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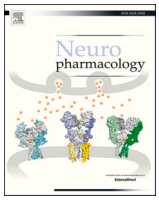

Targeting IL-6 as a novel therapeutic approach for alcohol abstinence – related mechanical allodynia

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ABSTRACT

Alcohol use disorder (AUD) is defined by the emergence of negative affective symptoms during withdrawal. Neuroinflammation is a key contributor to AUD, and it is well known to play an essential role in the pathogenesis of pain states. The chronic-intermittent ethanol two-bottle choice (CIE-2BC) paradigm is well-established to generate alcohol-dependent (Dep) and non-dependent (Non-Dep) mice. Our recent work demonstrated that the CIE-2BC model promotes mechanical allodynia in Dep mice, with these mice developing mechanical allodynia during withdrawal. In this study, we examined the role of interleukin-6 (IL-6) in the development of mechanical allodynia by adapting the CIE-2BC mouse model and employing the von Frey test, *in situ* hybridization (RNAscope), and Multiplex protein analysis of the spinal cord, examining changes in this target including an array of cytokines associated with IL-6 signaling. CIE-2BC escalated alcohol drinking and enhanced mechanical allodynia in Dep versus Non-Dep mice, with Dep females displaying greater alcohol intake. Dep mice displayed increased IL-6 protein in the spinal cord while males additionally had increased *Il6*$^{+}$ cell expression versus Non-Dep controls. Systemic treatment of an IL-6 receptor antibody (IL-6R Ab) did not decrease mechanical allodynia during abstinence. Collectively, these data suggest that alcohol exerts sex-dependent effects on spinal IL-6 levels. However, in our study, blocking IL-6 signaling did not reduce alcohol-associated pain sensitivity in a mouse model of comorbid pain and alcohol dependence.

1. Introduction

Mechanical allodynia, defined as a painful sensation caused by innocuous stimuli, is a hypersensitivity state that can be related to the misuse of numerous substances, including alcohol (Lolignier et al., 2015; Xu et al., 2015). The combination of pain hypersensitivity with negative emotional state during alcohol withdrawal may contribute to relapse in patients with alcohol use disorders (AUD) (Koob, 2021). While both clinical and preclinical studies suggest sex differences in pain sensation during abstinence from drug or alcohol use, few studies have focused on these potential differences in the development of allodynia associated with alcohol withdrawal and the underlying biological mechanisms (Adrienne McGinn et al., 2020; Bergeson et al., 2016a; Brandner et al., 2023; Fillingim and Gear, 2004; Gabel et al., 2023; Kest et al., 2000; Lopez et al., 2023; Morgan et al., 2022; Tonetto et al., 2023; Wakley et al., 2014).

Neuroimmune signaling pathways contribute to the adaptive processes associated with the negative affect phase of the addiction cycle (Cruz et al., 2023; Roberto et al., 2018). Immune cells, including microglia, play a critical role in the trajectory and pathology of AUD (Cruz et al., 2023; Marshall et al., 2013; Warden et al., 2020). Genetic predispositions for high alcohol consumption in humans and rodents have been associated with alteration of both microglial activity and expression of related immune factors, some of which affect synaptic function and plasticity (Bajo et al., 2014; Blednov et al., 2012; Farris et al., 2015; Iancu et al., 2013; Marshall et al., 2013; Pastor et al., 2009; Patel et al., 2019, 2022; Ponomarev et al., 2012; Roberts et al., 2019). We have previously shown that IL-1β, a pro-inflammatory cytokine in

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the interleukin family, is increased in both microglia and neurons of alcohol dependent mice (Patel et al., 2019). While multiple pro-inflammatory cytokines have been associated with alcohol use, interleukin-6 (IL-6) has gained particular interest as a neuroimmune regulator of alcohol effects. A meta-analysis of 23 studies found significantly higher concentrations of IL-6 in people with AUD when compared to healthy controls while there were no significant differences found in the other 13 cytokines (i.e., TNF-α, IL-1, IL-1RA, IL-2, IL-4, IL-5, IL-7, IL-8, IL-10, IL-13, IL-15, IFNγ and sCD14) examined (Moura et al., 2022). A separate systematic review and meta-analysis of changes in circulating cytokines in people with AUD found the largest effect size for increased IL-6 (Adams et al., 2020). Furthermore, alcohol drinking has also been shown to alter IL-6 expression in various tissues across humans and rodents (Blaine et al., 2023; Cruz et al., 2023; Jiang et al., 2021; Roberts et al., 2019).

In addition to its role in AUD, mounting evidence has suggested that IL-6 may play a critical role in the development of pathological pain using various models including cancer pain, neuropathic pain, peripheral nerve injury, spinal cord injury, chemotherapy-induced peripheral neuropathy and inflammatory pain (Guptarak et al., 2013; Zhou et al., 2016). Evidence has shown that blocking IL-6 pathways reduces symptoms associated with several rodent models of peripheral neuropathy (Barney et al., 2019; Huehnchen et al., 2020; Lee et al., 2010; Quarta et al., 2011; Sery et al., 2003). In addition, injection of recombinant IL-6 evoked tactile allodynia in rats, an effect that was attenuated by intrathecal supplement of TGF-β or IL-10 (Yang et al., 2022). Furthermore, IL-6 knockout mice exhibit delayed onset and reduced severity of mechanical allodynia after nerve injury (Ramer et al., 1998; Zhou et al., 2016). At the clinical level, humanized anti-IL-6R monoclonal antibody has exhibited efficacy for numerous disease states such as rheumatoid arthritis, neuromyelitis optica and polyarticular-course juvenile idiopathic arthritis (Araki et al., 2014; Imagawa et al., 2012; Jiao and Guo, 2022; Miyagawa et al., 2014; Zhou et al., 2016). However, while the role of IL-6 has been demonstrated in AUD and pain models separately, it has not been examined in AUD-related pain.

While an abundance of work has investigated brain mechanisms that modulate AUD-related pain, the spinal cord remains largely overlooked. In the absence of alcohol, the spinal cord serves as a critical region in the behavioral output of chronic pain which exerts sex-dependent modulation of pain sensitivity (Lee et al., 2023b; Mogil et al., 2024). Limited information is available on the modulation of neuroimmune factors, including IL-6, as well as microglia activation associated with alcohol at the spinal cord level. Recent reports have demonstrated microgliosis in the spinal cord of mice subjected to excessive alcohol intake (Alexander et al., 2023; Borgonetti et al., 2023b). However, the relationship between this AUD-induced microgliosis and the development of mechanical allodynia has not been characterized. Thus, the present study assessed an array of microgliosis related cytokines to understand their relationship with AUD and pain hypersensitivity.

