17590164]
- [6]. Guo RF, Ward PA. Role of C5a in inflammatory responses. *Annual review of immunology*. 2005; 23:821–852.
- [7]. Markiewski MM, DeAngelis RA, Strey CW, Foukas PG, Gerard C, Gerard N, Wetsel RA, Lambris JD. The regulation of liver cell survival by complement. *Journal of immunology*. 2009; 182:5412–5418.
- [8]. Markiewski MM, Mastellos D, Tudoran R, DeAngelis RA, Strey CW, Franchini S, Wetsel RA, Erdei A, Lambris JD. C3a and C3b activation products of the third component of complement (C3) are critical for normal liver recovery after toxic injury. *Journal of immunology*. 2004; 173:747–754.
- [9]. Strey CW, Markiewski M, Mastellos D, Tudoran R, Spruce LA, Greenbaum LE, Lambris JD. The proinflammatory mediators C3a and C5a are essential for liver regeneration. *The Journal of experimental medicine*. 2003; 198:913–923. [PubMed: 12975457]
- [10]. Mastellos D, Papadimitriou JC, Franchini S, Tsonis PA, Lambris JD. A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration. *Journal of immunology*. 2001; 166:2479–2486.
- [11]. Pritchard MT, McMullen MR, Stavitsky AB, Cohen JI, Lin F, Medof ME, Nagy LE. Differential contributions of C3, C5, and decay-accelerating factor to ethanol-induced fatty liver in mice. *Gastroenterology*. 2007; 132:1117–1126. [PubMed: 17383432]
- [12]. Bykov I, Junnikkala S, Pekna M, Lindros KO, Meri S. Complement C3 contributes to ethanol-induced liver steatosis in mice. *Annals of medicine*. 2006; 38:280–286. [PubMed: 16754259]

- [13]. Hopken UE, Lu B, Gerard NP, Gerard C. The C5a chemoattractant receptor mediates mucosal defence to infection. *Nature*. 1996; 383:86–89. [PubMed: 8779720]
- [14]. Strainic MG, Shevach EM, An F, Lin F, Medof ME. Absence of signaling into CD4(+) cells via C3aR and C5aR enables autoinductive TGF-beta1 signaling and induction of Foxp3(+) regulatory T cells. *Nature immunology*. 2013; 14:162–171. [PubMed: 23263555]
- [15]. Emmanuilidis K, Weighardt H, Maier S, Gerauer K, Fleischmann T, Zheng XX, Hancock WW, Holzmann B, Heidecke CD. Critical role of Kupffer cell-derived IL-10 for host defense in septic peritonitis. *Journal of immunology*. 2001; 167:3919–3927.
- [16]. Gibbons MA, MacKinnon AC, Ramachandran P, Dhaliwal K, Duffin R, Phythian-Adams AT, van Rooijen N, Haslett C, Howie SE, Simpson AJ, Hirani N, Gaudie J, Iredale JP, Sethi T, Forbes SJ. Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *American journal of respiratory and critical care medicine*. 2011; 184:569–581. [PubMed: 21680953]
- [17]. Barnes MA, McMullen MR, Roychowdhury S, Pisano SG, Liu X, Stavitsky AB, Bucala R, Nagy LE. Macrophage migration inhibitory factor contributes to ethanol-induced liver injury by mediating cell injury, steatohepatitis, and steatosis. *Hepatology*. 2013; 57:1980–1991. [PubMed: 23174952]
- [18]. Bakhautdin B, Das D, Mandal P, Roychowdhury S, Danner J, Bush K, Pollard K, Kaspar JW, Li W, Salomon RG, McMullen MR, Nagy LE. Protective role of HO-1 and carbon monoxide in ethanol-induced hepatocyte cell death and liver injury in mice. *Journal of hepatology*. 2014; 61:1029–1037. [PubMed: 24946281]
- [19]. Yaman I, Fernandez J, Sarkar B, Schneider RJ, Snider MD, Nagy LE, Hatzoglou M. Nutritional control of mRNA stability is mediated by a conserved AU-rich element that binds the cytoplasmic shuttling protein HuR. *The Journal of biological chemistry*. 2002; 277:41539–41546. [PubMed: 12196519]
- [20]. Pritchard MT, Cohen JJ, Roychowdhury S, Pratt BT, Nagy LE. Early growth response-1 attenuates liver injury and promotes hepatoprotection after carbon tetrachloride exposure in mice. *Journal of hepatology*. 2010; 53:655–662. [PubMed: 20615570]
- [21]. Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology*. 2006; 44:27–33. [PubMed: 16799979]
- [22]. Roychowdhury S, McMullen MR, Pisano SG, Liu X, Nagy LE. Absence of receptor interacting protein kinase 3 prevents ethanol-induced liver injury. *Hepatology*. 2013; 57:1773–1783. [PubMed: 23319235]
- [23]. Ge X, Leung TM, Arriazu E, Lu Y, Urtasun R, Christensen B, Fiel MI, Mochida S, Sorensen ES, Nieto N. Osteopontin binding to lipopolysaccharide lowers tumor necrosis factor-alpha and prevents early alcohol-induced liver injury in mice. *Hepatology*. 2014; 59:1600–1616. [PubMed: 24214181]
- [24]. Smathers RL, Galligan JJ, Shearn CT, Fritz KS, Mercer K, Ronis M, Orlicky DJ, Davidson NO, Petersen DR. Susceptibility of L-FABP-/- mice to oxidative stress in early-stage alcoholic liver. *Journal of lipid research*. 2013; 54:1335–1345. [PubMed: 23359610]
- [25]. Cohen JJ, Roychowdhury S, McMullen MR, Stavitsky AB, Nagy LE. Complement and alcoholic liver disease: role of C1q in the pathogenesis of ethanol-induced liver injury in mice. *Gastroenterology*. 2010; 139:664–674. 674, e661. [PubMed: 20416309]
- [26]. Roychowdhury S, McMullen MR, Pritchard MT, Hise AG, van Rooijen N, Medof ME, Stavitsky AB, Nagy LE. An early complement-dependent and TLR-4-independent phase in the pathogenesis of ethanol-induced liver injury in mice. *Hepatology*. 2009; 49:1326–1334. [PubMed: 19133650]
- [27]. Hoek JB, Pastorino JG. Cellular signaling mechanisms in alcohol-induced liver damage. *Seminars in liver disease*. 2004; 24:257–272. [PubMed: 15349804]
- [28]. Lee H, Whitfeld PL, Mackay CR. Receptors for complement C5a. The importance of C5aR and the enigmatic role of C5L2. *Immunology and cell biology*. 2008; 86:153–160. [PubMed: 18227853]

