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Alcohol intake alters immune responses and promotes CNS viral persistence in mice

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Abstract

Chronic hepatitis C virus (HCV) infection leads to progressive liver disease and is associated with a variety of extrahepatic effects, including central nervous system (CNS) damage and neuropsychiatric impairments. Alcohol abuse can exacerbate these adverse effects on brain and behavior, but the molecular mechanisms are not well understood. This study investigated the role of alcohol in regulating viral persistence and CNS immunopathology in mice infected with lymphocytic choriomeningitis virus (LCMV), a model for HCV infections in humans. Female and male BALB/c mice (n = 94) were exposed to alcohol (ethanol; EtOH) and water (or water only) using a two-bottle choice paradigm, followed one week later by infection with either LCMV clone 13 (causes chronic infection similar to chronic HCV), LCMV Armstrong (causes acute infection), or vehicle. Mice were monitored for 60 days post-infection and continued to receive 24-hour access to EtOH and water. Animals infected with LCMV clone 13 drank more EtOH, as compared to those with an acute or no viral infection. Six weeks after infection with LCMV clone 13, mice with EtOH exposure evidenced higher serum viral titers, as compared to mice without EtOH exposure. EtOH intake was also associated with reductions in virus-specific CD8⁺ T cell frequencies (particularly CD11a^{hi} subsets) and evidence of persistent CNS viremia in chronically infected mice. These findings support the hypothesis that EtOH use and chronic viral infection can result in combined toxic effects accelerating CNS damage and neuropsychiatric dysfunction and suggest that examining the role of EtOH in regulating viral persistence and CNS immunopathology in mice infected with LCMV can lead to a more comprehensive understanding of comorbid alcohol use disorder and chronic viral infection.

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Keywords

Blood brain barrier; ethanol; hepatitis C virus; lymphocytic choriomeningitis virus; neurotoxicity; T-cells

1. Introduction

In addition to being hepatotropic, HCV is also lymphotropic, and it is the virus' ability to infect lymphocytes that appears to contribute to the etiology of HCV's extrahepatic symptoms, including central nervous system (CNS) abnormalities and neuropsychiatric impairments [1, 2]. Fatigue, mood disturbances, and cognitive dysfunction are frequent in patients with chronic HCV infection, including those who have mild liver disease [3–6]. These findings raise the possibility that HCV infection of the brain could be directly related to psychiatric and cognitive impairments, as has been demonstrated with other chronic viral infections [*e.g.*, human immunodeficiency virus (HIV) [7]]. Increasingly, reports of HCV infection of the CNS [8–11] are allowing investigators to test the theory that HCV neuroinvasion may induce CNS inflammation, brain dysfunction, and behavioral deficits (*e.g.*, [12]).

Alcohol and HCV are hypothesized to act synergistically to accelerate extrahepatic consequences, including neuropsychiatric sequelae—symptoms that adversely affect quality of life, potentially alter the courses of disease, and make the addiction and viral infection more challenging to treat [13]. Shared molecular mechanisms underlying the pathological interactions between alcohol and HCV involve: 1) altered T cell responses, 2) compromised blood brain barrier (BBB) function, 3) modulation of glial cell activation, cytokine production and immune signaling, 4) neuronal loss, 5) myelin damage, and 6) brain region-specific metabolic abnormalities [2, 6, 14–18]. Recent studies from our and other laboratories show that either chronic HCV or prolonged use of alcohol and other drugs of abuse augment inflammatory responses (*e.g.*, via T cell mediated mechanisms) and result in increased pro-inflammatory activation and impaired neuropsychiatric function, which has resulted in a paradigm shift linking immune response to addictive behavior (*e.g.*, [19, 20]). The potential synergistic effects of a chronic viral infection and alcohol exposure on CNS immunopathology and behavior are not well defined. Given that the prevalence of HCV infection is up to 30-fold higher in alcoholics compared with the general population [21, 22], a greater understanding of the mechanisms through which alcohol abuse alters immune and CNS response to chronic infection is needed.

