Effects of alcohol withdrawal on monocyte subset defects in chronic alcohol users

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ABSTRACT

Excessive alcohol consumption has a modulating effect on immune functions that may contribute to decreased immunity and host defense. It is associated with increased intestinal permeability to endotoxins that is normalized after 14 d of abstinence. Whether and how blood monocyte subsets are impaired in patients with an AUD and what their evolution is after alcohol withdrawal are the paper’s objectives. With the use of flow cytometry, blood monocyte subsets were quantified in AUDs and compared with HC donors (n = 20). Expression of TLR2 and TLR4 on monocyte subsets was also quantified. Cytokine response of monocytes was monitored following PGN and LPS stimulation. The CD14^+CD16^- subset was decreased, whereas the CD14^{dim}CD16^+ subset was expanded (P < 0.001) in AUD compared with HC. The frequencies of TLR2- and TLR4-expressing monocytes were reduced in AUD compared with HC. Although the basal production of IL-1, IL-6, and TNF by monocytes in AUD was compared with HC, the PGN- and LPS-mediated IL-6 and TNF production was increased in AUD. Frequencies of IL-6-expressing monocytes were higher in AUD than HC. Alcohol withdrawal partially restored the distribution of monocyte subsets and the frequency of IL-6-producing monocytes and increased the frequency of TNF-producing cells in response to LPS and PGN stimulation to levels compared with those in HC. Our findings indicate that chronic alcohol use alters the distribution as well as the phenotypic and functional characteristics of blood monocyte subsets, which are partially restored following 2 wk of alcohol withdrawal. J. Leukoc. Biol. 100: 000–000; 2016.

Introduction

Alcohol consumption is a major risk factor for morbidity and mortality worldwide. Alcohol-related deaths are secondary to cancer, cardiovascular diseases, digestive system diseases, and behavioral disorder [1]. AUDs consist of alcohol dependence and alcohol abuse. The lifetime risk of AUD for men is >20% [2]. Alcohol abuse has negative effects on immune functions that may contribute to decreased immunity and host defense and thus, increased susceptibility to certain diseases that are life threatening. These immune dysfunctions share an initial common cause characterized by an increase in gut permeability [3] that is restored after only 14 d of alcohol withdrawal [4, 5]. Subjects with an AUD are known to have significantly increased plasma endotoxin levels compared with HCs [6] secondary to the increase in microbial translocation independently on liver disease [7]. Endotoxins are pathogen-associated molecular patterns that are recognized by TLRs and that promote the activation of leukocytes. TLR2 and TLR4 are involved in the recognition of specific bacterial endotoxins, sensing PGNs of gram-positive bacteria or LPS products of gram-negative bacteria, respectively [8]. A major consequence of TLR2 and TLR4 binding is the activation of the NF-κB pathway and production of inflammatory cytokines [9, 10]. Therefore, as a first line of host defense against pathogens, TLR2 and TLR4, expressed by monocytes, may be involved in the pathogenesis of AUD through bacterial endotoxin-induced inflammatory responses.

The negative impact of alcohol and its metabolites on monocytes have been studied for many years in animal and human models of acute and chronic alcohol exposure, and evidences show that defects in monocytes could contribute to alcohol-induced disorders [11]. Monocytes represent 5–10% of peripheral leukocytes, circulate for several days, and migrate into tissues under specific conditions to give rise to macrophages, dendritic cells, and osteoclasts, representing key regulators of...
both inflammation and tissue homostasis. Monocytes are heterogeneous and comprise several subtypes committed to different functions, such as clearance of pathogens and dead cells, initiation of adaptive immunity, and tissue healing. Human monocytes can be divided into 3 subsets, based on their differential expression of CD14 and CD16 cell surface markers: CD14+CD16− (also named inflammatory or classic), CD14+CD16+ (also named intermediate), and CD14dimCD16+ (also named patrolling or nonclassic) monocytes. These subsets are different in their capacity for phagocytosis, become activated, secrete inflammatory cytokines, and migrate to inflamed tissues in response to different stimuli [12]. Although accumulating evidence suggests distinct changes in monocyte subsets in several pathologic conditions, the composition of monocyte subsets and the endotoxin-induced inflammatory responses in patients with AUD remain to be investigated.