This study examined spinal neuroimmune changes in alcohol-dependent mice with abstinence-related mechanical allodynia and compared sex differences. We used an established chronic-intermittent ethanol two-bottle choice (CIE-2BC) paradigm to mimic different phenotypes in AUD that include non-dependent (Non-Dep; moderate alcohol consumption) and dependent (Dep; escalated alcohol intake after alcohol vapor exposure) groups in mice (Bajo et al., 2019; Bloch et al., 2022; Gorini et al., 2013; Huitron-Resendiz et al., 2018; Okhuarobo et al., 2023; Patel et al., 2021; Warden et al., 2020). We previously reported increased spinal IL-6 protein levels in Dep mice when compared to Non-Dep mice (Borgonetti et al., 2023b). Thus, herein, we characterized IL-6's potential role in drinking-related mechanical allodynia. Additionally, we examined changes in microglia expressing *Il6* in spinal cord tissue of Non-Dep and Dep mice. We also analyzed various cytokines associated with microgliosis to assess their disruptions. Lastly, we evaluated the efficacy of an IL-6 receptor antibody (IL-6R Ab) to alleviate mechanical allodynia probing the modulation of IL-6 as a potentially attractive strategy to address the development of mechanical allodynia associated with AUD.

2. Material and methods

2.1. Animals

We used adult male (~30 g) and female (~20 g) C57BL/6J mice (10-11 weeks old at the start of our paradigm; The Jackson Laboratory, N = 108). All mice were housed 3-4 per cage in individually ventilated caging under sanitary conditions in light (12 h on, 12 h off; lights on 8:00 p.m.) and temperature (21 ± 2 °C) controlled vivarium rooms with food and water available *ad libitum*. All procedures involving the housing and use of experimental animals were approved by the Scripps Research Institutional Animal Care and Use Committee (IACUC; protocol no. 09-0004 and 09-0006) and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with reporting in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020).

2.2. Experimental design

These experiments included 2 cohorts of mice: Cohort 1 (male n = 33; female n = 15) and Cohort 2 (male n = 31; female n = 29). Cohort 1 underwent the CIE-2BC protocol and von Frey testing without any injections and distinct subsets were used for multiplex cytokine assays (26M/7F) and RNAscope experiments (6M/6F). Cohort 2 underwent the CIE-2BC protocol and von Frey testing with IL-6R Ab or IgG2b isotype control injections in the last round of CIE-2BC. A subset of 16 isotype control animals from Cohort 2 (6M/10F) was used for the multiplex cytokine assay. All Cohorts were tested with von Frey monofilaments to measure the mechanical sensitivity during alcohol withdrawal and after alcohol exposure. Female mice were allowed to cycle freely throughout the study to capture a more physiological relevant effect of IL-6R Ab in females. Estrous cycles were not monitored to maintain sufficient power within current sample sizes.

2.3. Chronic intermittent ethanol vapor exposure - two-bottle choice (CIE-2BC)

The CIE-2BC protocol was conducted as reported previously in two cohorts (Cohorts 1 and 2) (Gorini et al., 2013). Briefly, to establish baseline drinking, 2BC testing was performed 5 days per week for 4 consecutive weeks. The mice were singly housed 30 min before the lights went off and given 2 h access to two drinking tubes (15 % alcohol in water). Following this baseline phase, the mice were divided into two balanced groups with equal alcohol and water consumption. Mice in the dependent (Dep) group were injected intraperitoneally (i.p.) with 1.75 g/kg alcohol + 68.1 mg/kg pyrazole (alcohol dehydrogenase inhibitor) and placed in alcohol vapor chambers (La Jolla Alcohol Research, La Jolla, CA, USA) for 4 days (16 h vapor on, 8 h off). These pyrazole + alcohol injections prior to each exposure to the vapor chambers stabilize blood alcohol levels (BALs) in the target range and decrease variability across exposure and between mice (Griffin, 2014). BALs were determined immediately upon vapor chamber removal on the third day of exposure. Alcohol drip rates were manipulated to maintain BALs of 150–200 mg/dL. Non-dependent (Non-Dep) mice were injected with 68.1 mg/kg pyrazole in saline, placed into identical caging, and exposed to air. Vapor/air exposure was followed by 72 h of abstinence and 5 days of 2BC testing. This regimen was repeated an additional three to four more times for a total of four or five full rounds. In Cohort 2, IL-6R Ab or the isotype control IgG2b were injected i.p. (150 μg/day) daily starting 24 h after the last vapor/air exposure session for 7 days, 30 min prior to the 2BC session (or the time when the 2BC session would have occurred for injections during withdrawal). Before euthanasia, Dep mice were exposed to a single alcohol vapor exposure (16 h), and trunk blood was collected to determine terminal BALs.

This CIE-2BC paradigm produces two groups of mice differing in their alcohol experience: 1) alcohol experienced but not dependent (2BC only, Non-Dep) and 2) alcohol dependent (CIE-2BC, Dep). Previous studies have shown that Dep mice present signs of dependence relative to the other groups, including increased alcohol drinking as well as effects on behavior and sleep (Bajo et al., 2019; Bloch et al., 2022; Gorini et al., 2013; Huitron-Resendiz et al., 2018; Okhuarobo et al., 2023; Patel et al., 2021; Warden et al., 2020).

2.4. Mechanical sensitivity

Mechanical sensitivity was measured using von Frey filaments (North Coast Medical, Gilroy, CA, USA) of ascending force, as previously reported (Borgonetti et al., 2023b). Briefly, mice were first habituated for 5–10 min to the testing apparatus by placing them on a metal mesh stand. A series of monofilaments (0.07, 0.16, 0.4, 0.6, 1.0, 1.4 and 2.0 g) were then applied to the plantar surface of the hind paws with increasing force until a positive withdrawal response was elicited. A positive withdrawal response was recorded if the paw was lifted at least three times in five replicate measurements of the same force. The lowest von Frey filament force that provoked a positive response was considered as the mechanical threshold (g). In Cohort 1, the measurements were conducted 24-, 48-, and 72 h after the last vapor session (labeled as "24H", "48H" and "72H" withdrawal) and then immediately after the last 2BC session (alcohol re-exposure; labeled as "Alcohol".) In Cohort 2, the measurements were conducted immediately after the last 2BC session (labeled as "Alcohol") and then 24-, 48-, and 72 h into withdrawal. Measurements were made by the same blinded experimenter throughout the study and animals were tested under red lights, during their dark (active) phase.

2.5. Multiplex assay of the spinal cord cytokine levels

A total of 49 mice (a subset from Cohort 1 [26M/7F] and a subset of controls from Cohort 2 [6M/10F]) were used to assess cytokine levels in the spinal cord. Mice were anesthetized with 3–5 % isoflurane, transcardially perfused with phosphate-buffered saline (PBS) and decapitated, and the spinal cords were rapidly removed, snap-frozen in isopentane, and stored at −80 °C. Samples were homogenized in 200 μL of complete homogenate RIPA buffer consisting of protease and phosphate inhibitors (1:100; Thermo Fisher, catalog #s 78429 and 78420) in RIPA buffer (Millipore Sigma, catalog #R0278). The homogenate was left seated on ice for 10 min and then centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was collected, and total protein concentration was measured using the DC protein assay (Bio-Rad, Hercules, CA, USA). The samples were aliquoted and stored at −80 °C until use. Duplicates of each sample were tested using a Mouse Cytokine/Chemokine Magnetic Bead Panel kit that included 8 of the 32 preset analyte options, IFN-γ, IL-6, IL-1β, IL-1α, TNF-α, IL-17, IL-10, and MCP-1, (MCYTOMAG-70K; MilliporeSigma, Burlington, MA; Kit lot # 4049592) and the Luminex xMAP System using xPONENT software (Luminex Corporation, Austin, TX), as previously reported (Cruz et al., 2024). The assay was conducted following the manufacturer's protocol. Results are presented as mean ± SEM (pg/mL). The intra-assay coefficients of variation were <10 %.

