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Alcohol Use Disorder is Associated with Enhanced Sensitivity to Cellular Lipopolysaccharide Challenge

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Abstract

Background: Inflammation is implicated in alcohol use disorder (AUD). A novel method to characterize AUD-related immune signaling is by probing Toll-like receptor (TLR)-4 stimulated monocyte production of intracellular cytokines (ICCs) via lipopolysaccharide (LPS). We evaluated relationships between AUD and ICC production at rest and after LPS stimulation.

Methods: This study analyzed blood samples from 36 participants (AUD N=14; Controls N=22), collected across five timepoints, with ICC expression assessed at rest (i.e., unstimulated) and following stimulation with LPS (5 repeated measures/participant for unstimulated and stimulated). Markers assessed included tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), TNF-α and IL-6 co-expression, and interferon (IFN). For each, linear mixed models were constructed with AUD, LPS, and timepoint as fixed effects (BMI as covariate), allowing for random slope and intercept. AUD×LPS was included as an interaction.

Results: For TLR4-stimulated monocyte production of TNF-α, there were effects for AUD (p<0.01), LPS (p<0.001), and AUD×LPS interaction (p<0.05), indicating that individuals with AUD showed greater unstimulated- and stimulated monocyte expression of TNF-α. Similarly, for TLR4 stimulated monocyte co-expression of TNF-α and IL-6, there were effects of AUD (p<0.01), LPS (p<0.001), and AUD x LPS interaction (p<0.05). No AUD or LPS effects were found for IL-6. Timepoint effects were observed on IL-6 and TNF-α/IL-6 co-expression (p<0.001). Finally for IFN, AUD (p<0.05), LPS (p<0.001), and AUD×LPS (p<0.001) effects were found.

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Conclusions: Individuals with AUD showed elevated resting or unstimulated levels of intracellular monocyte expression of TNF-α and IL-6/TNF-α co-expression compared to controls. AUD was associated with increases in TLR4-stimulated monocyte production of TNF-α, and co-production of IL-6 and TNF-α. This is, to our knowledge, the first study to investigate relationships between AUD and monocyte production of proinflammatory cytokines, at rest and in response to TLR4 stimulation with LPS. This study extends previous findings on the roles of proinflammatory cytokines in AUD and serves as a critical proof-of-concept for this method in probing neuroimmune mechanisms underlying AUD.

Keywords

Alcohol Use Disorder; Inflammation; Cytokine; Lipopolysaccharide

Introduction

Inflammation has been implicated in the development and maintenance of alcohol use disorder (AUD), termed the neuroimmune hypothesis of AUD (Cui et al., 2011; Mayfield and Harris, 2017). In preclinical models, chronic alcohol exposure has been shown to increase both central and peripheral markers of inflammation (Mayfield et al., 2013, Crews et al., 2015). Preclinical research also indicates that inflammation heightens motivation for alcohol consumption, enhances alcohol-related reward, and contributes to substance use-related cognitive impairment and depression-like behavior (Alfonso-Loeches et al., 2010, Briones and Woods, 2013). In humans, post-mortem brain tissue of individuals with AUD shows increased levels of proinflammatory gene expression (He and Crews, 2008, Liu et al., 2006), and individuals with AUD have heightened levels of peripheral proinflammatory biomarkers relative to healthy controls (Achur et al., 2010, Adams et al., 2020). A prolonged or excessive proinflammatory response can have detrimental effects on health and, in populations with AUD, is suggested to contribute to compulsive alcohol intake and other AUD symptomatology (Cui et al., 2011; Leclercq et al., 2014; Lee et al., 2021).

Essential for survival, innate and adaptive immune mechanisms serve as the human body's primary defense against pathogens (Bonilla and Oettgen, 2010; Slavich and Irwin, 2014). When the innate immune system is activated, inflammatory responses are provoked by the detection of pathogen-associated molecular patterns (PAMPs) such as the bacterial ligand lipopolysaccharide (LPS). *In vitro* LPS stimulation has been shown to induce microglial expansion and increase microglial TSPO binding, a clinical marker of neuroinflammation used in positron emission tomography (PET) (Tournier et al., 2020). LPS also serves as a biomarker of AUD such that individuals with AUD are shown to have elevated LPS levels but may re-normalize after abstinence (Leclercq et al., 2012; Qin et al., 2008).

Toll-like receptors (TLRs) are widely implicated in neuroimmune signaling processes related to alcohol use (Meredith et al., 2021). Commonly found on immune cells, TLRs recognize PAMPs. When TLR4 is bound by LPS, activation of transcription factors, such as interferon (IFN) regulatory factors, nuclear factor-κB (NF-κB), and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (Aurelian et al., 2016; Balan et al., 2018). These activated transcription factors drive the expression of

proinflammatory cytokines, immune protein molecules released by immune cells. Cytokines coordinate inflammatory cellular functions and, with the ability to cross the blood-brain barrier (Banks et al., 1995), have been shown to affect physiological and behavioral responses (Dinarello, 2000).

