

Gender differences in the inflammatory cytokine and chemokine profiles induced by binge ethanol drinking in adolescence

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ABSTRACT

Heavy binge drinking in adolescence can cause long-term cognitive and behavioral dysfunctions. Recent experimental evidence indicates the participation of immune system activation in the effects of ethanol in the adolescent brain and suggests gender differences. The present study aims to assess plasma cytokine and chemokine levels in male and female adolescents and young adults during acute alcohol intoxication and to correlate these results with the toll-like receptor 4 (TLR4) response. The potential role of the TLR4 signaling response was also assessed in plasma and prefrontal cortex (PFC) of adolescent wild-type and TLR4-knockout male and female mice with binge ethanol treatment. The results showed that alcohol intoxication increased the plasma levels of several cytokine and chemokine [interferon- γ , interleukin (IL)-10, IL-17A, IL-1 β , IL-2, IL-4, IL-6, IL-8, fractalkine, monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1 α (MIP-1 α)] and the upregulation of TLR4 mRNA levels occurred in intoxicated females, while elevation of colony-stimulating factor was only observed in the plasma of males. In wild-type female adolescent mice, intermittent ethanol treatment increased the levels of several cytokines (IL-17A and IL-1 β) and chemokines (MCP-1, MIP-1 α and fractalkine) in PFC and in serum (IL-17A, MCP-1 and MIP-1 α), but significant differences in the fractalkine levels in PFC were observed only in male mice. No changes in serum or prefrontal cortex cytokine and chemokine levels were noted in ethanol-treated male or female TLR4-knockout mice. Our findings revealed that females are more vulnerable than males to inflammatory effects of binge ethanol drinking and suggested that TLR4 is an important target of ethanol-induced inflammation and neuroinflammation in adolescence.

Keywords Adolescent humans, adolescent mice, gender difference, inflammation, TLR4.

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INTRODUCTION

Heavy alcohol binge drinking is increasing in adolescents from different European countries (Danielsson, Wennberg, Hibell, & Romelsjo 2012), including some Mediterranean ones where a shift from natural moderate wine alcohol consumption to heavy binge drinking has been noted (Peretti-Watel, Beck, & Legleye 2006; Tur, Puig, Pons, & Benito 2003), which can lead to many alcohol-related problems among adolescents (Danielsson *et al.* 2012). Evidence obtained from human and experimental animals has shown the harmful consequences of

alcohol binge drinking during a developmental stage of adolescent brain maturation. Thus, alterations in cortical and white matter structure (Squeglia, Jacobus, & Tapert 2014), deficits in memory, poor academic performance (Hanson, Cummins, Tapert, & Brown 2011), as well as visual learning (Sanhueza, Garcia-Moreno, & Exposito 2011) and executive function impairments (Goudriaan, Grekin, & Sher 2007; Scaife & Duka 2009) have been reported in adolescents with alcohol abuse. Alcohol consumption in adolescence continues into adulthood and is also associated with later alcohol problems and alcohol dependence (Hermos, Winter, Heeren, & Hingson 2008).

Data on experimental animals have also revealed that binge-like ethanol treatment in adolescence impairs both prefrontal cortex (PFC) maturation (Montesinos *et al.* 2015) and hippocampal neurogenesis (Vetreno & Crews 2015), induces structural changes in the hippocampus and cerebellum (Vetreno, Yaxley, Paniagua, & Crews 2015), alters the myelin structure (Montesinos *et al.* 2015) and causes long-term cognitive and behavioral dysfunctions (Montesinos *et al.* 2015; Schulteis, Archer, Tapert, & Frank 2008; Vetreno *et al.* 2015).

Although the molecular mechanisms of alcohol actions in the adolescent brain remain elusive, our previous studies have shown that ethanol is capable of activating immune receptors toll-like receptor 4 (TLR4) by triggering signaling pathways, which induce the release of inflammatory mediators and consequent brain damage in the adult (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri 2010) and adolescent (Montesinos *et al.* 2015) brain. Using adolescent rats (Pascual, Blanco, Cauli, Minarro, & Guerri 2007) and mice (Montesinos *et al.* 2015), we have demonstrated that intermittent binge-like alcohol treatment triggers pro-inflammatory cytokines and mediators (iNOS and COX-2) in the brain, which cause inflammatory damage in the PFC, and impairs synaptic and myelin structures (Montesinos *et al.* 2015) and long-term cognitive dysfunctions (Montesinos *et al.* 2015; Pascual *et al.* 2007) in young adult mice treated with alcohol in adolescence. The role of neuroinflammation and the TLR4 response in ethanol actions on the adolescent brain has been supported by data that have indicated that anti-inflammatory compounds (Pascual *et al.* 2007) or the genetic elimination of TLR4 prevents neuroinflammation, synaptic and myelin disarrangements and long-term cognitive alterations (Montesinos *et al.* 2015; Pascual *et al.* 2007). Neuroimmune gene expression activation has also been suggested to contribute to the risk of alcoholism or to other brain diseases associated with neuroinflammation and linked to adolescent drinking (Vetreno, Qin, & Crews 2013). Some studies have demonstrated that binge drinking differentially affects adolescent male and female brain morphometry and greater deficits in visual memory have been shown in female than in male adolescents (Squeglia *et al.* 2012). Animal studies have also revealed that ethanol-induced neuroinflammatory damage is greater in females than in males (Alfonso-Loeches, Pascual, & Guerri 2013).

Neuroinflammation is associated with increased levels of brain cytokines and chemokines. We have recently shown that some cytokines/chemokines can be used as biomarkers of alcohol-induced brain inflammation and behavioral response in animals after chronic ethanol intake (Pascual, Balino, Aragon, & Guerri 2015). However, whether plasma cytokines and/or chemokines can also

be used as markers of neuroinflammation in adolescents with binge drinking habits is presently unknown.

By using samples from adolescent and young individuals and mice adolescents, the present study was designed to assess if acute alcohol intoxication differently affects the plasma immune response (cytokines and chemokines) in males and females and whether the immune response is associated with the TLR4 response. We herein demonstrated that alcohol intoxication induced higher plasma levels of cytokines/chemokines in females than in males. We found an association between elevated cytokines/chemokines and the upregulation of blood TLR4 mRNA levels in females, but not in males. We also provide evidence that intermittent ethanol treatment increases the levels of several cytokines/chemokines in serum and in the PFC, along with the upregulation of the TLR4 expression of adolescent female mice. However, no significant differences in the cytokines and chemokines levels were observed in adolescent male mice, when compared with untreated counterparts. The present work further showed how female and male adolescent TLR4-knockout (KO) mice were protected against the ethanol-induced immune response in both plasma and the brain. These findings suggest that human female adolescents and young adults are more vulnerable than males to the inflammatory effects of binge ethanol drinking and confirm that TLR4 is an important target of ethanol-induced inflammation.

