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Toll-like receptor 3 dynamics in female C57BL/6J mice: Regulation of alcohol intake

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Abstract

Although there are sex differences in the effects of alcohol on immune responses, it is unclear if sex differences in immune response can influence drinking behavior. Activation of toll-like receptor 3 (TLR3) by polyinosinic:polycytidylic acid (poly(I:C)) produced a rapid proinflammatory response in males that increased alcohol intake over time (Warden et al., 2019). Poly(I:C) produced a delayed and prolonged innate immune response in females. We hypothesized that the timecourse of innate immune activation could regulate drinking behavior in females. Therefore, we chose to test the effect of two time points in the innate immune activation timecourse on every-other-day two-bottle-choice drinking: (1) peak activation; (2) descending limb of activation. Poly(I:C) reduced ethanol consumption when alcohol access occurred during peak activation. Poly(I:C) did not change ethanol consumption when alcohol access occurred on the descending limb of activation. Decreased levels of MyD88-dependent pathway correlated with decreased alcohol intake and increased levels of TRIF-dependent pathway correlated with increased alcohol intake in females. To validate the effects of poly(I:C) were mediated through MyD88, we tested female mice lacking *Myd88*. Poly(I:C) did not change alcohol intake in *Myd88* knockouts, indicating that poly(I:C)-induced changes in alcohol intake are dependent on MyD88 in females. We next determined if the innate immune timecourse also regulated drinking behavior in males. Poly(I:C) reduced ethanol consumption in males when alcohol was presented at peak activation. Therefore, the timecourse of innate immune activation regulates drinking behavior and sex-specific dynamics of innate immune response must be considered when designing therapeutics to treat excessive drinking.

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Keywords

alcohol use disorder; toll-like receptors; sex; females; cytokines; poly(I:C); alcohol; neuroimmune; drinking

1. Introduction

Alcohol increases the expression of proinflammatory cytokines that are transported from blood to brain and the release of inflammatory neuromodulators from glial and neuronal cells within the brain (1). We have hypothesized that positive feedback loops of proinflammatory systemic and central nervous system immune signaling promote excessive alcohol drinking. In support of this hypothesis, alcohol craving and consumption are positively correlated with elevated plasma levels of inflammatory cytokines in human alcoholics (2). Moreover, expression of innate immune components are increased in brains of human alcoholics and alcohol-exposed rodents, with expression correlating with total lifetime consumption and age of drinking onset (1). Therefore, understanding the interplay between immune responses and behavior in the progression to alcohol dependence may reveal new therapeutic targets for treatment of excessive drinking (see (3) for review).

Alcohol affects females differently than males. For instance, females progress from use to addiction more rapidly than males and are more vulnerable to developing neurotoxic and medical consequences of chronic drinking (see (4) for review). Animal models reflect many of the sex differences observed in the human population. Female rats, in general, acquire self-administration of alcohol and escalate their drinking more rapidly than males and show greater reinstatement (4–10). Specifically, in two-bottle choice testing procedures, female mice and rats of various strains show greater alcohol intake and preference than males and females do not decrease intake as much as males when alcohol concentrations are increased (6, 7, 11). Estrogenization of females, which confers a male phenotype on a genetically female brain, reduces ethanol intake compared with normal female rats and results in drinking patterns that are indistinguishable from those of normal male rats (8, 12). Together these studies indicate that sex differences in alcohol drinking behavior are partially attributable to biologic differences between males and females.

Males and females also differ in their innate immune responses (13). For example, innate detection of microorganisms and viruses by toll-like receptors (TLRs) differs between sexes (14). TLRs initiate inflammatory responses via two intracellular signaling transduction cascades: (1) Myeloid differentiation response gene 88 (MyD88)-dependent and (2) TIR-domain-containing adapter-inducing interferon β (TRIF)-dependent. Only TLR3 initiates inflammatory responses solely through the TRIF-dependent pathway. Endosomal TLR3 and TLR7 are more highly expressed in females compared with males, potentially due to lack of X-inactivation resulting in higher expression (15, 16). In contrast, TLR4 is more highly expressed in males (15, 17). After immunization or viral challenge, female peripheral blood mononuclear cells have higher transcript abundance of components of both the TRIF-dependent and MyD88-dependent pathways (e.g. *Ticam1*, *Myd88*, *Irf3*) (18, 19). Yet to date, only a handful of studies have directly examined sex differences in the effects of alcohol on

inflammatory and immune responses (20–25). These reports show that in the absence of alcohol exposure, inflammatory and immune responses are stronger in females than in males (20–22). Chronic ethanol intake also produces a greater inflammatory response in both prefrontal cortex and peritoneal macrophages in intoxicated females compared with males (23, 25, 26).

These sex-specific innate immune differences may also explain sex-dependent drinking behaviors. Knockout of various chemokines, immune receptors, and TLR pathway components result in sex-specific changes in drinking behavior (27–30). For instance, genetic deletion of the chemokine CCL2 lowers ethanol preference only in female mice (27). Moreover, binge alcohol intoxication selectively increases expression of several cytokines and chemokines in the prefrontal cortex and plasma of female adolescent mice—indicating that females are more vulnerable than males to inflammatory effects of binge ethanol drinking (25). Together these studies support the hypothesis that sex-dependent innate immune responses can regulate drinking behavior (31).