Lymphocytic choriomeningitis virus (LCMV) clone 13 has been postulated as a murine model for chronic viral infections such as HCV in humans [23]. The LCMV clone 13 model has led to the development of experimental therapies to treat persistent infections, including HCV [24]. Similar to HCV and HIV, LCMV infection also has effects on brain and behavior. In mice, LCMV injected (either systemically or centrally) at birth or as adults results in long-lasting CNS and behavioral abnormalities, including cognitive deficits [25–27]; however, to date, there have been no studies investigating the effects of alcohol on viral persistence and CNS immunopathology in LCMV-infected mice. Despite considerable

prevalence of alcohol abuse and concurrent HCV infection [28], few studies have examined mechanisms that potentially contribute to heightened burden of disease co-morbidity *in vivo*. Through human studies [29, 30] and the establishment of an animal model of co-morbid viral infection and alcohol abuse, our goal is to identify specific mechanisms by which chronic viral infection and alcohol induce abnormalities in T cell function and contribute to persistent neuropsychiatric impairments.

2. Materials and methods

2.1 Animals and experimental design

The house mouse (*Mus musculus*) represents the natural host for LCMV and provides one of the best-characterized models for studying antiviral T cell responses [31, 32]. In three experiments, female (n = 24) and male (n = 70) BALB/c mice [purchased from the Jackson Laboratories (Bar Harbor, ME); average baseline body weights of 18.78 g and 22.39 g, respectively] were singly-housed (with environmental enrichment) and exposed to alcohol (ethanol; EtOH) and water (or water only) using a two-bottle choice paradigm, followed one week later by infection with either LCMV clone 13 or LCMV Armstrong (an LCMV strain that results in acute infection). Unlike LCMV Armstrong, which causes an acute infection that is cleared within 8–10 days, LCMV clone 13 establishes a chronic infection that persists for one to three months [33]. Control mice were sham infected with vehicle. Sample sizes consisted of 3–6 mice per treatment group, per experiment. Mice were monitored for 60 days post-infection and continued to receive 24-hour access to EtOH and water. Blood samples were collected weekly post-infection to assess changes in viral load liver and liver function over time. Mice were humanely euthanized at the end of the experiments for the collection of splenocytes, blood, and brain samples. All experimental procedures were approved by the Oregon Health & Science University and Veterans Affairs Portland Health Care System Institutional Animal Care and Use Committees. Experiments complied with the ARRIVE guidelines [34] and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2 EtOH intake during 24-h free access, two-bottle choice paradigm

After habituation (water only in both tubes), mice were offered 3% EtOH (v/v) versus water for 4 days. Immediately following 3% EtOH, a choice between 6% (v/v) EtOH and water was offered for 4 days, then 9% (v/v) EtOH for 4 days, then 12% (v/v) EtOH for 4 days, then 15% (v/v) EtOH for 4 days, and then 20% (v/v) EtOH for the duration of the experiment (40 days of 20% EtOH). To facilitate 20% EtOH consumption, mice were exposed to a modified sucrose-fading procedure [35, 36]. During the sucrose-fading procedure, mice were offered sucrose in EtOH drinking tubes according to the following schedule: 10% sucrose for 4 days, 5% sucrose for 4 days, and 2% sucrose for the remainder of the experiment. Control animals (drinking water only) received sucrose in one of their water tubes according to the same schedule. Water and EtOH consumption volumes were recorded daily.

2.3 LCMV infections and determination of viral titers

Mice were infected with either LCMV clone 13 [injected intravenously (IV) at 2×10^6 plaque forming units (PFU)], LCMV Armstrong [injected intraperitoneally (i.p.) at 2×10^5 PFU], or treated with vehicle (DMEM, 2 mM L-Glutamine, 100 U/mL Pen/Strep). Body weights were monitored every other day during the course of infection and blood samples were collected weekly. Serum LCMV levels were calculated by plaque assay on African green monkey kidney (Vero) cell monolayers [37]. Briefly, dilutions of mouse serum were used to infect Vero cells for five days. Plaques were stained using crystal violet and counted to determine viral load.

