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Toll-like receptor 2 activation in monocytes contributes to systemic inflammation and alcohol-associated liver disease in humans

Luca Maccioni[1] | Joyce Kasavuli[1] | Sophie Leclercq[2,3] | Boris Pirlot[1] | Géraldine Laloux[4] | Yves Horsmans[5] | Isabelle Leclercq[1] | Bernd Schnabl[6,7] | Peter Stärkel[1,5]

[1]Institute of Experimental and Clinical Research, Laboratory of Hepatogastroenterology, UCLouvain, Université Catholique de Louvain, Brussels, Belgium

[2]Metabolism and Nutrition Research Group, Louvain Drug Research Institute, UCLouvain, Université Catholique de Louvain, Brussels, Belgium

[3]Institute of Neuroscience, UCLouvain, Université Catholique de Louvain, Brussels, Belgium

[4]de Duve Institute, UCLouvain, Université Catholique de Louvain, Brussels, Belgium

[5]Department of Hepatogastroenterology, Cliniques Universitaires Saint-Luc, Brussels, Belgium

[6]Department of Medicine, University of California San Diego, La Jolla, California, USA

[7]Department of Medicine, VA San Diego Healthcare System, San Diego, California, USA

Correspondence
Peter Stärkel, Department of Hepatogastroenterology, Cliniques Universitaires Saint-Luc, Av. Hippocrate 10,1200 Brussels, Belgium.
Email: peter.starkel@saintluc.uclouvain.be

Abstract

Background and Rationale: In the context of gut leakiness and translocation of microbial products in alcohol-associated liver disease (ALD), it is possible that systemic and liver inflammation involve the activation of circulating monocyte through gut-derived factors. We explored the association between monocytes, microbial translocation, systemic inflammation, and ALD.

Methods: Patients with alcohol use disorder following a rehabilitation program were compared with healthy controls. We determined the circulating number and proportion of monocyte subsets by FACS. The activation of signaling pathways by gut-derived microbes was analyzed by quantitative PCR in isolated monocytes. Cytokines secretion by monocytes and phagocytosis were assessed *in vitro*. Serum microbial translocation markers and cytokines were measured by ELISA and multiplex assay, respectively. ALD severity and liver inflammatory responses were analyzed in liver biopsies by various methods.

Results: In patients with alcohol use disorder, the number of blood monocytes increased compared with controls. Monocytes from patients with alcohol use disorder upregulated IL-1β and IL-8 together with toll-like receptor 2 and downstream AP-1, while fungal sensor CARD9 was downregulated. IL-1β and IL-8 were actively secreted upon stimulation *in vitro* with the toll-like receptor 2 ligand peptidoglycan. Exposure with *Escherichia coli* confirmed preserved bacterial phagocytic activity. In contrast, *Candida albicans* stimulation leads to downregulation of IL-1β and TNFα compared with controls. Systemic cytokines and monocyte changes correlated with microbial translocation. Hepatic

Abbreviations: ALD, alcohol-associated liver disease; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; AUD, alcohol use disorder; BMI, body mass index; CK18, cytokeratin 18; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharides; MCP-1, monocyte chemoattractant protein 1; mRNA, messenger RNA; PBMCs, peripheral blood mononuclear cells; PGN, peptidoglycans; PGRPS, peptidoglycan-recognition proteins; sCD14, soluble CD14; TLR, toll-like receptor; TREM1, triggering receptor expressed on myeloid cells 1.

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IL-1β and IL-8 increased with ALD severity together with liver macrophage activation and upregulation of chemokines involved in monocyte attraction.
Conclusions: Our results point to the contribution of activated monocytes to systemic inflammation and ALD. Monocytes likely infiltrate the liver, transform into monocyte-derived macrophages and release IL-1β and IL-8 in response to peptidoglycan and toll-like receptor 2 activation.

INTRODUCTION

Alcohol abuse is a major public health problem and alcohol-associated liver disease (ALD) is the most prevalent type of chronic liver disease worldwide. Most individuals with an alcohol use disorder (AUD) develop simple steatosis which can progress to alcoholic steatohepatitis characterized by hepatic inflammation and hepatocyte ballooning.[1] Chronic alcoholic steatohepatitis eventually leads to fibrosis and cirrhosis and in some cases HCC.[2] The mechanisms leading to ALD progression remain incompletely understood.

Recent reports have highlighted the pivotal role of the gut-liver axis in the pathogenesis of ALD. In humans, alterations in the gut microbiome and mycobiome together with elevated systemic translocation of either microbial products and/or microbes themselves are associated with ALD.[3,4] Increased systemic translocation of bacterial products, such as lipopolysaccharides (LPS) and peptidoglycans (PGN), are associated with elevated plasma levels of inflammatory cytokines in AUD patients.[5]

Mechanistic analyses have shown that in AUD patients, LPS and to a greater extent PGN can contribute to the activation of peripheral blood mononuclear cells (PBMCs). PBMCs are likely contributing to the PGN-triggered release of IL-1β, IL-8, and IL-18 into the blood of AUD patients.[5] Intriguingly, a short period of abstinence was able to restore normal blood LPS levels, while the expression of PGN-associated receptors remained increased in the PBMCs, suggesting that PGN could contribute to the persistence of an elevated systemic inflammatory response observed in sober patients. In contrast, TNFα and IL-6 were not elevated in PBMCs indicating that their increased plasma levels might originate from other sources.[6] Among PBMCs, monocytes represent the cell type with the highest potential to secrete these cytokines.[7]

Circulating inflammatory monocytes are recruited to the liver during inflammation and fibrosis, principally involved in ALD.[8] Once in the liver, monocytes transform into monocyte-derived macrophages which have been shown to play a key role in ALD development in animal models.[9,10] However, animals only develop mild forms of ALD upon chronic alcohol feeding and do not resume the liver damage pattern observed in humans.[11] Therefore, investigations in human ALD are needed to evaluate the role of monocytes and monocyte-derived macrophages in systemic inflammation and liver disease.

Human studies generally focused on the role of monocytes and monocyte-derived macrophages in late stages of liver disease such as decompensated cirrhosis and severe alcohol-associated hepatitis.[12,13] Little is known about the role of monocytes and monocyte-derived macrophages in the development of systemic inflammation and ALD in response to bacterial and fungal antigens in earlier stages of ALD.