Previous studies indicate a role for TLR signaling in the regulation of alcohol intake, specifically through TLR4 (31–35). For instance, activation of TLR4 with lipopolysaccharide increased alcohol intake, whereas siRNA inhibition of downstream TLR4 mediators decreased alcohol consumption (31, 34). However, despite evidence that TLR4 may be important for alcohol responses, a recent study using multiple genetic and pharmacological manipulations showed that TLR4 is not the critical determinant of excessive drinking (29, 36). Therefore, we hypothesize that another innate immune pathway may be important for regulation of excessive alcohol consumption, specifically TLR3-dependent signaling. In the frontal cortex of human alcoholics and mice subjected to chronic voluntary alcohol consumption TLR3 and its downstream signaling components are increased (37–39). TLR3 transcript abundance also correlated with lifetime alcohol consumption in human alcoholics (38). In a recent study we showed that activation of TLR3 in male mice increased alcohol consumption in a TLR3-dependent manner (40). Moreover, we have shown that inhibition of TRIF-dependent signaling components IKKI and TBK1 reduce ethanol consumption in male mice (37). It is not known if TLR3 regulates drinking behaviors similarly in female mice.

In this study, we tested the hypothesis that activation of TLR3-dependent signaling alters drinking behavior differently in female mice compared with male mice. We activated TLR3-dependent signaling by administering the TLR3 agonist polyinosinic-polycytidylic acid (poly(I:C)) to male and female C57BL/6J mice. We found that females showed no change or decreased consumption of ethanol and a delayed and prolonged immune response to poly(I:C). This was very different from what we found in males, which increased their drinking and showed peak activation of immune responses at 3 hours post-injection (40). We established that the time between TLR3 activation and ethanol access can change drinking behavior in both sexes and that this change in behavior is not correlated with a poly(I:C)-induced sickness response or a change in saccharin preference. We also documented changes in innate immune transcripts after chronic poly(I:C) administration during alcohol intake that may mediate changes in alcohol consumption in females. These results establish that

TLR3/TRIF-dependent signaling produces sex-specific effects on alcohol consumption and confirm that sex-dependent innate immune responses can regulate drinking behavior.

2. Materials and Methods

2.1. Mice

Generation of *Myd88* (B6.129P2(SJL)-*Myd88tm1.1DefrJ*, stock #009088) knockout (KO) mice was described previously (41). Mutant strains were purchased from The Jackson Laboratory (Bar Harbor, ME) and were backcrossed onto a C57BL/6J background for 6 generations. Female C57BL/6J mice were purchased from the Jackson Laboratory at 8-10 weeks of age and then bred to maintain our colony. Food and water were available ad libitum. The vivarium was maintained on a 12:12 hour light/dark cycle with lights on at 7:00 a.m. The temperature and humidity of the rooms were kept constant. Behavioral testing began when the mice were at least 2 months old, and mice were weighed every 4 days. All experiments were conducted in isolated behavioral testing rooms. All experiments were approved by the University of Texas at Austin Institutional Animal Care and Use Committee.

2.2 Poly(I:C) administration

To determine the time course of poly(I:C) in females, we injected 5mg/kg poly(I:C) and then sacrificed mice at 3, 24, 48, 72 and 96 hours post injection before qRT-PCR analysis (n=6 per group). For ethanol drinking studies, poly(I:C) was administered intraperitoneally (i.p.) every fourth or fifth day during no alcohol access periods. For the control injection, 0.9% saline (volume matched) was administered to control groups. Single use, sterile needles (27.5 gauge) were used to administer treatment. All injections we made between 8 am and 9 am to animals 8 -16 weeks of age.

2.3. Two-bottle choice every-other-day procedure

Intermittent (every-other-day [EOD]) access to ethanol increases voluntary drinking in rats (42, 43) and mice (44-46). Mice were given EOD access to ethanol (15 or 20% v/v) and water for 24-hour sessions, and water only was offered on off days. The side placement of the ethanol bottles was alternated with each alcohol session. The quantity of ethanol consumed was calculated as g/kg body weight/24 h. Total fluid intake was calculated as g/kg body weight/24h, (n=10/group).

2.4. Preference for saccharin

Mice were tested for saccharin consumption using an every-other-day 2BC protocol in which one bottle contained water and the other contained saccharin solution. Mice were offered saccharin (0.0008%). Saccharin was offered for a series of four poly(I:C) injections (16 days). Bottle positions were changed for each saccharin session (n=10/group).

2.5. Brain Collection

For qRT-PCR experiments brains were quickly harvested and the prefrontal cortex rapidly dissected before being snap frozen in liquid nitrogen. For immunohistochemistry

experiments, mice were anesthetized with isoflourane, transcardially perfused with 0.9% saline until cleared of blood, and then perfused with freshly prepared 4% paraformaldehyde (47) in phosphate-buffered saline (PBS). Then the brain was removed and post fixed in 4% PFA at 4 °C for 24 h followed by cryoprotection for 24 h at 4 °C in 20% sucrose. Brains were then placed in a plastic mold containing optimum cutting temperature compound (OCT, VWR, Radnor, PA) and quickly frozen in isopentane on dry ice.

2.6. qRT-PCR

Total RNA was isolated using the MagMAX-96 Total RNA Isolation Kit (Life Technologies, Grand Island, NY). Total RNA was quantified using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Grand Island, NY) and assessed for quality using the Agilent TapeStation (Agilent Technologies, Santa Clara, CA). All samples passed quality control measures (RIN >8). Reverse transcription was performed using the Applied Biosystems High Capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY). PCR amplification was performed using TaqMan Universal PCR Master Mix and primer pairs and probes (Thermo Fisher Scientific, Grand Island, NY). Relative quantification of mRNA levels was determined using BIORAD software as previously described (48, 49). *Gusb* was selected as an endogenous control to normalize target gene mRNA levels. For all qRT-PCR, six animals were used per group, unless otherwise noted.

