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Neuroinvasion and cognitive impairment in comorbid alcohol dependence and chronic viral infection: An initial investigation

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ABSTRACT

Viruses that invade the central nervous system (CNS) can cause neuropsychiatric impairments. Similarly, chronic alcohol exposure can induce inflammatory responses that alter brain function. However, the effects of a chronic viral infection and comorbid alcohol use on neuroinflammation and behavior are not well-defined. We investigated the role of heavy alcohol intake in regulating inflammatory responses and behavioral signs of cognitive impairments in mice infected with lymphocytic choriomeningitis virus (LCMV) clone 13. LCMV-infected mice exposed to alcohol had increased peripheral inflammation and impaired cognitive function (as indicated by performance on the novel object recognition test). Initial findings suggest that brain region-specific dysregulation of microglial response to viral infection may contribute to cognitive impairments in the context of heavy alcohol use.

1. Introduction

A number of chronic viral infections [e.g., with the hepatitis C virus (HCV) or the human immunodeficiency virus (HIV)] are associated with a range of neuropsychiatric symptoms, including cognitive and mood impairments (Huckans et al., 2009; Kramer et al., 2002; Loftis et al., 2008; Munjal et al., 2017; Nelligan et al., 2008; Thames et al., 2015; Wang et al., 2017). Viruses that can enter the central nervous system (CNS), such as HCV and HIV, contribute to pathological changes in brain (Forton et al., 2001, 2002; Heeren et al., 2011). Microglia, the resident macrophages of the CNS, mediate key innate immune responses against many viruses [reviewed in (Kaushik et al., 2011)], and microglia-induced neuroinflammation may be particularly responsible for the adverse neuropsychiatric consequences associated with chronic viral infection. For example, in the CNS, HCV infects mainly microglia (Wilkinson et al., 2009) and induces their activation (Grover et al., 2012; Wilkinson et al., 2010). The HCV non-structural 3 (NS3) protein (one of the potent antigens of this virus) activates microglia (Rajalakshmy et al., 2015), and the HCV core protein induces the expression of pro-inflammatory cytokines in microglia (Vivithanaporn et al., 2010). Concurrent with neuroimmune activation, animals implanted with the HCV core protein also display neurobehavioral deficits (Vivithanaporn et al., 2010). Further, microglia/macrophages can become latent viral reservoirs, as has been demonstrated with HIV, making viral eradication more challenging to achieve (Castellano et al., 2017).

Like viral infection with HCV or HIV, chronic alcohol exposure induces inflammatory responses that contribute to the drug's adverse CNS and neuropsychiatric effects. The brain is one of the major target organs of alcohol's actions, and its chronic use results in significant alterations in brain structure and function, including the blood brain barrier (Haorah et al., 2008). This growing body of research shows, for example, that neuroinflammation is evident in the brains of adults with a history of alcohol abuse, with increased activation of microglia and elevated expression of central and peripheral inflammatory factors (Achur et al., 2010; He and Crews, 2008). Animal studies also show that alcohol induces gliosis, and specifically, activation of immune receptors that stimulate microglia and the induction of pro-inflammatory factors (Alfonso-Loeches et al., 2016). Thus, these glial cells are vital players in CNS immune response, and dysregulation of this response, by chronic

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CHOL, total cholesterol; CNS, central nervous system; EtOH, ethanol (alcohol); HCV, hepatitis C virus; HIV, human immunodeficiency virus; Iba1, ionized calcium binding adaptor molecule 1; LCMV, lymphocytic choriomeningitis virus; NORT, novel object recognition test; PBS, phosphate-buffered saline; PFU, plaque forming units; TBIL, total bilirubin

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viral infection or excessive alcohol use, can contribute to brain damage and neuropsychiatric impairments. However, whether microglia activation initiates or exacerbates the neuroinflammatory effects of alcohol in the brain has not yet been determined [reviewed in (Henriques et al., 2018)].

Inflammation induced in the CNS has remote effects on other organs, including the liver. For example, administration of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) to the brain results in the release of CC chemokine, CCL-2, by the liver (Campbell et al., 2005) and increased recruitment of neutrophils in the liver (Campbell et al., 2010), respectively. Moreover, chronic viral infection [e.g., with LCMV clone 13 (Liang et al., 2015) or HCV (Welsch et al., 2017)] and alcohol abuse (Chen et al., 2012; Kim et al., 2005) have direct effects on the liver, including inflammatory processes that likely contribute to related impairments in mood and cognition (D'Mello et al., 2017; Szabo and Lippai, 2014). In other words, inflammation induced in the liver can also impact brain function. Studies in humans report that elevated liver enzymes are associated with brain volume shrinkage (Chen et al., 2012) and increased risk of intracerebral hemorrhage (Kim et al., 2005). In mice with liver inflammation, platelets play a key role in facilitating the activation of microglia and in the development of sickness behavior (also described as depressive-like behavior) (D'Mello et al., 2017). Although studies from our and other laboratories show that either chronic viral infection (e.g., HCV and/or HIV) or prolonged use of alcohol and other drugs of abuse increase inflammatory responses and can result in increased impairments in thinking and mood [e.g., (Loftis et al., 2018)], the effects of a chronic viral infection and alcohol exposure on neuroimmune responses and behavioral outcomes are not well-defined.