MATERIALS AND METHODS

Human study

Subjects

Our clinical sample included 27 adolescent and young adults (48.1 percent females) who were admitted to the Emergency Department of the University Hospital of Salamanca (Salamanca, Spain) with moderate to severe acute alcohol intoxication, as previously reported (Lopez-Moreno *et al.* 2015). Acute alcohol intoxication was defined by clinical signs and symptoms (e.g. motor incoordination, unsteady gait, impaired reasoning, slurred speech, confusion or disorientation), blood alcohol levels (BALs) >1 g/l and consumption of at least five (50 g, males) or four (40 g, females) standard drinks during the 6 hours before admission, although the total amount of drinking or the time from first and last ethanol intake was not exactly known for each individual. Patients were excluded if they had any other acute (e.g. trauma or infection) or chronic illness, took any medication or if their toxicological urinary analysis or clinical data demonstrated that they had used illegal drugs (apart from cannabis). Individuals' clinical, epidemiological and analytical characteristics are shown in Table 1.

Table 1 Characteristics of healthy control individuals and alcohol-intoxicated individuals.

	Healthy control individuals		Alcohol-intoxicated individuals	
	Male (n = 8)	Female (n = 9)	Male (n = 14)	Female (n = 13)
Age (years)	23.4 (1.1)	24.3 (1.1)	20.4 (1.2)	19.8 (1.1)
BALs (g/l)	0	0	2.5 (0.1)**	2.2 (0.2)**
AST levels (IU/l)	20.8 (2.0)	16.6 (1.3)	34.6 (4.5)*	18.5 (1.0)***
ALT levels (IU/l)	23.2 (5.5)	14.6 (1.7)	25.8 (5.8)	13.4 (0.9)
ALP levels (IU/l)	58.4 (5.2)	57.0 (9.6)	92.7 (11.8)	70.4 (8.3)
GGT levels (IU/l)	16.4 (3.0)	11.3 (1.2)	25.9 (6.1)	14.0 (1.4)
White blood cell count/ μ l	6830.0 (1190.1)	7302.5 (786.9)	9337.1 (606.3)	7870.8 (454.1)
Individuals who reported weekend drinking (%) ^a	0	0	6 (60.0%)	2 (28.6%)

Quantitative variables are presented as the mean (SEM) and qualitative variables are presented as absolute frequencies (percentage). ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BALs = blood alcohol levels; GGT = γ -glutamyl transpeptidase; IU = international units; SEM = standard error of the mean. * $P < 0.05$. ** $P < 0.001$, as compared with the healthy control group. *** $P < 0.01$, as compared with the alcohol-intoxicated males. ^aFour male individuals and six female individuals refused to answer the questionnaire regarding drinking patterns.

Seventeen healthy controls (eight males and nine females) were also included in the study and were recruited among medical and nursing students. Controls did not consume alcohol apart from light sporadic drinking, did not report alcohol consumption during the 72 hours prior to blood extraction and reported no binge drinking episodes during the three previous months. These subjects showed normal hematological and plasma biochemical parameters (Table 1) and reported no chronic or acute illness. The study was approved by the Ethics Committee of the University Hospital of Salamanca, and written informed consent was obtained from each participant. Blood samples were obtained from the patients upon admission for use in standard care and also for research purposes. These blood samples were used to determine the BAL, blood count and liver function tests and for the mRNA and cytokine analyses. Samples were processed and analyzed for this study only after the patients were able to provide informed consent.

Quantitation of cytokines and chemokines in human plasma samples

Whole blood samples (2.5 ml) from each individual were drawn into lithium heparin tubes and PAXgene Blood RNA tubes (Qiagen, Valencia, CA, USA). Plasma samples were obtained by centrifuging the lithium heparin tubes immediately after collection and were stored at -80°C . Samples were analyzed simultaneously to determine the levels of several cytokines and chemokines using the Milliplex MAP kit premixed human cytokine/chemokine magnetic bead panel (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The analyzed cytokines and chemokines included colony-stimulating factor (CSF), interferon (IFN)- γ ,

interleukin (IL)-10, IL-17A, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, tumor necrosis factor (TNF)- α , fractalkine, monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1 α (MIP-1 α). Data were acquired in a Luminex 100 System and analyzed by the MASTERPLEX CT software, version 1.2.0.7 (Hitachi MiraiBio, USA).

Blood RNA isolation and quantitative reverse transcription polymerase chain reaction

The PAXgene Blood RNA tubes with each subject's blood were kept at room temperature for at least 2 hours and were stored at -20°C . Total RNA was isolated using the PAXgene Blood miRNA Kit (Qiagen) following the manufacturer's instructions and was stored at -80°C . RNA concentration and purity were examined in a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For mRNA expression analysis, complementary DNA was synthesized by reverse transcription using a commercial High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's manual. Relative quantitative real-time polymerase chain reaction was performed using SYBR Green PCR master mix (Applied Biosystems) and gene-specific primer sets: TLR4, 5'-CGA GGA AGA GAA GAC ACC AGT-3' (forward) and 5'-CAT CAT CCT CAC TGC TTC TGT-3' (reverse); actin, 5'-CCA ACC GCG AGA AGA TGA-3' (forward) and 5'-CCA GAG GCG TAC AGG GAT AG-3' (reverse). PCR experiments were performed in duplicate on a StepOnePlusTM Real-Time PCR System (Applied Biosystems), and primer specificity was verified by melt curve analysis. Threshold cycle (C_T ; number of cycles to reach threshold of detection) was determined for each

reaction, and gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

Animal study

Animals and treatments

Male and female C57BL/6 wild-type (WT) (Harlan Ibérica, Barcelona, Spain) and TLR4-KO mice (C57BL/6 background, kindly provided by Dr. S. Akira, Osaka University, Suita, Japan), aged 30 days, were used. All the animals were kept under controlled light and dark (12/12 hours), temperature (23°C) and humidity (60 percent) conditions. All the experimental procedures were approved by the Ethical Committee of Animal Experimentation of the Principe Felipe Research Center (Valencia, Spain) and were carried out in accordance with the guidelines approved by the European Communities Council Directive (2010/63/EU) and by Spanish Royal Decree 1201/2005.

For the binge ethanol treatment, both the male and female WT and TLR4-KO mice were housed (four animals per cage) and maintained with water and solid diet ad libitum. Morning doses of either saline or 25 percent (v/v) ethanol (3 g/kg) in isotonic saline (15 µl/g) were administered intraperitoneally to the 30-day-old mice on two consecutive days with 2-day gaps without injections for 2 weeks (PND30 to PND43), as previously described (Pascual *et al.* 2007). For the biochemical studies, animals were anesthetized with sodium pentobarbital (0.06 mg/kg) 24 hours after the eight ethanol administration and whole blood was collected from the hepatic portal vein. After centrifugation, the separated serum was stored at -80°C until further analysis. Although the reproducibility of the biochemical value was similar in both the plasma and serum samples, we collected serum samples in mice because serum contains higher metabolite concentrations than plasma (Yu *et al.* 2011), which make it possible to provide more sensitive results in biomarker detections. Brains were removed, and the PFC was dissected and stored at -80°C until used.

Quantitation of cytokines and chemokines in mouse serum and PFC

The serum and the homogenized extracts from the PFC were used to determine the levels of IL-1 β (eBioscience, Vienna, Austria), fractalkine (R&D Systems, Abingdon, UK), IL-17A, MCP-1 and MIP-1 α (Peprotech, Barcelona, Spain) with an enzyme-linked immunosorbent assay kit, following the manufacturer's instructions.