2.7. Immunohistochemistry

Thirty-micron sections from brain were permeabilized in 0.1% Triton-X-100 and then blocked in 10% donkey serum (Equitech-Bio, Kerrville, TX) for 1 h at room temperature. Sections were then incubated with primary antibodies overnight at 4 °C. Following three washes in PBS, sections were incubated with secondary antibodies for 2 h at RT. Finally, sections were mounted in 0.2% gelatin, dehydrated, and cover slipped with a DAPI (4',6-diamidino-2-phenylindole)-containing mounting medium (Vector Labs, Burlingame, CA). See Supplemental Table 1 for list of all antibodies used.

2.8. Microscopy

Quantification of immunopositive cells was performed using a Zeiss Axiovert 200 M fluorescent light microscope (Zeiss, Thornwood, NY) equipped with an AxioCam b/w camera. Bilateral images of the PFC (Bregma +2.8 to +2.24) were captured using a 20× objective. For all immunohistochemistry experiments, parameters used for image acquisition were identical across treatments.

2.9. Quantitative Image Analysis

To determine the number of immunopositive cells for each protein type, the 20× images of the prefrontal cortex were separated into quadrants and overlaid with a 10-mm grid. All immunohistochemistry was quantified bilaterally within fixed area frames: PFC (box, 645 μm × 645 μm). Within each fixed area frame, four representative grids were chosen randomly for counting. Total cell counts for each animal were then averaged to give #immunopositive cells/area as previously described (37, 50). Cell counts were performed within each grid by ImageJ plug-in ITCN (<http://rsb.info.nih.gov/ij/plugins/itcn.html>).

2.10 Statistical analysis

Data are reported as mean $\pm$ SEM values, unless otherwise noted. The statistics software program GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) was used to perform 2-way ANOVAs, Pearson correlations and Student's *t*-tests. Drinking data were analyzed by repeated-measures 2-way ANOVA followed by Bonferroni post-hoc tests. Transcript abundance data were analyzed by two-way ANOVA followed by Tukey's HSD post-hoc tests. The Pearson correlation ($\alpha = 0.05$) was used to evaluate correlations between ethanol consumption and transcript abundance. Grubbs test ($\alpha = 0.05$) was used to detect potential outliers. Student's *t*-tests (two-tailed) were used to analyze raw qRT-PCR data and immunohistochemical data.

3. Results

3.1 C57BL/6J females have an extended timecourse for innate immune activation after poly(I:C) in prefrontal cortex

Male and female mammals differ in their innate immune responses, suggesting sex-dependent differences in immune activation could directly regulate behavior (51–53). Therefore, we hypothesized there was a sex-dependent difference in innate immune activation after poly(I:C) in C57BL/6J females, which would be reflected in either the transcript abundance or timecourse for immune activation. To capture changes over time, we measured a time course of poly(I:C) effects on prefrontal cortical transcripts for TRIF- and MyD88-dependent pathway members and for select cytokines and chemokines implicated in behavioral responses to ethanol (see review (54)). In females, poly(I:C) (5mg/kg) dynamically changed transcript levels across time. There were two distinct patterns of change in transcript levels: (1) peak abundance occurring between 24–48 hours post-injection; (2) an initial decrease in abundance at 3 hours post-injection followed by a return to baseline (Fig 1, Supplemental Fig 1). Unlike in studies performed in males, toll-like receptors and signaling components (*Tlr2*, *Tlr3*, *Tlr4*, *Ticam1*, *Myd88*, *Ikk β*) showed no change in abundance at 3 hours post-injection (38, 40, 55). Instead there was a large increase at 24–48 hours post-injection that dissipated by 96 hours post-injection [$F_{\text{treatment} \times \text{time}(4,50)}=49.85$, ($p<0.0001$) for *Tlr2*; 33.25, ($p<0.0001$) for *Tlr3*; 15.32, ($p<0.0001$) for *Tlr4*; 17.13, ($p<0.0001$) for *Ticam1*; 31.05; ($p<0.0001$) for *Myd88*; 17.53, ($p<0.0001$) for *Ikk β*]. Similarly peak abundance for proinflammatory mediators such as *Il6*, *Il1 β* , *Ifnb*, *Ccl2*, and *Ccl5*, occurred at 24–48 hours post-injection [$F_{\text{treatment} \times \text{time}(4,50)}=25.99$, ($p<0.0001$) for *Il6*; 66.55, ($p<0.0001$) for *Il1 β* ; 11.62, ($p<0.0001$) for *Ifnb*; 75.87, ($p<0.0001$) for *Ccl2*; 74.32; ($p<0.0001$) for *Ccl5*]. In contrast, TRIF-dependent pathway components *Irf3* and *Ikke* showed an initial decrease 3 hours post-injection before returning to baseline [$F_{\text{treatment} \times \text{time}(4,50)}=22.97$, ($p<0.0001$) for *Irf3*; 6.06 ($p=0.0005$) for *Ikke*]. These findings suggest that activation of TLR3 signaling in females produced a delayed and prolonged innate immune response.

3.2 Alcohol access at peak innate immune response decreases alcohol intake in females

Since females have a prolonged innate immune response, we hypothesized that time-dependent activation of the innate immune response could regulate drinking behavior. Therefore, we chose to test the effect of two time points in the innate immune activation

timecourse on drinking behavior: (1) peak activation and (2) descending limb of activation after poly(I:C). To determine how alcohol access at peak innate immune activation affects drinking behavior, we administered poly(I:C) (5mg/kg, i.p.) every four days during EOD drinking (Fig 2A). Poly(I:C) on a 4-day injection schedule significantly decreased ethanol intake [Fig 2B, $F_{\text{treatment} \times \text{time}}(9,171)=1.885$, $p=0.057$; $F_{\text{treatment}}(1,19)=24.83$, $p<0.0001$, $F_{\text{time}}(9,171)=23.93$, $p<0.0001$] and preference [Fig 2C, $F_{\text{treatment} \times \text{time}}(9,171)=1.824$, $p=0.06$; $F_{\text{treatment}}(1,19)=11.62$, $p=0.003$, $F_{\text{time}}(9,171)=14.77$, $p<0.0001$]. There was no main effect of poly(I:C) on total fluid intake [Fig. 2D, $F_{\text{treatment} \times \text{time}}(9,171)=2.879$, $p=0.003$; $F_{\text{treatment}}(1,19)=0.397$, $p=0.53$, $F_{\text{time}}(9,171)=4.29$, $p<0.0001$]. The interaction between time and treatment on total fluid intake was attributed to variability in fluid intake across time. Together this suggested that at peak innate immune activation, poly(I:C) decreases alcohol intake.