The primary aim for this research study was to begin to identify the effects of alcohol use on inflammatory processes that can contribute to impairments in cognitive function during chronic viral infection. It was hypothesized that heavy alcohol exposure would exacerbate the adverse effects of viral infection on brain function, particularly in brain regions known to be vulnerable to the adverse neuroinflammatory effects of viral infection or alcohol exposure [e.g., frontal cortex (Loftis et al., 2016; McCarthy et al., 2017) and hippocampus (Bryant et al., 2017; Ward et al., 2009)]. To test this hypothesis, we investigated the role of heavy alcohol intake in regulating: 1) peripheral and CNS changes in inflammatory responses, and 2) behavioral signs of cognitive impairments in mice infected with lymphocytic choriomeningitis virus (LCMV; a single-stranded RNA virus from the Arenaviridae family) clone 13. LCMV clone 13 is a laboratory-adapted strain of the virus used to model chronic viral infections, such as HCV infection. LCMV clone 13 establishes a chronic infection that persists for up to three months (Matloubian et al., 1990) and is a versatile model system for studying key aspects of CNS immunobiology (Coles et al., 2017; Kunz et al., 2006). We found that mice exposed to alcohol and infected with LCMV clone 13 had increased peripheral inflammation (as measured by liver profile assessment) and impaired cognitive function (as indicated by performance on a memory task), compared to mice with LCMV clone 13 and no alcohol exposure. In LCMV-infected frontal cortex, a history of alcohol exposure may be associated with altered microglia response to viral infection.

2. Materials and methods

2.1. Animals

Thirty, male BALB/cJ mice [purchased from the Jackson Laboratories (Bar Harbor, ME, USA); average baseline body weights (SD) of 28.88 (2.67) grams (g)] were tested in a series of experiments that investigated the role of heavy alcohol intake on peripheral (cohort 1) and CNS (cohort 2) inflammatory effects in mice infected with LCMV clone 13. For cohort 1, mice were assigned to one of three treatment groups: 1) EtOH-/LCMV-: mice without ethanol (EtOH) exposure or LCMV clone 13 infection ($n = 6$), 2) EtOH + /LCMV-: mice with EtOH exposure and without LCMV clone 13 infection ($n = 6$), and 3) EtOH + /LCMV + : mice with EtOH exposure and LCMV clone 13 infection ($n = 6$). For cohort 2, mice were assigned to one of two treatment groups: 1) EtOH-/LCMV + ($n = 6$) and 2) EtOH + /LCMV + ($n = 6$). For cohort 1, attrition within the EtOH + /LCMV + group was due to morbidity and mortality ($n = 2$). For cohort 2, attrition within the EtOH + /LCMV + group was due to morbidity ($n = 2$).

2.2. Ethics

All experimental procedures were approved by the Veterans Affairs Portland Health Care System Institutional Animal Care and Use Committee. Experiments complied with the ARRIVE guidelines (Kilkenny et al., 2010) and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were humanely euthanized during and at the end of the experiments for the collection of blood and brain samples.

2.3. Ethanol exposure

Ethanol was self-administered via a liquid diet, as previously described (Bertola et al., 2013; Lieber et al., 1989) and specifically for BALB/c mice (Mason et al., 2004; Porretta et al., 2012). Mice were singly housed and given 24-h access to liquid EtOH diet [increasing in EtOH concentrations from 1% (2 days) to 2.5% (2 days) and 5% (19 days)] or pair-fed the isocaloric liquid control diet. Both diets were made by mixing Shake and Pore Lieber-DeCarli '82 powder (F1259SP and F1258SP, BioServ, Flemington, NJ, USA) with tap water or tap water with EtOH. The Lieber-DeCarli liquid EtOH diet contained 1 Kcal/ml, of which 35% were fat derived, 11% derived from carbohydrate, 18% derived from protein, and 36% derived from EtOH [5% *w/v* concentration of EtOH (5 g/dL)]. The isocaloric liquid control diet that was used as a control for the Lieber-DeCarli EtOH diet contained 1.0 Kcal/ml–of which 35% was derived from fat, 47% from carbohydrate, and 18% from protein. Liquid diet consumption was measured daily. This method of EtOH exposure induces immunosuppression [e.g., reduced peripheral $CD4^+$ and $CD8^+$ lymphocyte responses (Mason et al., 2004)] similar to effects observed in humans with alcohol use disorders (Zaldivar Fujigaki et al., 2015). In order to ensure the health of all experimental mice and to calculate g/kg of EtOH intake, body weights were monitored and recorded daily.