The aim of our present study is to assess the potential role of monocytes in the development of systemic inflammation in relation to elevated systemic microbial translocation and its potential involvement in ALD in humans.

METHODS

Patients

AUD patients (*Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* criteria) admitted for elective alcohol withdrawal to a dedicated alcohol withdrawal unit followed a highly standardized and controlled 3-week detoxification and rehabilitation program (Supplementary Figure 1, http://links.lww.com/HC9/A218). They were compared with healthy volunteers (social drinkers consuming <20 g of alcohol/d) matched for sex, age, and body mass index. All patients reported long term (> 1 y) alcohol consumption (> 60 g/d) and were actively drinking until the day of admission. Clinical and baseline biochemical data were collected prospectively for all patients, and they routinely underwent a large panel of investigations. Additional inclusion and exclusion criteria are available in Supporting Material (http://links.lww.com/HC9/A218).

Examinations and sample collections (Supplementary Figure 1, http://links.lww.com/HC9/A218)

On the day of admission, transient elastography (Fibroscan; Echosense) was performed and a fasting blood

sample (serum, plasma) was drawn. A transjugular liver biopsy was routinely proposed to all patients with suspicion of significant liver disease [FibroScan ≥ 7.6 kPa (≥ F2), or Doppler ultrasound with dysmorphic liver or splenomegaly or alterations of portal vein flow].

Due to ethical restrictions, methodological reasons, and limited material available, some analyses were only performed in representative cohorts of patients, as indicated in the flowchart in Supplementary Figure 1 (http://links.lww.com/HC9/A218).

Ethical aspects

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and Istanbul and was approved by the institution's human research and ethical committee (B403201422657) Comité d'éthique hospitalo-facultaire, Université Catholique de Louvain. Written informed consent was obtained from all patients and healthy volunteers.

Isolation of human monocytes

The PBMCs were isolated from blood by centrifugation on a Ficoll-Paque Plus gradient medium (GE Healthcare Biosciences AB) (Supplementary Material, http://links.lww.com/HC9/A218). $CD14^+$ monocytes were isolated by using anti-human CD14 MicroBeads (Miltenyi Biotec), according to the manufacturer's protocol. Quality control by flow cytometry showed that at least 90% of the isolated cells were $CD14^+$.

RNA extraction, reverse transcription, and real-time quantitative PCR

RNA extraction from cells and tissue was performed by using TRIzol reagent (Invitrogen). RNA was then purified by using columns of the Direct-zol RNA Microprep kit (Zymo Research, BaseClear Lab). Messenger RNA (mRNA) was assessed by quantitative real-time PCR and quantified using the $\Delta\Delta C_t$ method normalized for the internal standard riboprotein L19. Data are presented as fold changes compared with controls set at 1 unless otherwise stated. Primers are depicted in Supporting Material (http://links.lww.com/HC9/A218).

Immunohistochemistry/ immunofluorescence

Five microlitres formalin-fixed liver sections were routinely stained with hematoxylin and eosin and primary and secondary antibodies using standard methods followed by quantification (details in Supporting Material, http://links.lww.com/HC9/A218).

Flow cytometry evaluation of monocyte subsets

Flow cytometric analysis was performed on isolated PBMCs stained with antibodies against CD14, CD16, CCR2, HLA-DR, and CX3CR1, toll-like receptor (TLR) 2 (listed in Supporting Material, http://links.lww.com/HC9/A218) by using a BD FACSCanto II equipped with BD FACSDiva software.

Short-term cell culture and *in vitro* stimulation

To test *in vitro* TLRs activation upon LPS and PGN stimulation, monocytes from AUD patients and matched healthy controls were cultured in 96-well plates in RPMI-1640 culture medium (50,000 cells/well) containing L-glutamine, 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 5% CO_2-humidified incubator at 37°C, and exposed to 1 ng/mL of either LPS (InvivoGen) or PGN (Invivogen) or with increasing doses of *Candida albicans* extracts (InvivoGen) (10^6, 500×10^6, and 10^7 cells/mL) for 24 hours. Cells cultured in culture medium alone served as controls. The effect of TLR2 inhibition was tested by using the TLR2 inhibitor C29 at a final concentration of 50 μM for 1 hour[14] and then stimulating monocytes with PGN 1 ng/mL for 24 hours. Culture medium was recovered for measurements of IL-1β, IL-8, and TNFα levels. RNA was extracted from stimulated monocytes as described above.

Phagocytosis assay

Phagocytosis was assessed by using dTomato-expressing *Escherichia coli* generated by transforming *E. coli* with the plasmid vector ptdTomato (details in Supporting Material, http://links.lww.com/HC9/A218). Bacteria and monocytes isolated from AUD patients or healthy controls were cocultured in a ratio 1:10 in RPMI-1640 medium supplemented with heat-inactivated fetal bovine serum, L-glutamine, and penicillin/streptomycin for 15 minutes to assess bacterial engulfment. Bacterial engulfment was evaluated by flow cytometry as described[15] using a BD FACSCanto II equipped with BD FACSDiva software.

Determination of serum biomarkers of liver cell damage and microbial translocation

In addition to aspartate transaminase and alanine transaminase, serum cytokeratin 18 (CK18) was used to assess liver cell damage (CK18-M65 ELISA kit; TECOmedical AG). Serum levels of CK18-M30

(CK18-M30 ELISA kit; TECOmedical AG) were used to assess hepatocyte's apoptosis. Microbial translocation was determined using soluble CD14 (Human CD14 Quantikine ELISA kit sCD14; Bio-techne Ltd) and PGRPs (human PGRPs ELISA kit; Thermofisher), respectively. All assays were performed in duplicate following the manufacturer's instructions.

Assessment of plasma levels of cytokines

Plasma concentrations of cytokines (IL-8, IL-18, IL-6, and TNFα) were determined using the Meso Scale Discovery (MSD) U-Plex assay, IL-1β levels which were assessed by using the IL-1 beta human ELISA kit, high sensitivity (Thermofisher), following the manufacturer's instructions.

Statistics

Data were analyzed using GraphPad Prism 8 (GraphPad Software) and presented as mean ± SEM unless otherwise indicated. Normality was assessed by the Kolmogorov-Smirnov test followed by *t* tests for normally distributed data, or the Wilcoxon test for nonnormally distributed data. Data were compared by 1-way ANOVA for multiple groups, followed by Bonferroni post hoc test for pairwise comparisons. Wilcoxon or paired *t* tests compared data of patients before and after abstinence. The Pearson or Spearman correlation tests were used for correlations between data sets. A *p*-value <0.05 was considered as statistically significant. Flow cytometry data were analyzed using FlowJo (TreeStar).