3.3 Increasing time between TLR3 activation and alcohol access prevents poly(I:C)-induced decreases in alcohol intake in females

To determine how alcohol access at the descending limb of innate immune activation affects drinking behavior we administered poly(I:C) (5mg/kg, i.p.) every five days during EOD drinking, with two days of “water only” between poly(I:C) injection and alcohol access (Fig 3A). In this experiment, poly(I:C) did not decrease ethanol consumption [Fig 3B, $F_{\text{treatment} \times \text{time}}(9,144)=1.256$, $p=0.26$; $F_{\text{treatment}}(1,16)=2.02$, $p=0.17$; $F_{\text{time}}(9,144)=15.47$, $p<0.0001$] or preference [Fig 3C, $F_{\text{treatment} \times \text{time}}(9,144)=1.29$, $p=0.26$; $F_{\text{treatment}}(1,16)=0.19$, $p=0.66$; $F_{\text{time}}(9,144)=18.95$, $p<0.0001$]. Although there was a trend towards increased total fluid intake with poly(I:C) treatment, this effect was not statistically significant [Fig 3D, $F_{\text{treatment} \times \text{time}}(9,144)=0.69$, $p=0.71$; $F_{\text{treatment}}(1,16)=4.60$, $p=0.053$; $F_{\text{time}}(9,144)=3.57$, $p<0.001$]. Because ethanol preference was nearly 0.8 at 15% ethanol, we reran the experiment with 20% ethanol to decrease ethanol preference and avoid a potential ceiling effect. Switching to a higher percentage ethanol dropped preference as expected, but there was still no effect of poly(I:C) on ethanol consumption or preference (Supplemental Fig 2).

Poly(I:C) can induce a transient sickness response; therefore, we estimated the severity of this response by measuring changes in body weight and water intake (31, 55). Poly(I:C) prevented increases in body weight over time for both injection schedules (Supplemental Fig 3A-B). Additionally, poly(I:C) treatment resulted in a small but significant weight loss for the 4-day injection schedule [Supplemental Fig 3A, $F_{\text{treatment} \times \text{time}}(9,171)=4.66$, $p<0.0001$; $F_{\text{treatment}}(1,19)=4.6$, $p=0.045$; $F_{\text{time}}(9,171)=10.12$, $p<0.0001$]. No weight loss was observed for the 5-day injection schedule [Supplemental Fig 3B, $F_{\text{treatment} \times \text{time}}(9,162)=3.07$, $p=0.002$; $F_{\text{treatment}}(1,18)=2.87$, $p=0.10$; $F_{\text{time}}(9,162)=33.67$, $p<0.0001$]. Because we observed poly(I:C)-induced weight loss on the 4-day injection schedule, we next tested if the severity of this sickness behavior was related to changes in alcohol intake. There was no correlation between change in body weight and average alcohol intake in poly(I:C)-treated animals (Supplemental Fig 3C). Taken together, this suggested that changes in alcohol intake due to poly(I:C), for either injection schedule, were not due to a sickness response.

3.4 Time between TLR3 activation and alcohol access produces directional changes in innate immune transcript abundance in females

Since the TLR3/TRIF-dependent pathway regulates escalations in alcohol intake in males (40), we hypothesized that TRIF-dependent pathway transcript abundance would be positively associated with alcohol intake in poly(I:C)-treated females that did not decrease drinking (i.e. 5-day injection schedule). To test this hypothesis, we measured changes in the abundance of transcripts for TRIF-dependent genes, as well as for MyD88-dependent genes and proinflammatory mediators in female mice from both drinking procedures. The values for each transcript, represented as the fold-change from the respective saline group, are shown as a heatmap in Fig 4A. Raw qRT-PCR data for each transcript are available in Supplemental Figure 4. Poly(I:C)-treated females that did not decrease alcohol intake (5-day injection schedule) showed an increase in transcript abundance of TRIF-dependent pathway components (*Tlr3*, *Ticam1*, *Ikke*, *Irf3*), interferon signaling components (*Ifnb*, *Ifnar1*) and MyD88-dependent pathway components (*Tlr2*, *Tlr4*, *Myd88*, *Ikkβ*). In contrast, in females that decreased alcohol intake after poly(I:C) (4-day injection schedule), there was no change in mRNA expression of TRIF-dependent pathway components (*Tlr3*, *Ticam1*, *Ikke*, *Irf3*). Instead, we observed a decrease in transcript abundance for interferon signaling components (*Ifnb*, *Ifnar1*) and MyD88-dependent signaling components (*Myd88*, *Ikkβ*).