As phenotypic heterogeneity of monocytes may imply differences in function, the studying of monocyte subset distribution in subjects with AUD and whether excessive alcohol consumption has an impact on monocytes functionality after stimulation of TLR2 and TLR4, as well as the consequences of alcohol withdrawal, represent major issues. The understanding of monocyte subset imbalance in AUD is indeed a first step to treat better alcoholic-related, disease-like ALD; sleep disturbance; or mood disorders [3, 13]. It may also help to determine the delay required to reduce the risk associated with immune dysfunction in patients with AUD.

Therefore, the aim of the present study was to characterize the composition and endotoxin-induced inflammatory responses of monocyte in subjects with AUD, as well as the effect of alcohol withdrawal, by performing in-depth phenotypic and functional analyses at baseline and after TLR2 and TLR4 stimulation. We also assessed monocyte subset expression of TLR2 and TLR4 stimulation and their relation to markers of monocyte activation and patient characteristics before and 2 wk after alcohol withdrawal.

**MATERIALS AND METHODS**

**Patients and controls**

The study protocol was reviewed and approved by the Institutional Ethic Committee, University Hospital of Montpellier (2013.03.08bis). All 40 patients were enrolled in the study between September 2013 and September 2014 at the Department of Internal Medicine and Addictology (University Hospital of Montpellier, France) and gave their informed consent. The inclusion criteria were AUD (meeting 2 of the 11 DSM-5 criteria during a 12 mo period), daily drinking, and hospitalization for an alcoholic withdrawal in the Clinical Department, and participants must have been adults. The exclusion criteria for patients and controls included the following: a history of chronic inflammatory disease, the presence of current infection, a positive HIV serology or an immunomodulatory or immunosuppressive treatment, decompensated cirrhosis, clinical signs of liver disease, or alteration of liver bioptic markers (i.e., lowered the time of prothrombine or albumin, increased bilirubin). Questionnaires were anonymous and completed by the principal investigator. The following information was recorded: age, gender, and quantity of alcohol per day using a Timeline Followback questionnaire. A complete physical examination was performed and standard blood parameters measured: aspartate amino transferase/alanine amino transferase, γ glutamyl transferase, bilirubin, complete blood count, cholesterol and triglycerides, C and B hepatitis serology, and C-reactive protein.

Twenty HCs were selected on a voluntary basis and matched with subjects in frequency on age and gender. Two blood samples were collected, according to the standard care in the department: at baseline (±1 d before withdrawal) and 2 wk after (±3 d) alcohol withdrawal. A 2 wk period of abstinence is sufficient to restore normal intestinal permeability [4, 5].

**Monocyte subsets immunophenotyping**

For the identification of the different monocyte subsets, 50 μl fresh peripheral blood was incubated with a combination of 7 fluorochrome-conjugated mAbs: CD14/FITC (mouse IgG2b), CD16/APC-A750 (mouse IgG1), CD45/KO (mouse IgG1), CD3/ECD (mouse IgG1), CD56/ECD (mouse IgG1), CD20/ ECD (mouse IgG1), and HLADR/Pacific Blue (mouse IgG1; all from Beckman Coulter, Villepinte, France); and TLR2/PE (mouse IgG2b) and TLR4/APC (mouse IgG2a; both from R&D Systems, Abingdon, United Kingdom) and their respective isotype controls. RBCs were then lysed (Immunoprep; Beckman Coulter) and washed out twice with PBS.

