Role of Inflammatory Pathways, Blood Mononuclear Cells, and Gut-Derived Bacterial Products in Alcohol Dependence

Sophie Leclercq, Christine De Saeger, Nathalie Delzenne, Philippe de Timary, and Peter Stärkel

Background: Inflammation might play a role in the development of several psychiatric diseases. However, the origins of processes that mediate inflammation are unknown. We previously reported increased intestinal permeability, elevated blood lipopolysaccharide levels, and low-grade systemic inflammation associated with psychological symptoms of alcohol dependence in alcohol-dependent subjects. In this study, we tested inflammatory responses of peripheral blood mononuclear cells (PBMCs) to gut-derived bacterial products during detoxification and the relationship to alcohol craving.

Methods: In 63 actively drinking noncirrhotic alcohol-dependent subjects, testing was performed at the beginning (day 2) and end (day 18) of alcohol detoxification and compared with testing in 14 healthy subjects. Activation of various intracellular signaling pathways by gut-derived bacterial products was analyzed by quantitative polymerase chain reaction, Western blotting, and DNA binding assays (for transcription factors). Toll-like receptor activation was assessed by cell cultures.

Results: In addition to lipopolysaccharides, we showed that peptidoglycans may also cross the gut barrier to reach the systemic circulation. Both activate their respective Toll-like receptors in peripheral blood mononuclear cells. Chronic alcohol consumption inhibited the nuclear factor kappa B proinflammatory cytokine pathway but activated the mitogen-activated protein kinase/activator protein 1 pathway, together with the inflammasome complex. This activity resulted in increased messenger RNA and plasma levels of interleukin (IL)-8, IL-1β, and IL-18. Activated proinflammatory pathways, in particular, IL-8 and IL-1β, were positively correlated with alcohol consumption and alcohol-craving scores. Short-term alcohol withdrawal was associated with the recovery of lipopolysaccharide-dependent receptors but not peptidoglycan-dependent receptors.

Conclusions: Lipopolysaccharides and peptidoglycans from the gut microbiota stimulate specific inflammatory pathways in peripheral blood mononuclear cells that are correlated with alcohol craving.

Key Words: Alcohol craving, inflammasome, lipopolysaccharides, MAP kinases, peptidoglycans, proinflammatory cytokines

Studies suggest a role for inflammation in the development of several psychiatric diseases (1), including alcohol dependence (2), a disorder that affects 5%–7% of the population in developed countries (3). Inflammation in alcohol dependence (AD) has been ascribed to a local proinflammatory effect of ethanol, either in the brain or in the liver (2). However, heavy chronic alcohol consumption induces gut mucosal damage, increases intestinal permeability (4–6), induces changes in the composition of the gut microbiota (7,8), and induces bacterial overgrowth in the small intestine (9,10). Gut bacteria are classified into one of two major groups, according the multilayered structure of their cell envelopes. Gram-negative bacteria are surrounded by a thin peptidoglycan (PGN) cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide (LPS). Gram-positive bacteria lack the LPS-associated outer membrane but are surrounded by layers of PGN many times thicker than is found in gram-negative bacteria (11). The view that systemic inflammation could play a role in alcohol dependence and be induced by increased intestinal permeability and permeation of LPS is supported by more recent data in humans (6). Nevertheless, the possibility of an inflammatory effect of other bacterial components such as PGN has not been tested to date.

Gut-derived bacterial LPS and PGN interact with receptors on lymphocytes and monocytes, the membrane-bound Toll-like receptors (TLR4 and TLR2, respectively), and the cytosolic Nod-like receptors to elicit inflammatory responses (12,13). On receptor activation, a signal transduction cascade converges toward a common set of signaling molecules, leading to the activation of various transcription factors that drive the production of proinflammatory cytokines and type I interferons (14,15).

The Toll-like receptor TLR4 interacts with coreceptors CD14 and MD2 to activate signal transduction pathways through adapter molecules, including myeloid differentiation primary-response protein 88 (MyD88) and TIR domain-containing adapter inducing interferon-β (16–18). The MyD88-dependent pathway, which is common to most Toll-like receptors, leads to the activation of two distinct intracellular pathways: the inhibitor of nuclear factor kappa B (NFκB) kinase pathway, culminating in the activation of the transcription factor NFκB (14), and the mitogen-activated protein kinase (MAPK) pathway inducing another transcription factor, activator protein 1 (AP-1) (19,20). The receptor TLR4 is also able to activate a second MyD88-independent pathway, which results more specifically in the induction of the interferon regulatory factor IRF3, leading to increased production of type I interferons, in particular interferon-β (21). The receptor TLR2 is directly stimulated by PGN, whereas muramyl dipeptide (MDP), which is the minimal bioactive cytosolic structure of PGN, interacts with Nod-like receptor

From the Institute of Neuroscience and Department of Adult Psychiatry (SL, PdT), Institute of Experimental and Clinical Research, Laboratory of Hepato- and Gastroenterology (PS, CDS), and Louvain Drug Research Institute, Metabolism and Nutrition Research Group (ND), Université Catholique de Louvain, Brussels, Belgium.

Authors PdT and PS contributed equally to this work.

Address correspondence to Peter Stärkel, M.D., Ph.D., Department of Gastroenterology, Cliniques Universitaires St. Luc, Avenue Hippocrate 10, B-1200 Brussels, Belgium; E-mail: peter.starkel@uclouvain.be.

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proteins NOD2 and NLRP3 (22,23). The receptors TLR2 and NOD2 also activate the MyD88-dependent pathway. The receptor NLRP3 forms the inflammasome, which is a multiprotein complex that also comprises the enzyme caspase-1. This converting enzyme cleaves the precursor forms of interleukin (IL)-1β and IL-18 into mature and active cytokines (24).