To identify potential targets that regulate poly(I:C)-induced directional changes in drinking behavior, we examined correlation between transcript abundance and average ethanol consumption for each injection schedule (Fig 4B). Several transcripts were significantly affected by poly(I:C) and alcohol exposure for each injection schedule, but only five transcripts significantly correlated with ethanol consumption overall. In females that decreased drinking (4-day injection schedule), there was a significant positive correlation between Myd88-dependent transcript levels and alcohol intake in poly(I:C)-treated female mice (Fig 4C-D, *Myd88*: $r = 0.87$, $p = 0.004$; *Ikkβ*: $r = 0.72$, $p = 0.04$). Interestingly, type-1 interferon receptor 1 (*Ifnar1*) was also positively correlated ($r = 0.80$, $p = 0.0018$) with alcohol intake for this injection schedule (Fig 4E). In contrast, in females that did not decrease alcohol intake (5-day injection schedule), there was no significant correlation between Myd88-dependent pathway transcript levels and alcohol intake. However, we observed a significant positive correlation between TRIF-dependent pathway transcript abundance and alcohol intake for this schedule (Fig 4F-G, *Ticam1* $r = 0.93$, $p = 0.0008$; *Ikke* $r = 0.94$, $p = 0.0004$). We verified that changes in transcript levels were associated with corresponding changes in proteins that they encode by immunohistochemistry (Supplemental Figures 5-6). Together this suggested that the TRIF-dependent pathway may regulate increased alcohol intake and the MyD88-dependent pathway be involved in suppression of alcohol intake in females.

3.5 Poly(I:C) decreases alcohol intake in a MyD88-dependent manner in females.

Male and female global *Myd88* knockouts display different drinking behaviors, with increased alcohol consumption in male knockouts but no change in consumption in female knockouts (29), suggesting sex-dependent differences in how MyD88-dependent pathway may be influencing drinking behavior. The strong positive correlation between poly(I:C)-induced decreases in alcohol intake and MyD88-dependent transcript abundance suggested

that decreases in MyD88 may be necessary for poly(I:C)-induced changes in alcohol intake in females. If this were true, then administration of poly(I:C) should not decrease alcohol intake in females on a 4-day injection schedule. To test this hypothesis, we injected female *Myd88* knockout mice (−/−) with poly(I:C) (5mg/kg) every four days while they were consuming 15% ethanol in an EOD procedure for a total of 28 days. Poly(I:C) produced no significant effect on ethanol consumption [Fig 5A, $F_{\text{treatment} \times \text{time}}(6,78)=0.80$, $p=0.57$; $F_{\text{treatment}}(1,13)=0.28$, $p=0.60$; $F_{\text{time}}(6,78)=2.58$, $p=0.02$] or preference [Fig 5B, $F_{\text{treatment} \times \text{time}}(6,78)=0.38$, $p=0.88$; $F_{\text{treatment}}(1,13)=2.2$, $p=0.16$; $F_{\text{time}}(6,78)=3.27$, $p=0.006$]. Although there was a trend towards decreased total fluid intake with poly(I:C) treatment, this effect was not statistically significant [Fig 5C, $F_{\text{treatment} \times \text{time}}(6,78)=0.44$, $p=0.85$; $F_{\text{treatment}}(1,13)=4.69$, $p=0.05$; $F_{\text{time}}(6,78)=1.14$, $p=0.34$]. These results indicate that in females, poly(I:C)-mediated decreases in alcohol intake are dependent on MyD88. One limitation of this knockout study is that wildtype controls were not used in conjunction with *Myd88* knockout mice, making the null effect more difficult to interpret. One interpretation of the data is that poly(I:C)-induced decreases in alcohol intake did not replicate, negating the biological interpretation of the *Myd88* knockout data. However, we have replicated the poly(I:C)-induced decreases in alcohol intake in a separate study (Supplemental Figure 7), increasing the likelihood that poly(I:C) decreases alcohol intake through mechanisms dependent on MyD88 in females.

3.6 Poly(I:C) does not alter saccharin consumption in C57BL/6J females

We also studied consumption of saccharin using an EOD procedure (0.008% saccharin) to determine whether altered taste perception could account for poly(I:C)-induced decreases in alcohol intake in females on a 4-day injection schedule. Poly(I:C) did not change saccharin consumption [Fig 6A, $F_{\text{treatment} \times \text{time}}(3,54)=1.89$, $p=0.14$; $F_{\text{treatment}}(1,18)=0.004$, $p=0.94$; $F_{\text{time}}(3,54)=7.633$, $p<0.001$] or preference [Fig 6B, $F_{\text{treatment} \times \text{time}}(3,54)=0.07$, $p=0.97$; $F_{\text{treatment}}(1,18)=0.38$, $p=0.54$; $F_{\text{time}}(3,54)=24.59$, $p<0.001$]. Total fluid intake was unchanged by poly(I:C) treatment [Fig 6C, $F_{\text{treatment} \times \text{time}}(3,54)=2.87$, $p=0.05$; $F_{\text{treatment}}(1,18)=0.13$, $p=0.71$; $F_{\text{time}}(3,54)=15.07$, $p<0.0001$]. These findings indicate that poly(I:C) does not change detection of sweet taste, suggesting that poly(I:C) does not decrease alcohol intake by perturbing saccharin taste perception in females.

3.7 Alcohol access at peak innate immune response prevents escalation of alcohol intake in males

Presentation of alcohol at peak innate immune activation decreased alcohol intake in females, therefore, we hypothesized that the timecourse of innate immune activation could also regulate drinking behavior in males. If this were true, then males given alcohol access at their peak innate immune response (3 hr post-injection) should decrease alcohol intake. To test this hypothesis, we injected C57BL/6J male mice with poly(I:C) (5mg/kg, i.p) every four days and allowed access to 15% ethanol 3 hours post-injection in an EOD procedure for a total of 32 days. Access to alcohol 3 hours post-injection prevented poly(I:C)-induced escalation of alcohol intake and caused a trend toward decreased alcohol consumption [Fig 7B, $F_{\text{treatment} \times \text{time}}(7,126)=1.38$, $p=0.21$; $F_{\text{treatment}}(1,18)=3.29$, $p=0.08$; $F_{\text{time}}(7,126)=5.04$, $p<0.0001$]. Access to alcohol 3 hours post-injection did not change ethanol preference for poly(I:C)-treated compared with saline-treated animals [Fig 7C, $F_{\text{treatment} \times \text{time}}(7,126)=2.39$,

$p=0.02$; $F_{\text{treatment}}(1,18)=1.16$, $p=0.30$; $F_{\text{time}}(7,126)=2.23$, $p=0.04$]. Total fluid intake was unchanged by treatment [Fig 7D, $F_{\text{treatment} \times \text{time}}(7,126)=1.33$, $p=0.24$; $F_{\text{treatment}}(1,18)=3.78$, $p=0.06$; $F_{\text{time}}(7,126)=8.53$, $p<0.0001$]. These results indicate that access to alcohol at peak innate immune activation prevents previously reported poly(I:C)-induced escalation of alcohol intake in males (40).