**Intracellular cytokine production**

PBMCs were isolated by Ficoll-Hypaque density gradient (Pancoll human, PAN-Biotech, Aidenbach, Germany) and seeded at 10^7/well in a 24-well plate. PBMCs were stimulated (or not) for 20 h with 100 ng/ml LPS (Sigma-Aldrich, Saint Quentin-Fallavier, France) and 1 mg/ml PG (Sigma-Aldrich). Brefeldin A (Sigma-Aldrich) was added for 4 h before the end of the culture. Subsequently, cells were labeled with CD5/ECD, CD20/ECD, CD56/ECD, HLADR/Pacific Blue, and CD45/KO mAb, permeabilized (PerFix-nc kit; Beckman Coulter), and incubated with IL-1β/AF647 (mouse IgG1; BioLegend, London, United Kingdom), IL-6/FITC (mouse IgG1; Beckman Coulter), and TNF-α/PE (mouse IgG1; Beckman Coulter) mAb or their respective isotype controls.

**Data acquisition and analysis**

Cell acquisition was performed on a 10-color flow cytometer (Navios; Beckman Coulter), and data analysis was performed using Kaluza software (Beckman Coulter). The entire monocyte population was identified based on morphologic parameters (forward/side-scatter); absence of CD5, CD56, and CD20 surface expression; and presence of HLADR surface expression.

**Statistical analysis**

The characteristics of subjects included in the study were described using frequency and proportion for categorical variables and means and standard deviation for continuous variables. Comparison of characteristics between AUD and HC subjects at baseline was performed using the ch^2 test for categorical variables (or Fischer exact test when one of the expected values of the cells of the contingency tables was <5) and using the Student’s t test for continuous variables (or the Mann-Whitney test when data distributions were skewed). Comparisons of monocyte subset distributions, TLR expression, and cytokine production profile were performed using the nonparametric test of Mann-Whitney. When comparisons were performed in AUD subjects between baseline and after 14 d of withdrawal, we used the paired-sample Wilcoxon signed rank test. Analyses were performed with a bilateral level of 0.05 using SAS software, version 9.1 (SAS Institute, Cary, NC, USA).

**RESULTS**

**Demographic and baseline characteristics**

Forty patients diagnosed for AUD and 20 HCs were included in the study. No difference was found between patients and HCs with respect to demographic or clinical criteria analyzed, including age, sex, BMI, systolic blood pressure, and frequency of diabetes (Table 1). During the study, 7 out of the 40 AUD patients were eliminated (17.5%), as a result of either alcohol relapse or premature exit of hospitalization. Clinical, biologic,
and addictological traits of AUD subjects are detailed in Table 2. AUD subjects drank 292 ± 125 g pure alcohol per day, and 87.5% of them were active tobacco smokers. Five patients (13%) had a positive serology for HCV. Four of them (4/5) were negative HCV-RNA, and the fifth was not tested.

Changes in monocyte subset distributions in AUD patients at baseline and after alcohol withdrawal
We examined whether the frequencies of the 3 main monocyte subsets were changed in AUD patients compared with HC. Based on the expression of the phenotypic CD14 and CD16 surface molecules, human peripheral blood monocytes were segregated into CD14+CD16–, CD14+CD16+, and CD14dimCD16+ subsets (Fig. 1A). There was no significant difference in the percentage of total monocytes between AUD and HC groups (Fig. 1B). Compared with HC, changes in the distribution of monocyte subsets were found in AUD subjects (Fig. 1C). The frequency of classic CD14+CD16– monocytes was decreased significantly in the blood of AUD patients compared with HC (median of 78.6% vs. 86.5%, P < 0.0001). In contrast, the nonclassic CD14dimCD16+ monocyte subset was significantly (3.6-fold) expanded in AUD patients compared with HC (median of 8.6% vs. 2.5%, P < 0.0001). The frequency of intermediate CD14+CD16+ monocytes that typically consists of 3-5% of the circulating monocytes showed no difference between AUD subjects and HC (median of 3.9% vs. 3.7%).