2.6. RNAscope in situ hybridization on lumbar spinal cord

RNAscope was performed as reported previously (Agoglia et al., 2020; Borgonetti et al., 2023a; Khom et al., 2020; Wolfe et al., 2019). Briefly, a total of 12 mice (Cohort 1; n = 3/sex and experimental group; 1-2 spinal cord sections from each mouse; 4-8 images for each mouse) were deeply anesthetized with 3–5 % isoflurane and transcardially perfused with Z-fix for 5 min. The spinal cord was removed and put in Z-fix solution for 24 h at 4 °C, cryoprotected in sterile 30 % sucrose in PBS for 24-48 h at 4 °C, snap frozen in isopentane and stored at −80 °C until use. Spinal cords were sliced on a cryostat in 20 μm-thick sections. The sections were mounted on SuperFrost Plus slides (Fisher Scientific, catalog no. 1255015) and stored at −80 °C until further processing. *In situ* hybridization (ISH) was performed using the RNAscope multiplex fluorescent detection kit (ACD, catalog no. 323110), including a target retrieval protocol under RNase-free conditions as outlined in the manufacturer's manual (ACD, catalog no. 32200 and 322381). The slides were incubated with the following probes: *Il6* (ACD, catalog no. 315891; encoding interleukin-6) and *Itgam* (ACD, catalog no. 311491-C3; encoding integrin alpha M; microglial marker) for 2 h at 40 °C. A negative control (ACD, catalog no. 320871) was run in tandem. The signal was amplified to detect RNA transcripts for a total of 90 min at 40 °C according to the manufacturer's instructions. Finally, the slides were mounted with DAPI-containing Vectashield (Fisher Scientific, catalog no. NC9029229).

2.7. Imaging and analysis

A total of 4–8 images of the lumbar spinal cord for each mouse were taken in similar points using a Zeiss LSM 780 laser scanning confocal microscope (40 × oil immersion, 1024 × 1024, tile scanning of spinal cord 5 μm z-stacks) and all microscope settings were held constant between the experiments during image acquisition. Analysis and quantification were performed as previously described using CellProfiler (Jones et al., 2008; Wolfe et al., 2019). The software CellProfiler was used to identify nuclei based on DAPI staining. Nuclei were considered positive (+) for the probe if the corresponding fluorescent signal was present after background subtraction (negative control). The percentage of positive nuclei was calculated and then normalized to the Non-Dep group. To calculate the percentage of microglial cells ($Itgam^+$) that co-expressed IL6 ($Il6^+$), the number of co-labeled nuclei ($Il6^+/Itgam^+$) were divided by the number of $Itgam^+$ cells. To confirm the data obtained on CellProfiler, a random set of images were quantified blindly using Fiji by hand counting. Brightness, contrast and pixel dilation were kept consistent across all representative images.

2.8. Drugs

For behavioral experiments, anti-mouse IL-6R antibody (BioXCell, Catalog# BE0047, Clone 15A7, Lot #795921D1) or isotype control rat IgG2b antibody (BioXCell, Catalog# BE0090, Clone LTF-2, Lot #767921D1) were prepared fresh in dilution buffer (BioXCell, Catalog# IP0700, Lot #762122D1) on each injection day to a concentration of 750 μg/mL and administered at 150 μg/200 μL intraperitoneally (i.p.). The IL-6R dose and frequency of administration were consistent with prior work (Murakami et al., 2013). Additionally, the intraperitoneal route of administration has been previously employed to examine the effects of IL-6R Ab in spinal-cord related models (Guptarak et al., 2013; Mukaino et al., 2010; Okada et al., 2004). Alcohol for 2BC drinking sessions was prepared as 15 % ethanol (v/v, 200 proof ethanol in drinking water).

2.9. Statistical analysis

Drinking and von Frey time course results were analyzed with two-way repeated measures (within [time] – between [experimental group]) ANOVA or mixed-effects model (REML) as appropriate and followed by Dunnet's *post hoc* test where appropriate. Average weekly alcohol intake during the last round of CIE exposure (CIE5), mechanical allodynia after 72 h of withdrawal, multiplex cytokine assays, and RNAscope data were analyzed using two-way ANOVA with sex and experimental group as factors and followed by Fisher's LSD *post hoc* test where appropriate. The number of animals (n) used for each experiment are described in the corresponding figure legends. All data were analyzed and graphed using GraphPad Prism 10 software (La Jolla, California). Statistical significance was defined *a priori* as $p < 0.05$. See

Supplemental Table S1 for all statistical tests and factors.

3. Results

3.1. Alcohol dependence induced mechanical allodynia in both male and female mice

We used the CIE-2BC paradigm (Fig. 1A) to generate alcohol dependent (Dep) mice, characterized by a significant escalation in the average weekly alcohol intake (g/kg) when compared to baseline before CIE exposure in male (Fig. 1B; Time: $F_{3.2,98.6} = 3.27$, $p = 0.0223$; Time x Experimental Group Interaction $F_{5,155} = 8.18$, $p < 0.0001$) and female (Fig. 1C; Time: $F_{3.2,41.3} = 3.25$, $p = 0.0289$; Time x Experimental Group Interaction: $F_{5,65} = 6.61$, $p < 0.0001$) groups. Notably, females drank significantly more than males during the last week of 2BC and Dep mice drank significantly more than Non-Dep mice (Fig. 1D; Sex: $F_{1,44} = 10.08$, $p = 0.0027$; Experimental Group: $F_{1,44} = 32.56$, $p < 0.0001$). While average weekly alcohol intake varied between males and females as well as the Non-Dep and Dep groups, regardless of alcohol intake, weight remained steady within sex and between experimental groups (Non-Dep and Dep) across the study (data not shown).

We used von Frey filaments to measure the development and progression of mechanical allodynia (Fig. 1A). There was a significant main effect of experimental group wherein both male (Fig. 1E; Experimental Group: $F_{1,100} = 62.21$, $p < 0.0001$) and female (Fig. 1F; Experimental Group: $F_{1,13} = 18.67$, $p = 0.0008$) Dep mice developed more mechanical sensitivity compared to their Non-Dep controls. We also tested if alcohol re-exposure via 2BC ("Alcohol" timepoint in Fig. 1) alcohol drinking could ameliorate withdrawal-induced mechanical allodynia. There was a trend toward normalized mechanical sensitivity upon alcohol re-exposure (Fig. 1E and F). At 72 h of withdrawal, both male and female Dep mice had increased pain sensitivity when compared to Non-