4. Discussion

These results, along with our recent study (40), reveal pronounced sex differences in how TLR3 activation regulates drinking behavior. Females have a delayed and prolonged immune response after poly(I:C) in prefrontal cortex. Females decreased drinking when alcohol access occurred at the peak of innate immune activation after poly(I:C). Females did not decrease drinking when alcohol access occurred on the descending limb of innate immune activation. To further support our hypothesis that the time between alcohol access and immune activation regulates behavior, we found that poly(I:C) can prevent escalation of alcohol intake in males when alcohol access occurs at peak innate immune activation. Importantly, these findings support the conclusion that females require different treatment strategies than males due to sex-dependent differences in innate immune signaling.

Several reports have demonstrated clear sex differences in the neurotoxic and inflammatory effects of ethanol. For instance, binge drinking has been associated with sex-specific differences in the frontal, temporal, and cerebellar brain activation during spatial working memory tasks, with females exhibiting poorer sustained attention and working memory performance (56, 57). Animal models confirmed that alcohol preferentially damages the cortex and hippocampus of female compared with male adolescent rats (23). Chronic ethanol intake also causes a greater inflammatory response in both prefrontal cortex and peritoneal macrophages in intoxicated females than in males (23, 25, 26). Moreover, females of many species have sex-dependent expression of innate immune components and launch more robust immune responses than males (13, 58, 59). For instance, TLR3 and TLR7 and its downstream mediators (e.g. IRF5) are more highly expressed in human and mouse female peripheral immune cells (15-17). Immune activation with specific toll-like receptor agonists produce sex-dependent responses with higher expression of TLR7 in female immune cells leading to greater cytokine and interferon production—which is regulated by sex chromosome expression (13). These results indicate that (1) females are more vulnerable to the neurotoxic and cognitive effects of heavy alcohol intake than males, and that (2) inflammatory and immune responses are more marked in females than in males after ethanol or immune stimulation.

Consistent with these findings, we observed sex-dependent differences in immune activation in our study. Following stimulation by poly(I:C), we observed a delayed and prolonged innate immune activation in females, with peak production of proinflammatory mediators (e.g. *Il1 β* , *Il6*) 24-48 hours post-injection. Recent studies in males demonstrated that poly(I:C) significantly upregulated proinflammatory mediators in brain at much earlier timepoints—between 3-8 hours post-injection (55, 60, 61). To exemplify the immune activation delay between sexes we plotted *Il6* mRNA expression across time (Fig 8). The observed delayed immune response for females is consistent with studies on sex-dependent

differences in microglial production of proinflammatory cytokines. After binge-drinking, adolescent females display heightened and different cytokine responses compared to males (25). After traumatic brain injury, males have a more immediate production of proinflammatory cytokines (e.g. *Il1 β* , *Tnfa*) and females have a biphasic and delayed immune response (53). Moreover, at baseline, females show a greater proportion of ramified microglia relative to males alongside heightened basal proinflammatory gene expression (62-64). These findings suggest that females have a lower threshold for activation and launch a delayed but more robust proinflammatory response compared with males.

Poly(I:C)-injected females given access to alcohol at peak innate immune activation decreased alcohol intake. This decrease in drinking was not accompanied by a decrease in body weight or total fluid intake, suggesting that this result was not due to a general sickness response. A drug can alter taste perception, which can change the taste of ethanol leading to changes in consumption. A reduction in the perception of sweet taste or an increase in aversion to bitter taste can reduce ethanol consumption (65). A previous study linked deletions in genes expressed in taste buds and critical to taste transduction to changes in alcohol consumption, revealing that perception of sweet taste, not bitter aversion, is important for voluntary alcohol consumption (65). Therefore, we measured if poly(I:C) decreased alcohol consumption by altering sweet taste perception. Saccharin consumption was unchanged by poly(I:C) on this injection schedule, suggesting poly(I:C) has an ethanol-specific effect.

We hypothesized that the timing between activation of immune signaling and alcohol access could regulate drinking behavior. Extending the time between poly(I:C) injection and alcohol access to accommodate delayed immune activation in females prevented poly(I:C)-induced decreases in alcohol intake. Supporting our hypothesis, males given access to alcohol during peak innate immune activation decreased alcohol intake. Taken together, these findings indicate that the timing of innate immune activation and alcohol access regulates drinking behavior *via* sex-dependent differences in immune activation.

There are two possibilities for how poly(I:C) activates TLR signaling to change alcohol consumption in females: (1) poly(I:C) enters the brain and directly activates TLR3 signaling without involvement of MyD88-dependent signaling; (2) poly(I:C) activates peripheral TLR3, which then leads to induction of inflamlogens that can cross the blood brain barrier and activate central neuroimmune receptors. Our recent study demonstrated that synthetic dsRNA can be detected in both prefrontal cortex and liver after peripheral injection of poly(I:C) (5mg/kg), with the greatest increase in dsRNA signal observed in the liver (40). This suggests that poly(I:C) can directly activate central TLR3 but peripheral immune signaling most likely plays a larger role in regulating poly(I:C)-induced changes in drinking behavior. Therefore, we hypothesized that inflamlogens from the periphery could activate the MyD88-dependent to produce changes in drinking behavior. Moreover, activation of MyD88-dependent signaling has already been shown to regulate alcohol intake in C57BL/6J males and females, making it a probable pathway to regulate drinking behavior after poly(I:C) (31).