2.4. LCMV infection

Mice were infected with either LCMV clone 13 [injected intravenously (IV) via the tail vein at 2×10^6 plaque forming units (PFU) in 0.2 mL sterile phosphate-buffered saline (PBS)] or treated with vehicle (0.2 mL of sterile PBS, administered IV). LCMV clone 13 or vehicle injections were administered seven days after the start of either Lieber-DeCarli liquid EtOH diet or isocaloric liquid control diet.

2.5. Liver profile assessment

The VetScan Mammalian Liver Profile reagent rotor (500–1040, Abaxis, Inc., Union City, CA, USA) was used to measure alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP), total bilirubin (TBIL), total cholesterol (CHOL), and blood urea nitrogen (BUN) in mouse serum. Standard procedures provided by Abaxis were used to run the disks utilizing a VetScan Chemistry Analyzer (model 200–1000, Abaxis).

2.6. Immunohistochemistry

Immunohistochemistry on brain sections was performed using a diaminobenzidine (DAB) staining protocol similar to previous reports

Table 1
Liver profiles of mice exposed to liquid EtOH or control diets, with and without infection with LCMV clone 13.

Liver profile[a]	EtOH-/LCMV-(n = 6)	EtOH + /LCMV-(n = 6)	EtOH + /LCMV + (n = 4)	p-value[b]
ALB (g/dL)	2.87 (0.10)	3.13 (0.08)	2.85 (0.37)	0.05
ALP (U/L)	111.50 (27.11)	78.33 (10.97)[c]	70.50 (11.27)[c]	0.008
ALT (U/L)	103.80 (20.27)	167.70 (33.49)[c]	151.30 (39.18)[c]	0.009
BUN (mg/dL)	16.17 (3.37)	17.50 (1.38)	14.0 (1.63)	0.12
CHOL (mg/dL)	96.67 (17.75)	112.70 (25.55)	127.80 (29.24)[c]	0.05
TBIL (mg/dL)	0.27 (0.05)	0.25 (0.06)	0.30 (0)	0.29

[a] Data shown in the table are mean values (SD). Liver profile abbreviations: ALB = albumin, ALP = alkaline phosphatase, ALT = alanine aminotransferase, BUN = blood urea nitrogen, CHOL = total cholesterol, TBIL = total bilirubin, U/L = SI (Système international) units.

[b] p-values from one-way ANOVA are shown; post hoc analyses using Holm-Sidak's multiple comparisons test were conducted for group differences with p-values $\leq .05$.

[c] Post hoc comparisons between control group (i.e., EtOH-/LCMV-) and treatment groups (EtOH + /LCMV- or EtOH + /LCMV +) that were statistically significant ($p < .05$).

[e.g., (Loftis and Janowsky, 2002)], with the exception that sections were pretreated with a 0.05% trypsin antigen retrieval protocol and then stained with primary antibodies against either LCMV [1:1000, VL-4 antibody reacts with LCMV nucleoprotein (NP, a 63 kDa structural protein), BE0106; BioXCell, West Lebanon, NH, USA] or ionized calcium binding adaptor molecule 1 (Iba1, a 17-kDa EF hand protein that is specifically expressed in macrophages/microglia and is upregulated during the activation of these cells) (1:1000, ab17884; Abcam, Eugene, OR, USA). Antibody detection was performed using Vectastain ABC and DAB kits (PK-6100 and SK4100; Vector Laboratories, Inc., Burlingame, CA, USA). LCMV- and Iba1-positive cells within regions of the frontal cortex, hippocampus, and cerebellum were initially visualized and analyzed using a Motic BA200 microscope (Motic, British Columbia, Canada) and Fiji Is Just ImageJ (Fiji) (Schindelin et al., 2012). Each region of interest was selected manually using anatomical reference points from a mouse brain atlas (Franklin and Paxinos, 2008), and analysis was performed by an experimenter blinded to treatment group assignment. The numbers of LCMV- and Iba1-postitive cells were counted in 6-μm serial sagittal sections collected from 0.12 to 0.24 mm lateral to the brain midline and visualized under a 20× objective, according to the mouse brain atlas (Franklin and Paxinos, 2008). Sections were photographed under the microscope and digitized photomicrographs were analyzed using a standardized background subtraction and the threshold function in Fiji. Cell counts were normalized by the area analyzed and are reported as a percent of total area for each brain region. A Leica DM750 upright light microscope (Leica Microsystems, Wetzlar, Germany) was used to capture images for presentation, and image annotation was done using Leica Application Suite X (LAS X) version 3.0.4 software and GNU Image Manipulation Program version 2.10.6 (www.gimp.org).