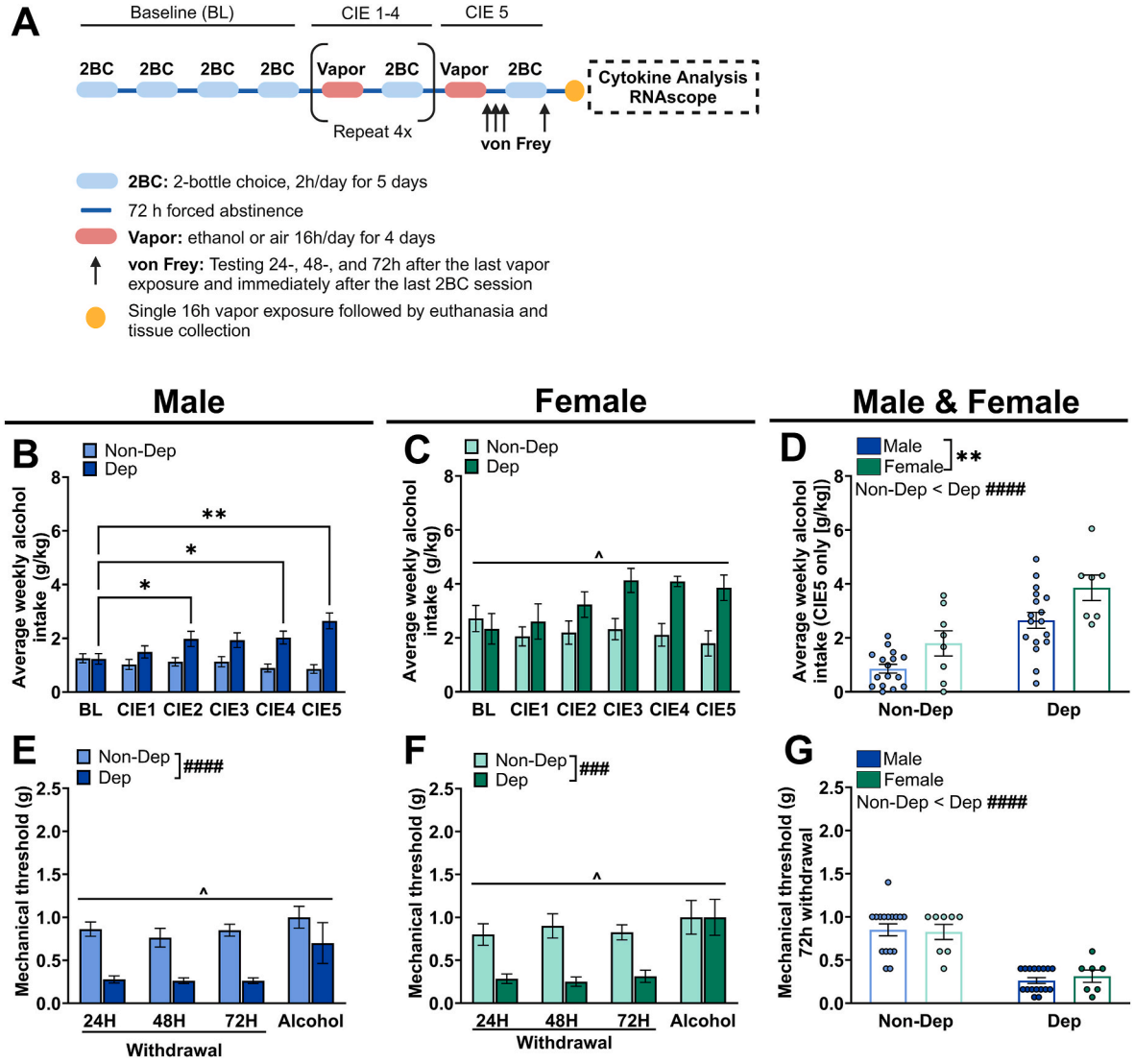

Fig. 1. Alcohol dependence induced mechanical allodynia in both male and female mice.
(**A**) Experimental timeline for Cohort 1 Male (Non-Dep: n = 16, Dep: n = 17) and female (Non-Dep: n = 8, Dep: n = 7) mice. See Methods Sections 2.2-2.4 for full experimental details. (**B-C**) Average weekly alcohol intake (g/kg) measured during baseline (BL), and after rounds 1-5 of vapor exposure (CIE 1-5) during the 2BC drinking procedure in males (**B**) and females (**C**). (**D**) Comparison of the average alcohol intake during the last round of CIE exposure between male and female non-dependent and dependent groups. (**E-F**) Mechanical allodynia measured during withdrawal (24-, 48-, and 72 h after the last vapor exposure) and immediately after the last 2BC session ("Alcohol" timepoint) in male (**E**) and female (**F**) mice. (**G**) Comparison of mechanical allodynia after 72h of withdrawal between male and female non-dependent and dependent groups. Results are expressed as mean ± SEM. Significant differences are denoted by $*p < 0.05$ and $**p < 0.01$. # Denotes a main effect of experimental group: ###$p < 0.001$ and ####$p < 0.0001$. ^ Denotes a main effect of time: ^ $p < 0.05$. See Table S1 in Supplemental Information for statistics relevant to each panel.

Dep mice (Fig. 1G; Experimental Group: $F_{1,44} = 64.97$, $p < 0.0001$).

3.2. Alcohol dependence alters cytokine levels in the spinal cord

To assess spinal immune activation associated with withdrawal-induced mechanical allodynia, we measured levels of the cytokines identified to mediate the neuroimmune responses induced by excessive alcohol use in the brain regions involved in alcohol use disorder (Bajo et al., 2019; Cruz et al., 2023; Vetreno and Crews, 2014). We observed a significant main effect of sex for all cytokines except IL-10 and MCP-1 (Fig. 2A; IL-6: Sex: $F_{1,45} = 7.62$, $p = 0.008$; Fig. 2B; IL-1β: Sex: $F_{1,45} = 13.63$, $p = 0.0006$; Fig. 2C; IFN-γ: Sex: $F_{1,45} = 9.39$, $p = 0.0037$; Fig. 2D; IL-1α: Sex: $F_{1,45} = 9.82$, $p = 0.003$; Fig. 2E; TNF-α: Sex: $F_{1,45} = 7.46$, $p = 0.009$; Fig. 2F; IL-17: Sex: $F_{1,45} = 6.60$, $p = 0.0136$). Females showed higher levels of IL-6 (Fig. 2A), IL-1β (Fig. 2B), IFN-γ (Fig. 2C), IL-1α (Fig. 2D), TNF-α (Fig. 2E) and IL-17 (Fig. 2F) when compared to males. Moreover, there was a main effect of the experimental group for IL-6 (Fig. 2A; Experimental Group: $F_{1,45} = 7.64$, $p = 0.0082$) and IL-1β (Fig. 2B; Experimental Group: $F_{1,45} = 4.86$, $p = 0.0037$). No significant differences were observed in IL-10 (Fig. 2G) or MCP-1 (Fig. 2H) levels. These findings indicate a sex difference in the neuroimmune response at the spinal level produced by excessive alcohol consumption. Dep mice had higher levels of both IL-6 and IL-1β than Non-Dep mice; however, due to greater significance and the current scientific interest in the role of IL-6 related mechanisms in several forms of allodynia, the rest of this report will focus on IL-6. The potential role of IL-1β in mechanical allodynia associated with AUD will be studied in due course.