In females that decreased alcohol intake, there was no change in TRIF-dependent pathway mRNA or protein levels. However, we did observe a strong positive correlation between MyD88-dependent pathway levels and ethanol consumption in poly(I:C)-treated mice, implying that decreased MyD88-dependent signaling decreases alcohol consumption in females. Poly(I:C) can downregulate TLR4 signaling via a TLR3-dependent mechanism, implying that the protective effects of poly(I:C) may be mediated by decreased TLR4/MyD88-dependent signaling (66, 67). Therefore, we considered whether TLR3-induced decreases in MyD88-dependent signaling could explain the decrease in alcohol intake that we observed in female mice treated with poly(I:C). Poly(I:C) did not change alcohol intake in *Myd88* knockout females, suggesting that poly(I:C) decreases alcohol intake through mechanisms dependent on MyD88.

Poly(I:C)-treated females that escalated drinking similar to saline-treated animals showed increased innate immune transcript abundance for both TLR pathway branches and proinflammatory mediators. Increased abundance of TRIF-dependent pathway transcripts correlated with increased alcohol intake, suggesting that the TRIF-dependent pathway may drive escalations in alcohol intake in females as well. In our recent study, we showed poly(I:C)-induced increases in alcohol intake were, at least partially, dependent on TLR3 in males, supporting a role for TRIF-dependent signaling in excessive drinking behavior in both sexes (40). There is currently no literature on how ethanol treatment itself changes TRIF-dependent signaling in females. However, we hypothesize that chronic alcohol treatment increases expression of TLR3 and TRIF-dependent signaling components (37, 60). Because TLR3 activation resulted in either increased or decreased drinking depending on each sex's innate immune activation timecourse, we hypothesize that drugs like amlexanox, which inhibits IKKI and TBK1 and reduced ethanol consumption in males, may not be as effective in females due to differences in the TLR3 activation timecourse (37).

One limitation of the current study is that the role of sex steroids as was not addressed in either the context of drinking behavior or immune activation. Studies investigating the role of the normal human menstrual cycle in regulation of alcohol consumption are conflicting and indeterminate (4, 68), thus the precise role that the estrous cycle has on drinking behavior in females remains unknown. Some effects of alcohol are dependent on phase of the estrous cycle. Decreases in alcohol consumption during estrus have been identified in hormone-synchronized female rats (69). A previous microdialysis study showed that alcohol caused the greatest increase in dopamine levels in the medial prefrontal cortex during estrus (70). The sedative effects of alcohol are less pronounced in proestrus and diestrus (71). However, the effect of estrous cycle on alcohol consumption, even in hormone-synchronized rodents, is modest, accounting for (at maximum) only 35% of variation in alcohol intake across the cycle (68, 69). Moreover, multiple studies report that estrous cycle does not substantially impact alcohol intake in naturally cycling rodents (10, 72–74). A recent study showed that hormonal fluctuations had little impact on alcohol intake in models of non-dependent drinking and escalated drinking under free-cycling conditions in which female rodents were single-housed (voluntary two-bottle choice drinking in the home cages) and cohabitated in the same housing room as males (74). These are the same experimental parameters used in our study. Taken together, these results suggest that in rodent models of

voluntary alcohol consumption, the estrous cycle is unlikely to significantly impact drinking behavior.

Humans do show strong sex differences in innate immunity, (13). Indeed, receptors for estrogens and estradiol regulate various cells and pathways involved in innate immunity (13). Estradiol has a well-documented biphasic effect on immune function in both preclinical and clinical studies (75, 76), and its pro- or anti-inflammatory effects are dependent upon dose, time and method of testing. For example, high levels of estradiol inhibit NF- κ B and decrease proinflammatory signaling (77, 78). In contrast, low doses of estradiol, comparable to normal circulating levels, increase peripheral concentrations of proinflammatory cytokines (79). A previous study demonstrated in primary endometrial epithelial cells that TLR3 expression is cycle-dependent and treatment with 17 β -estradiol suppressed cytokine and chemokine production after TLR3 stimulation with poly(I:C)—although this effect was not dependent on TLR3 but rather on estrogen receptor alpha expression (80, 81). Putative response elements and oestrogen response elements are also present in the promoters of several innate immunity genes including those in the TLR3 pathway, suggesting that sex steroids may directly cause dimorphic innate immune responses (13, 18). For instance, the lower level of cell surface TLR4 expression observed on female-derived macrophages following LPS challenge relative to that seen on similarly treated male-derived cells might contribute to the reported sex-based differences in LPS tolerance (17). Moreover, sex steroids may regulate innate immune activity in brain. Production of inflammatory cytokines within the brain following intracerebral injection of LPS is attenuated in ovariectomized animals, which is reversed following exogenous estrogen administration (82). Therefore, it is possible that during different phases of estrous, estrogens may suppress the proinflammatory effect of poly(I:C) leading to a reduction in drinking, accounting for at least a portion of the differences in alcohol intake between the 4- and 5-day poly(I:C) injection schedules. If this were true, then the sex differences in innate immune timecourse could not regulate drinking behaviors in males. However, we observed a trend toward reduced alcohol consumption in males when alcohol access was permitted during peak cytokine activation, suggesting that the innate immune timecourse regulates direction of drinking behavior after TLR3 activation. Future studies will need to address how sex steroids influence the observed poly(I:C) innate immune activation sexual dimorphism as well as the role of sex steroids in poly(I:C)-induced changes in alcohol intake.