Chronic ethanol consumption has been shown to result in increased levels of plasma cytokines such as tumor necrosis factor α (TNF-α) and interleukins IL-1β, IL-17 in wild-type mice (Pascual et al., 2015). Mice with TLR system (i.e., TLR4, TLR2) knockouts were protected from these effects, however, providing evidence in support of the TLR system's importance in alcohol-related neuroinflammation (Crews et al., 2017). In humans, a recent meta-analysis of 17 clinical studies (Adams et al., 2020) found increased cytokine concentrations (e.g., IL-6, TNF-α, IL-8) among individuals with AUD compared to healthy controls; these abnormalities were more prominent during active drinking and acute withdrawal periods compared to periods of early or prolonged abstinence. In sum, preclinical and clinical evidence indicate that the immune and neuroimmune system is related to AUD symptomatology, but specific mechanisms remain unclear.

A novel method to characterize mechanisms of AUD-related immune signaling is to probe monocyte production of intracellular cytokines at rest and following *in vitro* TLR4 stimulation with LPS. This method provides insight into the source of systemic inflammation, independent of extracellular levels (Cho et al., 2019). Monocytes comprise approximately 5% of circulating leukocytes and are a major contributor to proinflammatory cytokine production in peripheral blood (O'Connor et al., 2007). The acute inflammatory state induced by LPS stimulation is thought to be reflective of stress, as physiological and psychological stressors both activate inflammatory processes (Black, 2002), and TLR4-stimulated monocyte expression reflects the inflammatory responsivity of cells to these stressors (Bale, 2006). Whereas higher levels of TLR4 stimulated production of TNF-α has been found to correlate with depression symptom severity in mixed cell populations (Suarez et al., 2004, 2003), no study to our knowledge yet used this method to examine the associations between alcohol use and monocyte expression of inflammatory cytokines at rest and in response to LPS challenge, even though systemic inflammation is reported to occur in AUD.

The current study evaluated relationships between alcohol use disorder and monocyte intracellular cytokine production at rest or unstimulated and following ligation of the TLR4 receptor with LPS. Proinflammatory cytokines of interest included tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interferon (IFN). Participants with AUD were hypothesized to show higher resting, unstimulated levels of monocyte intracellular expression of inflammatory cytokines, and also to show greater response to TLR4 stimulation with LPS, consistent with an interaction between AUD and LPS on monocyte production of inflammatory markers.

Materials and Methods

Participants

Participants included 36 volunteers (AUD N=14; Controls N=22). Subjects in the AUD group had a DSM-IV diagnosis of current, primary alcohol dependence with or without secondary depression and no other primary affective or other psychiatric disorders; controls were diagnosed as having no history of any major psychiatric disorder via structured clinical interview for DSM-IV (American Psychiatric Association, 1994). Inclusion criteria also required that participants be free of active medical disorders that might compromise interpretation of the inflammatory measures. Specifically, a review of symptoms was performed, along with a systematic review of medical history. In addition to this structured interview, we also used structured questionnaire to inquire about medication use related to a variety of medical disorders including cardiovascular disease, hypertension, inflammatory disorders such as rheumatoid arthritis or psoriasis, cancer, or chronic infections such as human immunodeficiency virus or hepatitis C. Finally, laboratory screening tests were performed, and both AUD and control groups had liver function tests in the normal range, which were similar between control and AUD participants respectively (mean±SD, alkaline phosphatase IU/l, 59.6±15.3 vs. 57.2±23.2, p=0.8; aspartate aminotransferase, 23.5±7.5 vs. 26.9±10.4, p=0.7; alanine transferase, 20.8±6.8 vs. 26.5±11.5, p=0.1; bilirubin total 0.8±0.6 vs. 0.7±0.3, p=0.3). Exclusion criteria included suicide risk, immunosuppression from neoplastic disease, corticosteroids, or other immunosuppressive therapy, and use of psychotropic or anti-hypertensive medications. Those with active infections within the last week were also excluded.

Subjects were 70% male, between the ages of 25 and 55, and had an average body mass index (BMI) of 25.35 kg/m^2 ± 4.46 kg/m^2. Sample demographics broken down by AUD status can be found in Table 1. All participants provided informed consent. This study was approved by the Institutional Review Board of the University of California Los Angeles.