As a 2 wk period of abstinence is sufficient for intestinal permeability to return to healthy levels [4], we investigated whether 14 d of alcohol withdrawal changed the frequency and functional phenotype of monocyte subsets in AUD patients. The frequency of CD14dimCD16+ monocytes was significantly reduced in the peripheral blood of AUD patients after 2 wk of alcohol withdrawal (10.3 ± 7.7 at d 0 vs. 6.7 ± 6.4 at d 14, P < 0.01), approaching levels compared with age-matched HCs (Fig. 1C). Compared with patients with and without HCV, there was no difference in the frequency of the CD14dimCD16+ monocyte subset after 14 d of withdrawal. Although slightly increased, the frequency of CD14CD16+ monocytes did not significantly differ between d 14 and d 0 (75.3 ± 11.9 at d 0 vs. 78.1 ± 13.4 at d 14, P = 0.09).

Differential expression of TLR2 and TLR4 on peripheral monocyte subsets of AUD patients and evolution with alcohol withdrawal
As alcoholics display elevated plasma endotoxin levels compared with HCs [6], and monocytes sense these microbial endotoxins, i.e., LPS and PGNs specifically via TLR2 and -4, we analyzed the expression of TLR2 and TLR4 on monocyte subsets. Flow cytometric quantification showed that frequencies of total blood monocytes expressing TLR2 and TLR4 were both reduced in AUD patients compared with HC (Fig. 2A and B). The distribution of TLR2-expressing monocytes was much more homogenous in both AUD patients and HCs than the distribution of TLR4-expressing monocytes.

In-depth analysis of monocyte subsets revealed that the reduced percentage of TLR2-expressing monocytes was attributable to the CD14dimCD16+ monocyte subset (Fig. 2C), whereas the 3 monocyte subsets contributed to the observed reduced percentage of TLR4-expressing monocytes in AUD (Fig. 2D). We noticed 2 subgroups of AUD subjects for TLR4-expressing CD14+CD16+ and CD14dimCD16+ monocytes. Statistical analyses

<table>
<thead>
<tr>
<th>TABLE 1. Demographic characteristics of the study groups</th>
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<td>Characteristic</td>
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<td>Men, n (%)</td>
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<td>Age, mean (±sd)</td>
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<td>BMI, mean (±sd)</td>
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<td>Systolic blood pressure, mean (±sd)</td>
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<td>Diabetes, n (%)</td>
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*a*Mean and so.

<table>
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<th>TABLE 2. Clinical, addictological, and biologic features of alcohol-dependent subjects</th>
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<tr>
<td>Clinical examination</td>
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<tr>
<td>Sign of hepatic failure</td>
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<td>Diabetes (type 2)</td>
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<td>Addictological characteristics</td>
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<tr>
<td>DSM-5 (2 positive criteria for at least 1 yr)</td>
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<tr>
<td>Type of alcohol drunk</td>
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<td>Predominantly Aperitifs</td>
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<td>Beer</td>
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<td>Liquor</td>
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<td>Alcohol drunk</td>
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<td>Quantity of alcohol, b mean (±sd)</td>
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<td>Active tobacco smokers</td>
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<tr>
<td>Number of packs/yr, mean (±sd)</td>
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<td>Regular cannabis smokers</td>
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<td>Biologic characteristics</td>
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<td>HCV-positive serology</td>
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<td>HIV-positive serology</td>
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<td>Albumin, mean (±sd)</td>
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<td>C-Reactive protein, mean (±sd)</td>
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*a*Quantity of alcohol drunk in grams of pure alcohol per day. bMean and so.
Figure 1. Altered distribution of monocyte subsets in AUD subjects and effect of alcohol withdrawal. Leukocytes were harvested from the blood, labeled with antibodies, as described in Materials and Methods, and monocyte subsets quantified by flow cytometry in HCs \( n = 20 \) and in patients with an AUD before \([d 0 (D0), n = 40]\) and after \([d 14 (D14), n = 33]\) 2 wk of alcohol withdrawal. (A) Gating was first performed after exclusion of doublets, and monocytes were defined within an SSC \( / \mathrm{CD} 45^a \) gate, as HLA-DR \( ^b \), CD3 \( ^{-} \), CD56 \( ^{-} \), CD20 \( ^{a} \) (Lin \( ^{-} \)), and were further divided into CD14 \( ^{+} \)/CD16 \( ^{-} \) (Lin \( ^{-} \)) and CD14 \( ^{+} \)/CD16 \( ^{+} \) (red) monocyte subsets. (B) Percentage of total monocytes. SSC, Side-scatter; PB, Pacific Blue; AA;APC-A750 (APC-Alexa Fluor 750). (C) Frequencies of CD14 \( ^{+} \)/CD16 \( ^{-} \) (left), CD14 \( ^{+} \)/CD16 \( ^{+} \) (middle), and CD14 \( ^{+} \)/CD16 \( ^{+} \) (right) monocyte subsets were expressed as the percentage of total monocytes. Bars indicate means \( \pm \) sd. Nonparametric Mann-Whitney test and paired-sample Wilcoxon signed rank test were used for statistical analyses d 0 vs. HC and d 0 vs. d 14, respectively (**\( P \leq 0.001 \), ***\( P \leq 0.001 \)).