5. Conclusions

In summary, we demonstrate a novel role for TLR3-dependent signaling in regulation of escalation of ethanol consumption in both males and females. Our study also highlights the role of the MyD88-dependent pathway in suppression of alcohol intake in females. An implication of this study is that the timecourse of innate immune activation regulates drinking behavior. The ability innate immune activation to control alcohol intake highlights an important question that will be addressed in future studies, namely at what time point during innate immune activation does ethanol become rewarding versus aversive? Our data suggests that some level of proinflammatory response is necessary for escalation of alcohol intake, but too much cytokine response can lead to decreased alcohol consumption. Specific pathways and the balance between them seem to be critical regulators of drinking behavior.

Therefore, indiscriminant inhibition of inflammatory pathways may not provide a viable strategy to limit excessive drinking. We conclude that being able to target specific timepoints in innate immune activation—for inhibition of either TRIF- or MyD88-dependent pathways—will permit development of targeted treatment strategies for excessive drinking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights:

- Females have a delayed and prolonged proinflammatory response after poly(I:C).
- Timecourse of cytokine activation regulated alcohol intake in both sexes.
- Increased TRIF-dependent signaling enables escalation of alcohol intake.
- Decreased MyD88-dependent signaling enables suppression of alcohol intake.
- Decreased alcohol intake due to poly(I:C) is dependent on MyD88 in females.

Figure 1: Poly(I:C) produces delayed and prolonged innate immune activation in prefrontal cortex of female C57BL/6J mice.

Heat map showing transcript abundance at five time points after poly(I:C) (5mg/kg) injection. The transcript levels are presented using fold-change values (Log2 format) normalized to endogenous control. The red and green colors indicate high and low expression, respectively. The scale representing the relative signal intensity values is shown on the right. Hierarchical clustering was used in the data analysis. (n=6 per group).

Figure 2: Poly(I:C) decreases alcohol intake when alcohol access occurs at peak innate immune activation in females.

C57BL/6J female mice were injected with saline or poly(I:C) (5mg/kg) every fourth day for a total of ten injections during two-bottle choice, every-other-day drinking (EOD, 15% v/v). (A) 4-day injection schedule; (B) Ethanol (EtOH) intake (g/kg/24 h); (C) preference for EtOH; (D) total fluid intake. Arrows indicate days when animals received injection; (n=10 per group).

Figure 3: Poly(I:C) prevents reductions in alcohol intake when alcohol access occurs after peak innate immune activation in females.

C57BL/6J female mice were injected with saline or poly(I:C) (5mg/kg) every fifth day for a total of ten injections during two-bottle choice, every-other-day drinking (EOD, 15% v/v).

(A) 5-day injection schedule; (B) Ethanol (EtOH) intake (g/kg/24 h); (C) preference for EtOH; (D) total fluid intake. Arrows indicate days when animals received injections; (n=10 per group).

Figure 4: Innate immune transcript abundance correlates with directional changes in alcohol intake in females.

(A) Heat map showing transcript abundance after both the 4-day poly(I:C) injection schedule and 5-day poly(I:C) injection schedule drinking experiments. The transcript levels are presented using fold-change values (Log2 format) normalized to endogenous control and saline-treated controls for each injection schedule. (B) Correlation heatmap of individual transcripts with average ethanol intake. The red and green colors indicate high and low transcript abundance or correlation strength, respectively. For the 4-day injection schedule, there were significant positive correlations between *Myd88* (C) and amount of ethanol

consumed, between *Ikk β* (D) and amount of ethanol consumed and *Ifnar1* (E) and amount of ethanol consumed in poly(I:C)-treated mice. For the 5-day injection schedule, there were significant positive correlations between *Ticam1* (F) and amount of ethanol consumed and between *Ikk ϵ* (G) and amount of ethanol consumed in poly(I:C)-treated mice. ****p<0.0001, ***p<0.0002, **p<0.0021, *p<0.05 for Tukey post-hoc tests and correlations.

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Figure 5: Poly(I:C)-induced decreases alcohol intake are dependent on MyD88.

Mutant female mice (*Myd88*^{-/-}) were injected with saline or poly(I:C) (5mg/kg) every four days for a total of seven injections during a two-bottle choice every-other-day drinking procedure (EOD, 15% v/v). (A) Ethanol (EtOH) intake (g/kg/24 h), (B) preference for EtOH, and (C) total fluid intake (n=10 per group).

Figure 6: Poly(I:C) does not change saccharin taste perception.

C57BL/6/J female mice were injected with saline or poly(I:C) (5mg/kg) every four days for a total of four injections during a two-bottle-choice every-other-day saccharin procedure (EOD, 0.008%). (A) Saccharin consumption (g/kg/24 h), (B) preference for saccharin, and (C) total fluid intake (g/kg/24 h) (n=10 per group).

Figure 7: Poly(I:C) prevents escalation of alcohol intake when alcohol access occurs at peak innate immune activation in males.

C57BL/6J male mice were injected with saline or poly(I:C) (5mg/kg) every fourth day for a total of eight injections during two-bottle choice, every-other-day drinking (EOD, 15% v/v). (A) 3hr delay injection schedule; (B) Ethanol (EtOH) intake (g/kg/24 h); (C) preference for EtOH; (D) total fluid intake. Arrows indicate days when animals received injection; (n=10 per group).

Figure 8: Males and females have different innate immune activation timecourses.

Expression of *Il6* mRNA after poly(I:C) (5mg/kg) injection. Note after poly(I:C), male peak innate immune activation occurs between 3-8 hours post-injection and female peak innate immune activation occurs between 24-48 hours post-injection. mRNA was normalized to saline treated animals as well as the endogenous control, *Gusb*.