2.7. Novel Object Recognition Test (NORT)

Cognitive function was assessed using the NORT, with modifications based on published methods (Leger et al., 2013). The behavioral testing occurred across three days and consisted of habituation, training, and retention sessions. During the habituation session, mice were individually habituated to an open-field box (29 cm × 36 cm), without objects for 10 min. Following habituation, two of three objects (i.e., objects of similar size but different in shape) were symmetrically attached to the floor of the box and mice were given 10 min to explore (i.e., training session). Twenty-four hours later mice were placed back in the box for the retention session, and one of the objects was replaced with one not previously encountered. During the retention session, mice were given five minutes to explore. Behavior was recorded and measured using the EthoVision XT 10 video-tracking system (Noldus Information Technology Inc., Leesburg, VA, USA). Exploration of an object was defined as directing the nose at a distance ≤1.5 cm to the object and/or touching it with the nose, while turning around or sitting on the object was not considered exploration. A preference index [i.e., the amount of time spent exploring the novel object over the total time spent exploring both objects (during the retention session)], was used to evaluate cognitive function (a measure of recognition memory). Exploratory preferences during the retention session were also calculated as the E2- and D2-indices according to the method described (Ennaceur and Delacour, 1988) [see also (Bianchi et al., 2006)]. The E2-index is the total exploration time of the retention trial (familiar object + novel object), and the D2-index [(novel object − familiar object)/(novel object + familiar object)] represents the ability to discriminate the novel from the familiar object.

2.8. Statistical analysis

Graphpad Prism, version 8.1.2 software (La Jolla, CA, USA) was used for all statistical analyses. One-way analysis of variance (ANOVA) was used to compare liver profile factors among the treatment groups, followed by Holm-Sidak's multiple comparison post hoc tests, when appropriate. Student's t-tests were used to evaluate between-group differences in immunopositive cell counts, EtOH intake, and cognitive function indices (i.e., NORT parameters). A p-value of < 0.05 denoted a statistically significant difference. Bar graphs shown in the figures illustrate group means ± SEM.

3. Results

3.1. LCMV clone 13 infection and alcohol (ethanol, EtOH) consumption alters liver enzymes and lipids

Mice self-administered the Lieber-DeCarli liquid EtOH diet or the isocaloric liquid control diet seven days before and sixteen days after infection with LCMV clone 13 or control treatment. To evaluate the effects of EtOH consumption and a chronic viral infection on indicators of peripheral inflammation and liver damage (similar to factors assessed in humans), we measured serum levels of ALT, CHOL, and related factors (e.g., ALB). EtOH-exposed mice had higher ALT and lower ALP concentrations when compared to control animals, regardless of LCMV clone 13 infection status (Table 1). LCMV clone 13 infection in EtOH drinking mice was associated with higher levels of CHOL as compared with control animals, an effect not observed in the uninfected mice with EtOH exposure (Table 1). Mice in the EtOH + groups consumed similar amounts of EtOH ($p = .62$) [mean daily consumption (SD): EtOH + /LCMV- = 35.20 (8.36) g/kg, EtOH + /LCMV + = 32.30 (11.28) g/kg].

Table 2
Percentages of immunopositive cells[a] for Iba1 and LCMV in mice with LCMV clone 13 infection, with and without EtOH exposure.

Brain region	Protein	EtOH-/LCMV + (n = 5–6)[c]	EtOH + /LCMV + (n = 4)	p-value[b]
Frontal cortex	Iba-1	5.3 (2.7)	5.6 (2.8)	0.89
	LCMV NP	6.7 (1.7)	3.5 (1.2)	0.01
Hippocampus	Iba-1	4.4 (1.6)	3.2 (3.3)	0.48
	LCMV NP	6.5 (3.4)	4.5 (2.7)	0.39
Cerebellum	Iba-1	4.0 (1.1)	4.6 (2.0)	0.55
	LCMV NP	7.3 (3.5)	6.2 (3.3)	0.57

[a] Cell counts were normalized by the area analyzed, and the data shown are the percentages of immunopositive cells within each brain region of interest (SD).
[b] p-values from t-tests are reported.
[c] Immunostaining of the hippocampus from the EtOH-/LCMV + group was quantifiable in five of six animals.

3.2. Effects of EtOH exposure on brain and behavior during chronic viral infection

3.2.1. Immunodetection of microglia and LCMV in discrete brain regions

Viral neuroinvasion and heavy EtOH use can induce neurodegeneration, which is accompanied by an inflammatory response, including activation of microglia (Potula et al., 2006; Silverstein et al., 2014). To evaluate the effects of EtOH during chronic viral infection with LCMV clone 13, immunohistochemistry was performed using brain sections from mice that self-administered the Lieber-DeCarli liquid EtOH diet or the isocaloric liquid control diet 7 days before and sixteen days after infection with LCMV clone 13. Table 2 shows the percentages of immunopositive cells for the ionized calcium binding adaptor molecule 1 (Iba1; a microglia/macrophage-specific calcium-binding protein) and LCMV nucleoprotein (NP) in discrete brain regions (i.e., frontal cortex, hippocampus, and cerebellum). Statistically significant differences in the numbers of Iba1-immunopositive cells were not detected between LCMV-infected mice with and without EtOH exposure and LCMV. Unexpectedly, within the frontal cortex, mice exposed to EtOH had significantly less LCMV NP immunoreactivity than those without EtOH exposure, an effect not observed in the hippocampus or cerebellum (Table 2). When microglia cells were analyzed relative to LCMV-immunopositive cells, mice with a history of EtOH consumption had higher numbers of Iba1-immunopositive cells in frontal cortex, compared to mice without EtOH exposure (Fig. 1). Group differences in the expression of microglia, relative to LCMV-infected cells, were not observed in the hippocampus or cerebellum (p = .52 and p = .56, respectively).