RESULTS

Study population

Overall, 138 middle-aged, predominantly male AUD patients and 36 healthy volunteers were included in the study. Demographic and biochemical data are provided in Table 1. All patients reported alcohol consumption until the evening before their admission. In 62 AUD patients with suspicion of significant liver disease [FibroScan ≥ 7.6 kPa (≥ F2), or Doppler ultrasound with dysmorphic liver or splenomegaly or alterations of portal vein flow] a liver biopsy was performed. Forty-eight percent (n = 30) had early-stage ALD with a Metavir fibrosis score of F0/F1 and various degrees of steatosis or steatohepatitis on histology. Seven patients had a Metavir score of F2 and 25 patients presented with advanced fibrosis (≥ F3) (Table 1). All patients had a preserved synthetic liver function and showed no clinical signs of liver decompensation.

Circulating monocytes and systemic microbial translocation in AUD patients

Increased number of circulating monocytes together with increased proportions of intermediate and nonclassical subsets characterized AUD patients

We first assessed whether there was a difference between number of circulating monocytes in AUD patients and healthy controls and found a significant ($p < 0.0001$) increase in the number of monocytes in AUD patients (Figure 1A). We then determined the 3 main subsets of monocytes by flow cytometry (Supplementary Figure 2, http://links.lww.com/HC9/A218). We observed a significant diminution of classical monocytes accompanied by a significant increase in both intermediate and nonclassical monocytes (Figure 1B).

Expression of IL-1β and IL-8 is upregulated in monocytes of AUD patients

A previous report has shown that PBMCs from AUD patients were characterized by upregulation of IL-1β, IL-8, and IL-18, while TNFα and IL-6 were not increased compared with PBMCs from healthy controls.[5] We isolated monocytes to verify whether these transcriptional changes also characterize monocytes in AUD patients. IL-1β and IL-8 mRNA were significantly upregulated (Figure 1C), while the gene expression of other proinflammatory cytokines remained unchanged in monocytes from AUD patients compared with healthy controls (Supplementary Figure 3A, http://links.lww.com/HC9/A218). In parallel, we found elevated blood levels of the inflammatory cytokines IL-8 (Figure 1C), IL-6, and TNFα (Supplementary Figure 3B, http://links.lww.com/HC9/A218) in our cohort of AUD patients compared with controls, while IL-1β could not be detected in blood in both groups. Monocytes can also produce anti-inflammatory cytokines, such as IL-10 and TGFβ1, implicated, for example, in immune suppression and liver fibrosis. We, therefore, evaluated their expression in isolated monocytes and found increased mRNA levels of IL-10, but not TGFβ1, in monocytes from AUD patients compared with controls at baseline (Figure 1E).

Since IL-1β and IL-8 are transcriptionally activated by the transcription factor AP-1, we then assessed gene expression of c-Fos and c-Jun as components of the transcription factor AP-1.[16] We found a significantly increased gene expression of c-Jun, but not c-Fos, in monocytes from AUD patients compared with controls, pointing to a potential involvement of the transcription factor AP-1 (Figure 1D). In contrast, gene expression of NLRP3, a component of the inflammasome involved in cleavage and activation of pro-IL-1β,[17] was similar between the 2 groups (Figure 1D). Since NF-κB

TABLE 1 Baseline demographic and biochemical data of the study population

	Healthy volunteers (n = 36)	AUD patients (n = 138)	*p* value
Demographics			
Sex (female/male)	14/22	42/96	0.3335
Age (mean ± SEM) (y)	47.6 ± 11	50.5 ± 8.2	0.1771
BMI (mean ± SEM)	25.4 ± 3.0	25.6 ± 3.8	0.7125
Biochemistry [mean ± SEM (normal range)]			
AST (IU/L)	ND	88.8 ± 62 (< 40)	—
ALT (IU/L)	ND	73.4 ± 47.6 (< 40)	—
γ-GT (IU/L)	ND	340.8 ± 315 (< 40)	—
ALP (IU/L)	ND	93.9 ± 34.8 (30–130)	—
Bilirubin (mg/dL)	ND	0.82 ± 0.37 (0.3–1.2)	—
Albumin (g/L)	ND	44.6 ± 3.9 (35–52)	—
Histology (n = 62)			
Fibrosis (≥ F2)[a] (N = 32)			
F2 (n)	7		
F3 (n)	10		
F4 (n)	15		
No fibrosis (F0/F1) (N = 30)			
Simple steatosis (n)	12		
Steatohepatitis (n)	18		

[a]Metavir classification.
Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; AUD, alcohol use disorder; BMI, body mass index; γ-GT, gamma-glutamyltransferase; ND, not done.

pathway controls the expression of IL-6 and TNFα, which did not change in monocytes from AUD patients, we next assessed the mRNA levels of I-kB, inhibitor of NF-κB, and found upregulation in AUD compared with controls (Supplementary Figure 3A, http://links.lww.com/HC9/A218), pointing to a possible restriction of the pathway.

These results indicate that an increased number of inflammatory-primed (ie, the ability to produce IL-1β and IL-8) monocytes accompanied by alterations in their subpopulations characterizes AUD patients as compared with healthy volunteers.

Elevated serum levels of Gram-positive translocation marker correlate with the proportion of intermediate monocytes

As anticipated from previous work, we also show a significant increase of soluble CD14 (sCD14) and peptidoglycan-recognition proteins (PGRPs), serum surrogate markers of microbial translocation for Gram-negative and Gram-positive bacteria, respectively [18,19] in this cohort of AUD patients (Figure 2A). We next evaluated whether the alterations observed in the monocytes correlated with serum levels of microbial translocation markers. We found a positive correlation between intermediate monocytes and serum levels of PGRPs (Figure 2B), while no correlations between alterations in the monocytes and sCD14 levels were observed (Supplementary Table. S1, http://links.lww.com/HC9/A218).

Because microbial translocation drives systemic inflammation and early disease pathogenesis,[20] we also correlated plasma levels of cytokines with surrogate markers of microbial translocation. All 3 cytokines significantly correlated with sCD14 but not with PGRPs (Supplementary Table. S2, http://links.lww.com/HC9/A218).