The aim of the present study is threefold: 1) to test whether gut-derived bacterial products activate peripheral blood mononuclear cells (PBMCs), which intracellular pathways are involved, and whether they contribute to the systemic proinflammatory cytokine response under natural conditions in noncirrhotic AD subjects; 2) to assess whether activation of specific pathways, and especially the proinflammatory cytokines, are related to the amount of alcohol consumed and to alcohol craving; and 3) to investigate the recovery of pathway activation after 18 days of detoxification.

Methods and Materials

Subjects and Study Design

We recruited 63 actively drinking AD inpatients from the alcohol-detoxification unit of the Departments of Gastroenterology and Psychiatry, Saint-Luc Academic Hospital, Brussels, Belgium. The following minimal eligibility criteria were required: alcohol dependence according to the DSM-IV (25) and alcohol drinking until the day of admission. Exclusion criteria were as follows: the use of antibiotics, probiotics, glucocorticoids, or nonsteroidal anti-inflammatory drugs currently or during the 2 months preceding enrollment and the presence of metabolic disorders such as diabetes and obesity (body mass index >30 kg/m^2), chronic inflammatory diseases (e.g., inflammatory bowel disease or rheumatoid arthritis), cancer, or other severe medical conditions, including cirrhosis or significant liver fibrosis (fibrosis ≥ 2 on transient liver elastography) (Supplement 1). Fasting blood was drawn from the antecubital vein on the day after admission (T1) and at the end of the detoxification program, on day 18 (T2). Among the 63 subjects, 41 participated to both time points (subjects who abandoned their treatment or resumed alcohol consumption were excluded at T2). The AD subjects were compared with 14 control (CT) subjects who were matched for age, gender, and body mass index and who consumed socially low amounts of alcohol (<20 g/day). The study protocol was approved by the ethical committee of the hospital (reference B40320096274), and written informed consent was obtained from all subjects.

Alcohol Consumption

At T1, subjects were asked to self-report the number of drinks that they were having each day before hospitalization. In a subset of 21 subjects, alcohol consumption was evaluated more carefully, with the timeline follow-back approach (26), as detailed in de Timary *et al.* (27).

Isolation of Human PBMCs and RNA

The PBMCs were isolated from blood by centrifugation on a Ficoll-Paque Plus gradient medium (GE Healthcare Biosciences AB, Uppsala, Sweden) (Supplement 1). RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, California).

Reverse Transcription and Real-Time Quantitative Polymerase Chain Reaction

Complementary DNA was synthesized, and quantitative polymerase chain reaction was performed with the Step One Plus device and software (Applied Biosystems, Inc, Carlsbad, California) by using the fluorogenic SYBR Green PCR Master Mix (Applied Biosystems, Inc), as previously described (28). The ΔΔCT method was used for quantification normalized to ribosomal protein L19 RNA (internal standard). Primers (Table S1 in Supplement 1) were designed with Primer Express design software (Applied Biosystems, Inc).

Quantification of Transcription Factor Activation

Activation of transcription factors (p65, c-Fos, phospho-c-Jun) in PBMCs was assessed in whole-cell extracts (Nuclear Extract Kit; Active Motif, la Hulpe, Belgium) by using a sensitive TransAM detection kit (Active Motif) according to the manufacturer's guidelines.

Western Blotting

Western blot analysis was performed on whole-cell extracts according to standard electrophoresis and transfer techniques. Membranes were revealed with the Western Lightning chemiluminescent detection system (PerkinElmer, Waltham, Massachusetts) before quantification of the blots with the Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Nazareth, Belgium). Membranes were stripped (Thermo Fisher Scientific, Erembodegem, Belgium) and reprobed with several antibodies (Table S2 in Supplement 1). β-Actin was used as a loading control.

Plasma PGN and Cytokine Measurements

Plasma was diluted 1:2, and detection of PGN was performed using a PGN enzyme-linked immunosorbent assay kit (CUSABIO Antibodies-online, Aachen, Germany) following the manufacturer's guidelines. Plasma cytokines including tumor necrosis factor (TNF)-α and IL-6, IL-1β, and IL-8 were assayed in duplicate with a multiplex immunoassay (Millipore, Molsheim, France) and Luminex xMap technology (Bio-Rad Laboratories) following the manufacturer's instructions.

Short-Term Cell Culture and In Vitro Stimulation

To test in vitro Toll-like receptor activation on LPS and PGN stimulation, PBMCs from four AD subjects and four healthy CT subjects were cultured (Supplement 1).

Assessment of Alcohol Craving

The Obsessive-Compulsive Drinking Scale (OCDS) questionnaire assesses the cognitive aspects of alcohol craving during the preceding 7 days (29) and provides a total craving score and two subscores: an obsessive and a compulsive subscore (Supplement 1). A validated French version was used in this study (30).

Statistical Analysis

Statistical analyses were performed with SPSS version 20.0 (IBM Corporation, Armonk, New York) after log transformation for non–normally distributed data. Independent *t* tests were performed to compare AD subjects with CT subjects, and paired *t* tests were performed to compare AD subjects at T1 and T2. Correlations were calculated by using the Pearson product-moment correlation coefficient and multiple regression by the stepwise method (Supplement 1). Statistical significance was defined as $p < .05$. Data presented in the graphs are nontransformed means ± SEM.