3.2.2. Behavioral signs of cognitive impairment associated with EtOH exposure and LCMV clone 13 infection

In mice, LCMV injected (either systemically or centrally) at birth or as adults results in long-lasting behavioral abnormalities, including greater latency to move in the open-field and impaired learning on spatial discrimination tasks (Brot et al., 1997; Hotchin and Seegal, 1977, 1978; Kunz et al., 2006). To evaluate the effects of a history of EtOH exposure on cognitive function in mice infected with LCMV clone 13, mice were tested using the NORT, a behavioral test that assesses recognition memory function (Leger et al., 2013). Mice self-administered the Lieber-DeCarli liquid EtOH diet or the isocaloric liquid control diet 7 days prior to infection with LCMV clone 13. Following viral infection, mice continued to receive free access to liquid diets for sixteen days, and behavioral assessments were conducted over a three-day period (habituation, training, and retention sessions). During the retention session of the NORT, analyses indicated that mice in the EtOH-/LCMV + and EtOH + /LCMV + groups spent similar amounts of time exploring the objects (Fig. 2A, B), but mice with EtOH exposure appeared to explore the novel object less frequently than mice with LCMV infection only (Fig. 2C). Unpaired t-tests detected a non-significant trending difference (p = .06) between the groups for the number of explorations and a significant difference in the time it took (latency) to initially explore the novel object (Fig. 2D). The groups did not show statistically significant differences in preference for the novel object (Fig. 2E) or in the ability to discriminate between the objects (Fig. 2F).

4. Discussion

Chronic alcohol abuse impairs not only liver function, but also multi-organ interactions, leading to CNS inflammation and brain pathology (Wang et al., 2010). In our previous work, we found that six weeks after infection with LCMV clone 13, mice with alcohol exposure evidenced higher serum viral titers, as compared to mice without alcohol exposure. Alcohol intake was also associated with reductions in virus-specific CD8 + T-cell frequencies (particularly CD11a^{hi} subsets) and persistent CNS viremia in chronically infected mice (Loftis et al., 2016). In this follow-up study, we conducted a preliminary investigation of the role of alcohol intake, using a paradigm with established inflammatory effects [e.g.,(Massey et al., 2015)] on peripheral and central indices of inflammation and behavioral signs of cognitive impairments in mice infected with LCMV clone 13. As anticipated and consistent with other research, (Massey et al., 2015; Zhang et al., 2017), mice consuming the liquid alcohol diet had higher ALT levels when compared to control animals, regardless of LCMV clone 13 infection status (Table 1). One recent study found that in mice consuming liquid diets of 4% *v*/v EtOH or 4% v/v EtOH plus 0.5% cholesterol, serum ALT, AST, and ALP levels were even higher in the mice with increased cholesterol intake (Li et al., 2019). In other animal models of liver disease, higher levels of ALT (and related enzymes) are accompanied by deficits in cognitive performance (Huang et al., 2009), providing evidence that inflammation induced in the liver can also impact brain function. In contrast to our ALT findings, [and unexpectedly (Zysset et al., 1985)], alcohol use was associated with lower ALP levels in mice with or without LCMV clone 13 infection. Although some animal studies find increased ALP levels following alcohol intake, clinical research indicates that ALP levels have low diagnostic abilities for chronic alcohol misuse and may provide “misleading information” (Pirro et al., 2011).

Cholesterol levels were also evaluated, and we found that concentrations were highest in the group of mice with LCMV clone 13 infection and a history of alcohol exposure (Table 1). Alcohol use is known to affect lipid metabolism. Alcohol consumption not only elevates levels of plasma cholesterol, but also the deposition and crystallization of cholesterol, which activates the NLRP3 inflammasome (Abdul-Muneer et al., 2017) - an inflammasome complex critical for sensing cellular stress signals and participating in the production and activation of pro-inflammatory cytokines including interleukin (IL)-1β and IL-18 (Strowig et al., 2012). Alcohol exposure together with cholesterol intake contributes to more severe liver injury by influencing cholesterol metabolism (Li et al., 2019). Clinical studies similarly show that cholesterol levels are positively associated with alcohol use measures [e.g., scores on the Alcohol Use Disorders Identification Test (AUDIT-C) (Bradley et al., 2016)], but less is known about the effects of alcohol abuse on cholesterol during chronic viral infection. In earlier studies, cholesterol lipid rafts were shown to influence the infectivity of