PGN activation of TLR2 primes monocytes for increased IL-1β and IL-8 production in AUD patients

We next evaluated gene expression of TLR2 and 4 TLR4, which bind the Gram-positive and Gram-negative products PGN and LPS, respectively.[21] TLR2 was upregulated in monocytes from AUD patients (Figure 3A), while TLR4 gene expression did not change (Supplementary Figure 4B, http://links.lww.com/HC9/A218). Gene expression of TLR1 and TLR6, co-receptors of TLR2, was similar in monocytes from AUD patients compared with controls (data not shown). Although not significant, flow cytometry showed increased mean surface expression of TLR2 in monocytes from AUD patients compared with controls

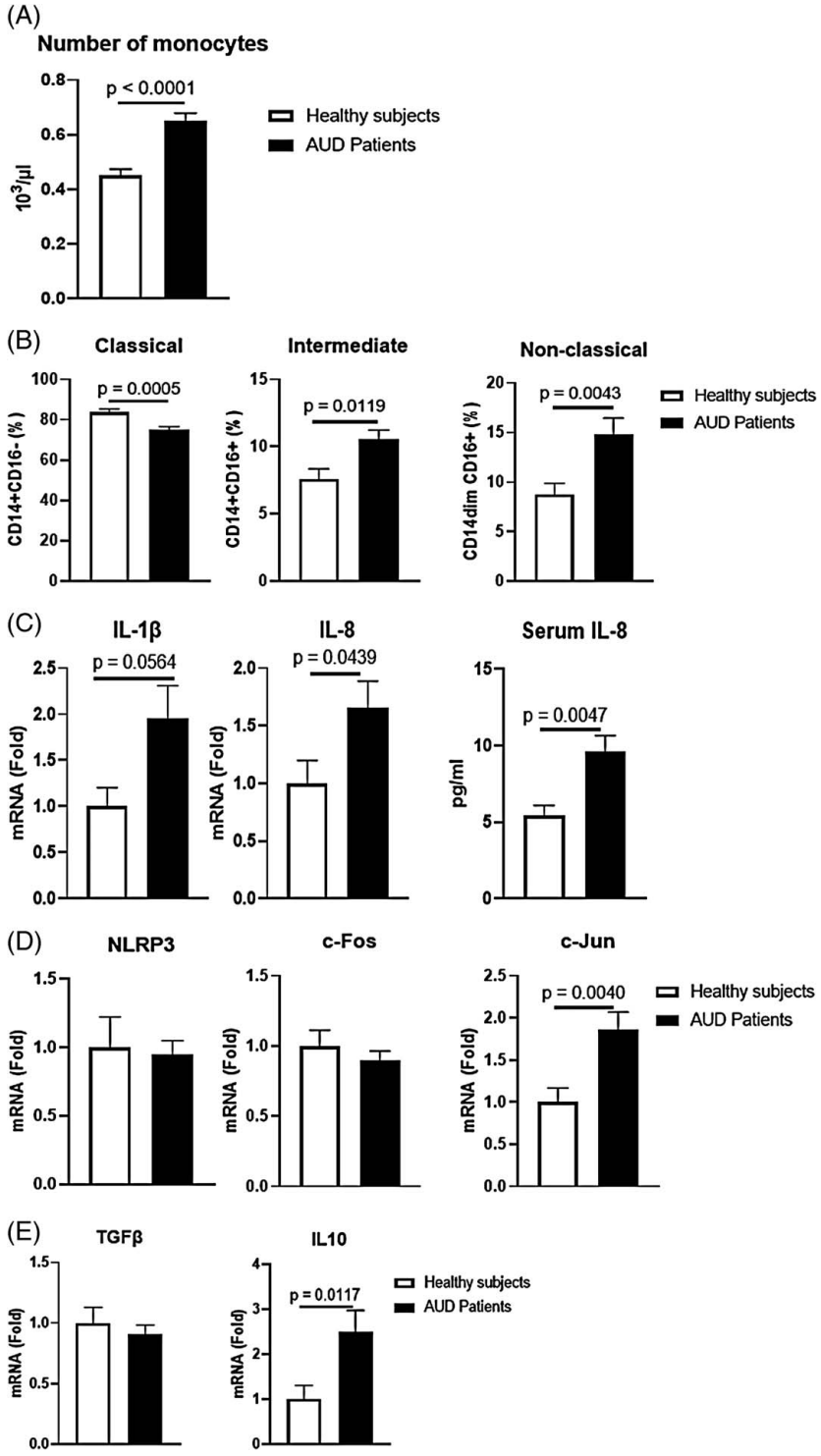

FIGURE 1 Evaluation of monocytes in alcohol use disorder (AUD) patients. Monocyte count in the systemic circulation (A) showed a significative ($p < 0.0001$) increased number of monocytes in AUD patients (n = 61) compared with matched healthy volunteers (n = 18). (B) Flow cytometry analysis revealed diminution in the proportion of classical monocytes accompanied by an increased proportion of both intermediate and nonclassical monocytes in AUD patients (n = 24) compared with healthy controls (n = 8). Gene expression of inflammatory cytokines in monocytes showed upregulation of IL-1β and IL-8 in AUD patients (n = 24) compared with controls (n = 8) associated with increased serum levels of IL-8 (n = 24). Serum IL-1β levels could not be detected with the ELISA kit used to assess IL-1β in the culture medium (C). Monocytes from AUD patients have similar gene expression of NLRP3 and analysis of the 2 components of the transcription factor AP-1, such as c-Fos and c-Jun, showed upregulated of c-Jun in monocytes from AUD patients (n = 24) compared with controls (n = 8), while c-Fos did not change (D). Monocytes from AUD patients upregulate IL-10, but not TGFβ1, compared with controls (E).