- [29]. Shen H, French BA, Liu H, Tillman BC, French SW. Increased activity of the complement system in the liver of patients with alcoholic hepatitis. *Experimental and molecular pathology*. 2014; 97:338–344. [PubMed: 25217811]
- [30]. Gobejishvili L, Barve S, Joshi-Barve S, Uriarte S, Song Z, McClain C. Chronic ethanol-mediated decrease in cAMP primes macrophages to enhanced LPS-inducible NF-kappaB activity and TNF expression: relevance to alcoholic liver disease, *American journal of physiology. Gastrointestinal and liver physiology*. 2006; 291:G681–688. [PubMed: 16751174]
- [31]. Yin M, Wheeler MD, Kono H, Bradford BU, Gallucci RM, Luster MI, Thurman RG. Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. *Gastroenterology*. 1999; 117:942–952. [PubMed: 10500078]
- [32]. Lu Y, Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. *Free radical biology & medicine*. 2008; 44:723–738. [PubMed: 18078827]
- [33]. Wang S, Ni HM, Dorko K, Kumer SC, Schmitt TM, Nawabi A, Komatsu M, Huang H, Ding WX. Increased hepatic receptor interacting protein kinase 3 expression due to impaired proteasomal functions contributes to alcohol-induced steatosis and liver injury. *Oncotarget*. 2016; doi: 10.18632/oncotarget.6893
- [34]. Wu XN, Yang ZH, Wang XK, Zhang Y, Wan H, Song Y, Chen X, Shao J, Han J. Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis. *Cell death and differentiation*. 2014; 21:1709–1720. [PubMed: 24902902]
- [35]. Humphries F, Yang S, Wang B, Moynagh PN. RIP kinases: key decision makers in cell death and innate immunity. *Cell death and differentiation*. 2015; 22:225–236. [PubMed: 25146926]
- [36]. Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N, Shapiro VS, Dubyak GR, Heeger PS, Medof ME. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity*. 2008; 28:425–435. [PubMed: 18328742]
- [37]. Perianayagam MC, Balakrishnan VS, King AJ, Pereira BJ, Jaber BL. C5a delays apoptosis of human neutrophils by a phosphatidylinositol 3-kinase-signaling pathway. *Kidney international*. 2002; 61:456–463. [PubMed: 11849385]
- [38]. Perianayagam MC, Balakrishnan VS, Pereira BJ, Jaber BL. C5a delays apoptosis of human neutrophils via an extracellular signal-regulated kinase and Bad-mediated signalling pathway. *European journal of clinical investigation*. 2004; 34:50–56. [PubMed: 14984438]
- [39]. Daveau M, Benard M, Scotte M, Schouft MT, Hiron M, Francois A, Salier JP, Fontaine M. Expression of a functional C5a receptor in regenerating hepatocytes and its involvement in a proliferative signaling pathway in rat. *Journal of immunology*. 2004; 173:3418–3424.
- [40]. Schlaf G, Schmitz M, Rothermel E, Jungermann K, Schieferdecker HL, Gotze O. Expression and induction of anaphylatoxin C5a receptors in the rat liver. *Histology and histopathology*. 2003; 18:299–308. [PubMed: 12507307]
- [41]. Li F, Huang Q, Chen J, Peng Y, Roop DR, Bedford JS, Li CY. Apoptotic cells activate the "phoenix rising" pathway to promote wound healing and tissue regeneration. *Science signaling*. 2010; 3:ra13. [PubMed: 20179271]
- [42]. Pastorino JG, Hoek JB. Ethanol potentiates tumor necrosis factor-alpha cytotoxicity in hepatoma cells and primary rat hepatocytes by promoting induction of the mitochondrial permeability transition. *Hepatology*. 2000; 31:1141–1152. [PubMed: 10796891]
- [43]. Yan AW, Schnabl B. Bacterial translocation and changes in the intestinal microbiome associated with alcoholic liver disease. *World journal of hepatology*. 2012; 4:110–118. [PubMed: 22567183]
- [44]. Kim MS, Ong M, Qu X. Optimal management for alcoholic liver disease: Conventional medications, natural therapy or combination? *World J Gastroenterol*. 2016; 22:8–23. [PubMed: 26755857]