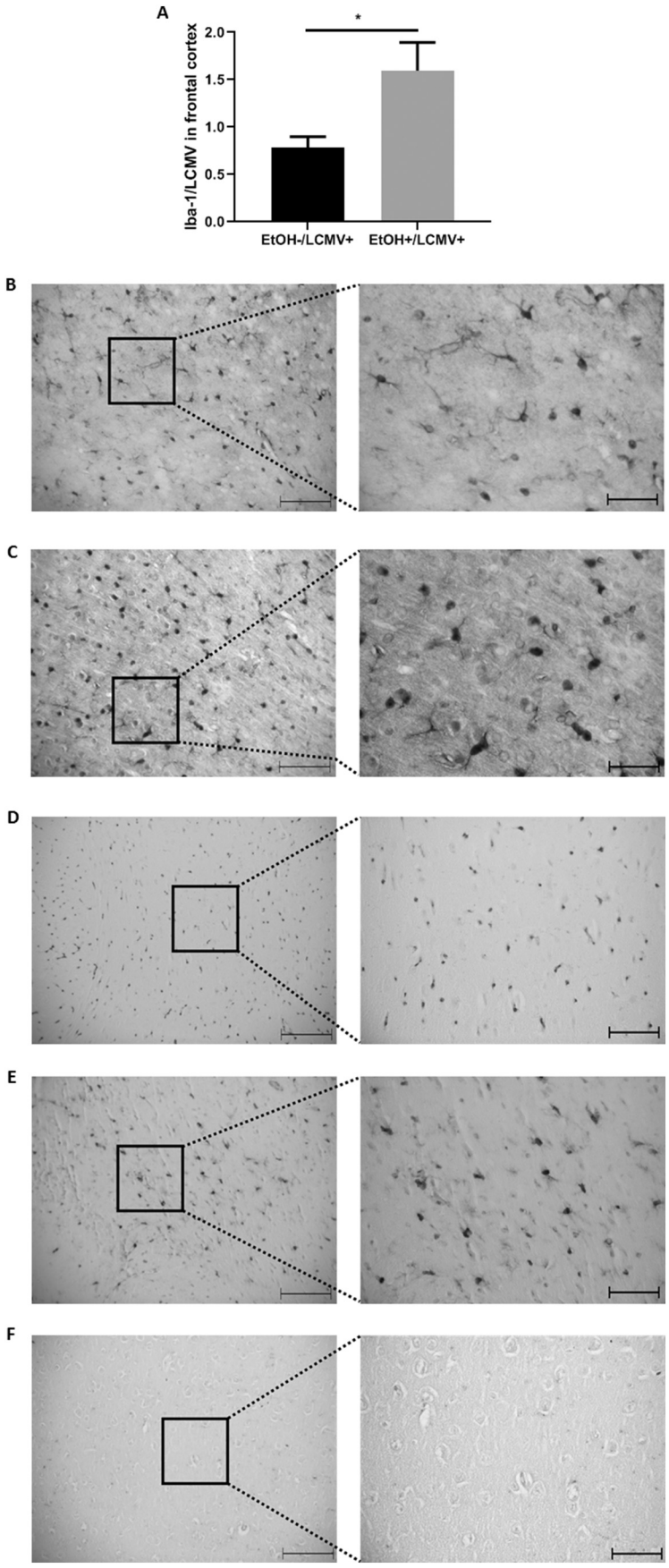

Fig. 1. Microglia expression and LCMV neuroinvasion associated with EtOH consumption during LCMV clone 13 infection. (A) Comparison of EtOH-/LCMV+ ($n = 6$) and EtOH+/LCMV+ ($n = 4$) groups by unpaired *t*-test detected a significant difference in the number of Iba1-immunopositive cells relative to LCMV-immunopositive cells within the frontal cortex ($p = .02$). (B-F) Representative photomicrographs illustrate Iba-1 and LCMV NP immunoreactivity in the frontal cortices from mice with and without LCMV clone 13 infection. 20× and 40× magnification images (left and right, respectively) are shown. (B, C) Iba-1 immunostaining from LCMV-infected mice without (B) and with (C) EtOH exposure. (D, E) LCMV NP immunostaining from LCMV-infected mice without (D) and with (E) EtOH exposure. (F) LCMV NP immunostaining from a control mouse without LCMV infection or EtOH exposure. Large (20×) and small (40×) scale bars within representative photomicrographs indicate 100 μm and 50 μm, respectively.