3.3. Alcohol dependence increases *Il6*$^+$ cells in the lumbar spinal cord of Dep males

We used the RNAscope protocol to assess whether elevated IL-6 levels are associated with an increased number of *Il6*$^+$ cells (Fig. 3A), particularly microglia (using *Itgam* as a microglial marker; Fig. 3B), as well as the percentage of microglial cells (*Itgam*$^+$) co-expressing *Il6*$^+$ (Fig. 3C), in the lumbar spinal cord of Dep mice. Representative RNAscope images are shown in Fig. 3D with negative control in Fig. 3E. There was a main effect of sex and significant sex x experimental group interaction observed for *Il6*$^+$ cells (Fig. 3A; Sex: $F_{1,56} = 6.03$, $p = 0.01$; Sex x Experimental Group Interaction: $F_{1,56} = 6.039$, $0 = 0.017$). More specifically, Dep males showed a higher number of *Il6*$^+$ cells compared to Non-Dep male controls, while no differences were observed between Dep and Non-Dep females. Thus, Dep males had more *Il6*$^+$ cells than Dep females. For microglia (*Itgam*$^+$ cells), no sex or experimental group differences were detected in the number of *Itgam*$^+$ cells (Fig. 3B). However, a main effect of sex was detected for the percentage of *Itgam*$^+$ cells co-expressing *Il6*$^+$ (*Il6*$^+$/*Itgam*$^+$ cells). There were more *Il6*$^+$/*Itgam*$^+$ co-expressing cells found in the spinal cord of females than males (Fig. 3C; Sex: $F_{1,56} = 5.03$, $p = 0.02$). Collectively, these data highlight sex-related differences in spinal IL-6 expression after varying levels of alcohol exposure.

3.4. IL-6R Ab does not reduce mechanical allodynia during alcohol withdrawal

As our findings showed increased IL-6 levels in the spinal cord of the Dep males during withdrawal, we assessed whether blocking the IL-6 signaling could alleviate the mechanical allodynia induced by alcohol abstinence according to the experimental design procedure in Fig. 4A. To block the IL-6 signaling, we injected male and female Non-Dep (Fig. 4B and C) and Dep (Fig. 4D and E) mice with the IL-6 receptor antibody (IL-6R Ab; 150 μg/day; i.p.), and as an isotype control, we used IgG2 antibody. There was a significant main effect of time on mechanical sensitivity in Non-Dep female (Fig. 4C; Time: $F_{2.3,32} = 13.61$, $p < 0.0001$), Dep male (Fig. 4D; Time: $F_{2.1,23.6} = 6.13$, $p = 0.006$) and Dep female (Fig. 4E; Time: $F_{2,21.5} = 4.64$, $p = 0.0218$) mice. There was no main effect of treatment (IgG2b vs IL-6R Ab) in Non-Dep or Dep mice of either sex (Fig. 4B–E). However, a trend in IL-6R Ab alleviation of mechanical allodynia induced by alcohol-withdrawal was observed further into alcohol withdrawal (48 and 72 h) in Dep male mice. These results do not support the hypothesis that IL-6 plays a critical role in the processes related to alcohol abstinence-induced mechanical allodynia.

4. Discussion

The present study investigated the expression of IL-6 in the spinal

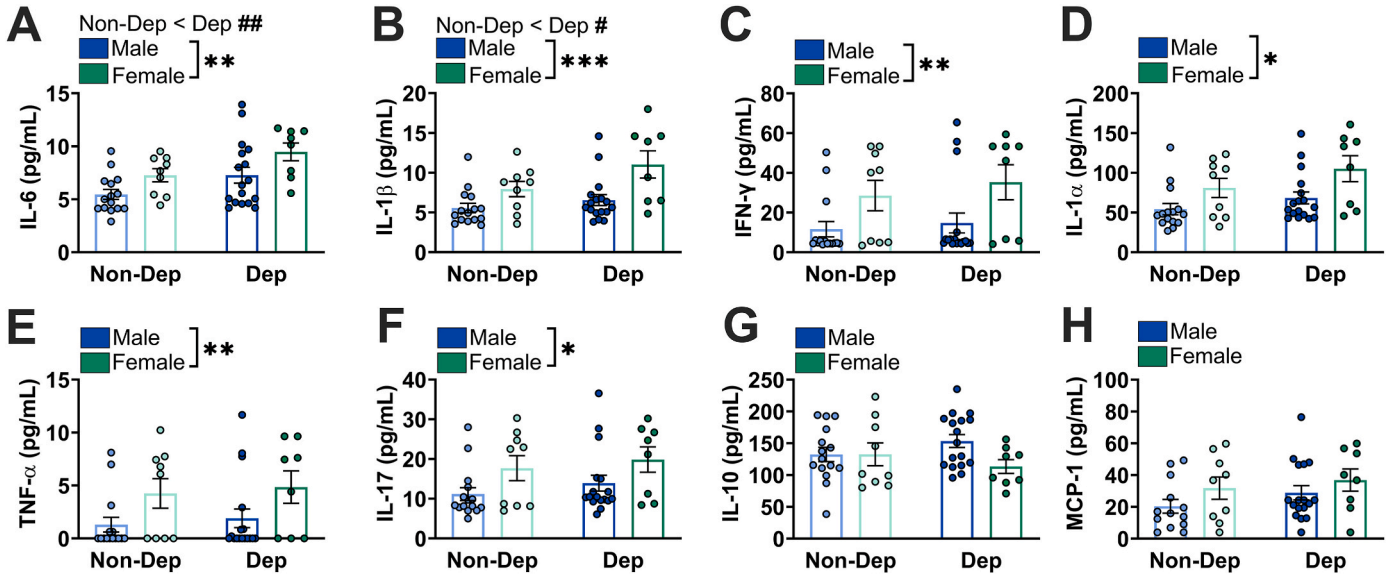

Fig. 2. Alcohol dependence alters cytokine levels in the spinal cord.
Multiplex assay cytokine levels in the spinal cord of male (Non-Dep: Cohort 1 n = 12, Cohort 2 n = 3; Dep: Cohort 1 n = 14, Cohort 2 n = 3) and female (Non-Dep: Cohort 1 n = 4, Cohort 2 n = 5, Dep: Cohort 1 n = 3, Cohort 2 n = 5) mice after the CIE-2BC procedure: IL-6 (**A**); IL-1β (**B**); IFN-γ (**C**); IL-1α (**D**); TNF-α (**E**); IL-17 (**F**); IL-10 (**G**); MCP-1 (**H**). Results are expressed as mean ± SEM. Significant main effects of sex are denoted by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. # Denotes main effect of experimental group: #$p < 0.05$ and ##$p < 0.01$. See Table S1 in Supplemental Information for statistics relevant to each panel.