Western blot analysis

The Western blot technique was performed in the PFC tissue lysates, as described elsewhere (Fernandez-Lizarbe,

Pascual, & Guerri 2009). The primary antibodies used were NF κ B p-p65 (1:500, Cell Signaling Technology, Leiden, The Netherlands), TLR4 (1:1000, Santa Cruz Biotechnology, Madrid, Spain), NF κ B p65 (1:500) (Santa Cruz Biotechnology) and GAPDH (1:2500, glyceraldehyde 3-phosphate dehydrogenase, Chemicon, CA, USA). Samples were incubated overnight with the corresponding antibodies.

Statistical analysis

The results are reported as mean \pm standard error of the mean. The biochemical data were analyzed by a two-way ANOVA (gender \times alcohol intoxication or treatment), followed by Tukey's multiple comparison test (GRAPHPAD PRISM v7.00, GraphPad Software Inc., CA, USA). The correlations of the data in Fig. 2 and Supporting Information Table S1 were determined by Pearson's coefficient (SPSS program v17.0, IBM, Madrid, Spain). A linear regression model was also generated with the BALs as a score predictor of the cytokine or chemokine levels (SPSS program, v17.0).

RESULTS

Gender differences in the plasma cytokine and chemokine levels after alcohol intoxication in human adolescents and young adults

The median age of females and males was 18.0 years [interquartile range (IQR) 18.0–22.5] and 19.0 years (IQR 16.8–24.5), respectively. These ages have been considered as late adolescence (ages 16–20 years) or young adulthood (ages 21–25 years) (Brown *et al.* 2008; NIAAA 2009). In addition, the biochemical analysis of plasma during the intoxication period demonstrated a median BAL of 2.10 g/l (IQR 1.80–2.50) for females and one of 2.40 g/l (IQR 2.28–2.73) for males. No other drugs of abuse were found. The data showed a greater dispersion of the BALs in females than in males. Control subjects were used, with median ages of 23.0 years (IQR 21.2–26.2) for females and 23.0 years (IQR 21.5–25.7) for males.

Two-way ANOVA with Tukey's *post hoc* test revealed that alcohol intoxication in the female subjects caused a significant upregulation in the levels of plasma cytokine, e.g. IL-1 β , IL-2, IL-6, IL-10, IL-8, IFN- γ , IL-17A, IL-4 and chemokine, e.g. MIP-1 α , fractalkine and MCP-1, compared with the control counterparts group (Fig. 1a). When the plasma cytokines levels were assessed in the ethanol-intoxicated males, only significant elevations were noted for CSF compared with the plasma samples of the control individuals. No differences were observed between healthy male and female subjects in the analyzed cytokines and chemokines. Two-way ANOVA

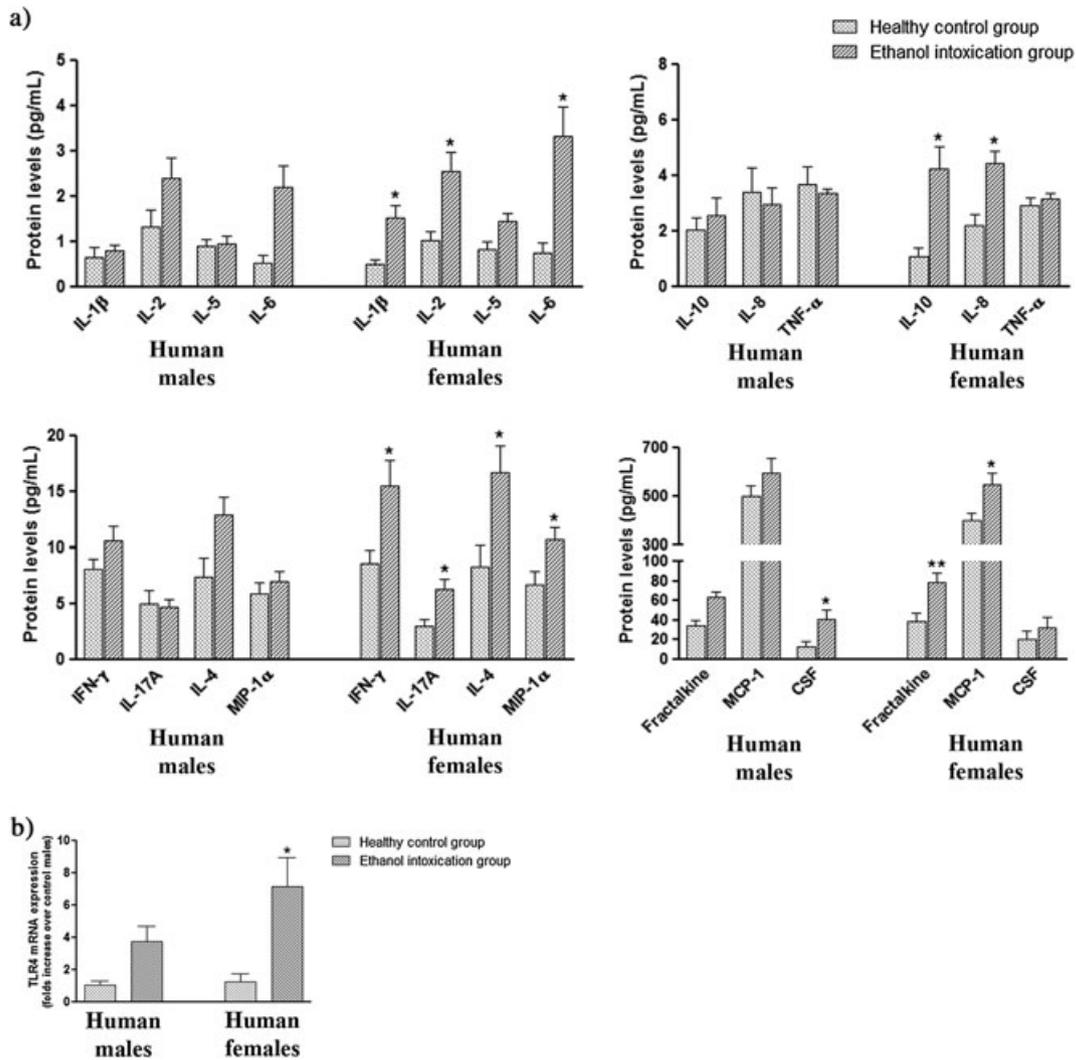


Figure 1 Human plasma levels of interleukin (IL)-1 β , IL-2, IL-5, IL-6, IL-10, IL-8, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , IL-17A, IL-4, macrophage inflammatory protein (MIP)-1 α , fractalkine, monocyte chemoattractant protein (MCP)-1 and colony-stimulating factor (CSF) of the males and females. (a) Graphs represent the plasma data of females and males after acute ethanol intoxication and the data of the corresponding control abstainers. Data represent mean \pm standard error of the mean (SEM), $n = 8-14$ individuals/group. * $P < 0.05$, ** $P < 0.01$ compared with their respective control abstainers. (b) Graphs represent the levels of the toll-like receptor 4 (TLR4) mRNA expression from the blood of female and male individuals after acute ethanol intoxication and also of the corresponding control abstainers. Results are expressed relatively to values of the control group (AU, arbitrary units; mean \pm SEM), $n = 8-14$ individuals/group. * $P < 0.05$ compared with their respective control abstainers

analyses were conducted to compare whether the effects of alcohol intoxication on the plasma cytokine and chemokine levels differed between the female and male subjects. These analyses revealed a significant interaction effect on IL-17A [$F(1, 39) = 4.24, P < 0.05$] and a main effect of alcohol intoxication on CSF [$F(1, 37) = 7.44, P < 0.01$], IFN- γ [$F(1, 39) = 7.15, P < 0.05$], IL-10 [$F(1, 34) = 5.63, P < 0.05$], IL-1 β [$F(1, 35) = 6.42, P < 0.05$], IL-2 [$F(1, 38) = 10.95, P < 0.01$], IL-4 [$F(1, 36) = 14.30, P < 0.001$], IL-6 [$F(1, 33) = 14.62, P < 0.001$], fractalkine [$F(1, 39) = 16.97, P < 0.001$], MIP-1 α [$F(1, 37) = 7.08, P < 0.05$], IL-8 [$F(1, 38) = 6.00, P < 0.05$] and MCP-1 [$F(1, 33) = 6.37, P < 0.05$]. No significant interactions were found on