Results

Demographic Data and Alcohol Consumption

The principal demographic data are summarized in Table 1. The average values of alcohol consumption obtained by using the

Table 1. Characteristics of Alcohol-Dependent and Control Groups

	AD-T1	AD-T2	CT
No. Participants	63	41	14
Age (Years)	48 ± 11[a]	49 ± 11[a]	43 ± 11[a]
Gender, *n* (%)			
Male	35 (56%)	21 (51%)	7 (50%)
Female	28 (44%)	20 (49%)	7 (50%)
BMI (kg/m^2)	25.6 ± 5.3[a]	ND	25.0 ± 5.4[a]
Alcohol Intake (g/day)	155 ± 90[a]	0	<20

AD-T1 and AD-T2 refer to AD subjects tested at the beginning and end of the detoxification program, respectively.

AD, alcohol-dependent; CT, control; BMI, body mass index; ND, not defined.

[a]Data are means ± SD.

self-reporting approach (160 ± 100 g/day) and the timeline follow-back approach (145 ± 69 g/day) were not statistically different ($p = .53$).

Mechanistic Analyses

Increased Expression and Activation of TLR4 Receptor Complex in PBMCs of AD Subjects. Because the TLR4 complex is principally involved in the recognition of bacterial LPS in immune cells, we first studied its expression in PBMCs. The messenger RNA (mRNA) levels of TLR4 and CD14, the coreceptor required for LPS recognition by TLR4, were significantly increased in AD subjects (Figure 1A). To test activation of the TLR4 complex, we designed a cell culture–based experiment in which PBMCs were treated with physiologic doses of LPS. After 6 hours of stimulation, TNF-α mRNA levels in PBMCs and TNF-α protein concentrations in culture medium were higher in AD subjects than in CT subjects, suggesting a higher reactivity of AD subjects to LPS, in line with upregulated activation of the TLR4 receptor complex (Figure S1A,B in Supplement 1).

Elevated Plasma PGN Levels and Increased Expression and Activation of PGN-Associated Receptors in PBMCs of AD Subjects. We hypothesized that PGN might also cross the gut barrier. Plasma PGN levels were elevated in AD subjects compared with CT subjects (Figure 1B). We determined the expression of receptors TLR2 and NOD2, which recognize PGN-associated components, and MDP, the minimal bioactive structure of PGN; mRNA levels of both were significantly increased in AD subjects compared with CT subjects (Figure 1C). In addition, mRNA levels of NLRP3, a component of a multiprotein complex called inflammasome known to be activated by MDP, were also strongly increased in AD subjects (Figure 1C). Interaction of adenosine triphosphate (ATP) with the P2X7 receptor may activate the inflammasome complex. We measured plasma ATP levels and P2X7 and pannexin1 mRNA expression, which is known to increase on P2X7 activation (31,32). Plasma ATP levels were lower in AD subjects, and the expression of P2X7 and pannexin1 remained unchanged and decreased, respectively, suggesting no involvement of the ATP/P2X7 complex in activating the inflammasome and IL-1β secretion (Figure S2 in Supplement 1). Finally, PBMCs were cultured in the presence of physiologically relevant doses of PGN. After 6 hours of stimulation, mRNA levels of IL-1β were higher in PBMCs from AD subjects than CT subjects. In particular, a very small dose of PGN (1 ng/mL) induced strong upregulation of IL-1β mRNA levels (Figure S3A in Supplement 1) and increased the IL-1β concentration in the cell culture supernatant from AD subjects (Figure S3B in Supplement 1). These results suggest that PGN also cross the gut barrier of AD subjects and likely contribute to the activation of PGN-specific receptors in PBMCs.

Activation of Toll-like Receptor–Dependent Downstream Signaling in PBMCs of AD Subjects. Toll-like receptor activates downstream signaling pathways through interaction with various molecules. We investigated the expression and activation of key components of the different pathways. First, we showed that the expression of MyD88 and the phosphorylated form of IL-1 receptor–associated kinase (IRAK-1) was higher in AD subjects, suggesting activation of the MyD88-dependent pathway (Figure 2A–C).

Inhibition of the NFκB Proinflammatory Cytokine Pathway in PBMCs of AD Subjects: The NFκB proinflammatory cytokine pathway is one of the principal pathways activated on Toll-like receptor stimulation. In AD subjects, mRNA levels of the NFκB subunits p65 and p105 (NFκB p50 precursor) did not differ from levels in CT subjects (Figure 3A). The mRNA levels and protein expression of IκBα, the principal inhibitor of NFκB, were more than threefold increased in AD subjects (Figure 3A–C). Subsequent analysis of the DNA binding activity of p65 showed that NFκB DNA binding was inhibited in AD subjects (Figure 3D). To confirm inhibition of NFκB, we also assessed mRNA expression of the cytokines TNF-α and IL-6, which are directly regulated by this transcription factor. The TNF-α levels were significantly lower in AD subjects, and the IL-6 levels did not differ significantly from CT subjects (Figure 3E). All of these observations demonstrate inhibition of the NFκB proinflammatory cytokine pathway in PBMCs of AD subjects.

Activation of MAPK and AP-1 Pathway in PBMCs of AD Subjects: The MyD88-dependent Toll-like receptor signaling also activates the MAPK signaling pathway, leading to activation of the transcription factor AP-1. We first assessed protein expression of MAPK p38 and JNK, known activators of AP-1. Western blot analysis showed increased phosphorylation of both proteins in