2.4 Flow cytometry

Single-cell suspensions of mouse splenocytes were stained to identify and analyze cell phenotypes, including LCMV-specific CD8⁺ T-cells. Cells were labeled with fluorescent conjugated anti-CD8 PerCP, anti-CD11a FITC, NP118-tetramer APC [LCMV-derived epitope nucleoprotein (NP) 118 with H-2L^d], and LIVE/DEAD aqua stain. Appropriate single stain controls were applied, and at the end of staining, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline. Antibodies and other flow cytometry reagents were obtained from eBioscience, BioLegend, and BD Biosciences Pharmingen (San Diego, CA); NP118-tetramers were generously provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Data were collected using an LSR Fortessa cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Treestar, Ashland, OR).

2.5 Reverse transcription-polymerase chain reaction (RT-PCR)

PCR reactions were carried out with modifications based on the protocol of Ciurea et al. [38]. Brains were collected immediately following euthanasia, transected to collect hemispheres or microdissected to collect frontal cortices, flash frozen on dry ice, and stored at -80°C until assayed. Brain samples were homogenized with disposable pestles in TRI Reagent (Zymo Research, Irvine, CA) and SV Total RNA Isolation and GoScript Reverse Transcription Systems (Promega, Madison, WI) were used to isolate RNA and create cDNA for downstream RT-PCR. Primers (Integrated DNA Technologies, Coralville, IA) were used to detect the presence of LCMV glycoprotein (Fwd- *TGCCTGACCAAATGGATGATT*; Rev- *CTGCTGTGTTCCCGAAACACT*) and/or nucleoprotein (Fwd- *GCGGCTGACCTTGAGAAGCTGA*; Rev- *TGAGCGCTTGTACAACGTCATT*) within the brain. cDNA created from LCMV clone 13 was used as a positive control. RT-PCR products were analyzed with gel electrophoresis (*i.e.*, run on 2% agarose gels for one hour at a constant voltage of 100V) and visualized using SYBR Safe DNA gel stain (ThermoFisher, Grand Island, NY).

2.6 Aspartate aminotransferase (AST) activity assay

Whole blood was collected from mice and centrifuged to separate serum. Serum samples were stored at -80°C until assayed. Activity levels of AST were determined from samples collected at weeks 2, 4, 6, and 8 using a commercially available colorimetric kit, according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO).

2.6 Statistical analyses

Graphpad Prism, version 6.04 software (La Jolla, CA) was used for all statistical analyses. Student's t-tests were used to evaluate between-group differences in plasma viral titers and brain LCMV expression. Analysis of variance (ANOVA) and post hoc t-tests or Holm-Sidak's multiple comparison tests were conducted to compare EtOH intake, T cell phenotypes, and liver enzymes among the treatment groups. A p-value of less than 0.05 denoted a statistically significant difference. Bar graphs shown in the figures illustrate group means $\pm$ SEM.

3. Results

3.1 Mice infected with LCMV clone 13 exhibit increased EtOH consumption

Mice were exposed to increasing concentrations of EtOH (3% – 20%) for 65 days using a two-bottle (EtOH vs. water) choice paradigm. Voluntary EtOH intake [grams (g) of EtOH consumed per kilograms (kg) of body weight] was measured in mice before and after LCMV infection (Armstrong or clone 13 strains) and in mice without viral infection (vehicle treated). Prior to LCMV or vehicle treatment, mice consumed similar amounts of EtOH (Fig. 1A); however, post-infection, there was a significant effect of LCMV infection on EtOH consumption. A one-way ANOVA detected statistically significant between-group differences in cumulative 20% EtOH consumption ($p = 0.04$). Post hoc tests indicated that mice infected with LCMV clone 13 had greater EtOH intake, as compared to mice infected with LCMV Armstrong ($p = 0.03$) or mice with no viral infection ($p = 0.059$; trend) (Fig. 1B). Given the recent increase in research highlighting sex differences in substance use disorders and the need to reduce gender disparity in preclinical studies, we conducted exploratory analyses to assess potential sex differences in EtOH consumption. Although female mice appeared to drink more EtOH than male mice, the differences were not statistically significant; both female and male mice showed similar patterns of EtOH consumption across treatment groups, as illustrated in the inset graph for Figure 1B.