CSF [$F(1, 37) = 2.29, P > 0.05$], IFN- γ [$F(1, 39) = 1.53, P > 0.05$], IL-10 [$F(1, 34) = 2.91, P > 0.05$], IL-1 β [$F(1, 35) = 3.65, P > 0.05$], IL-2 [$F(1, 38) = 0.35, P > 0.05$], IL-4 [$F(1, 36) = 0.07, P > 0.05$], IL-5 [$F(1, 38) = 2.37, P > 0.05$], IL-6 [$F(1, 33) = 0.27, P > 0.05$], TNF- α [$F(1, 39) = 0.88, P > 0.05$], fractalkine [$F(1, 39) = 0.45, P > 0.05$], MIP-1 α [$F(1, 37) = 1.27, P > 0.05$], IL-8 [$F(1, 38) = 2.03, P > 0.05$] and MCP-1 [$F(1, 33) = 2.33, P > 0.05$]. Similarly, no main effect of ethanol intoxication was observed on IL-17A [$F(1, 39) = 2.92, P > 0.05$], IL-5 [$F(1, 38) = 3.40, P > 0.05$], TNF- α [$F(1, 39) = 0.03, P > 0.05$] and gender on CSF [$F(1, 37) = 0.72, P > 0.05$], IFN- γ [$F(1, 39) = 2.26, P > 0.05$], IL-10 [$F(1, 34) = 0.21, P > 0.05$], IL-17A [$F(1, 39) = 0.04,$

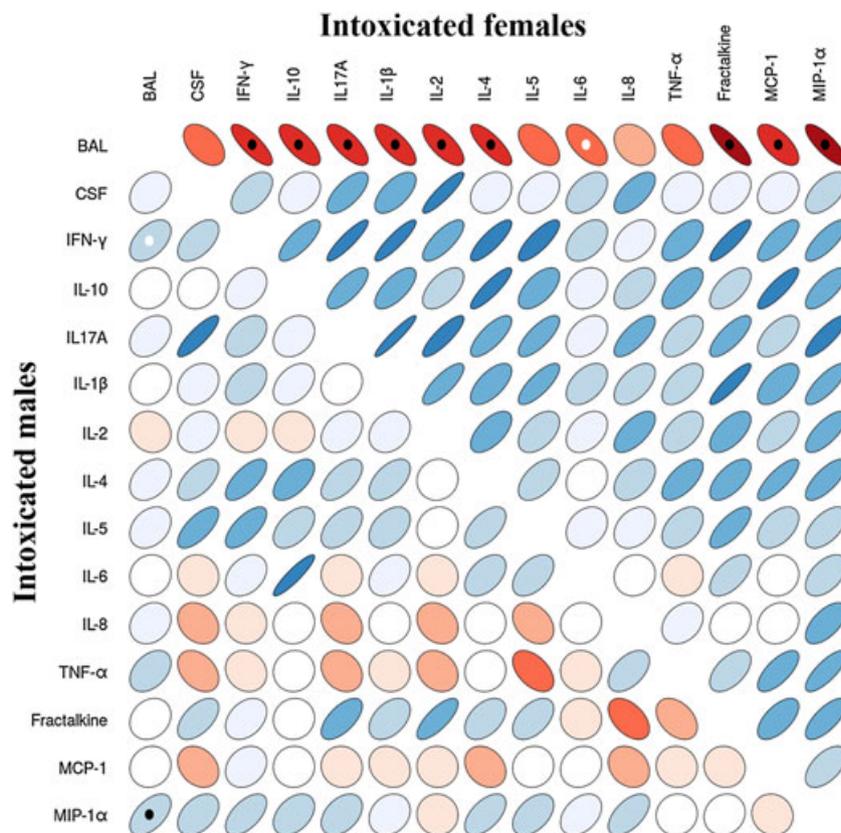


Figure 2 Pearson's coefficient between the plasma levels of cytokines or chemokines and the blood alcohol levels (BALs) in females and males with alcohol intoxication. Plot represents the correlation coefficient and the significant P value between the plasma BALs and the levels of cytokines or chemokines in females and males intoxicated with alcohol. The width of the ellipses and the color gradient indicate the strength of the correlation. Blue ellipses represent positive correlations, and negative ones are represented by red ellipses. Black points represent statistically significant correlations, and white ones denote a tendency to be significant

$P > 0.05$], IL-1 β [$F(1, 35) = 1.65, P > 0.05$], IL-2 [$F(1, 38) = 0.03, P > 0.05$], IL-4 [$F(1, 36) = 0.45, P > 0.05$], IL-5 [$F(1, 38) = 0.45, P > 0.05$], IL-6 [$F(1, 33) = 0.79, P > 0.05$], TNF- α [$F(1, 39) = 2.54, P > 0.05$], fractalkine [$F(1, 39) = 1.47, P > 0.05$], MIP-1 α [$F(1, 37) = 3.65, P > 0.05$], IL-8 [$F(1, 38) = \dots, P > 0.05$] and MCP-1 [$F(1, 33) = 0.29, P > 0.05$]. These results indicated that females were more vulnerable than males to alcohol effects.

To study whether there was a correlation between the high plasma levels of cytokines and chemokines and the BALs during the intoxicated period, we used a Pearson's correlations analysis. Figure 2 and Supporting Information Table S1 show a significant negative correlation in the intoxicated females for cytokines and chemokines, IFN- γ , IL-1 β , IL-2, IL-4, IL-10, IL-17A, fractalkine, MCP-1 and MIP-1 α and a significant tendency for IL-6, which became evident at high alcohol levels. Among the intoxicated male subjects, we observed a correlation only between the BALs and MIP-1 α , although a significant tendency was noted for IFN- γ (Fig. 2 and Supporting Information Table S1). We also performed a linear regression model in the BALs of both alcohol-intoxicated

females and males with the different cytokines and chemokines to estimate possible linearity between both variables. This analysis improved the evaluation of the relationship between the levels of each cytokine/chemokine with BALs. The linear regression analysis gave similar significant results to Pearson's correlation (Table 2 and Supporting Information Fig. S1).