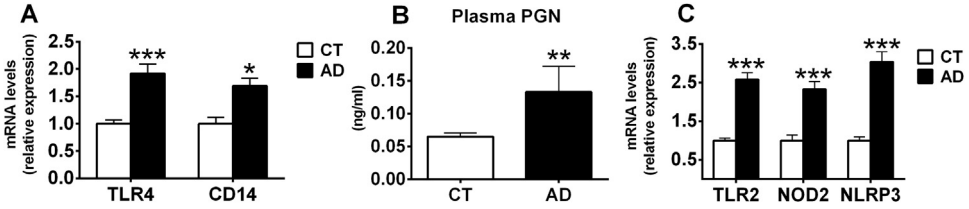

Figure 1. Increased expression of lipopolysaccharide- and peptidoglycan (PGN)-associated receptors in peripheral blood mononuclear cells of alcohol-dependent (AD) subjects. **(A)** Increased messenger RNA (mRNA) expression of TLR4 and CD14 in peripheral blood mononuclear cells of AD subjects under naturalistic conditions. **(B)** Increased plasma PGN concentration in AD subjects. **(C)** Increased mRNA levels of PGN-associated receptors (TLR2 and NOD2) and NLRP3 in peripheral blood mononuclear cells of AD subjects under naturalistic conditions. Data are means ± SEM. CT, control group. *$p < .05$, **$p < .01$, ***$p < .001$.

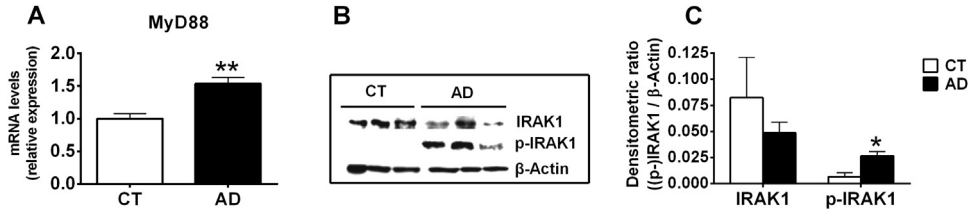

Figure 2. Activation of the myeloid differentiation primary-response protein 88 (MyD88)–dependent pathway in peripheral blood mononuclear cells of alcohol-dependent (AD) subjects. **(A)** Increased messenger RNA (mRNA) expression of MyD88 in peripheral blood mononuclear cells of AD subjects under naturalistic conditions. **(B)** Representative Western blots of total and phosphorylated forms of interleukin-1 receptor-associated kinase (IRAK1) protein expression. **(C)** Quantification of IRAK1 and phosphorylated IRAK1 (p-IRAK1) expressions in Western blots by densitometry normalized to the loading control β-actin, showing that the expression of the phosphorylated and activated form of IRAK1 is higher in AD subjects compared with healthy control (CT) subjects. Data are means ± SEM. $^{*}p < .05$, $^{**}p < .01$.

AD subjects compared with CT subjects, suggesting activation of the MAPK pathway (Figure 3F,G). In addition, mRNA levels of c-Fos and c-Jun, the two principal subunits of AP-1, were significantly upregulated in AD subjects (Figure 3H). Also, c-Fos and phospho c-Jun DNA binding activities were increased in AD subjects, confirming activation of AP-1 (Figure 3I). Finally, a 3.5-fold and a 6-fold upregulated expression of the cytokines IL-1β and IL-8, respectively, which are at least partly regulated by AP-1, and significantly higher levels of IL-18 (Figure 3J) add further arguments in favor of AP-1 activation in PBMCs of AD subjects.

Weak Activation of MyD88-Independent Pathway in PBMCs of AD Subjects: The MyD88-independent pathway classically leads to activation of the interferon pathway and production of type I interferons. Although assessment of the interferon regulatory factors IRF3 and IRF7 mRNA levels did not show any significant difference between AD subjects and CT subjects