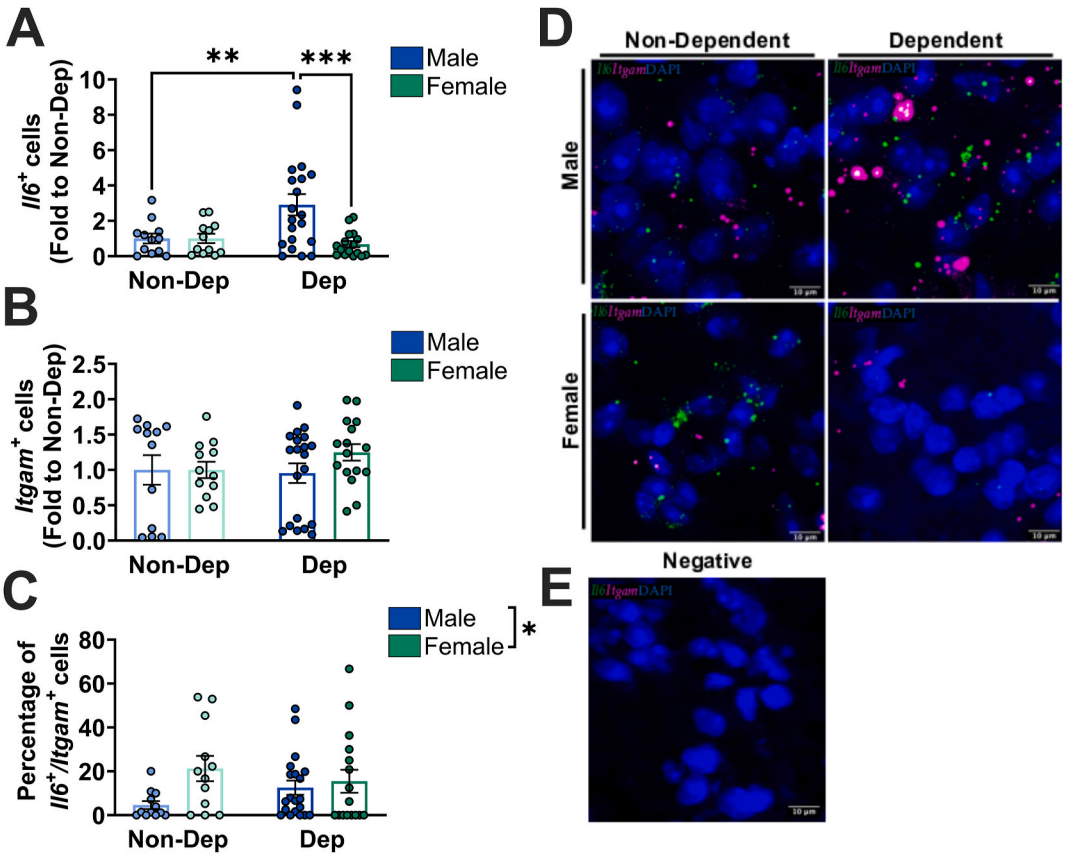

Fig. 3. Alcohol dependence increases *Il6*$^{+}$ cells in the lumbar spinal cord of Dep males.
(**A-B**) Number of positive nuclei expressing *Il6* (**A**) and *Itgam* (**B**) in male and female mice (Cohort 1: n = 3/sex/group). (**C**) Percentage of *Itgam*$^{+}$ cells co-expressing *Il6*$^{+}$ (for calculation see methods section 2.7). (**D**) Representative images for male and female Dep and Non-Dep mice displaying *Il6* (green), *Itgam* (magenta) and DAPI (blue). (**E**) Negative control images. Scale bar: 10 μm. Results are expressed as mean ± SEM. Significant differences are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. See Table S1 in Supplemental Information for statistics relevant to each panel. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cord and ability of this target to decrease alcohol abstinence-related mechanical allodynia in male and female mice using a mouse model of comorbid pain and alcohol dependence. Our results demonstrated that alcohol dependence (Dep) elevated spinal cord IL-6 protein expression versus Non-Dep mice. Interestingly, we observed an increase in *Il6*$^{+}$ cell expression in the spinal cord of Dep males only versus non-Dep mice. However, blocking IL-6 signaling by the administration of IL-6R Ab did not reduce mechanical allodynia during abstinence within our experimental parameters. These results do not support the hypothesis that targeting IL-6 may be an effective strategy to modulate pain sensitivity during alcohol withdrawal, however they do not reduce the potential for efficacy of IL-6R Ab in alternative experimental paradigms or as a combination therapy. Overall, these results suggest that while alcohol modulates IL-6 levels, targeting IL-6 to reduce alcohol – related mechanical allodynia warrants further study. These studies may additionally investigate the role of IL-6 as a therapeutic strategy for co-occurring pain and alcohol dependence more broadly.

The CIE-2BC paradigm is an established model to study alcohol dependence, which allows for precise assessment of the development of alcohol dependence by examining increases in escalation of drinking over a periods of weeks in dependent mice (Bloch et al., 2022; Okhuarobo et al., 2023; Patel et al., 2019). Using this model, we previously revealed that chronic alcohol exposure promotes mechanical allodynia in Non-Dep and Dep mice during abstinence when compared to naïve control mice (Borgonetti et al., 2023b). The current study uses the same experimental protocol as our previous study and the mechanical withdrawal thresholds that result are closely aligned as well as consistent with studies describing the negative effects of alcohol use on pain sensitivity in rodents (Aguilar et al., 2024; Alexander et al., 2024; Borgonetti et al., 2023b; Gilpin et al., 2021; Kelley et al., 2024). Importantly, we showed that male and female Dep mice developed mechanical allodynia 24 h after alcohol-removal, lasting up to 72 h, and this allodynia was reduced by consuming alcohol again (Borgonetti et al., 2023b). This animal model serves as an optimal tool to examine pain associated drinking phenotypes, and provides convergent validity to address key factors contributing to the maintenance of alcohol dependence and unrelieved pain sensitivity, which increases vulnerability to relapse during abstinence (Karimi et al., 2022).

The emerging role of neuroimmune regulators to study co-occurring pain and AUD has gained significant interest based on evidence that these systems regulate alcohol dependence and anxiety in rodents (Adams et al., 2023). For example, Dep male mice using a similar model to the one described in this report show an upregulation of neuroinflammatory genes and a decrease of anti-inflammatory cytokines including IL-10 (Bajo et al., 2019; Patel et al., 2019, 2021; Salem et al., 2024). Here, we examined the consequences of alcohol dependence on specific cytokines at the level of spinal cord tissue in Dep and Non-Dep mice, which extends previous work conducted in brain systems impaired by AUD (Adams et al., 2020; Cruz et al., 2023; Kazmi et al., 2022). We detected higher spinal cord IL-6 in Dep mice when compared to Non-Dep mice. We also observed increased spinal IL-1β in Dep versus Non-Dep mice. While the current manuscript focused on changes in IL-6, the finding on IL-1β warrants further study in due course. Other cytokines only showed increases by sex, where females displayed higher basal IFN-γ, IL-1α, IL-17, and TNF-α levels compared to males. These results are consistent with human studies suggesting female brains might be