3.2 Viral clearance is delayed in mice with EtOH exposure

To determine the effects of EtOH consumption on viral clearance, a series of LCMV plaque-forming assays were conducted (*i.e.*, post-infection at weeks 2, 6, and 9). Two weeks following infection with LCMV clone 13, mice consuming EtOH had similar serum levels of virus, as compared to mice drinking water only (Fig. 2A). By six weeks post-infection, EtOH-drinking mice had significantly higher viral titers, as compared to mice without EtOH exposure ($p < 0.05$; Fig. 2B), suggesting that EtOH consumption may have contributed to impaired immune function, and in particular, reduced virus-specific CD8⁺ T cell responses. At the conclusion of the experiment (week 9) average serum LCMV viral titers were not significantly different between the groups; however 75% of mice in the LCMV treatment group had no detectable viremia (fewer than 50 PFU/ml), as compared with 50% of the mice in the LCMV + EtOH treatment group (Fig. 2C).

3.3 Virus-specific CD8⁺ T cell frequencies are reduced in mice with EtOH exposure

Peptide-MHC class I tetramers were used to stain virus-specific CD8⁺ T cells and subsets (CD11a^{hi} cells) in order to assess the effects of EtOH consumption on viral persistence. The percentages of CD8⁺ lymphocytes across treatment groups are shown in Figure 3A. Post hoc analyses did not find a significant effect of EtOH exposure on the frequency of total CD8⁺ T cells in LCMV-infected mice. There was, however, a significant difference in CD8-gated, tetramer-positive CD11a^{hi} T cell subsets across treatment groups ($p < 0.0001$); mice with chronic infection and EtOH had significantly fewer LCMV-specific CD8⁺CD11a^{hi} cells, as compared to chronically infected mice without EtOH ($p < 0.05$) (Fig. 3B).

3.4 Detection of LCMV in brain is more frequent in mice with EtOH exposure

Mouse brain samples were analyzed 60 days post-infection (week 9) by RT-PCR to qualitatively determine whether or not EtOH intake contributed to viral persistence in brain. Brain hemispheres as well as frontal cortices were evaluated because during LCMV infection, the greatest number of persistently infected neurons are found in the cerebral cortex [39]. LCMV RNA detection was significantly more prevalent in brain samples from mice with a history of EtOH exposure, as compared to mice with access to water only ($p < 0.0001$). Figure 4 shows the percentages of mice with and without detectable levels of LCMV RNA in brain.

3.5 Liver AST activity levels are similar in mice with and without EtOH exposure and LCMV clone 13 infection

Table 1 summarizes results from the AST activity assay which was conducted to determine whether differences in EtOH consumption, viral load, and neuroinvasion might be associated with impaired liver function. Two-way ANOVA indicated that AST activity levels were not significantly across time ($p = 0.27$) or treatment groups ($p = 0.64$).

4. Discussion

Alcohol abuse can have a significant impact on clinical course and outcomes in patients with chronic viral infections, such as HCV, including adverse effects on antiviral therapy responses and physical and mental health comorbidities. It has been suggested that; “mouse models are potentially very useful in dissecting elements of the HCV-ethanol relationship” [40], as more research is needed to provide a better understanding of the pathologic and neurotoxic combination of alcohol use and HCV infection. We found that mice chronically infected with LCMV clone 13 drank more EtOH than mice with an acute or no infection (Fig. 1). These preclinical observations parallel reports from the Centers for Disease Control and Prevention which show that adults with HCV recount greater alcohol intake than other adults. After controlling for a number of covariates (*e.g.*, age, sex, race/ethnicity, and education), infection with HCV was significantly associated with both excessive current alcohol drinking and former drinking [41, 42]. Clinical studies also indicate that alcohol consumption is associated with increases in HCV replication and disease progression [43, 44], which is similar to the delayed viral clearance observed in our mouse model (Fig. 2) as well as to recently published work [16, 45].