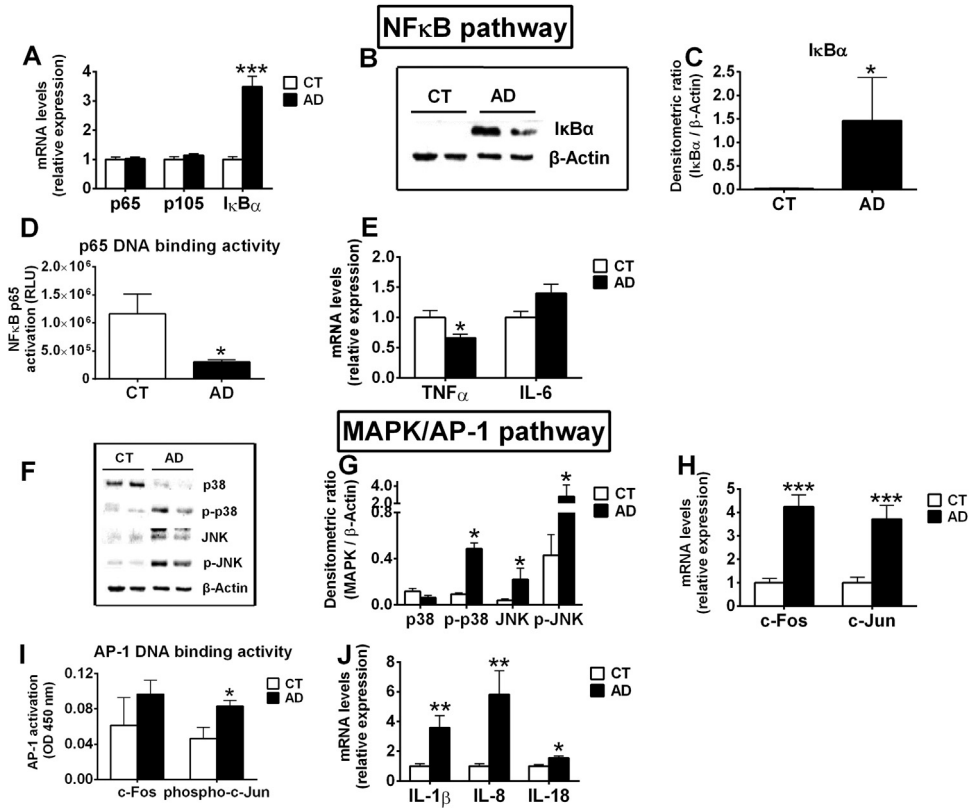

Figure 3. Inhibition of the nuclear factor kappa B (NFκB) proinflammatory signaling pathway and activation of the mitogen-activated protein kinase (MAPK)/activator protein 1 (AP-1) pathway in peripheral blood mononuclear cells (PBMCs) of alcohol-dependent (AD) subjects. **(A)** No induction of messenger RNA (mRNA) p65 and p105 NFκB subunit expression, but a large increase in the mRNA level of the NFκB inhibitor IκBα in PBMCs of AD subjects. **(B)** Representative Western blots of IκBα protein expression in PBMCs and quantification normalized to the loading control β-actin **(C)**, showing increased IκBα expression in AD subjects compared with control (CT) subjects. **(D)** NFκB p65 DNA-binding assay showing decreased p65 activation in AD subjects. **(E)** Decreased expression of tumor necrosis factor α (TNF α) and no significant change of interleukin (IL)-6 mRNA levels in PBMCs of AD subjects. (**F**) Representative Western blots of MAPK protein expression in PBMCs and quantification normalized to the loading control β-actin (**G**), showing an increased p38 phosphorylation (p-p38) and increased expression and phosphorylation (p-JNK) of the c-Jun amino-terminal kinase (JNK) in AD subjects compared with CT subjects. (**H**) Increased mRNA levels of both AP-1 subunits, c-Fos and c-Jun, in PBMCs of AD subjects. (**I**) Activator protein 1 DNA binding assay showing increased c-Fos and phospho c-Jun activation in AD subjects. (**J**) Increased mRNA levels of IL-1β, IL-8, and IL-18, which are at least partly regulated by transcription factor AP-1. Data are means ± SEM. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

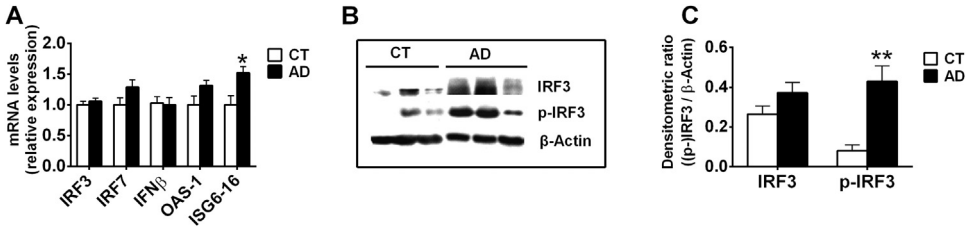

Figure 4. Myeloid differentiation primary-response protein 88–independent pathway in peripheral blood mononuclear cells of alcohol-dependent (AD) subjects. **(A)** No induction of interferon regulatory factors IRF3 and IRF7 and interferon-β (IFN-β) messenger RNA (mRNA) levels and no increase and weak increase of the interferon-induced genes *OAS-1* and *ISG6-16*, respectively, in peripheral blood mononuclear cells of AD subjects, suggesting that the myeloid differentiation primary-response protein 88–independent interferon pathway is not activated in AD subjects. **(B)** Representative Western blots of the total and phosphorylated forms of IRF3 protein expression in peripheral blood mononuclear cells. **(C)** Quantification of IRF3 and phosphorylated IRF3 (p-IRF3) expression in Western blots by densitometry normalized to the loading control β-actin, showing increased expression of p-IRF3 in AD subjects. Data are means ± SEM. CT, control group. $^{*}p < .05$, $^{**}p < .01$.

(Figure 4A), the phosphorylated form of IRF3 was more expressed in AD subjects (Figure 4B,C). We looked at the expression of the end product interferon-β and found no difference between groups (Figure 4A). In addition, *OAS-1* and *ISG6-16*, genes directly regulated by interferon-β, were not increased and only mildly increased in AD subjects, respectively (Figure 4A). Overall, these results suggest only a weak, if any, activation of the MyD88-independent pathway in PBMCs of AD subjects.

Induction of Plasma Proinflammatory Cytokines in AD Subjects. We measured plasma TNF-α, IL-6, IL-1β, and IL-8 concentrations and showed that they were all significantly higher in AD subjects than in CT subjects (Figure S4 in Supplement 1).

Clinical Analyses

Correlations between Alcohol Consumption and Expression of Proinflammatory Cytokines. In the subset of 21 subjects in whom alcohol consumption was evaluated with the timeline follow-back approach, correlations between the amount of alcohol consumed and the mRNA levels of proinflammatory cytokines IL-1β and IL-8 and signaling pathways contributing to their activation were observed at T1 (Table 2).

Correlations between Activated Inflammatory Pathways and Alcohol-Craving Scores. The OCDS was administrated to 38 subjects to assess alcohol craving. At T1, significant correlations were observed between the total score as well as the obsessive subscore of OCDS and the mRNA expression of proinflammatory

Table 2. Activation Status of Signaling Molecules and Correlations with Alcohol Consumption and Scores of Craving