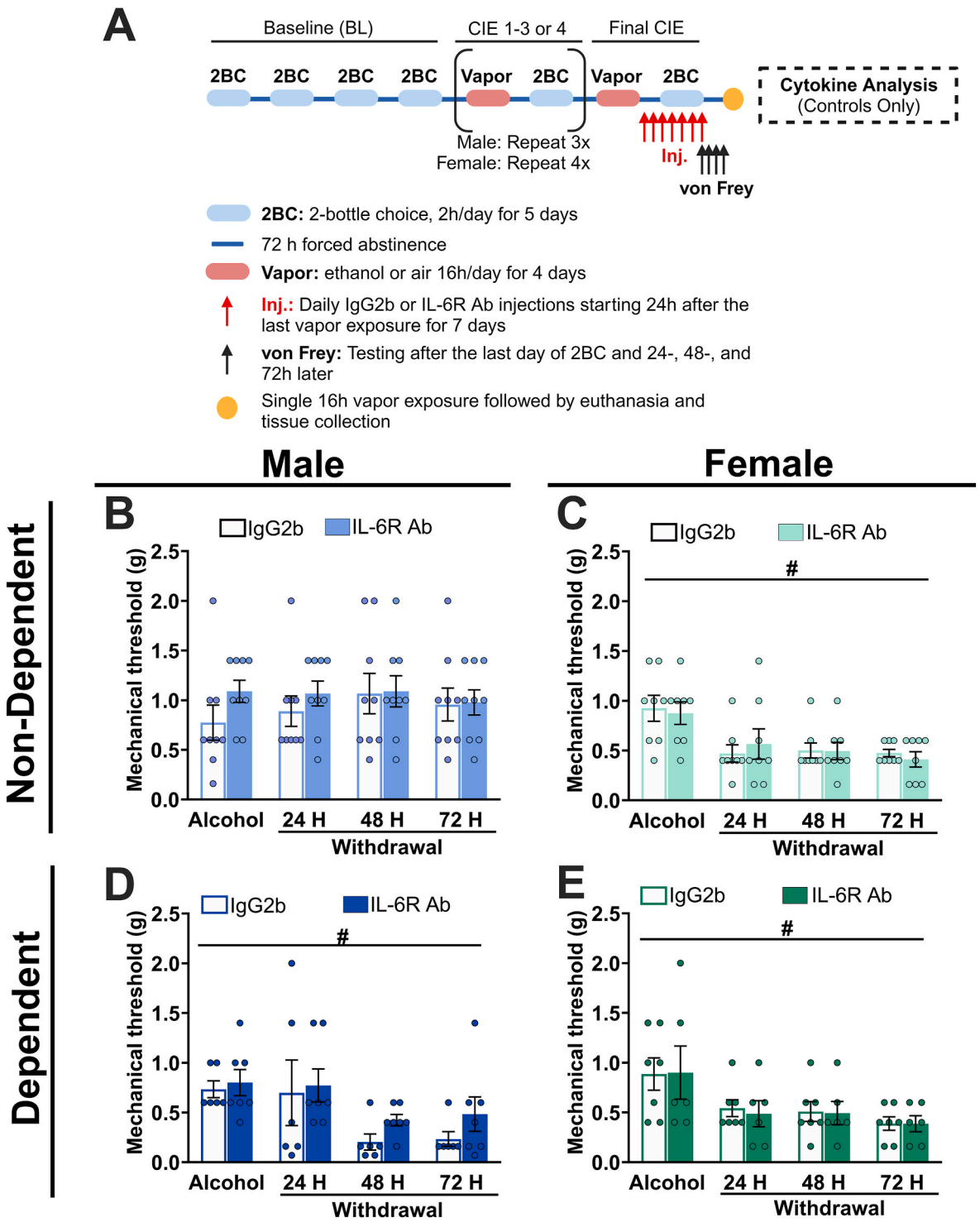

Fig. 4. IL-6R Ab does not reduce mechanical allodynia during alcohol withdrawal.
(**A**) Experimental timeline for Cohort 2 Male (Non-Dep: IL-6R Ab n = 9, IgG2b n = 9; Dep: IL-6R Ab n = 7, IgG2b n = 6) and female (Non-Dep: IL-6R Ab n = 8, IgG2b n = 8; Dep: IL-6R Ab n = 6, IgG2b n = 7) mice. See Methods Sections 2.2-2.4 for full experimental details. (**B-E**) Effect of IL-6R Ab on mechanical allodynia after the last day of 2BC (alcohol timepoint) and induced by alcohol-withdrawal (24-, 48- and 72h after the last 2BC session) in non-dependent (**B,C**) and dependent (**D,E**) male (**B,D**) and female (**C,E**) mice compared to the isotype control group (IgG2b). Results are expressed as mean ± SEM. # Denotes main effect of time $p < 0.05$. See Table S1 in Supplemental Information for statistics relevant to each panel.

more vulnerable to alcohol-induced damage compared to males (Hommer, 2003). Alfonso-Loeches et al. demonstrated that female mice are more susceptible to the neuroinflammatory changes from alcohol consumption and alcohol-exposed female rats show higher TNF-α hepatic levels than male rats, whereas males show higher IL-6 hepatic levels (Alfonso-Loeches et al., 2013; Colantoni et al., 2003). Our work reports that the response of males and females following chronic alcohol consumption may differ, and to our knowledge, this is the first report examining the contribution of spinal IL-6 in pain and alcohol dependence, highlighting the need for more work to examine spinal

mechanisms.

We found that alcohol dependence differentially affected expression of *Il6*$^{+}$ cells in spinal cord tissue, where Dep males showed increased IL-6 expression but not females and females showed a higher percentage of *Il6*$^{+}$ microglia than males regardless of treatment. This is congruent with a body of literature showing increased IL-6 and IL-6R levels with the development of neuropathy (for review please see (Kummer et al., 2021)). IL-6 is considered a potent inducer and modulator of microglial activation in pain. In a mouse spared nerve injury model of neuropathic pain, microglial STING/TBK1/NF-κB contributed to pain initiation via IL-6 signaling (Sun et al., 2022). The maintenance of mechanical allodynia after spinal cord injury has been attributed to the IL-6/JAK2/STAT3 pathway (Lee et al., 2023a). Microglia also play a significant role in the AUD trajectory and therapeutic interventions also exist to treat AUD (Marshall et al., 2013; Rasool et al., 2024; Warden et al., 2020). Systemic administration of microglia suppressing drugs (i.e., minocycline and tigecycline) decreased alcohol intake in both sexes (Bergeson et al., 2016b; Martinez et al., 2016). Alexander et al. demonstrated a female-specific role of TLR4 expression in microglia in the dorsal horn of the spinal cord after inducing alcohol-related allodynia using the Lieber-DeCarli alcohol diet (Alexander et al., 2024). Consistent with this, our research group has also found increases in IBA-1, and CSF-1 in male and female mice with pain-related hypersensitivity from alcohol abstinence in spinal cord tissue (Borgonetti et al., 2023b). This evidence supports the hypothesis that the increase of IL-6 at the microglial level in the spinal cord may be linked to the development of mechanical allodynia during abstinence from alcohol dependence. Of note, IL-6 is abundantly expressed in various tissue. While unpublished preliminary results indicate that increased IL-6 is due to proliferation of immune cells within the spinal cord rather than macrophagic infiltration from surrounding tissues, further studies that investigate the specific type of microglia present at the spinal level in alcohol-induced neuropathy are needed to clarify the mechanism of increased IL-6.