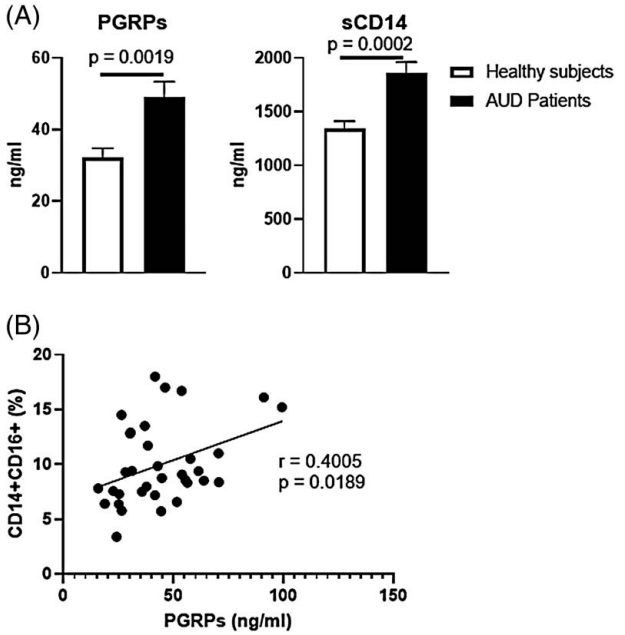

FIGURE 2 Evaluation of microbial translocation in relation to monocytes from alcohol use disorder (AUD) patients. Analysis of serum peptidoglycan-recognition proteins (PGRPs) and soluble CD14 (sCD14), a marker of Gram-positive and Gram-negative translocation, respectively, showed significantly increased levels in AUD patients (n = 24) compared with controls (n = 8) (A). Analysis showed a significant positive correlation between proportion of $C14^{+}CD16^{+}$ intermediate monocytes and serum levels of PGRPs microbial translocation marker (B).

(Supplementary Figure 4A, http://links.lww.com/HC9/A218). In addition, TLR2 expression correlated with mRNA levels of IL-1β and IL-8 ($r = 0.3698$, $p = 0.0406$; $r = 0.6036$, $p = 0.0003$, for IL-1β and IL-8, respectively) indicating a potential link between TLR2 ligands and activation of monocytes.

To test the possible differential activation of monocytes from AUD patients by Gram-positive and Gram-negative bacteria, we performed in vitro stimulation tests in monocytes isolated from AUD patients. We first evaluated the overall phagocytic capacity of monocytes to live *E. coli* and observed no differences between monocytes from AUD patients and healthy controls (Figure 3B) revealing a preserved bacterial phagocytic function.

The concept of trained immunity describes the long-term functional reprogramming of innate immune cells, which is evoked by exogenous or endogenous insults and which leads to an altered response towards a second challenge after the return to a nonactivated state.[22] We first assessed whether monocytes from AUD patients were "trained" against bacterial antigens by stimulating them with bacterial products PGN (Gram-positive bacteria) and LPS (Gram-negative bacteria). Monocytes from AUD patients secreted higher levels of IL-1β and IL-8 in response to the TLR2 ligand PGN (Figure 3C) compared with control monocytes. Intriguingly, PGN stimulation also increased gene expression of TLR2 and the inflammasome component NLRP3 in monocytes from AUD patients (Figure 3D). In contrast, stimulation with LPS lead to modest increase of TNFα secretion (Supplementary Figure 4D, http://links.lww.com/HC9/A218) without stimulating gene expression of its receptor TLR4 (Supplementary Figure 4C, http://links.lww.com/HC9/A218). Stimulation with PGN and LPS neither increase TGFβ1 mRNA in AUD patients and controls nor further enhance IL-10 in monocytes from AUD patients as compared with controls after 24 hours of stimulation (Supplementary Figure 4E, http://links.lww.com/HC9/A218).

To finally confirm the implication of TLR2 in IL-1β and IL-8 expression, we stimulated isolated monocytes with PGN in the presence or absence of C29, a selective TLR2 inhibitor at a fixed concentration retrieved from the literature.[14] We found reduced gene expression of both IL-1β and IL-8, but not TNFα, upon inhibition of TLR2 compared with monocytes treated only with PGN, indicating that TLR2 is implicated in increased IL-1β and IL-8 expression (Figure 3E).

Reduced CARD9 is associated with a blunted response to fungi in monocytes of AUD patients

AUD patients are characterized by fungal overgrowth and a specific increased proportion of *C. albicans*.[4,23] C-type lectin receptors bind and respond to different fungal species. We, therefore, evaluated C-type lectin receptors (eg, Dectin1, Dectin2, and Mincle) mRNA levels and found no differences in monocytes of AUD compared with controls (Figure 4A).

CARD9 is a signaling adaptor protein downstream of a variety of C-type lectin receptors and plays a critical role in fungal immune surveillance. Following receptor engagement and recruitment of Syk kinase, CARD9 forms a complex with BCL10 and MALT1, which transduces non-TLR signaling to the canonical NF-κB pathway.[24] We next assessed CARD9 gene expression and found downregulation in monocytes from AUD patients compared with controls (Figure 4A), pointing to a potential immune dysregulation against fungi.

To test this hypothesis, we stimulated monocytes with different doses of *C. albicans* extracts. We found a downregulation of IL-1β and TNFα in monocytes from AUD patients compared with controls with the highest dose of the extracts, while IL-8 mRNA did not change between the 2 groups (Figure 4B), indicating a reduced immune response against this fungus.

Overall, we found a reduced immune response to *C. albicans* with downregulation of IL-1β and TNFα. In contrast, upregulation of IL-1β and IL-8 occurred upon stimulation with the TLR2 ligand PGN, which in turn was abrogated by selective TLR2 blockage. Taken together, these observations indicate that the production of IL-1β and IL-8 in circulating monocytes seems to be primarily activated through TLR2 and its ligands in AUD patients.