Prior to entry into the protocol, alcohol and nicotine use was evaluated via the clinician-administered Semi-Structured Assessment for Genetics of Alcoholism (SSAGA) (Bucholz et al., 1994). Depression symptomatology was also assessed via the Beck Depression Inventory (BDI-II) (Beck et al., 1996), Hamilton Depression Rating Scale (HRS-D) (Hamilton, 1960), and the depression subscale of the Profile of Mood States (POMS) (McNair et al., 1971). Alcohol use and depression symptomatology statistics can be found in Table 2. Participants in the AUD and control groups were then required to be abstinent for between 14 and 21 days after baseline screening assessment and entry into the protocol.

Procedures

As previously described for control participants (Irwin et al., 2006), AUD and control participants in this study spent 4 days (24-hour periods) in the National Institutes of Health General Clinical Research Center. After adaptation to the sleep laboratory with screening for sleep apnea and nocturnal myoclonus, subjects underwent baseline testing (i.e., prior to partial sleep deprivation) and 1 day of testing after sleep deprivation. Only data collected from the baseline condition were used in the present analyses; results from the

sleep deprivation are reported separately. These methods were performed concurrently in the present sample of controls and AUD participants. Of the 22 controls reported herein, baseline (i.e., prior to sleep deprivation) TLR-4 stimulated monocyte production of IL-6 and TNFα for 17 controls has been previously reported (Irwin et al. 2006). For the AUD participants (n=14) and the other controls (n=5), monocyte intracellular cytokine production has not been previously reported. Of the 30 controls previously reported (Irwin et al. 2006), 13 controls were not included in this analysis because their assessments were not performed concurrently with AUD participants.

Sample Collection

Blood samples were collected from participants across five timepoints (0800, 1200, 1600, 2000, and 2300) over the span of a 15-hour period via an indwelling venous forearm catheter. All samples were collected from the baseline condition, prior to sleep deprivation. Samples were assessed for expression of intracellular proinflammatory cytokine production in peripheral blood mononuclear cell (PBMC) populations at rest or unstimulated and after stimulation with LPS for a total of 10 repeated measures per participant (5 unstimulated timepoints, 5 stimulated timepoints), or approximately 360 total observations.

Intracellular monocyte assay

As noted above, methods for the assessment of sleep and blood sampling were performed concurrently in the present sample of controls and AUD participants. On the day of blood sampling, intracellular monocyte assays were performed; blood sample were not stored prior to assay methods. Briefly, monocyte intracellular cytokine production at rest and in response to whole-blood LPS stimulation was assessed by flow cytometry using peridinin chlorophyll protein (PerCP)-labeled CD14 mAb and phycoerythrin (PE)-labeled anti-IL-6 Ab, as previously described (Collado-Hidalgo et al., 2006; Irwin et al., 2006; Prussin and Metcalfe, 1995). For stimulated measures, heparin-treated blood (1mL) was mixed with 100 pg/mL of LPS (Sigma, St. Louis, MO) and 10 μg/mL brefeldin A (Sigma, St. Louis, MO) and incubated for 4 h at 37°C in a platform mixer followed by an overnight incubation at 4°C. Red blood cells were lysed in FACS lysing solution (BD Biosciences, San Jose, CA), remaining cells were permeabilized in FACS permeabilizing buffer (BD Biosciences, San Jose, CA), and fluorescence-conjugated antibodies were added for 30 min at room temperature in the dark. Cells were then washed and resuspended in 1% paraformaldehyde for flow cytometry. Three-color flow cytometric analysis was conducted on a Coulter Elite flow cytometer using Coulter Elite software. Forward and side scatter were used to gate on the target population (on the population consisting of monocytes and granulocytes). For the monocyte population, the percentage of cytokine-secreting (PE positive) cells among CD14-PerCP-positive population was determined by counting about 12,000 CD14+ events. Proinflammatory cytokines of interest included tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), TNF-α / IL-6 co-expressing monocytes, and interferon (IFN). Results for cytokine-positive monocytes were expressed as percentages of the total CD14+ cells. For the purposes of this study, “unstimulated” refers to this percentage within resting whole blood; “stimulated” refers to the total level of percentage cytokines expressed after stimulation with LPS. The flow cytometric data collected after assay and cytometric analysis was archived for analysis in the present study.

Statistical Analysis

Data were analyzed using R statistical software (RStudio 1.2.5001). For each marker of interest, a linear mixed model was constructed with AUD status (i.e. AUD vs. control), LPS stimulation status (i.e. unstimulated vs. stimulated), and time point (i.e. hour 08, 12, 16, 20, or 23) as fixed effects, along with BMI as a covariate, allowing for random slope and intercept based on participant effects. AUD × LPS Stimulation status was included as an interaction term. Four individual models were run, one for each biomarker of interest. We did not correct for multiple comparisons, due to the exploratory nature of the study.