Acute ethanol intoxication increases the TLR4 mRNA levels in the blood of adolescent and young females, but not of males

To gain further insights into the actions of ethanol on the innate immune response and as we have previously demonstrated the role of TLR4 in the ethanol-induced immune response in mice (Alfonso-Loeches *et al.* 2010; Fernandez-Lizarbe *et al.* 2009; Montesinos *et al.* 2015), we assessed the mRNA expression of TLR4 in the whole blood samples of the females and males after acute alcohol intoxication. Two-way ANOVA with Tukey's *post hoc* test indicated that alcohol intoxication significantly increased the blood mRNA levels of TLR4 in females compared with the control group's values (Fig. 1b).

Table 2 Linear regression analysis between the plasma levels of cytokines or chemokines and the BALs in females and males with alcohol intoxication.

	Intoxicated females			Intoxicated males		
	Slope	Constant	P	Slope	Constant	P
CSF	-28.58	106.14	0.180	31.88	-37.35	0.388
IFN- γ	-8.54	34.61	0.006	7.45	-7.57	0.091
IL-10	-2.59	10.06	0.024	1.01	0.09	0.658
IL-17A	-3.39	14.13	0.021	2.84	-2.26	0.264
IL-1 β	-1.03	3.91	0.016	0.26	0.13	0.572
IL-2	-1.41	5.57	0.022	-0.62	3.89	0.701
IL-4	-6.77	30.16	0.007	6.11	-2.01	0.255
IL-5	-0.51	2.60	0.121	0.63	-0.58	0.311
IL-6	-2.27	8.68	0.070	0.38	1.28	0.823
IL-8	-0.75	5.91	0.412	3.56	-3.94	0.512
TNF- α	-0.51	4.28	0.103	0.81	1.35	0.175
Fractalkine	-37.94	162.81	0.002	14.04	28.65	0.517
MCP-1	-158.20	849.56	0.039	83.85	359.32	0.758
MIP-1 α	-4.44	20.83	0.005	5.71	-7.41	0.050

Data marked in dark grey are statistically significant, while data in light grey denote a tendency to be significant. CSF = colony-stimulating factor; IFN = interferon; IL = interleukin; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; TNF = tumor necrosis factor.

However, no significant differences in the gene expression levels of this receptor was noted in the blood of either intoxicated males versus healthy males or healthy control males versus female subjects. The two-way ANOVA analyses revealed a main effect of alcohol intoxication [$F(1, 28) = 10.73, P < 0.01$], but no significant effects in either gender [$F(1, 28) = 1.84, P > 0.05$] or interaction [$F(1, 28) = 1.42, P > 0.05$].

Gender differences in the cytokine and chemokine levels in the serum and PFC of the WT mice after intermittent ethanol exposure

To further investigate gender differences in the ethanol response in adolescent and young individuals, we used an experimental model of intermittent ethanol treatment in adolescence. Adolescent female and male WT mice, intermittently treated with ethanol or saline, were used to mimic the drinking pattern of adolescents. We determined BALs after a single ethanol dose to evaluate possible gender differences. After this single ethanol dose, BALs peaked at 164.5 ± 2.5 mg/dl in the WT female mice and at 161.6 ± 1.5 mg/dl in the WT male mice at 30 minutes post-injection with similar ethanol clearance at 5 hours post-injection (data not shown). We also evaluated the BAL levels in both male and female mice after eight intermittent ethanol doses. No statistical variations in the BAL levels in either the female and male mice were observed after the eighth ethanol injection (at 30 minutes post-injection, 165.4 ± 1.1 mg/dl in the WT female mice and 170.1 ± 0.8 mg/dl in the WT male mice).

The levels of cytokines and chemokines were also assessed in the serum and PFC of the saline-treated or ethanol-treated WT mice. Two-way ANOVA with Tukey's *post hoc* test revealed that intermittent ethanol treatment significantly increased the serum levels of IL-17A, MCP-1 and MIP-1 α of the WT adolescent female mice compared with the saline WT female mice (Fig. 3a). However, the same ethanol treatment did not significantly alter the serum cytokine/chemokine levels in the adolescent male mice compared with the saline control group. In addition, significant differences in the serum MIP-1 α levels were noted between the male and female saline-treated WT mice (Fig. 3a). To determine the differences between the WT male and female mice in the serum cytokine and chemokine levels, two-way ANOVAs were assessed. These analyses revealed a significant effect of interaction on IL-17A [$F(1, 28) = 10.22, P < 0.01$] and a main effect of gender [$F(1, 28) = 6.83, P < 0.05$] and interaction [$F(1, 28) = 4.30, P < 0.05$] on MIP-1 α . No significant effects were found on IL-1 β {gender [$F(1, 28) = 2.34, P > 0.05$], treatment [$F(1, 28) = 0.06, P > 0.05$], gender and treatment [$F(1, 28) = 0.01, P > 0.05$]}, IL-17A {treatment [$F(1, 28) = 2.14, P > 0.05$], gender [$F(1, 28) = 0.03, P > 0.05$]}, MCP-1 {gender [$F(1, 28) = 0.45, P > 0.05$], treatment [$F(1, 28) = 3.91, P > 0.05$], gender and treatment [$F(1, 28) = 1.78, P > 0.05$]}, MIP-1 α {treatment [$F(1, 28) = 0.70, P > 0.05$] and fractalkine {gender [$F(1, 28) = 2.45, P > 0.05$], treatment [$F(1, 28) = 0.01, P > 0.05$], gender and treatment [$F(1, 28) = 0.58, P > 0.05$]}.

We next assessed whether the differences in the serum levels of cytokines and chemokines observed