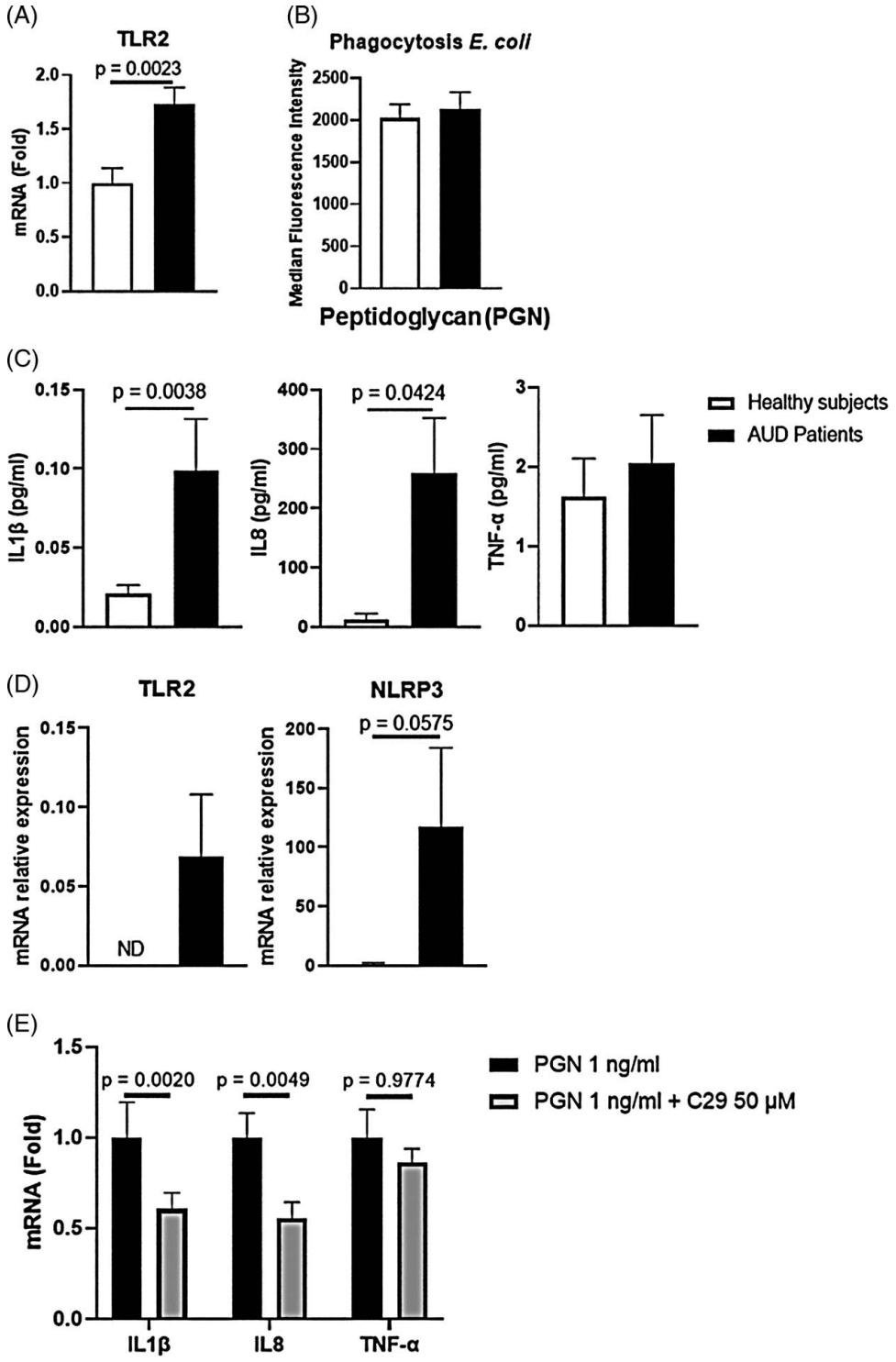

FIGURE 3 Assessment of monocytes activation by bacterial products in alcohol use disorder (AUD) patients. Toll-like receptor 2 (TLR2) gene expression was increased in monocytes from AUD patients (n = 24) compared with controls (n = 8) (A). (B) Phagocytosis capacity was similar in monocytes from AUD patients with steatohepatitis (n = 4) as compared with healthy controls (n = 4). Monocytes from AUD patients (n = 8) and controls (n = 8) were stimulated with 1 ng/mL peptidoglycan (PGN) for 24 hours. Results shown are the delta compared with the unstimulated cells. PGN stimulation (C) promoted increased secretion in the medium of IL-1β and IL-8, but not TNFα, in monocytes from AUD patients (n = 8) compared with controls (n = 8). (D) PGN stimulation leads to upregulation (increased delta mRNA relative expression compared with unstimulated monocytes) of TLR2 and NLRP3 in monocytes from AUD patients. (E) The effect of TLR2 inhibition was tested by incubating the cells with the selective Toll-like receptor 2 inhibitor C29 at a final concentration of 50 μM for 1 hour followed by stimulation of monocytes with PGN 1 ng/mL for 24 hours. TLR2 inhibition with C29 in isolated monocytes reduced expression of IL-1β and IL-8, but not TNFα, compared with monocytes treated with PGN alone.