Increased EtOH consumption in LCMV-infected mice was not associated with evidence of liver damage. Mice infected with LCMV clone 13 with or without a history of EtOH intake had similar AST levels (Table 1), as would be expected based on the cumulative EtOH intake amounts. This finding is also consistent with experiments using the LCMV Armstrong strain [46], as well as with clinical studies which show that approximately 25–30% of adults with HCV have normal levels of serum alanine aminotransferase (ALT) and AST [47–49]. Chronic HCV is associated with fatigue, depression, and cognitive impairment regardless of the stage of liver disease or viral load (ruling out hepatic encephalopathy as a confounding factor); even after HCV can no longer be detected in the periphery, fatigue persists in about one third of adult [50] (reviewed in [51])—highlighting the extrahepatic CNS effects of HCV, including neuropsychiatric impairments.

Within the CNS, LCMV is found primarily in neurons [39, 52]. Kappes et al. [50] demonstrated that LCMV infection of the meninges and ependyma precedes infection of neurons and proposed that a critical parameter in the pathogenesis of neurotropic viruses is the rate of immune activation. Thus, EtOH-induced damage to blood brain barrier integrity [53] and delays or impairments in T cell responses, as our data suggests (*e.g.*, Figs. 2 and 3), may increase vulnerability to persistent CNS infections [54]. Indeed, we observed a greater prevalence of LCMV RNA in brain samples from mice with a history of EtOH exposure, as compared to mice without EtOH exposure (Fig. 4). Our initial experiments focused on the frontal cortex [39], as HCV infects and damages this region, too [11], and alcohol abuse is associated with gray matter reductions in corticostriatal-limbic circuits (see recent meta-analysis; [55]). We found that EtOH consumption exacerbates viral persistence in the frontal cortex—a brain region that when damaged can contribute to functional deficits and hinder addiction recovery efforts.

During infection with LCMV as well as with HCV, evidence supports an important role for CD8⁺ T cell subsets in controlling infection [56, 57]. Given that alcohol has suppressive effects on the generation of viral specific CD8⁺ T cell responses [58], we evaluated the effects of EtOH intake on virus-specific CD8⁺ T cell frequencies. In the absence of EtOH exposure, LCMV infection upregulates CD11a (LFA-1 α chain; effector marker) on murine splenocytes [59] (Fig. 3). However, following EtOH consumption, mice infected with LCMV clone 13 had significantly fewer LCMV-specific CD8⁺CD11a^{hi} cells, as compared to mice without EtOH (Fig. 3B), which suggests that EtOH may impair CD8⁺ responses directed against NP118 epitopes. EtOH consumption in BALB/c mice (without viral infection) also affects CD11c⁺CD8⁺ dendritic cells and alters the pattern of cytokine secretion (*e.g.*, decreases in IL-17A and IFN- γ and increases in IL-13) and effector cell responses [60]. These findings are particularly important in the context of a viral infection, as such changes could contribute to CNS viral invasion and persistence. For instance, immunosuppression (via anti-thymocyte antibody treatment) of LCMV-infected rats resulted in significant increases in viral antigen in the cerebral cortex and hippocampus [61]. Thus, alcohol use may exacerbate the adverse effects of chronic viral infections, such as HCV, on brain and behavior and facilitate access of infected monocytes into the CNS (possibly via a CD8⁺ T-cell mediated mechanism similar to that postulated for alcohol and HIV-1 [62]).

The cause of the reduction in virus-specific CD8⁺ T cells in EtOH-drinking mice remains unclear. Future mechanistic studies will evaluate this pathology further by analyzing the number of virus-specific CD8⁺ T cells over a time course following infection to determine, for example, the potential contribution of impaired proliferation and/or enhanced death of virus-specific CD8⁺ T cells to neuroinvasion. It will similarly be important to characterize EtOH's contribution to LCMV neuroinvasion and persistence on other immune cell types (particularly, CD4⁺ T cells and dendritic cells [63–65]), the rate and duration of infection, brain region-specific vulnerabilities, and behavioral correlates. Given that persistent CNS infection can lead to the generation of CNS autoimmune responses (reviewed in [66]), more research is needed to provide a better understanding for the molecular basis of the neurotoxic combination of alcohol abuse and chronic viral infection (such as HCV).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AST	aspartate aminotransferase
BBB	blood brain barrier
CNS	central nervous system
EtOH	ethanol
HCV	hepatitis C virus
LCMV	lymphocytic choriomeningitis virus, PFU, plaque forming units

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Figure 1.