revealed that subjects with <50% of CD14 \( ^{+} \)/CD16 \( ^{-} \) and CD14 \( ^{+} \)/CD16 \( ^{+} \) monocytes expressing TLR4 were older (50 \( \pm \) 11 vs. 41 \( \pm \) 11 yr old; \( P = 0.015 \)) and smoked less marijuana (23% vs. 69%; \( P = 0.032 \)) than AUD subjects who displayed >50% of TLR4-expressing CD14 \( ^{+} \)/CD16 \( ^{-} \) and CD14 \( ^{+} \)/CD16 \( ^{+} \) monocytes. Interestingly, the percentage of monocyte subsets expressing TLR4 was comparable in young AUD subjects smoking marijuana and HCs. The expression levels of TLR2 and TLR4 on monocyte subsets remained unchanged in AUD patients compared with HCs (Supplemental Fig. 1). Neither the percentage of TLR2- and TLR4-expressing monocytes nor the expression levels of both TLRs on monocyte subsets were altered after 2 wk of alcohol withdrawal (Fig. 2 and Supplemental Fig. 1).

Cytokine profile in AUD patients and evolution with alcohol withdrawal

We studied the production of proinflammatory cytokines of downstream TLR2 and TLR4 activation by monocytes. The expression of TNF-\( \alpha \), IL-1\( \beta \), and IL-6 was determined in patients and controls, before and after TLR stimulation by PGN and LPS, respectively. At baseline, the percentage of TNF-\( \alpha \)- and IL-1\( \beta \)-producing monocytes showed no difference between AUD patients and HCs (Fig. 3A and B). The percentage of IL-6-producing cells was, however, significantly higher in AUD patients than in HCs (Fig. 3C). The levels of TNF-\( \alpha \), IL-1\( \beta \), and IL-6 expression were similar between AUD patients and HCs in basal conditions (Supplemental Fig. 2). Interestingly, alcohol withdrawal significantly reduced the percentage of IL-6-producing monocytes at baseline but to levels that remain different from HC percentages (Fig. 3C).

Upon TLR2 or TLR4 engagement, although the percentage of cytokine-producing cells in AUD patients was similar to HCs (Fig. 4), both PGN and LPS significantly increased the expression level of TNF-\( \alpha \) and IL-6 by monocytes in AUD patients as compared with HCs (Fig. 5). No significant difference in the TLR4-mediated, endotoxin-induced IL-1\( \beta \) expression was observed between AUD and HC following TLR2 and TLR4 engagement (Fig. 5B and E).

Importantly, following 2 wk of alcohol withdrawal, the percentage of TNF-\( \alpha \)-producing monocytes was significantly increased upon LPS and PGN stimulation (Fig. 4A and D). The percentage of IL-6- and IL-1\( \beta \)-producing monocytes upon LPS and PGN stimulation remained, however, unchanged, as well as the expression levels of TNF-\( \alpha \), IL-1\( \beta \), and IL-6 by monocytes of AUD patients (Figs. 4 and 5).