Results

AUD and control groups did not significantly differ on demographic variables (see Table 1), other than current smoking status [$X^2 = 17.039$; $p < 0.001$]. Participants in the AUD group reported consuming a total of 556.38 (SD: 601.63) drinks over the last 3 months, or an average of 11.67 (SD: 9.18) drinks per day; participants in the control group reported consuming a total of 16.18 (SD: 33.68) drinks in the last 3 months [$t=-3.234$; $p < 0.01$], or an average of 0.70 (SD: 0.98) drinks per day [$t=-4.124$; $p < 0.01$] (see Table 2).

Participants with AUD had significantly greater levels of depressive symptomatology than controls, as assessed by the BDI-II [$t=-4.439$; $p < 0.001$], HRS-D [$t=-3.189$; $p < 0.01$], and POMS [$t=-2.188$; $p < 0.05$]; both groups remained, on average, under clinical thresholds for depressive symptoms (see Table 2). Due to collinearity between AUD diagnosis and depression symptomatology, as well as the lack of clinical significance, these measures were not included as factors in the mixed model analysis.

Results from the linear mixed models for each inflammatory marker of interest are presented in Table 3. For monocyte expression of TNF-α (Figure 1), effects of AUD ($p < 0.01$), LPS ($p < 0.001$), and an AUD x LPS interaction effect ($p < 0.05$) were found, such that individuals with an AUD had greater resting monocyte expression of TNF-α levels and also greater TLR4 stimulated monocyte production of TNF-α. While no AUD or AUD × LPS effects were seen for IL-6 alone (Figure 2), there was an effect of AUD ($p < 0.01$), LPS ($p < 0.001$), and AUD x LPS interaction ($p < 0.05$) on monocytes co-expression of TNF-α and IL-6 (Figure 3), driven by the effects seen in the TNF-α model alone. Of note, a diurnal pattern of expression was seen for both IL-6 and TNF-α / IL-6 co-expressing monocytes (driven by the effects seen in the IL-6 model), with a significant effect of time point ($p < 0.001$) on both models. Finally, similar AUD ($p < 0.05$), LPS ($p < 0.001$), and AUD × LPS ($p < 0.001$) effects were found on IFN levels (Figure 4).

Discussion

This study investigated the relationship between alcohol use disorder and monocyte intracellular cytokine production at rest and following a cellular inflammatory challenge with lipopolysaccharide. As hypothesized, alcohol use disorder status (i.e., AUD versus controls) and LPS challenge were both associated with higher levels of proinflammatory markers. Importantly, interaction effects between these two factors were found, such that LPS ligation of TLR4 yielded greater monocyte expression of TNF-α and IFN in blood

samples from participants with AUD compared to controls, indicating that AUD was associated with enhanced sensitivity to cellular LPS challenge.

Chronic alcohol consumption is associated with increased TNF-α levels in rodents and humans (Heberlein et al., 2014; Leclercq et al., 2014); elevated TNF-α levels are also correlated with liver dysfunction and can be used as an early indicator of alcohol-associated hepatitis (Gonzalez-Quintela et al., 2008). Alcohol intake has also been shown to promote a systemic proinflammatory IFN response in mice resulting from chronic ethanol exposure and subsequent alcohol dependence (Frank et al., 2020). Previous studies on alcohol's effects on IFN provide inconsistent conclusions, with some studies showing alcohol consumption leading to increased IFN levels and others showing the opposite (Laso et al., 1999; Song et al., 2002; Starkenburg et al., 2001; Zhang et al., 2015). In the present study, stimulated levels of IFN across AUD groups were much lower than that of TNF-α and IL-6 results. Although there was no overlap between unstimulated and stimulated ICC levels, it was also apparent that only a small percentage of stimulated monocytes expressed IFN. While monocytes can express IFN, this cytokine mainly comes from T-cells (Pang et al., 2011; Parmar and Platanias, 2003), and as such, LPS may not be the best stimulus to affect IFN expression, especially in monocytes.

IL-6 is well-studied as an inflammatory marker in psychiatric disorders. Previous studies, especially in the depression literature, have demonstrated effects of LPS stimulation on IL-6 (Cho et al., 2019; Irwin et al., 2006). In the alcohol field, IL-6 has been emphasized as an important marker associated with chronic alcohol exposure (Moura et al., 2022) and withdrawal (Gruol et al., 2018; Roberts et al., 2019). Therefore, it was counter to our original hypothesis that there was neither a main effect of AUD nor an AUD × LPS stimulation effect on IL-6 observed in our study. Our results indicate that IL-6 was more sensitive to time course than other markers of interest, showing a distinct diurnal pattern of expression (Vgontzas et al., 2005). We hypothesize that due to the limited sample size, possible IL-6 effects may have been affected by the marker's sensitivity to time course. Although timepoint × alcohol interactions may be interesting to probe, especially considering the observed diurnal pattern of IL-6 expression, our statistical model did not converge due to lack of power for the inclusion of these additional multiple comparisons. We suggest that a larger sample size be used to study potential interactions between timepoint, alcohol, and LPS stimulation. Importantly, previous work has also shown that serum IL-6 levels decrease steeply in early withdrawal (over the course of 14 days of abstinence from alcohol), declining to a level non-significantly differentiable from controls by day 14. In comparison, TNF-α levels were shown to remain at an elevated state throughout withdrawal (Heberlein et al., 2014). As our participants were required to have abstained from alcohol for at least 14 days, it is possible that they had experienced a similar level of recovery in IL-6 effects. In particular, these findings speak to the effects of protracted withdrawal on inflammatory markers, as compared to the acute alcohol withdrawal phase.