Highlights

- Chronic ethanol-induced liver injury was studied in global C5aR^{-/-}, myeloid specific C5aR^{-/-} and non-myeloid specific C5aR^{-/-} mice.
- Global C5aR^{-/-} mice were protected from ethanol-induced pro-inflammatory cytokine production but still had elevated markers of liver injury
- Non-myeloid specific C5aR^{-/-} had exacerbated liver injury, expression of pro-inflammatory mediators and hepatocyte apoptosis following chronic ethanol feeding.
- C5a increased activation of cell-protective signaling pathways and protected cultured hepatocytes from cytotoxicity induced by ethanol and TNF α

Figure 1. C5aR deficient mice were protected from chronic ethanol-induced increases in cytokines and chemokines

WT and C5aR^{-/-} were allowed free access to ethanol (25d,32%) or pair-fed control diets as described in Materials and Methods. (A) Paraffin-embedded liver sections were deparaffinized followed by immunodetection of TNFα. Nuclei were counterstained with hematoxylin. All images were acquired using a 20X objective. Black arrows indicate nonparenchymal cells, whereas white arrows indicate hepatocytes; solid are strong positive, open are non-stained cells. (B) TNFα-stained areas were quantified using Image-Pro Plus software and analyzed. Values with different alphabetical superscripts were significantly different from each other ($P<0.05$). (C) Expression of TNFα, MCP-1, and IL-6 mRNA was detected in mouse livers using qRT-PCR. * $P<0.05$ for ethanol-fed compared to pair-fed within each genotype.

Figure 2. Chronic ethanol feeding induced liver injury in C67BL/6 and C5aR^{-/-} mice
 WT and C5aR^{-/-} were allowed free access to ethanol containing diets or pair-fed control diets for 25d. (A) ALT activity was measured in plasma. (B) Hepatic triglyceride content was measured in whole liver tissue. (C) CYP2E1 was detected by Western blot. Paraffin-embedded liver sections were deparaffinized followed by immunodetection of (D) CYP2E1 and (E) 4-hydroxynonenal protein adducts. Images were analyzed and quantified using Image-Pro Plus software. Values with different alphabetical superscripts were significantly different from each other ($P < 0.05$).

Figure 3. C5aR deficiency did not prevent markers of ethanol-induced cell death in mouse liver
 WT and C5aR^{-/-} were allowed free access to ethanol containing diets or pair-fed control diets for 25d. Paraffin-embedded livers were deparaffinized followed by (A) M30 (B) RIP3, (C) pMLKL staining. (A/D) M30-positive cells were counted and represented as M30+ cells/20X field. Open black arrows indicate M30+ hepatocytes. Images were acquired using (A/B) 20X and (C) 10X objectives and quantified using Image-Pro Plus software. (B/D) RIP3 positive areas were normalized and expressed as percentage of the total area of the liver. (C/D) pMLKL positive areas were expressed as the sum total area positive/10X field. (D) Values with different alphabetical superscripts were significantly different from each other ($P < 0.05$).