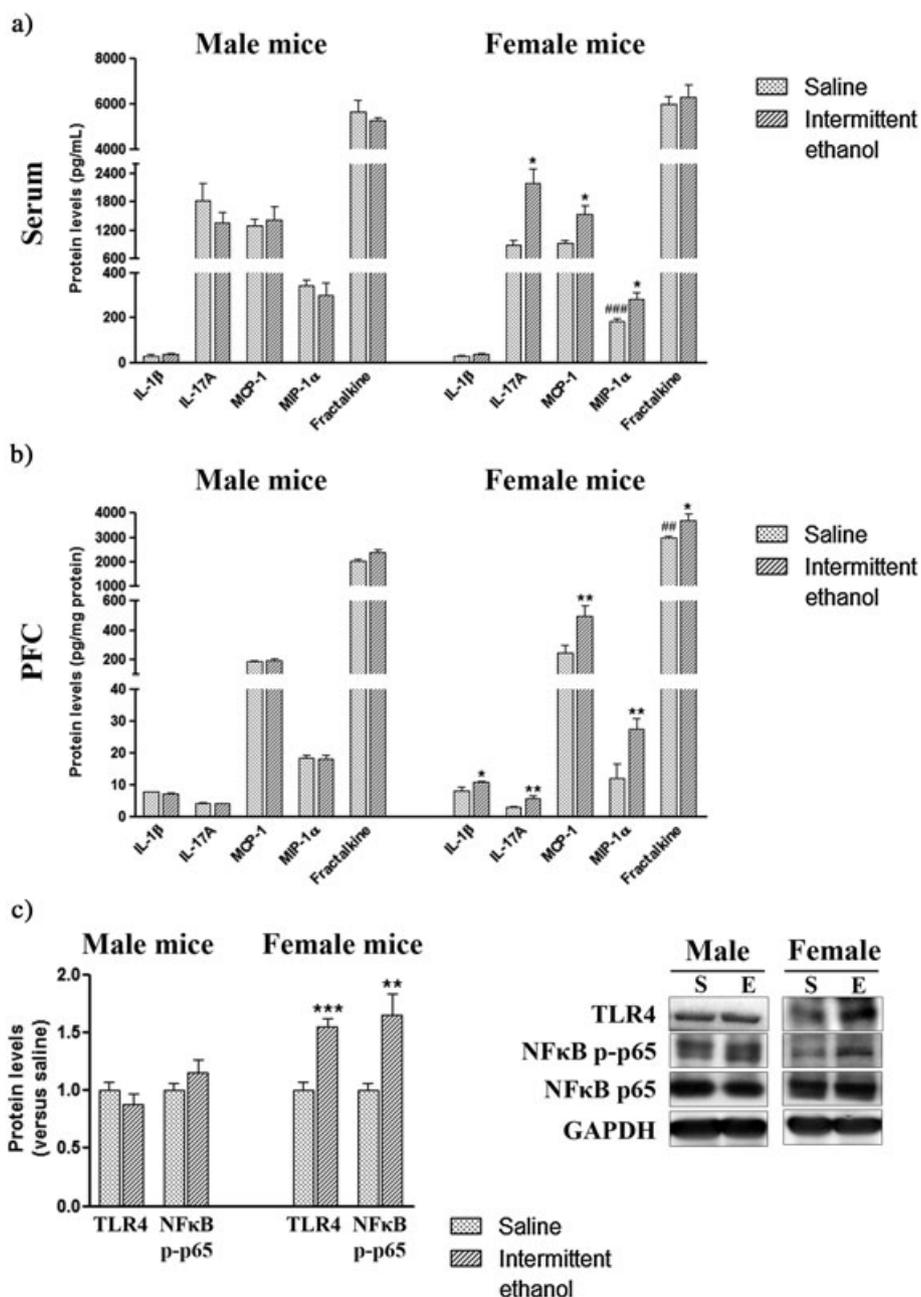


Figure 3 The prefrontal cortex (PFC) levels of interleukin (IL)-17A, IL-1β, fractalkine, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α of the male and female WT adolescent mice. Mice at PND 30 were treated intraperitoneally with eight intermittent ethanol doses (3 g/kg) or physiological saline. Graphs represent data from the (a) serum and (b) PFC of the female and male adolescent WT mice, treated or not with ethanol. Data represent mean ± standard error of the mean (SEM), $n = 8$ mice/group. * $P < 0.05$, ** $P < 0.01$ compared with their respective saline-treated group, ## $P < 0.01$, ### $P < 0.001$ compared with the saline-treated male group. (c) Immunoblot analysis and quantification of TLR4 and NFκB p-p65 from the PFC of the female and male adolescent WT mice treated intermittently, or not, with ethanol. A representative immunoblot of each protein is shown. Data represent mean ± SEM, $n = 6$ mice/group. ** $P < 0.01$, *** $P < 0.001$ compared with their respective saline-treated group

between the ethanol-treated WT adolescent male and female mice were associated with the changes in these pro-inflammatory molecules in the PFC. Figure 3b shows how intermittent ethanol treatment upregulated the levels of IL-1β, IL-17A, MCP-1, MIP-1α and the fractalkine levels in the PFC of the WT adolescent female mice compared with the untreated adolescent female

WT mice, as revealed by using two-way ANOVA with Tukey's *post hoc* test. Significant differences were also noted in the fractalkine levels of PFC between the male and female saline-treated WT mice (Fig. 3b). Two-way ANOVAs were assessed to determine the differences between the adolescent WT male and female mice in the cytokine and chemokine levels of the PFC. The overall

analyses revealed a significant effect of variables gender, treatment and the gender–treatment interaction on IL-1 β {gender [$F(1, 28) = 8.26, P < 0.01$], gender and treatment [$F(1, 28) = 5.87, P < 0.05$]}, IL-17A {treatment [$F(1, 28) = 7.07, P < 0.05$], gender and treatment [$F(1, 28) = 7.71, P < 0.01$]}, MCP-1 {gender [$F(1, 28) = 16.04, P < 0.001$], treatment [$F(1, 28) = 8.08, P < 0.01$], gender and treatment [$F(1, 28) = 7.22, P < 0.05$]}, MIP-1 α {treatment [$F(1, 28) = 6.79, P < 0.05$], gender and treatment [$F(1, 28) = 7.08, P < 0.05$] } and fractalkine {gender [$F(1, 28) = 49.76, P < 0.001$] and treatment [$F(1, 28) = 11.63, P < 0.01$]}, with no significant effects on IL-1 β {treatment [$F(1, 28) = 3.23, P > 0.05$]}, IL-17A {gender [$F(1, 28) = 0.42, P > 0.05$]}, MIP-1 α {gender [$F(1, 28) = 0.24, P > 0.05$] } and fractalkine {gender and treatment [$F(1, 28) = 1.35, P > 0.05$]}.

We further assessed the protein expression of TLR4 and the phosphorylation of the transcription factor NF κ B p65 subunit involved in the TLR4 signaling pathway, in the PFC of the WT male and female mice treated with saline or intermittent ethanol treatment. Two-way ANOVA with Tukey's *post hoc* test revealed that the levels of TLR4 and NF κ B p65 phosphorylation significantly increased in the WT female mice, but not in males, treated intermittently with ethanol (Fig. 3c). For both proteins, the two-way ANOVAs revealed a significant effect of treatment {TLR4: [$F(1, 20) = 8.30, P < 0.01$], NF κ B p-p65: [$F(1, 20) = 12.43, P < 0.01$]}, gender {TLR4: [$F(1, 20) = 20.10, P < 0.001$], NF κ B p-p65: [$F(1, 20) = 4.83, P < 0.05$] } and interactions {TLR4: [$F(1, 20) = 20.10, P < 0.001$], NF κ B p-p65: [$F(1, 20) = 4.83, P < 0.05$]}.

We also analyzed the cytokine and chemokine levels after an acute ethanol dose (1.5 hours post-injection). Unpaired Student's *t*-test analyses indicated that while an upregulation of IL-17A, IL-1 β and MCP-1 took place in the serum of the WT female mice after one ethanol dose, no changes were observed in the cytokine/chemokine levels of the PFC, although both the TLR4 expression and NF κ B p65 phosphorylation increased (Supporting Information Fig. S2).

Finally, we assessed the cytokine levels in the TLR4-KO adolescent female and male mice treated with either saline or ethanol. As shown in Fig. 4, ethanol treatment did not significantly change the cytokine/chemokine levels in either the serum (Fig. 4a) or PFC (Fig. 4b) in the TLR4-KO female and male mice.

These results indicated that the WT female mice were more vulnerable than the WT male mice to ethanol-induced immune activation, and also suggested the involvement of the innate immune receptors TLR4 response in ethanol effects.

DISCUSSION

The present findings indicated major gender differences in the immune response of ethanol. Thus, at the equivalent BALs, both the human and mice adolescent females had higher levels of plasma cytokines and chemokines than the adolescent males after acute alcohol intoxication. These results also provide evidence for the role of the TLR4 response in these effects because the TLR4 mRNA levels increased in the blood of the human adolescent females, but not in the males. Accordingly, the TLR4-KO adolescent female and male mice were protected against the inflammatory/neuroinflammatory response of ethanol.