This study has several strengths and limitations that should be considered when interpreting its results. Study strengths include the probing of monocyte production of ICCs following LPS stimulation, which captures an acute cellular immune response and reduces extracellular background. Strengths also include demographically comparable AUD

and control groups, and multiple repeated measures within subjects. Limitations include the small sample size and lack of representation of some ethnicities, particularly for the AUD group; future studies should enroll larger, more diverse samples with repeated measures to confirm the findings herein. We considered including depression symptomology in our analysis; however, such analyses were limited both by depression symptoms being highly collinear with alcohol use status in our sample and by our sample on average not reaching clinical significance on depressive symptom metrics. In future studies, we recommend investigating the relationships between alcohol use, inflammation, and depression with the inclusion of participants who may have AUD but not clinically significant depressive symptoms as well as participants who may have clinical depression but not AUD. Relatedly, we were unable to examine the effect of smoking in our analysis, as only individuals in the AUD group were current smokers. Future studies should recruit control participants who are also smokers. As mentioned above, participants in this sample were required to have abstained from alcohol for 14-21 days. Therefore, these participants may have already experienced some recovery of baseline inflammatory markers and were likely in a state of protracted withdrawal (Heberlein et al., 2014). It is likely that greater baseline effects may be seen in participants who were actively drinking and/or undergoing acute withdrawal, as opposed to protracted withdrawal, and we suggest that future studies include such a population. Finally, previous work has shown sex differences in LPS challenge-induced monocyte cytokine production (O'Connor et al., 2007). However, the current study was underpowered to examine sex effects. Future studies with a greater sample size should explore these effects.

In conclusion, this is, to our knowledge, the first study to investigate the relationships between alcohol use disorder and monocyte ICC production at rest and in response to cellular LPS challenge. This study extends previous preclinical and clinical findings on the roles of proinflammatory cytokines in AUD. Main effects of AUD and LPS stimulation, as well as AUD × LPS stimulation interaction effects, were observed on monocyte intracellular expression of TNF-α, IFN, and TNF-α / IL-6 co-expression indicating elevated levels of cellular inflammation at rest and response to TLR4 activation in AUD. In other words, monocytes from individuals with AUD show higher resting levels of inflammatory cytokines and greater expression of these cytokines in response to inflammatory challenge, as compared to controls. Insofar as the cellular LPS challenge mimics a stress response, these findings suggest that individuals with AUD may mount a more robust inflammatory response to systemic stress than healthy controls without AUD or heavy drinking. This differential response, in turn, may render individuals with AUD more vulnerable to chronic alcohol use. These analyses serve as a critical proof-of-concept for the use of this novel method in probing the neuroimmune mechanisms underlying AUD.