		Correlations with Alcohol Consumption T1		Correlations with OCDS-Obs T1		Correlations with Δ OCDS-Obs	
	Activation	*r* Pearson	*p* Value	*r* Pearson	*p* Value	*r* Pearson	*p* Value
Receptors							
TLR4	Activated	.47	<.05				
CD14	Activated	.62	<.01				
TLR2	Activated	.65	<.01				
NOD2	Activated	.55	<.05	.44	<.05	.03	NS
NLRP3	Activated	.68	<.001	.53	<.01	.65	<.01
Signal transduction							
MyD88	Activated	.73	<.001				
MAPK	Activated	ND					
IκBα	Activated	.66	<.01	.52	<.01	.51	<.05
Transcription factors							
p65	Inhibited	.40					
p105	Inhibited	.31					
c-Fos	Activated	.71	<.001	.58	<.001	.59	<.01
c-Jun	Activated	.59	<.01	.62	<.001	.28	NS
IRF3	Controversial	.30					
Cytokines							
TNF-α	Inhibited	.19					
IL-6	Inhibited	.20					
IL-1β	Activated	.65	<.01	.58	<.001	.57	<.01
IL-8	Activated	.55	<.05	.65	<.001	.52	<.05
IL-18	Activated	.16					
IFN-β	Inhibited	.10					

Recap chart of signaling molecule status and correlations with alcohol consumption (expressed in g/day) at T1, with the Obs subscore of alcohol craving at T1 and correlations between the difference scores (Δ = T1 − T2) of inflammatory signaling molecules and the Obs subscore of craving.

IFN, interferon; IL, interleukin; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary-response protein 88; ND, not determined; NS, not significant; Obs, obsessive; OCDS, Obsessive Compulsive Drinking Scale; TNF, tumor necrosis factor; T1, beginning of withdrawal; T2, end of withdrawal.

Table 3. Multiple Regression Report (Stepwise Method)

Model	B	SE B	β	*t* Test	Significance
Constant	8.83	.52		16.86	.000
mRNA IL-8	.16	.03	.697	4.86	.000

Multiple $R = .697$, $R^2 = .49$ (IL-8 mRNA accounted for 49% of the variance in craving with a significant association of the *t* test with the β value). B and SE B represent the unstandardized β values and the associated standard errors. β is the standardized coefficient with the associated *t* test and significance.

IL, interleukin; mRNA, messenger RNA.

cytokines IL-1β and IL-8 and the intracellular signaling pathways involved in their activation. As the OCDS total scores decrease during withdrawal (T1, 18 ± 6 vs. T2, 5 ± 4, $p < .001$) in parallel with reduction in inflammation, we also tested correlations between the difference scores (Δ) calculated by subtracting "inflammation T1 − inflammation T2" and "OCDS T1 − OCDS T2." Significant correlations were found between Δ for IL-1β and IL-8 and Δ for total and obsessive OCDS scores (Table 2).

Because several inflammatory parameters were associated with alcohol craving, we used a multiple linear regression to detect which parameters were the best predictors of craving. Using the stepwise method, we found that the IL-8 mRNA level was the best predictor of the outcome over and above all other predictors and accounted for 49% of the variance in craving (Table 3).

Effects of Short-Term Alcohol Withdrawal on Inflammatory Pathways

To test the effect of alcohol withdrawal on the recovery of inflammatory pathways in PBMCs of AD subjects, data at T1 and T2 for the subjects who completed the entire study were compared (Table 4). Concerning the LPS activated pathway, TLR4 and CD14 mRNA levels decreased significantly with abstinence to values observed in CT subjects.

For the PGN pathway, TLR2 and NLRP3 levels decreased from T1 to T2 but remained high in AD subjects at T2. High NOD2 expression also persisted and was not affected by withdrawal. The AP-1 subunits c-Fos and c-Jun decreased significantly from T1 to T2, but both remained higher than in CT subjects at T2. Despite evidence that stimulation of the TLR2 and MAPK/AP-1 pathways was not completely abrogated after 18 days of abstinence, the mRNA levels of IL-1β, IL-8, and IL-18 decreased to CT levels at T2.

Alcohol withdrawal reduced MyD88, but it remained above CT levels at T2. High IκBα levels persisted, and no change in the mRNA expression of NFκB subunits, TNF-α levels, and IL-6 levels was found during withdrawal. Plasma cytokines (TNF-α, IL-6, IL-1β, and IL-8) remained higher at T2 in AD subjects than in CT subjects (not shown).

Table 4. Changes in Expression of Inflammatory Signaling Molecule Messenger RNA Levels During Alcohol Withdrawal

Signaling Molecules	AD-T1	AD-T2	CT
TLR4	1.82 ± 1.42	1.31 ± .90[a]	1.00 ± .26
CD14	1.65 ± 1.07	1.28 ± .79[a]	1.00 ± .44
MyD88	1.61 ± .80	1.33 ± .60[b,c]	1.00 ± .30
c-Fos	5.13 ± 5.30	3.55 ± 2.98[b,d]	1.00 ± .69
c-Jun	3.02 ± 2.41	2.51 ± 3.14[b,c]	1.00 ± .62
IL-1β	4.00 ± 7.34	2.06 ± 2.91[b]	1.00 ± .59
IL-8	7.32 ± 13.44	1.33 ± 1.08[e]	1.00 ± .57
IL-18	1.58 ± .93	1.29 ± .80[b]	1.00 ± .42
TLR2	2.69 ± 1.53	2.25 ± 1.05[b,d]	1.00 ± .25
NOD2	2.40 ± 1.64	2.35 ± 1.52[d]	1.00 ± .49
NLRP3	3.02 ± 2.24	2.32 ± 1.39[b,d]	1.00 ± .36

Data are means ± SD normalized for CT subjects. AD-T1 and AD-T2 refer to the AD group at the beginning and at the end of withdrawal, respectively.

AD, alcohol-dependent; CT, control group; IL, interleukin; MyD88, myeloid differentiation primary-response protein 88.

[a] $p < .01$ compared with AD-T1.

[b] $p < .05$ compared with AD-T1.

[c] $p < .05$ compared with CT subjects.

[d] $p < .001$ compared with CT subjects.