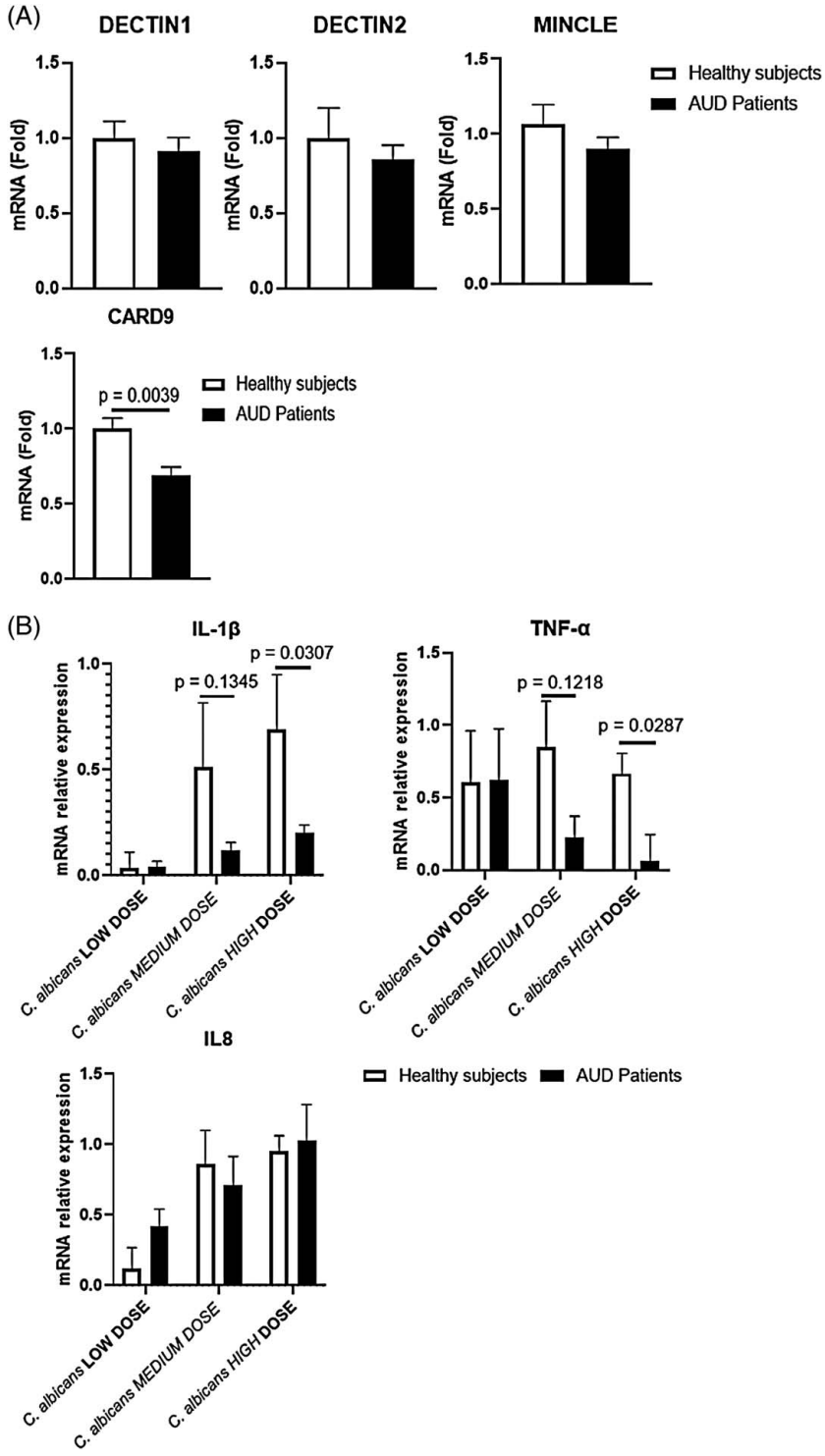

FIGURE 4 Assessment of monocytes activation by fungi in alcohol use disorder (AUD) patients. Monocytes from AUD patients (n = 24) have similar gene expression of receptors Dectin1, Dectin2, and Mincle compared with healthy volunteers (n = 8), while downstream CARD9 was downregulated in AUD patients (A). Monocytes from AUD patients (n = 7) and controls (n = 7) were stimulated for 24 hours with increasing doses of *Candida albicans* extracts. Results shown are the delta mRNA levels compared with the unstimulated cells. Stimulation with increasing doses of *Candida albicans* showed reduced gene expression of IL-1β and TNFα, but not IL-8, in a dose-dependent manner (B) in monocytes from AUD patients compared with controls (n = 7/group).

Monocyte-derived macrophages and ALD

Intrahepatic IL-1β and IL-8 correlated with ALD severity

Chronic liver diseases including ALD, have been associated with the activation of infiltrating macrophages with a massive release of proinflammatory cytokines in the liver.[12] We, therefore, assessed whether intrahepatic cytokines and markers of macrophages polarization correlated with markers related to liver disease severity. We subdivided AUD patient according to liver disease severity based on histology and we evaluated intrahepatic cytokines/chemokines and markers of macrophages activation in liver biopsies by quantitative real-time PCR and immunohistochemistry analysis. Based on histology, AUD patients were subdivided into 3 groups: steatosis (Metavir F0/F1, presence of steatosis, normal transaminases; n = 12); steatohepatitis (Metavir F0/F1, steatosis, hepatocyte ballooning, and elevated transaminases; n = 18), significant fibrosis (Metavir ≥ F2; n = 32) (Supplementary Figure 5, http://links.lww.com/HC9/A218). To get a better appreciation of liver disease severity, we further measured serum levels of markers linked to liver cell damage and apoptosis, such as CK18-M65 and CK18-M30, respectively (Table 2).

Liver gene expression of IL-1β increased significantly in patients with steatohepatitis and significant fibrosis compared with steatosis. IL-8 mRNA raised progressively in the different stages of ALD. In addition, increased serum IL-8 levels were only observed in ALD (steatohepatitis and fibrosis) but not in minimal liver disease (eg, simple steatosis) and healthy controls (Figure 5A). Moreover, IL-8 positively correlated with the liver damage markers aspartate transaminase, CK18-M65, and CK18-M30, while IL-1β correlated with ALT levels and CK18-M30 (Supplementary Table S3, http://links.lww.com/HC9/A218), indicating that IL-1β and IL-8 could potentially contribute to ALD development. No differences were found for TNFα (Supplementary Figure 6, http://links.lww.com/HC9/A218). We next evaluated liver chemokines involved in the recruitment of monocytes, such as monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) and CX3CL1. Monocytes from AUD patients had an overall 5 times increase of MCP-1 mRNA levels (Figure 5B), but not CX3CL1 (data not shown), compared with controls at baseline. After stimulation of those monocytes with PGN (Figure 5B), but not with LPS (Supplementary Figure 7, http://links.lww.com/HC9/A218), MCP-1 expression further increased by 7.5 times in AUD patients compared with controls, indicating that circulating monocytes in AUD patients do already express high levels of MCP-1 before eventually homing into tissue. In parallel, liver MCP-1 but also CX3CL1 were upregulated especially in AUD subjects with fibrosis (Figure 5B). We then assessed whether they correlated with hepatic IL-1β and IL-8 and found a positive correlation between MCP-1, but not CX3CL1, with both inflammatory mediators ($r = 0.5253$ and $r = 0.7156$, $p < 0.0001$, for IL-1β and IL-8, respectively).

We finally assessed markers of macrophage activation such as inducible nitric oxide synthase (iNOS) and CD163. Liver gene expression of iNOS increased significantly in patients with steatohepatitis and especially in patients with significant fibrosis compared with steatosis (Figure 5C). Immunofluorescence showed that iNOS is principally found in $CD68^+$ macrophages (Figure 5D). Similar results were observed for $CD163^+$ macrophages (Supplementary Figure 8A, B, http://links.lww.com/HC9/A218). Since liver macrophages could also contribute to chemoattraction, we then evaluated the potential relationship between macrophage activation markers (eg, iNOS and CD163) and liver chemokines. Both chemokines (MCP-1 and CX3CL1) positively correlated with iNOS ($r = 0.4460$, $p = 0.0009$; $r = 0.4895$, $p = 0.0002$, for MCP-1 and CX3CL1, respectively) but not with CD163 (data not shown). Since iNOS is primarily expressed in macrophages on immunofluorescence, those correlations indicate a potential relationship between liver macrophage activation, chemokine production in the liver, and subsequently monocytes chemoattraction. We then correlated the number of monocytes with liver chemoattractants/inflammatory mediators as well as with activation markers of macrophages. Despite no differences in total monocytes count, monocyte IL-1β and IL-8 mRNA levels in relationship to ALD stages (Supplementary Figure 9, http://links.lww.com/HC9/A218), we found inverse correlations between number of circulating monocytes and the liver expression of both CX3CL1 and iNOS (Figure 5E). Although correlations are not formal proofs, one is tempted to speculate that those