In this study, intraperitoneal injection of IL-6R Ab did not reduce alcohol abstinence-related mechanical allodynia in mice of either sex. In contrast, reports on administration of similar IL-6R antibodies have shown reduced spinal cord injury-related allodynia in rats and mice (Guptarak et al., 2013; Murakami et al., 2013). In humans, the epidural administration of tocilizumab, an anti-IL-6R monoclonal antibody, reduced radicular leg pain, numbness, and low back pain in lumbar spinal stenosis-induced sciatica (Ohtori et al., 2012). Multiple considerations may contribute to the discrepancy in these results, the first of which is administration timing. A single IL-6R Ab injection administered 28 days after spinal cord injury had an analgesic effect when monitored at one- and two-weeks post administration (Guptarak et al., 2013). Thus, the ideal timing of IL-6R Ab injection in relation to our CIE-2BC model where there is no precise timepoint of "injury" as well as the minimum time post-injection needed to observe an effect on mechanical sensitivity remains unclear. Additionally, contributions from other cytokines or potential compensatory mechanisms for a homeostatic immune response may mask effects. Injection of recombinant IL-6 in male rats resulted in elevation of not only IL-6 but also IL-1β and TNF-α in the spinal dorsal horn as well as reduction on IL-10 and TGF-β (Yang et al., 2022). It may be possible that IL-6 alone is not driving the mechanical allodynia seen at the time points examined and thus IL-6R Ab as a monotherapy is not sufficient to attenuate mechanical allodynia. A time course of changes in inflammatory cytokine levels after IL-6R Ab administration as well as a combinatorial approach would provide additional insight.

While there was no significant effect of IL-6R Ab on our assessment of mechanical allodynia related to alcohol abstinence herein, there was a trend for IL-6R Ab reduction of mechanical allodynia further into withdrawal (particularly 48 and 72 H) in Dep male mice. Thus, testing an alternative route of administration such as local administration at the level of the spinal cord (i.e., intrathecal) or potentially a larger dose of IL-6R Ab may reveal efficacy of IL-6R Ab to reduce mechanical allodynia during alcohol withdrawal as well, potentially in a sex-dependent manner. Future studies may also consider the role of IL-6 in the development of other pain manifestations that may arise from AUD (such as thermal allodynia) as well as other comorbidities including stress and anxiety. Studies have shown that alcohol use differentially affects the development of anxiety-like behavior in male and female rodents (Aguilar et al., 2023; Athanason et al., 2023; Jury et al., 2017; Serrano et al., 2018). Thus, examining the link between alcohol-induced anxiety and allodynia as well as the potential role of IL-6 is of interest.

We considered some limitations in this study that would further improve this conceptual framework but are beyond the scope of this report. First, our study examined a single dose of IL-6R Ab administered through repeated i.p. injections based on previous work (Guptarak et al., 2013; Mukaino et al., 2010; Murakami et al., 2013; Okada et al., 2004). However, a larger dose of IL-6R Ab may be needed to exert a significant effect on alcohol abstinence – related mechanical allodynia. While not feasible in the current study due to the experimental timeline, generation of a complete dose-response curve is warranted. Second, the potential for IL-6R Ab to alleviate mechanical allodynia was examined in short term abstinence herein. Previous work has shown efficacy of IL-6R Ab to alleviate mechanical allodynia weeks after spinal injury (Guptarak et al., 2013; Murakami et al., 2013). Thus, further studies are needed to examine the potential of IL-6R Ab to reduce alcohol-abstinence related mechanical allodynia in a protracted abstinence state. Finally, previous reports have shown that the expression of IL-6 correlated with phases of the estrous cycle (Chahal and Kabra, 2024; Prabhala and Wira, 1995). More work is required to understand changes in IL-6 patterns and other cytokine levels across the female estrous cycle in this mouse model of alcohol abstinence – related mechanical allodynia as well as responsivity to treatment with IL-6R Ab.

Lastly, while it may not be efficacious on its own, a monoclonal antibody targeting IL-6 (e.g., Tocilizumab) has the potential to complement one targeting IL-1β (e.g., Canakinumab) or a microglial inhibitor (e.g., minocycline) to effectively alleviate alcohol abstinence – related mechanical allodynia. Thus, future studies are warranted as this combination may be particularly beneficial for people with AUD that experience greater neuroinflammatory effects.

5. Conclusions

Our work highlights an initial role of sex-dependent involvement of IL-6 at the level of the spinal cord in Dep mice developing mechanical allodynia. Moreover, we tested the IL-6R Ab in a model that increases pain sensitivity associated with escalated alcohol consumption. Herein, intraperitoneal administration of IL-6R Ab did not improve alcohol withdrawal-associated allodynia while further studies are needed to fully assess the potential of IL-6R Ab to ameliorate alcohol – related mechanical allodynia as well as related pain states. This work lays the foundation for new studies to promote the mechanistic framework of spinal cord IL-6 in co-occurring pain and alcohol dependence.

CRediT authorship contribution statement

Vittoria Borgonetti: Writing – original draft, Methodology, Formal analysis, Conceptualization, Writing – review & editing, Visualization, Investigation, Data curation. **Celsey M. St. Onge:** Visualization, Funding acquisition, Data curation, Writing – review & editing, Investigation, Formal analysis. **Bryan Cruz:** Visualization, Funding acquisition, Data curation, Writing – review & editing, Investigation, Formal analysis. **Cristina Zalfa:** Visualization, Data curation, Writing – review & editing, Investigation. **Tali Nadav:** Visualization, Data curation, Writing – review & editing, Investigation. **Amanda J. Roberts:** Writing – review & editing, Supervision, Methodology, Data curation, Visualization, Resources, Funding acquisition. **Nicoletta Galeotti:** Supervision, Writing – review & editing, Data curation. **Michal Bajo:** Writing – review &

editing, Visualization, Funding acquisition, Writing – original draft, Supervision. **Marisa Roberto:** Writing – original draft, Supervision, Funding acquisition, Writing – review & editing, Visualization, Resources, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Glossary

AUD: alcohol use disorder; 2BC: two-bottle choice; CIE-2BC: chronic-intermittent ethanol two-bottle choice; IL-6: interleukin-6; IL-6R Ab: interleukin-6 receptor antibody; Dep: dependent; Non-Dep: non-dependent; i.p.: intraperitoneally; BAL: blood alcohol level; RIPA: radioimmunoprecipitation; DC protein assay: detergent compatible protein assay; PBS: phosphate buffered saline; ISH: *in situ* hybridization; Itgam: integrin alpha M; DAPI: 4′,6′-diamidino-2-phenylindole; IgG2b: immunoglobulin G2b; IL-10: interleukin-10; MCP-1: monocyte chemoattractant protein-1; IFN-γ: interferon-gamma; IL-1β: interleukin-1 beta; IL-1α: interleukin-1 alpha; TNF-α: tumor necrosis factor-alpha; IL-17: interleukin-17; STING: stimulator of interferon genes; TBK1: TANK-binding kinase 1; TANK: TRAF family member-associated NF-κB activator; TRAF: tumor necrosis factor receptor-associated factor; NF-κB: nuclear factor-kappa B; JAK2: janus kinase 2; STAT3: signal transducer and activator of transcription 3; TLR4: toll-like receptor 4; IBA-1: ionized calcium binding adaptor molecule 1; CSF-1: colony-stimulating factor-1; MR16-1: anti-mouse interleukin-6 receptor antibody.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2025.110584.

Data availability

Data will be made available on request.

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