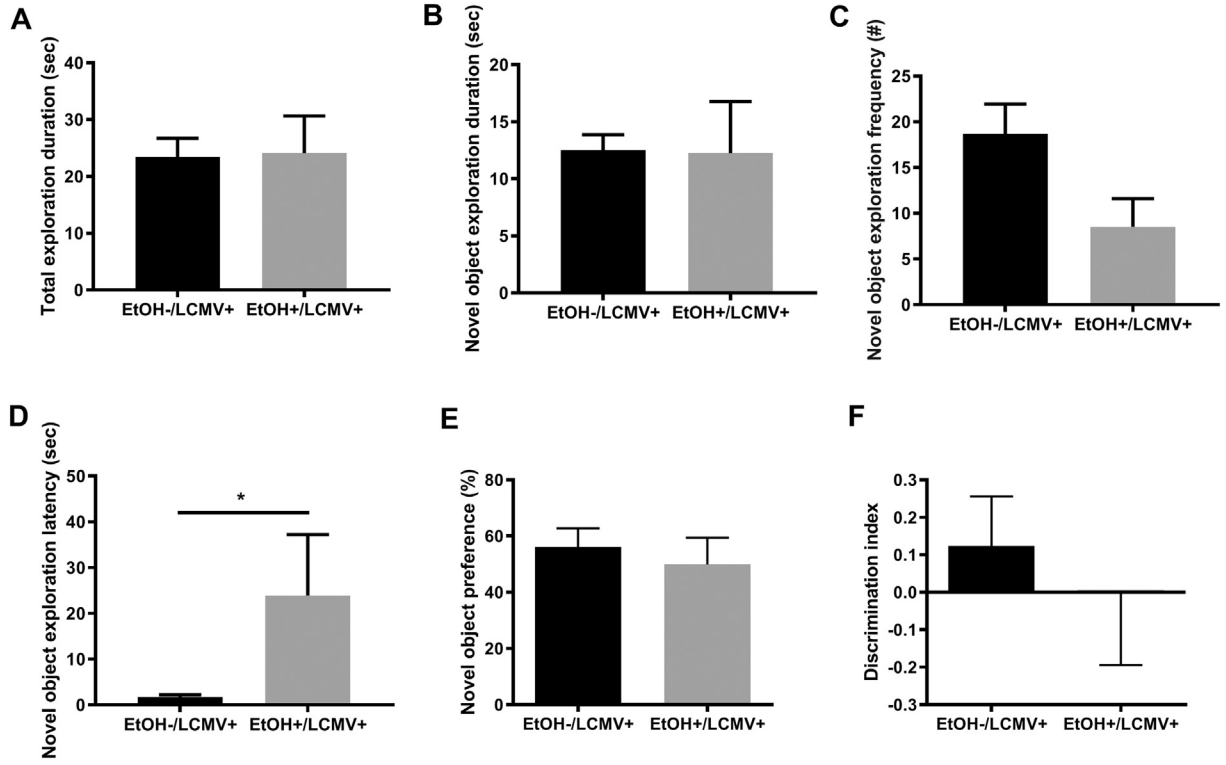

Fig. 2. Cognitive impairment associated with EtOH consumption during LCMV clone 13 infection. Panels show results from the retention session of the novel object recognition test (NORT) conducted with mice in the EtOH-/LCMV+ ($n = 6$) and EtOH+/LCMV+ ($n = 4$) treatment groups. Data were analyzed using unpaired *t*-tests. (A) The total exploration time (i.e., time spent exploring both the familiar and novel objects during the retention trial) was not significantly different between the groups ($p = .92$). (B, C) There was no difference in the duration of time spent exploring the novel object ($p = .95$), but there was a trending difference between the groups for the number of novel object exploration events (i.e., the number of times the mouse came within 1.5 cm of the novel object) ($p = .06$). (D) A statistically significant between-group difference was found for the time it took (latency) to initially explore the novel object ($p = .03$). (E, F) The groups did not show significant differences in preference for the novel object ($p = .59$) or in abilities to discriminate the novel from the familiar object ($p = .59$).

LCMV (Rojek et al., 2008; Shah et al., 2006), and more recently, the biosynthesis of cholesterol was suggested to control T-cell effector function in the CNS (Hoppmann et al., 2015). It is noteworthy that cholesterol and cholesterol oxides can differentially modulate microglia, resulting either in impairment of their immune functions or enhanced neurotoxic actions (Rackova, 2013).

The increased peripheral inflammation we observed in infected mice with a history of alcohol consumption was accompanied by fewer LCMV-immunopositive cells within the frontal cortex, as compared to mice without alcohol exposure. No between-group differences in Iba-1 expression were observed in the frontal cortex. However, when microglia staining was analyzed relative to LCMV infectivity, mice with a history of alcohol consumption had higher numbers of Iba1-immunopositive cells in frontal cortex, compared to mice without EtOH exposure, suggesting a dysregulation of microglial response to viral infection. Differences in Iba-1 and LCMV immunoreactivity were not observed in the hippocampus or cerebellum. Although different brain regions have different susceptibility to viruses (Chen et al., 2019), analysis of subregions within these structures (e.g., CA1, dentate gyrus) or other regions [e.g., subventricular zone (Sun et al., 2014)] and the use of methods to analyze changes in microglial morphology, such as the extent of ramification or the length of the dendritic processes, may provide further insights. Sources of microglial activation include pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), cytokines, chemokines, and other inflammatory mediators. Importantly, CNS glial cells are activated by LCMV and in response, produce pro-inflammatory cytokines and chemokines, such as TNF-α and monocyte chemoattractant protein-1 (MCP-1) (Zhou et al., 2008) - immune factors with putative effects on neuropsychiatric function, particularly in the context of chronic viral infection and alcohol abuse (Blednov et al., 2005; He and Crews, 2008; Huckans et al., 2014). Our observations suggest that, within the frontal cortex, heavy alcohol intake may be associated with greater microglia activation during LCMV neuroinvasion, which could have implications for exacerbating immunopathological damage. It was recently demonstrated that following LCMV infection, resident meningeal macrophages acquire viral antigens and interact with infiltrating cytotoxic T lymphocytes, while concurrently, inflammatory monocytes infiltrate the meninges and persist there for months after viral clearance (Rua et al., 2019), potentially affecting response to future inflammatory insults (e.g., heavy alcohol exposure). In addition to identifying mechanisms that control microglial activation, determining the downstream effects of microglia "over-activation" (e.g., neurodegeneration and cell death) is also crucial and may inform strategies to prevent or repair CNS damage and thereby alleviate the adverse cognitive effects. For example, deviations from microglial homeostasis have been identified as therapeutic targets for cognitive impairment (Wang et al., 2016), major depression (Yirmiya et al., 2015), and other neuropsychiatric disorders.