When measuring pro- and anti-inflammatory cytokine levels in the supernatant of PBMCs, AUD patients also displayed differences in cytokine production levels compared with HC, before and after LPS stimulation (Fig. 6). At baseline, PBMCs from AUD secreted significantly more TNF-\( \alpha \) and IL-6 than PBMCs from HCs (Fig. 6A and B), whereas the IL-10 secretion remained undetectable in both groups (Fig. 6C). Upon LPS stimulation, only PBMCs isolated from AUD patients significantly raised all 3 cytokine secretion levels. Finally, to get insights into deregulated endotoxin-induced cytokine production in AUD, pro- vs. anti-inflammatory cytokine balance was calculated. The TNF/IL-10 ratio was not significantly changed between HC and AUD, before and after treatment (Fig. 6D).

DISCUSSION

Chronic alcohol use is associated with immune dysfunctions that may contribute to decreased immunity and host defense, which are involved in the complications of AUD, including acute respiratory distress syndrome, liver cancer, and ALD [14]. To explore the distribution and function of monocytes in AUD, we investigated the phenotypic characteristics, as well as TLR2- and TLR4-mediated, endotoxin-induced inflammatory responses, of blood monocyte subsets and monitored their dynamic changes 14 d after alcohol withdrawal.
Monocytes are a true heterogeneous population, and a careful identification of specific subpopulations is necessary for understanding monocyte function in human disease. The performance of an in-depth phenotypic characterization allowed us to evidence, for the first time, an altered distribution of circulating monocyte subsets in AUD patients. In the present study, we demonstrate that the classic CD14+CD16− subset was decreased, whereas the nonclassic CD14dimCD16+ subset was expanded in the peripheral blood of AUD subject compared with HC. In addition, we show that 2 wk of alcohol withdrawal partially restored the abnormal distribution of nonclassic monocyte subsets.

Comparable abnormal distribution of circulating monocyte subsets was found in chronic systolic heart failure patients [15], and the CD16+ subpopulation has been shown increased in cases of infection or chronic inflammation [16–20]. Circulating CD16+ monocyte levels are positively correlated with levels of atherogenic lipids [21] and plaque vulnerability [22], whereas it is negatively correlated with cardiac function, such as left ventricular ejection fraction after acute myocardial infarction [23].

Figure 2. Reduced frequency of TLR2- and TLR4-expressing blood monocytes in AUD subjects and effect of alcohol withdrawal. Blood monocytes from HCs (n = 20) and patients with an AUD before (d 0, n = 40) and after (d 14, n = 33) 2 wk of alcohol withdrawal were stained for TLR2 and TLR4 surface markers. Percentages of TLR2 (A and C) and TLR4 (B and D)-expressing cells were plotted for either total blood monocytes (A and B) or each monocyte subsets (C and D). Bars indicate means ± so. Nonparametric Mann-Whitney test and paired-sample Wilcoxon signed rank test were used for statistical analyses d 0 vs. HC and d 0 vs. d 14, respectively (*P < 0.05; ns, nonsignificant).