[e] $p < .001$ compared with AD-T1.

Discussion

Inflammation might play a role in the development of several psychiatric disorders, including major depression, schizophrenia, and autism (1,33–36). Several preclinical and clinical studies in alcohol dependence have also suggested that inflammation plays a role in addictive behaviors (2,37–39).

To elucidate the contribution of PBMCs to inflammation, we analyzed proinflammatory cytokines and their intracellular signaling pathways in PBMCs of 63 noncirrhotic, actively drinking AD subjects. The AD subjects had elevated plasma PGN, IL-1β, and IL-8 levels and increased mRNA expression of IL-1β, IL-8, and IL-18 in PBMCs. Induction of these cytokines was likely related to increased expression and activation of the TLR2 receptors as well as activation of the transcription factor AP-1 and the NLRP3 inflammasome and to a lesser extent to LPS stimulation of the TLR4-MyD88-dependent pathway (Figure 5). Upregulation of IL-1β and IL-8 mRNA levels positively correlated with alcohol consumption and craving. Alcohol withdrawal was associated with recovery of LPS-dependent receptors but not PGN-dependent receptors.

In AD subjects, the origin and impact of inflammation was mainly attributed to a local effect of alcohol in the brain or the liver. We reported more recently increased intestinal permeability, elevated blood LPS, and low-grade systemic inflammation in AD subjects, findings that were also related to psychological factors (6), suggesting indirectly that gut-derived bacterial products could play a role in the development of inflammation.

Circulating PBMCs represent an essential defense barrier against gut-derived bacterial products entering the bloodstream that may potentially contribute to systemic inflammation. Because alcohol dependence naturally increases blood LPS (6) and PGN levels, inflammatory pathways were tested in PBMCs without culturing cells or applying pathogen-associated molecular pattern stimulation. The inflammatory response in PBMCs of AD subjects has previously been analyzed only in studies that included small numbers of patients with significant liver disease (40,41). Monocytes that were extracted from these patients were cultured and artificially stimulated with a high LPS dose and showed an increase in NFκB activity and TNF-α production, in contradiction to our findings. We included only patients who had not developed liver damage to test the effects of alcohol dependence itself and avoid potential bias linked to liver disease. The inflammation was tested under natural conditions. For these reasons, our results likely better reflect the physiologic impact of gut-derived bacterial products on immune responses of PBMCs in AD subjects.

The transmembrane Toll-like receptors and the cytoplasmic Nod-like receptors, responsible for sensing the presence of LPS and PGN, were upregulated in AD subjects except for TLR5, which recognizes flagellin from flagellated bacteria (data not shown).

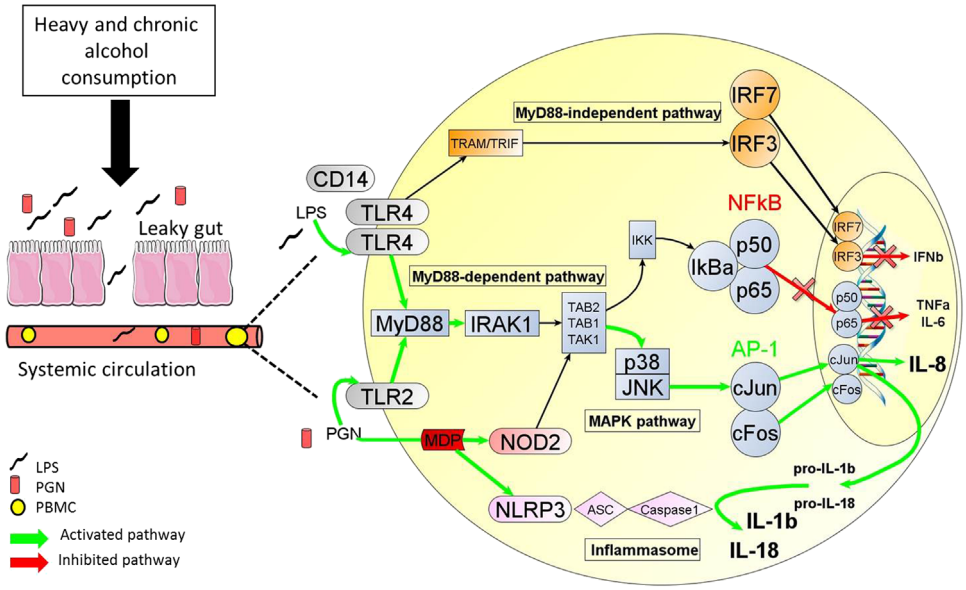

Figure 5. Proposed working model of the Toll-like receptor and Nod-like receptor signaling pathways in peripheral blood mononuclear cells (PBMCs) of noncirrhotic alcohol-dependent subjects. Alcohol-dependent subjects present with increased intestinal permeability (leaky gut) that favors the translocation of bacterial components (lipopolysaccharide [LPS] and peptidoglycan [PGN]) from the gut lumen to the systemic circulation. The gut-derived bacterial products interact with their respective receptors (TLR4 and TLR2, NOD2) located in PBMCs and activate downstream signaling. These events principally result in activation of the mitogen-activated protein kinase (MAPK)/activator protein 1 (AP-1) transcription factor pathway as well as in activation of the inflammasome leading to secretion of the proinflammatory cytokines interleukin (IL)-1β, IL-8, and IL-18. The cytokines IL-1β and IL-18 are first transcripted as precursors (pro-IL-1β and pro-IL-18) and then converted into mature and bioactive cytokines by the inflammasome complex. The nuclear factor kappa B (NFκB) pathway and the myeloid differentiation primary-response protein 88 (MyD88)–independent interferon pathway are not or only weakly activated. Green arrows represent activated pathways, and red arrows represent inhibited pathways. IFN, interferon; IRAK1, IL-1 receptor-associated kinase; IRF, interferon regulatory factor; JNK, c-Jun amino-terminal kinase; MDP, muramyl dipeptide; TNF, tumor necrosis factor.