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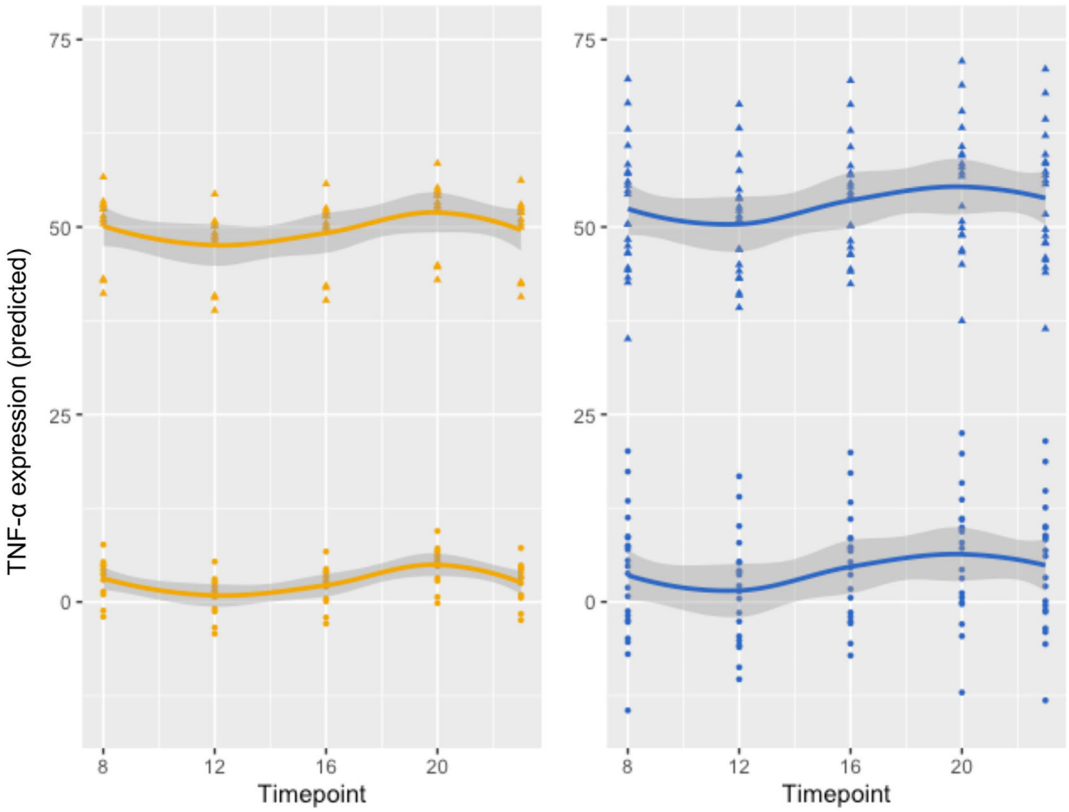

Figure 1.

Mixed model results predicting monocyte intracellular TNF-α expression. Yellow = control group; blue = AUD group. Circle points = unstimulated; triangle = stimulated.

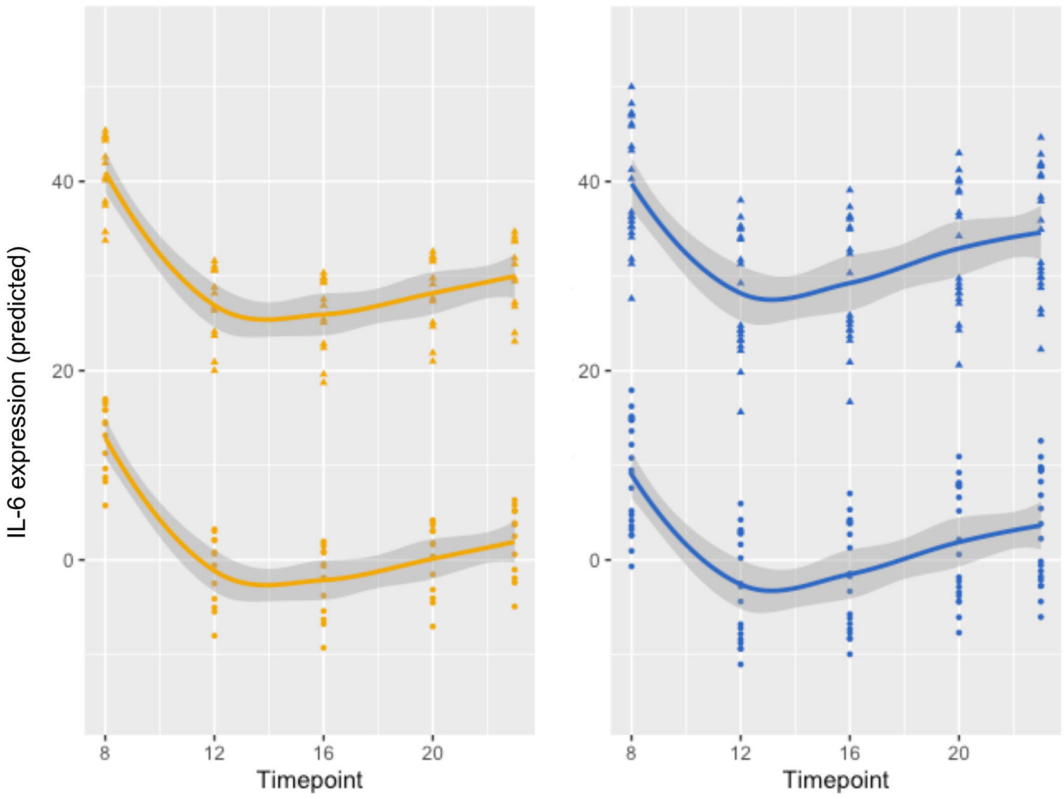

Figure 2.

Mixed model results predicting monocyte intracellular IL-6 expression. Yellow = control group; blue = AUD group. Circle points = unstimulated; triangle = stimulated.