Figure 3. Frequency of cytokine-expressing monocytes in AUD patients at baseline and effect of alcohol withdrawal. PBMCs were isolated from HCs (n = 20) and patients with an AUD (n = 40 at d 0, and n = 33 at d 14) and seeded at 100,000/well in a 24-well plate. After 24 h without stimulation, cells were labeled with CD3/ECD, CD20/ECD, CD56/ECD, HLA-DR/Pacific Blue, and CD45/KO mAb, permeabilized and incubated with IL-1β/AF647, IL-6/FITC, and TNF-α/PE mAb or their respective isotype controls. Intracellular expression of TNF-α (A), IL-1β (B), and IL-6 (C) was quantified by flow cytometry. Bars indicate means ± so. Nonparametric Mann-Whitney test and paired-sample Wilcoxon signed rank test were used for statistical analyses d 0 vs. HC and d 0 vs. d 14, respectively (**P < 0.01, *P < 0.05).
Significant increases in CD16+ monocyte levels have been described in human chronic pathologies, such as obesity [24] and multiple sclerosis [25]. None of these features were found in our studied cohort of AUD subjects. The peripheral imbalance between classic and nonclassic monocytes that we observed in AUD patients might be a result of differences in their respective kinetic of emigration from the bone marrow and/or migration toward inflamed tissues, as well as increased conversion of CD14+ into CD16+ monocytes. Indeed, CD16+ infiltrate the inflamed tissues later than CD14+ monocytes [26], and mouse studies suggest that classic monocytes might serve as nonclassic monocyte precursors under specific conditions [27–30]. The CD14dimCD16+ population demonstrates a patrolling behavior along blood vessel walls and accumulates in noninflamed peripheral tissues, such as spleen [31, 32]. They are involved in the innate local surveillance of tissues and the pathogenesis of autoimmune diseases [12]. Interestingly, liver subjected to chronic injury and inflammation, as observed in patients with liver diseases, displays increased frequency of the intermediate CD14+CD16+ subset [33]. Here, we evidence that this intermediate monocyte population is not affected in AUD patients, which is coherent with our exclusion criteria (i.e., decompensated cirrhosis or clinical signs of liver disease or alteration of liver biologic markers), and this tends to suggest that our AUD cohort does not contain patients with liver disease in the early stages. However, this cannot be expressly excluded, and thus, future replication studies shall propose adding a noninvasive test of fibrosis. Importantly, we show that the 3.6-fold increase in nonclassic CD14dimCD16+ blood monocytes can be reversed.
inflammation observed in AUD patients. Both TLRs activate a MyD88-dependent signaling pathway that leads to NF-κB activation and production of inflammatory cytokines, particularly TNF-α. Experimental models have demonstrated a role for the TLR4/MyD88 axis on alcohol-induced inflammatory responses in the brain and showed that the innate immunity receptor TLR4 regulating inflammatory cytokines plays a pivotal role in alcohol-induced neuro-inflammation and brain damage, and it is dispensable in TLR4-mediated liver injury in ALDs [35–37].

Here, we found increased cytokine responses to LPS and PGN in AUD patients that are likely to be a result of differences in the MyD88-dependent signaling pathway, as it is observed following TLR2 and TLR4 engagement. Indeed, we show that TLR2 and TLR4 ligands are more potent in AUD patients than in HCs to increase the expression levels of TNF-α and IL-6, but not of IL-1β, by monocytes. This impaired signaling is not attributable to higher expression levels of the TLRs, as AUD patients express TLR2 and TLR4 at levels compared with HC. This is consistent with a previous report showing that changes in the TNF-α production mediated by alcohol exposure are independent of TLR4 surface expression on human monocytes [34]. As AUD patients display a reduced frequency of TLR2- and TLR4-expressing monocytes, an augmented cytokine response is likely to be a result of an impaired signaling that leads to more potent functionality of the downstream TLR pathways in AUD monocytes, especially on CD14dimCD16+ monocytes, which are the primary subset for LPS-induced TNF-α production. Further studies are required for in-depth characterization of this hyper-responsiveness. Monocytes from patients with ALD are also more sensitive than control monocytes to stimulation with LPS [38]. Increased TNF-α production and NF-κB activation were also found in monocytes of patients with alcoholic steatohepatitis [39]. At baseline, frequencies of IL-6-producing monocytes in AUD patients are higher than in HCs and remained high after alcohol withdrawal, although significantly reduced. Together with the cytokine cross-regulation [40], which consists of the impairment of the IL-6-induced JAK/STAT signaling pathway by TLR4, high basal expression of IL-6 might be responsible for the lack of the IL-6 response to further TLR stimulation. Increased renal IL-6 levels and elevated hepatic production of TNF-α and IL-6 were reported in alcoholics [41]. Studies using animal models reveal that IL-6 protects against ethanol-induced liver injury [42].