Figure 4. Expression of C5aR in myeloid cells contributed to pro-inflammatory cytokine production in the liver

Bone marrow chimeras were created as described in Materials and Methods. Chimeras were allowed free access to ethanol (25d,32%) or pair-fed control diets. (A) Paraffin-embedded liver sections were deparaffinized followed by immunodetection of TNF α . Nuclei were counterstained with hematoxylin. All images were acquired using a 20X objective. Black arrows indicate non-parenchymal cells, whereas white arrows indicate hepatocytes; solid are strong positive, open are non-stained cells. (B) TNF α -stained areas were quantified using Image-Pro Plus software and analyzed. (C) Expression of TNF α and MCP-1 mRNA was detected in mouse livers using qRT-PCR. Values with different alphabetical superscripts were significantly different from each other ($P < 0.05$). (D) Adoptive transfer of donor marrow was validated from peripheral PBMC RNA using primers specific for C57BL/6 and C5aR $^{-/-}$ alleles.

Figure 5. Non-myeloid C5aR expression was critical for hepatocyte survival following chronic ethanol feeding

Bone marrow chimeras were created as described in Materials and Methods. Chimeras were allowed free access to ethanol containing diets or pair-fed control diets for 25d. (A) ALT activity was measured in plasma. (B) Hepatic triglyceride content was measured in whole liver tissue. (D) Paraffin-embedded liver sections were deparaffinized followed by immunodetection of M30. All images were acquired using a 20X objective; (C) M30-positive cells were counted and represented as M30+ cells/20X field. Open black arrows indicate M30+ hepatocytes. Values with different alphabetical superscripts were significantly different from each other ($P < 0.05$).

Figure 6. C5a suppressed TNF α cytotoxicity in ethanol-sensitized mouse hepatocytes
 AML12 cells and primary WT and C5aR $^{-/-}$ hepatocytes were cultured with or without 50 mM ethanol for 24hrs. (A) C5aR expression was determined in AML12 cells by immunocytochemistry and images were acquired using a 40X objective. Images are representative of 3 independent experiments (B) Expression of C5aR mRNA was detected in livers from WT mice using qRT-PCR. $n=4-6$, $*P<0.05$ for ethanol-fed compared to pair-fed within each genotype. (C) Cytotoxicity was determined in AML12 cells following exposure to 50ng/ml TNF α and/or 10 ng/ml C5a for an additional 24hrs by MTT assay. $n=5$, values with different alphabetical superscripts were significantly different from each other ($P<0.05$). (D) Cytotoxicity was determined in primary cultures of hepatocytes from WT and C5aR $^{-/-}$ mice following exposure to 50ng/ml TNF α and/or 10 ng/ml C5a for an additional 24hrs by MTT assay. $n=5$ $*P<0.05$ compared to WT mice. (E) Phosphorylation of Akt was assessed by Western blot in AML12 cells after culture in 50 mM ethanol for 24 h and then challenged with TNF α and C5a for 15 min. Phosphoproteins were normalized to GAPDH as a loading control. $n=3$, values with different alphabetical superscripts were significantly different from each other ($P<0.05$).

Table 1Animal body weight and food intake in WT and C5aR^{-/-} mice

	WT		C5aR ^{-/-}	
	Pair-fed	EtOH-fed	Pair-fed	EtOH-fed
Body weight (g)				
Initial	18.74 ± 0.52	19.06 ± 0.32	19.15 ± 0.31	19.23 ± 0.21
Final	22.83 ± 1.11 *	22.08 ± 1.28 *	23.31 ± 0.48 *	21.44 ± 0.40 *
Average daily food intake (ml/cage)	paired	21.54 ± 0.43	paired	21.24 ± 0.20

Data are presented as mean ± SEM. Statistical significance was determined by Student's t-test,

* $P < 0.05$ for ethanol-fed compared to pair-fed within each genotype.

Table 2

Animal body weight and food intake in chimeric mice

	WT→WT		C5aR-/- →WT		WT→C5aR-/-	
	Pair-fed	EtOH-fed	Pair-fed	EtOH-fed	Pair-fed	EtOH-fed
Body weight (g)						
Initial	17.03 ± 0.30	17.21 ± 0.38	16.21 ± 0.46	16.54 ± 0.27	15.00 ± 0.69	15.01 ± 0.32
Final	19.13 ± 0.62 *	17.29 ± .56	19.10 ± 0.71 *	17.78 ± 0.44 *	19.05 ± 0.48 *	16.44 ± 0.66
Average daily food intake (ml/cage)	paired	20.96 ± 1.04	paired	21.86 ± 0.51	paired	21.95 ± 2.03

Data are presented as mean ± SEM. Statistical significance was determined by Student's t-test,

* $P < 0.05$ for ethanol-fed compared to pair-fed within each genotype.