Long-term, heavy alcohol consumption is associated not only with alterations in microglia activation but also with a range of cognitive deficits, such as increased impulsivity and impaired learning, memory, and decision making (Ehrlich et al., 2012; Molnar et al., 2018; Yang et al., 2014). In mice with LCMV clone 13 infection, a history of heavy alcohol consumption was associated with impaired cognitive function, as compared to mice with no alcohol exposure (Fig. 2). Specifically, mice in the EtOH-/LCMV+ group explored the novel object more frequently and with less initial delay than mice in the EtOH+/LCMV+ group; however, the groups did not show significant differences in preference for the novel object. Given that infection with LCMV clone 13 induces cognitive impairments in the absence of prior alcohol

exposure [e.g., (Gold et al., 1994; Kunz et al., 2006)], it may be that the modest preference observed for the novel object (i.e., 56% for the EtOH-/LCMV+ and 50% for the EtOH+/LCMV+ group) limited our ability to detect further alcohol-induced impairments in cognition. The use of additional comparison groups could help to delineate the effects of alcohol exposure and viral infection on alterations in cognitive abilities and other neuropsychiatric functions. Future studies should also consider the role that alcohol- and virus-induced neuronal loss may have in contributing to these cognitive impairments (Dhanabalan et al., 2018; Sun et al., 2014).

5. Conclusions

Our initial findings suggest that during chronic viral infection, indices of peripheral inflammation are increased by heavy alcohol use and may contribute to cognitive impairments (e.g., as demonstrated by latency to explore novel objects during the NORT). Investigation of the molecular mechanisms of alcohol consumption by gene expression profiling reveals common consequences of alcohol intake and immune activation in both liver and brain (i.e., prefrontal cortex); however there are distinct genomic consequences associated with different types of alcohol consumption (Osterndorff-Kahanek et al., 2013).

The results reported here should be interpreted in consideration of the study limitations, which can be addressed in future research, and include: the lack of comparative controls groups (e.g., LCMV- groups were not included in the experiments that evaluated cognitive function), a relatively small sample size, the use of only male mice, and a single behavioral test to evaluate signs of neuropsychiatric dysfunction. A number of questions remain, such as the role of astrocytes in mediating the effects of viral neuroinvasion and alcohol. Astrocytes are targets for LCMV infection (Bonthius et al., 2002) and are vital for blood brain barrier function, infection control, and phagocytosis (Filous and Silver, 2016; Liddelow and Hoyer, 2016; Russell et al., 2017). Astrocyte pathology is also a common feature of alcohol exposure in humans and animal models [reviewed in (Adermark and Bowers, 2016)].

It is unclear whether viral clearance alone is sufficient to repair neuronal damage and improve brain function following chronic infection. One study tested cognitive behavior in mice following antiviral therapy and found no difference between mice that had been cleared of LCMV and those that remained persistently infected; thus, no reversal of the learning deficit was seen following viral clearance (Brot et al., 1997). In mice congenitally infected with LCMV, neuronal loss occurs after the virus has been cleared, but the mechanisms that contribute to this delayed-onset of cell loss are unknown [reviewed in (Bonthius and Perlman, 2007)]. Similarly, some reports in patients with HCV indicate that peripheral viral clearance is not always associated with improvements in neuropsychiatric function (Dirks et al., 2017; Kuhn et al., 2017; Marcellin et al., 2018) or reductions in liver inflammation (Welsch et al., 2017). Thus, to improve neuropsychiatric outcomes following chronic infection, future research should investigate the impact of peripheral and CNS viral clearance on brain function (under conditions of different levels of alcohol use) in order to clarify the effects of comorbid alcohol abuse and to identify the specific antiviral targets associated with neuropsychiatric impairments (Mathew et al., 2016).

Declaration of Competing Interest

None.

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