In AUD patients, the severity of liver disease is correlated with plasma level of endotoxins (notably LPS) originating from bacterial translocation [43]. Alcohol exposure increases susceptibility to infection because of compromised innate immune responses. However, there is a lack of consensus on the molecular mechanism by which alcohol mediates this immunosuppression. Our present work suggests a role for monocyte subsets by showing for the first time an increased frequency of CD14dimCD16+ monocytes associated with higher IL-6 and TNF responses to LPS in AUD patients compared with HCs. Importantly, cofactors, such as age, gender, diabetes, BMI, systolic blood pressure, tobacco smoking, type and quantity of alcohol drunk daily, or drug consumption could

with only 2 wk of alcohol withdrawal, although the frequencies remain significantly different from HC. There is also a trend to restore normal frequencies of classic CD14+CD16− monocytes.

In the present study, we also describe for the first time that the percentage of TLR2- and TLR4-expressing nonclassic CD14dimCD16+ monocytes was reduced in the blood of AUD patients compared with HCs and that 14 d of alcohol withdrawal does not modify this profile. Importantly, neither the reduced percentage of monocytes expressing TLR2 and TLR4 nor the alcohol status of AUD patients impacts the surface expression level of TLR2 and TLR4 on monocyte subsets. This is consistent with previous work showing that neither acute nor chronic in vitro exposure of CD14+ monocytes to alcohol alters the surface expression of TLR4 [34]. Our present study extends this finding by showing in vivo data on chronic exposure and by investigating in-depth monocyte subsets. Our data suggest that chronic alcohol exposure might induce a long-lasting and perhaps irreversible loss of TLR2 and TLR4 expression at the surface of monocytes in AUD patients. As we showed for the first time that AUD is associated with an expansion of CD14dimCD16+ monocytes, future studies might consider investigating the expression level of TLR7/8 and the response to viral recognition of this specific subset [12].

TLR4 is the major endotoxin receptor and together with TLR2, is likely to be important in the pathogenesis of systemic
not account for the observed modifications in function and distribution of the monocyte subset (data not shown). Our study also shows that 14 d of alcohol withdrawal were sufficient to initiate immunologic changes on monocyte dynamics and on basal frequencies of IL-6-producing monocytes, as well as endotoxin-induced frequencies of TNF-producing monocytes, but not sufficient to evidence an effect on cytokine production in response to endotoxins. Although this delay of 2 wk of abstinence is sufficient for intestinal permeability to return to healthy levels and to improve mucosa damages of the small intestine found in AUD patients [4, 5], a longer period of abstinence might be necessary to restore fully normal distribution and response of monocytes.

Our results suggest that imbalanced monocyte subsets in AUD could be used as a biomarker of excessive alcohol consumption and/or alcohol withdrawal and help to improve patient management, especially in case of programmed surgery. It may also yield significant insights into the contribution of each subset to immune-mediated disorders associated with AUD.

**AUTHORSHIP**

H.D.-R. provided the conception, design, and laboratory experimentations and writing of the manuscript and is the corresponding author. T.M. did the statistical analysis and direction, and writing of the manuscript. F.A. contributed the conception and editing of the manuscript before publication. P. Perney provided the conception and editing of the manuscript before publication. T.M. did the statistical analysis and writing of the manuscript and is the corresponding author. T.M. did the statistical analysis and writing of the manuscript. P. Perney provided the conception and editing of the manuscript before publication. F.A. contributed the conception, direction, and writing of the manuscript. All authors read and approved the final manuscript.

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**DISCLOSURES**

The authors declare no conflicts of interest.

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give rise to mucosal, but not splenic, conventional dendritic cells. J. Exp. Med. 204, 171–180.

KEY WORDS:
TLR - cytokines - immune function
Effects of alcohol withdrawal on monocyte subset defects in chronic alcohol users

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