The susceptibility of young females versus males to ethanol actions in adolescence has been pointed out in several studies. For instance, females who begin drinking in adolescence and who continue to drink heavily may be at a higher risk of brain damage and of behavioral problems later in life compared with male adolescents (Squeglia, Spadoni, Infante, Myers, & Tapert 2009). Accordingly, drinking in adolescence is associated with greater frontal cortical thinning in females than in males, effects that are linked to worse visuospatial and inhibition of attention performance (Squeglia *et al.* 2012; Squeglia *et al.* 2009). Executive functioning deficits have also been found in female substance-using teens (Giancola, Shoal, & Mezzich 2001; Moss, Kirisci, Gordon, & Tarter 1994). These results suggested that females could be much more susceptible than males to the neurodevelopmental dysfunction associated with heavy alcohol use in adolescence. In line with these results, a recent study performed in adult males and females has also indicated that after an acute ethanol dose, females had higher levels of BALs and circulating endotoxin levels than males (Bala, Marcos, Gattu, Catalano, & Szabo 2014). Although these gender differences in the immune response effects of ethanol are uncertain, differences in alcohol metabolism, hormonal changes and different alcohol distribution due to the body/fat ratio between females and males (Frezza *et al.* 1990; Wechsler, Dowdall, Davenport, & Rimm 1995) might explain some of the observed gender differences.

According to the human data, our results also showed how the cytokine and chemokine levels were higher in the serum and PFC of the adolescent female mice than in the male mice. These effects were associated with the TLR4 response because the TLR4 levels increased in the PFC of the WT female mice after different intermittent doses of ethanol in adolescence. These gender differences were not related to the differences found in BALs after administering ethanol because similar BALs levels were noted in the male and female mice. However, some studies (Middaugh, Frackelton, Boggan, Onofrio, & Shepherd

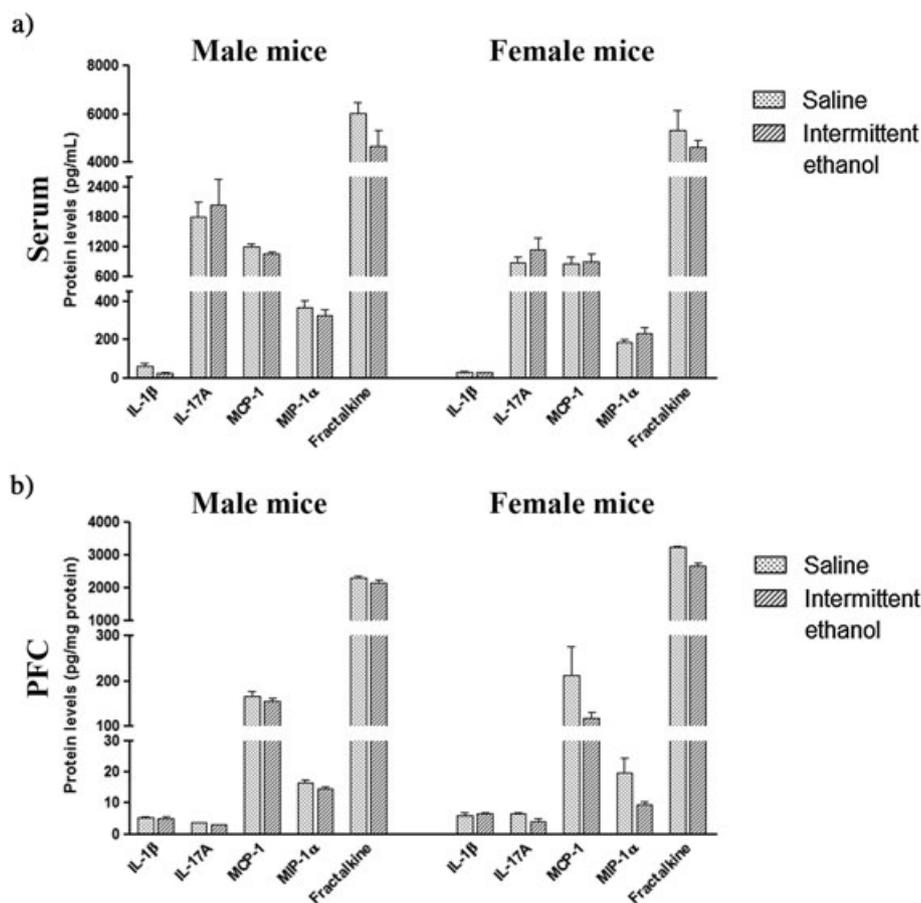


Figure 4 Serum levels of interleukin (IL)-17A, IL-1 β , fractalkine, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1 α of the male and female toll-like receptor 4-knockout (TLR4-KO) adolescent mice. Mice at PND 30 were treated intraperitoneally with eight intermittent ethanol doses (3 g/kg) or physiological saline. Graphs represent data from the (a) serum and (b) prefrontal cortex (PFC) of the female and male adolescent TLR4-KO mice treated, or not, with ethanol. Data represent mean \pm standard error of the mean (SEM), $n = 8$ mice/group

1992) report higher BALs in 2-month-old male mice than in female mice. We further showed that eliminating the TLR4, by using TLR4-KO mice, abolished the immune response in the serum and PFC of both male and female adolescent mice, which supports the role of these receptors in the ethanol-induced immune response. According to our results, the upregulation of both TLR4 immunoreactivity and pro-inflammatory cytokines in the PFC of adolescent rats treated intermittently with ethanol has previously been demonstrated (Vetreno *et al.* 2013), which supports the involvement of the immune response in ethanol effects.

In line with the results obtained in adolescent mice, we previously demonstrated some gender differences in adult mice because the neuroinflammation and astrogliosis associated with chronic alcohol intake were higher in female mice than in male mice and the TLR4-KO female mice were protected against neuroinflammatory ethanol actions (Alfonso-Loeches *et al.* 2013). The potential role of TLR4 in ethanol-induced immune activation was further supported by

the human data that showed an upregulation of TLR4 gene expression in the blood of females, but not of males. This suggests the participation of the TLR4 response in the gender differences of the ethanol-induced cytokine and chemokine immune response after acute ethanol intoxication. According with these findings, a recent study shows that women exhibited a more profound pro-inflammatory response (e.g. IL-6 and TNF- α) than men after an intravenous LPS (TLR4 ligand) injection (Engler *et al.* 2016). In fact, alveolar macrophages of the female rats treated with LPS spontaneously released significantly more nitric oxide than the male rats (Spitzer 1997). Conversely, other studies have shown that estradiol attenuates the LPS-induced expression of CXCL8 in human peripheral blood monocytes (Pioli *et al.* 2007). Spitzer & Zhang (1996) demonstrated that the LPS challenge elicits a milder phagocytic response in the ethanol-treated liver polymorphonuclear leukocytes and Kupffer cells of female rats than in male rats (Spitzer & Zhang 1996). Differences in species and immune cells response might account for these divergences.