LCMV clone 13 infection is associated with increased ethanol consumption. Mice were simultaneously offered an EtOH solution and water for 65 days, and consumption volumes were recorded daily. Prior to LCMV or vehicle treatment, mice consumed similar amounts of EtOH (A). Post-infection, there was a significant effect of LCMV infection on EtOH consumption. A one-way ANOVA detected statistically significant between group differences in 20% EtOH consumption. Post hoc tests indicated that mice infected with LCMV clone 13 had greater EtOH intake, as compared to mice infected with LCMV Armstrong or mice with no viral infection (trend) (B). Although female mice appeared to drink more than male mice, the differences were not statistically significant; both female and male mice showed similar patterns of EtOH intake across treatment groups, as illustrated in the inset graph (B).

Figure 2.

Ethanol exposure delays viral clearance in LCMV clone 13 infected mice. Two weeks following infection with LCMV clone 13, mice drinking EtOH had similar serum levels of virus, as compared to mice drinking water only (A). However, by six weeks post-infection, EtOH-drinking mice had significantly higher viral titers, as compared to controls (LCMV clone 13 infection, no EtOH consumption) (B). At the conclusion of the experiment (week 9) average serum LCMV viral titers were similar for both treatment groups, although 75% of mice in the LCMV treatment group had no detectable viremia, as compared with 50% of the mice in the LCMV + EtOH treatment group (C).

Figure 3.

Ethanol drinking alters the induction of virus-specific CD8⁺ T cells. Viable lymphocytes were analyzed 60 days post-infection (week 9) for the cell surface expression of CD8, CD11a, and NP118-tetramer. Cells were identified as CD8 single positive and then divided into NP118-tetramer⁺ T cell subsets. The percentages represent the net percentage (%) of positive cells in the appropriate quadrant. Post hoc analysis did not find a significant effect of EtOH exposure on the frequency of total CD8⁺ T cells in LCMV-infected mice (A). CD8⁺NP118-tetramer⁺CD11a^{hi} frequencies were significantly different across treatment groups; post hoc analysis indicated that mice with chronic infection and EtOH had significantly fewer LCMV-specific CD8⁺CD11a^{hi} cells, as compared to mice without EtOH exposure (B).

Figure 4.

EtOH intake contributes to CNS viral persistence in mice with LCMV clone 13 infection. LCMV detection was significantly more prevalent in brain samples from mice with a history of EtOH exposure ($n = 16$), as compared to mice with access to water only ($n = 17$). Pie charts illustrate the percentages of mice with and without LCMV detected in brain hemispheres (A, B) and LCMV glycoprotein detected in frontal cortices (C, D). Expression of LCMV nucleoprotein was detected in all cortical samples from mice infected with LCMV clone 13 and did not differ between the groups.

Table 1

Aspartate aminotransferase activity from mice with and without EtOH exposure and LCMV clone 13 infection

Treatment group	AST activity			
	Week 2	Week 4	Week 6	Week 8
EtOH	808.95 (240.75)	909.74 (20.75)	1093.08 (49.81)	1090.80 (28.81)
LCMV clone 13	1150.13 (30.44)	959.19 (37.33)	1116.66 (7.99)	964.14 (80.09)
EtOH + LCMV clone 13	891.87 (257.31)	943.98 (3.32)	1074.06 (31.98)	1071.02 (62.77)

Enzyme activity is expressed as mean (SEM) mU/mL; n = 3 mice per group, per time point.

Abbreviations: AST = aspartate aminotransferase; EtOH = ethanol; LCMV = lymphocytic choriomeningitis virus