Components associated with PGN stimulate TLR2 receptors on the plasma membrane, and MDP, the minimal bioactive structure of PGN, is commonly detected by the intracellular receptor NOD2 (22,42). From the observation of increased plasma PGN, TLR2, and NOD2 expression in AD subjects, it can be concluded that PGN likely crossed the gut barrier and stimulated specific inflammatory pathways in PBMCs, which has not been reported to date. Activation of the receptors was confirmed by exposure of PBMCs in culture to physiologically relevant doses of LPS or PGN during a short time period, showing increased TNF-α and IL-1-β gene transcription and protein concentrations in culture medium in AD subjects. These observations suggest that inflammatory pathways are more intensely activated by LPS and PGN in AD cells than in CT cells.

We next assessed the intracellular signaling pathways regulated by these receptors. Toll-like receptors activate MyD88-dependent and independent intracellular signaling pathways and usually involve the NFκB proinflammatory pathway leading to TNF-α and IL-6 transcription and the MAPK pathway culminating in AP-1 activation (19). Increased MyD88 mRNA levels and p-IRAK1 protein expression were found in PBMCs of actively drinking subjects, suggesting that chronic exposure to alcohol and Toll-like receptor ligands stimulates MyD88-dependent pathways. Several elements indicated inhibition of the NFκB proinflammatory cytokine pathway in AD subjects, as follows: 1) mRNA levels of NFκB p105 (the p50 precursor) and p65 subunits did not change; 2) IκBα, which inhibits NFκB nuclear translocation, was strongly upregulated; 3) DNA binding activity of the p65 subunit was strongly decreased; 4) mRNA levels of TNF-α and IL-6, which are cytokines tightly regulated by NFκB, were reduced and unchanged, respectively. In line with the inhibition of the NFκB proinflammatory pathway are reports of suppression of NFκB activity in response to LPS in animal and in vitro models of chronic alcohol exposure (43,44).

In AD subjects, mRNA levels of the cytokines IL-1β and IL-18 and of the chemokine IL-8 were upregulated, which could not be ascribed to NFκB activation. We observed activation of the MAPK (p38, JNK) and the AP-1 (c-Fos/c-Jun) pathways, known to be associated with IL-1β, IL-8, and IL-18 transcription (45–47). The cytokines IL-1β and IL-18, synthesized as nonfunctional pro-IL-1β and pro-IL-18, need to be cleaved into bioactive cytokines by caspase-1. Caspase-1 belongs to the multiprotein complex inflammasome, containing the adapter proteins ASC and NLRP3, which may serve as an inflammasome activation marker. Increased NLRP3, IL-1β, and IL-18 mRNA expression; plasma IL-1β protein upregulation; and secretion of the active form of the IL-1β protein in the culture medium of PBMCs from AD subjects provide evidence for activation of the inflammasome. However, in our study, the ATP/P2X7 receptor pathway did not seem to be involved in inflammasome activation. Finally, we did not find evidence in favor of the activation of the interferon pathway, which can be triggered by TLR4.

The strong correlations observed between these inflammatory factors and the amount of alcohol consumed as well as the total or partial recovery of several factors observed after 18 days of abstinence suggest that alcohol is likely the main factor in inducing the inflammatory response. The incomplete recovery of the PGN receptors TLR2 and NLRP3 and the absence of modification of NOD2 expression suggest that longer term abstinence might be required to obtain PGN clearance and concomitant downregulation of PGN receptors.

The mRNA expression of the molecules belonging to the activated inflammatory pathways was positively correlated with

alcohol craving and more particularly with the obsessive dimension of craving at T1. The best predictor of the outcome was IL-8. The correlations disappeared at T2, but positive correlations between difference scores (Δ = T1 − T2) of inflammation and craving were observed, suggesting that large recovery of inflammation during withdrawal is associated with a large improvement in alcohol craving (48). This specific relationship between the obsession of drinking and inflammation supports the general hypothesis of an involvement of inflammation in anxious symptoms of alcohol dependence (49), as previously observed in other anxious disorders (50).

Our data are consistent with involvement of these gut-activated inflammatory pathways, in particular, the PGN-activated pathway, in the pathophysiology of alcohol dependence, at least in active drinkers. It might be less relevant after they have stopped drinking. However, our observation of increased plasma levels of IL-6 and TNF-α but inhibition of mRNA levels of these cytokines in PBMCs suggests that PBMCs are not the only source of circulating cytokines. Inflammation likely also arises from other sources, such as in the liver and the gut wall itself. Additional analyses in liver or small bowel biopsies or both would be required to determine the respective contribution of each compartment to the inflammatory process.

Alcohol might also stimulate inflammation directly at the brain level (2). Previous studies performed on animal models supporting a role for inflammation in alcohol dependence all have supported a specific role for LPS, TLR4, and NFκB pathways (51,52). Alfonso-Loeches *et al.* (52) showed that heavy alcohol consumption failed to induce AD behaviors and to activate inflammation in the cortex of TLR4$^{-/-}$ knockout mice. However, our data suggest a stronger role for PGN than the LPS-activated pathway in human populations.

In conclusion, the results of this study indicate that gut-derived inflammatory pathways are interesting targets to study to improve understanding of the pathophysiology of alcohol dependence. These pathways also represent potent sites of interventions for the treatment of the disease, perhaps by the use of probiotics or prebiotics that might change the composition of the gut microbiota.

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