Our results also provide evidence that both human and mice adolescent females had higher levels of peripheral cytokines and chemokines than the adolescent males during or after acute alcohol intoxication. Changes and upregulation in plasma cytokine levels have also been reported in adolescent students with heavy binge drinking (Ward, Lallemand, & de Witte 2014), although no gender differences were assessed. Using the data of the human adolescents, we also examined the association between the BALs and the cytokine and chemokine circulating levels in both female and male adolescents with acute alcohol intoxication. When the linear regression analyses were used, we observed a potent linearity between the BALs and several cytokines and chemokines for both genders. Our results also showed that at the same BALs, e.g. 2 g/l, females displayed a stronger immune response than males. We also noticed that while 70 percent of the females with BALs of ~2 g/l (~45 mM) had higher levels of cytokines and chemokines than males, 30 percent of the females obtained very high BALs (3.2 g/l and ~75 mM) and displayed lower levels of cytokines and chemokines than at 2 g/l. This paradoxical effect could be explained by the biphasic effects of ethanol on the TLR4 response. Unfortunately, the negative correlation was not found in the males because they did not reach BALs over 75 mM. We previously showed that ethanol exerts biphasic action on TLR4; at low/moderate concentrations (10–50 mM), it interacts with membrane lipid rafts by activating the TLR4 response, while at a high concentration (75–100 mM), it disrupts lipid rafts and inhibits the receptor response and the release of cytokine (Blanco & Guerri 2007; Fernandez-Lizarbe, Pascual, Gascon, Blanco, & Guerri 2008). Likewise, corticoids have pro-inflammatory or anti-inflammatory effects in humans (Yeager, Pioli, & Guyre 2011). A single alcohol dose that resulted in a BAL of 100-mg percent activates the hypothalamo-pituitary-adrenal axis and leads to elevated cortisol levels (Richards, Fernandez, Caulfield, & Hawrylowicz 2000; Spencer & Hutchison 1999). It is therefore possible that high BALs could decrease the levels of cytokines/chemokines in ethanol-intoxicated females by inhibiting the TLR4 response or promoting the release of glucocorticoids and their anti-inflammatory response.

Activation of the immune system and the TLR4 might also occur through the release of gut bacteria components, as previously demonstrated in healthy adults after acute binge drinking (Bala *et al.* 2014). Interestingly, this study also shows that the levels of blood alcohol and circulating endotoxin were higher in women than in men, which corroborates the importance of gender differences in alcohol effects. To date, it remains unknown whether an ethanol-induced immune response is due to the release of endotoxin (Bala *et al.* 2014) and/or to direct ethanol action on the TLR4 response. However, if we

consider that alcohol in the absence of LPS can trigger the TLR4 response in microglia and in RAW 264.7 macrophage cells (Fernandez-Lizarbe *et al.* 2008; Fernandez-Lizarbe *et al.* 2009), it is conceivable to suggest that both ethanol and LPS could be involved in the TLR4-dependent peripheral immune response. Another question is whether the cytokines and chemokines induced by an acute ethanol dose in the PFC could originate in the brain and/or is the result of a peripheral response to ethanol. Because alcohol can reach the brain and can trigger the TLR4 signaling response in glial cells within minutes (Alfonso-Loeches *et al.* 2010; Fernandez-Lizarbe *et al.* 2009), a direct effect of alcohol on the TLR4 response could be considered before peripheral inflammation could reach the brain. Nonetheless, we cannot rule out the coexistence of other mechanisms.

Finally, the present results also suggested that cytokines and/or chemokines can be used as biomarkers of ethanol-induced neuroinflammation in female adolescents because alcohol intoxication and/or binge-drinking increases the peripheral levels of IL-17A, MCP-1 and MIP-1 α in both human and mice adolescent females and also because the levels of these cytokines/chemokines were also significantly high in the PFC of the adolescent female mice. In line with these results, we recently demonstrated that chronic ethanol consumption increased the levels of cytokines (IL-1 β , IL-17A and TNF- α) and chemokines (MCP-1, MIP-1 α and fractalkine) in the striatum and serum (MCP-1, MIP-1 α and fractalkine) of WT male mice (Pascual *et al.* 2015). These variations in cytokine and chemokine levels between both models could be related with the different ages of the animals (adolescents versus adults), ethanol treatment (binge drinking versus chronic treatment) or brain area analyzed.

It is important to point out the limitations of our results when using human samples, particularly the small sample size and the unknown drinking pattern of several participants. In fact, the poor statistical power associated with a small sample size could have prevented us from finding significant differences in the plasma cytokine/chemokine levels between the control and ethanol-intoxicated males. Available results from our sample, however, are in agreement with previous data showing that twice as many adolescent males reported heavy binge drinking (≥ 5 standard drinks on at least 1 day in the recorded diary week) compared with females (Olsson *et al.* 2016). If we take this into account, it is most likely that adolescent and young adult males in our study have had more episodes of binge drinking than females, although it is uncertain to which extent this could explain our results. Another limitation of this study is that the human ethanol samples were collected during the intoxication period, while the murine samples were collected 24 hours after the last ethanol dose. Human

studies indicate that a binge drinking episode causes a transient pro-inflammatory state, which is followed by an anti-inflammatory state (e.g. Afshar *et al.* 2015).

Taken together, these results indicate that female adolescents are more vulnerable than male adolescents to the inflammatory effects of binge alcohol drinking. The findings also suggest that circulating cytokines and chemokines might serve as clinical biomarkers of neuroinflammation and that the TLR4 response is an important target of the ethanol-induced immune and neuroimmune response in adolescence.

Acknowledgements

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Authors Contribution

CG, MP, JM, FJL and MM were responsible for the study concept and design. PCA and JLT contributed to the collection of human samples and clinical data and to the acquisition of the human TLR4 mRNA data. JM and MP contributed to the acquisition of animal samples and to carry on the enzyme-linked immunosorbent assay and Western blot experiments and to analyze all data. FGG contributed to the correlation and linear regression analyses. MP, JM and CG drafted the manuscript. MM, JLT, PCA and FJL provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table 1S: Pearson's correlation between the plasma levels of cytokines or chemokines and the BALs in females and males with alcohol intoxication. Data marked in dark grey are statistically significant, while data in light grey denote a tendency to be significant.

Figure 1S: Examples of dispersion data between BALs and fractalkine, IFN- γ and IL-4 in females and males with alcohol intoxication. It is also shown the linear regression between BALs and fractalkine, IFN- γ and IL-4 in intoxicated females, which their slope and constant are shown in Table 2.

Figure 2S: The PFC and serum levels of IL-17A, IL-1 β , MCP-1 and MIP-1 α of the female WT and TLR4-KO adolescent mice. Female mice at PND 30 were treated intraperitoneally with one ethanol dose (3 g/kg) or physiological saline. After 1.5 h of the injection, serum was collected and the PFC was removed. Graphs represent data from the (A) PFC and (B) serum of the female WT and TLR4-KO adolescent mice. Data represent mean \pm SEM, n = 8 mice/group. * p < 0.05 compared to their respective saline-treated group, and according to an unpaired Student's *t*-test. (C) Immunoblot analysis and quantification of TLR4 and NF κ B p-p65 from the PFC of the female WT mice treated with an acute ethanol dose (1.5 h). A representative immunoblot of each protein is shown. Data represent mean \pm SEM, n = 6 mice/group. * p < 0.05, *** p < 0.001 compared to their respective saline-treated group, and according to an unpaired Student's *t*-test.