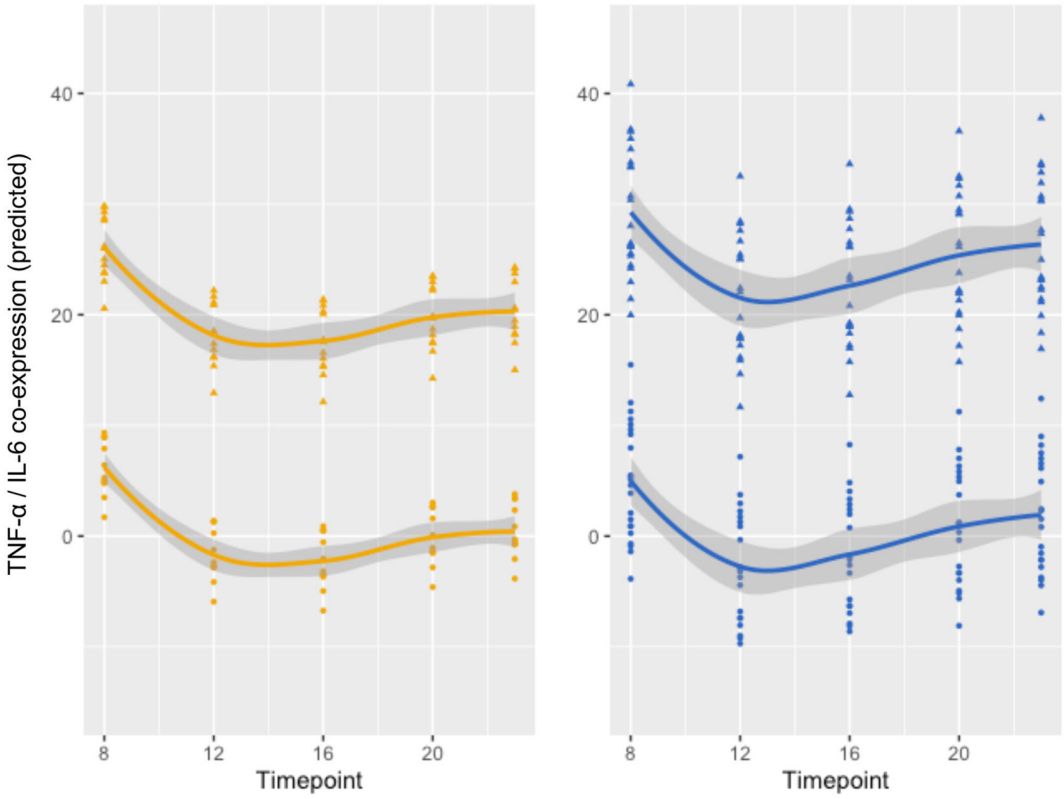

Figure 3.

Mixed model results predicting monocyte intracellular TNF-α / IL-6 co-expression. Yellow = control group; blue = AUD group. Circle points = unstimulated; triangle = stimulated.

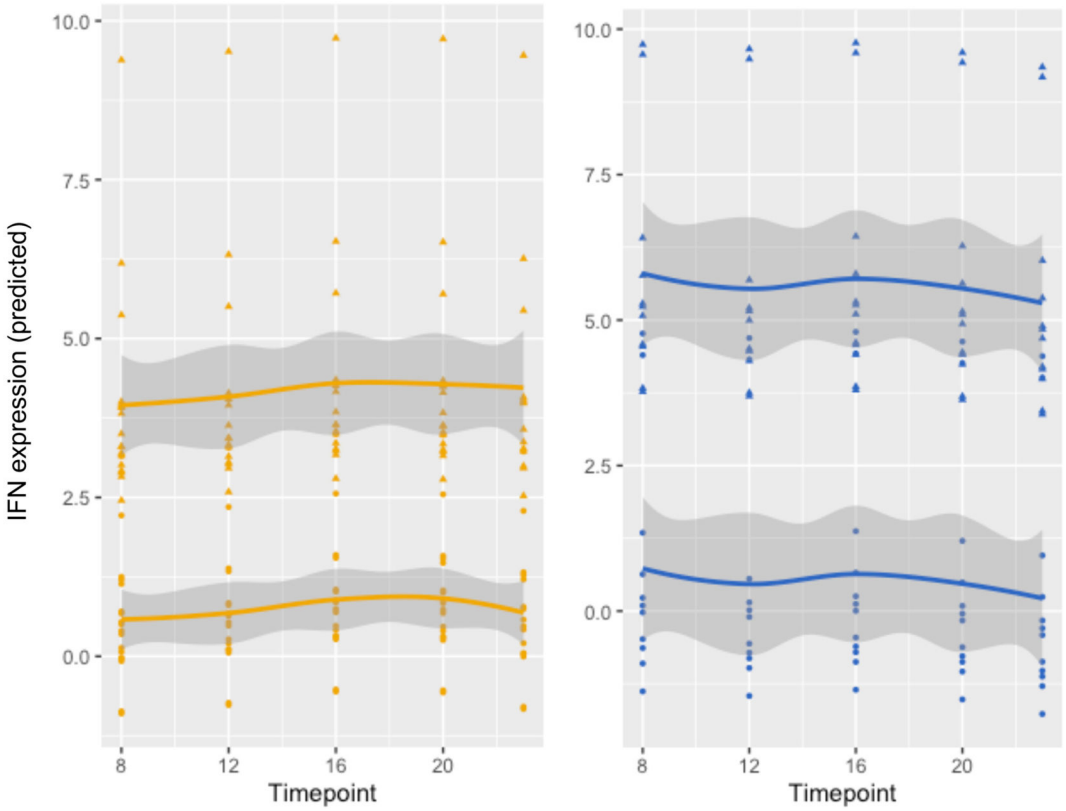

Figure 4.

Mixed model results predicting monocyte intracellular IFN expression. Yellow = control group; blue = AUD group. Circle points = unstimulated; triangle = stimulated.

Table 1.

Sample demographics, as separated by AUD vs. Control groups. ± indicates standard deviation. N.S. indicates $p > 0.05$.

	AUD (N=14)	Control (N=22)	Statistic	P-Value
Gender (%M)	71%	68%	$X^2 = 0.028$	*N.S.*
Age (Mean ± SD)	37.31 ± 5.42	37.05 ± 9.36	t=−0.105	*N.S.*
Ethnicity - Asian	0%	18.2%	X^2=6.592	*N.S.*
Pacific Islander	0%	4.5%		
Black/AA, Non-Hispanic	28.5%	22.7%		
Black/AA, Hispanic	14.3%	4.5%		
White, Non-Hispanic	35.7%	36.4%		
White, Hispanic	14.3%	4.5%		
Other	7.1%	9.1%		
% Current Smoker	64.3%	0%	$X^2 = 17.039$	***p* < 0.001**
BMI	26.09 ± 3.03	24.92 ± 5.14	t= −0.854	*N.S.*

Table 2.

Alcohol use and depression clinical characteristics, as separated by AUD vs. Control groups. ± indicates standard deviation. N.S. indicates $p > 0.05$.

	Alcohol (N=14)	Control (N=22)	Statistic	P-Value
Average Drinking Days / Month	22.57 ± 10.34	5.45 ± 14.06	t=−4.198	***p* < 0.001**
Average Drinks / Day	11.67 ± 9.18	0.70 ± 0.98	t=−4.124	***p* < 0.005**
Maximum Drinks / Day (last 3 months)	21.25 ± 13.40	1.50 ± 1.44	t=−5.269	***p* < 0.001**
Total Drinks (last 3 months)	556.38 ± 601.63	16.18 ± 33.68	t=−3.234	***p* < 0.01**
BDI-II Total	7.46 ± 4.47	1.65 ± 1.89	t=−4.439	***p* < 0.001**
Hamilton Total	6.85 ± 6.20	1.19 ± 1.97	t=−3.189	***p* < 0.01**
POMS - Depression Subscale	5.92 ± 8.10	0.90 ± 2.07	t=−2.188	***p* < 0.05**

Table 3.

Mixed model results: alcohol use, LPS stimulation status, time point, BMI, alcohol × stimulation effects on inflammatory marker outcomes. N.S. indicates p > 0.05.

Outcome	**TNF-α**	**IL-6**	**TNF/IL-6 Co-Expressing**	**IFN**
Alcohol (AUD vs. control)	t=2.604 ***p* < 0.01**	t=0.042 *N.S.*	t=3.005 ***p* < 0.01**	t=2.607 ***p* < 0.05**
LPS Stimulation (Unstimulated vs. stimulated)	t=34.505 ***p* < 0.001**	t=21.496 ***p* < 0.001**	t=26.676 ***p* < 0.001**	t=15.599 ***p* < 0.001**
Time point (hour 08, 12, 16, 20, or 23)	t=0.293 *N.S.*	t=7.111 ***p* < 0.001**	t=5.493 ***p* < 0.001**	t=0.563 *N.S.*
Body Mass Index (BMI)	t=0.404 *N.S.*	t=0.813 *N.S.*	t=0.070 *N.S.*	t=0.952 *N.S.*
Alcohol × LPS Stimulation	t=1.978 ***p* < 0.05**	t=1.219 *N.S.*	t=2.418 ***p* < 0.05**	t=2.879 ***p* < 0.001**

t = t-value