TABLE 2 Serum levels of liver cell damage markers according to ALD stage

	Mean ± SEM			
	AST (IU/L)	ALT (IU/L)	CK18-M65 (U/L)	CK18-M30 (U/L)
Steatosis	31.9 ± 5.9	30.6 ± 11.6	329.3 ± 96.3	193.9 ± 98
Steatohepatitis	133.4 ± 68.1	103.2 ± 48.2	718.8 ± 233	636.7 ± 408.5
Significant fibrosis	136.2 ± 89.6	89.8 ± 65.3	827.5 ± 575	813.4 ± 737.4

Abbreviations: ALD, alcohol-associated liver disease; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CK18, cytokeratin 18.

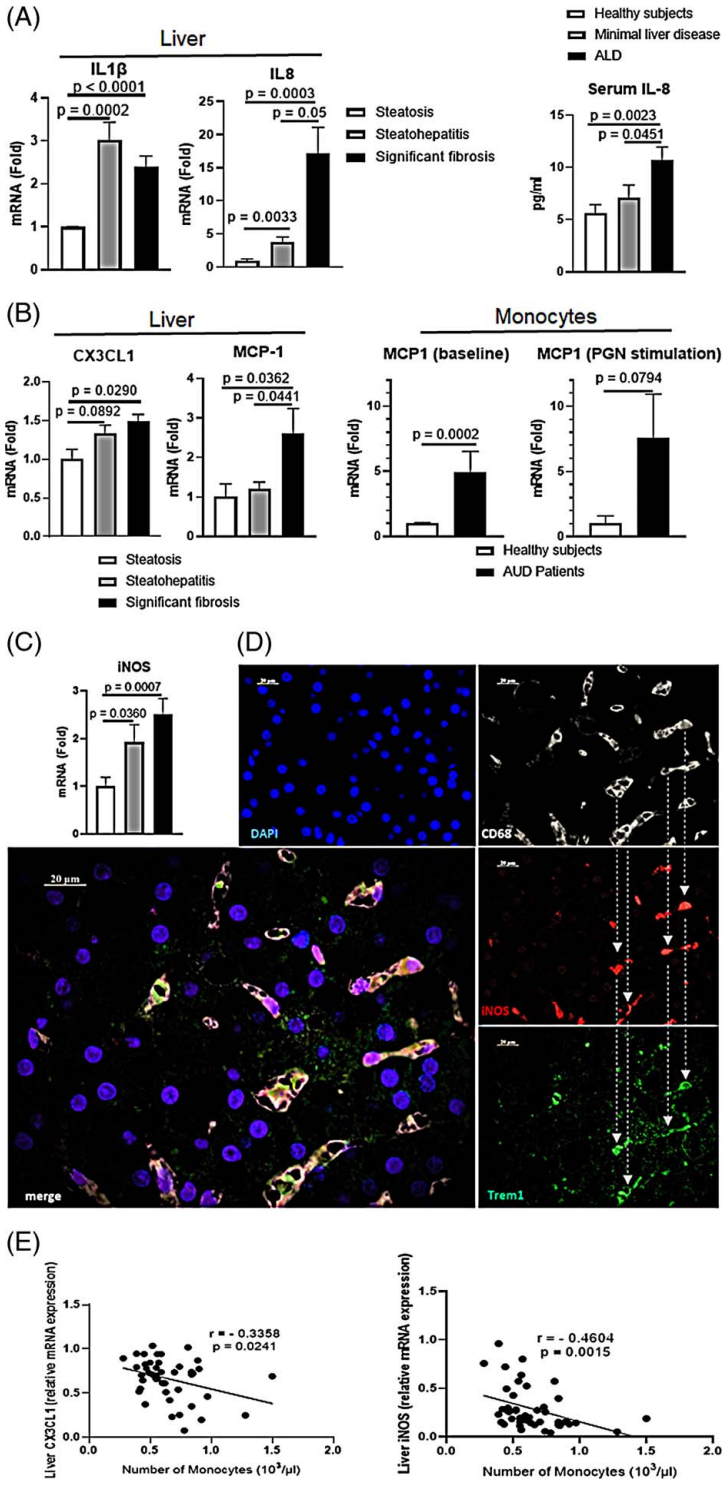

FIGURE 5 Evaluation of inflammatory markers related to monocyte-derived macrophages in livers of AUD subjects according to liver disease severity. (A) Hepatic gene expression (right) of IL-1β increased significantly in AUD patients with steatohepatitis (n = 18) and fibrosis (n = 29) compared of steatosis (n = 3), while IL-8 was progressively upregulated according to liver disease severity (right). Elevated serum IL-8 levels (left) in patients with ALD stages (steatohepatitis and significant fibrosis) compared with minimal liver disease (eg, simple steatosis) and healthy controls (B). Liver and monocyte mRNA expression of CX3CL1 and MCP-1. Hepatic expression of chemokines involved in monocytes chemoattraction (CX3CL1 and MCP-1) increased significantly, especially in AUD patients with fibrosis (left). Circulating monocytes from AUD patients upregulated MCP-1 already at baseline with an additional increase upon PGN stimulation compared with monocytes from healthy controls (right). (C) Increased iNOS mRNA expression in the liver of AUD subjects with steatohepatitis (n = 18) and fibrosis (n = 29) compared with steatosis (n = 3) (D). Representative immunofluorescence staining in liver biopsies of $CD68^+$ macrophages expressing iNOS and TREM1 activation markers. Arrows in the individual wavelength panels highlight $C68^+$ cells expressing concomitantly iNOS and TREM1 (E). Inverse correlations between number of circulating monocytes and liver CX3CL1 (left) and iNOS mRNA levels (right). Abbreviations: ALD, alcohol-associated liver disease; AUD, alcohol use disorder; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein 1.

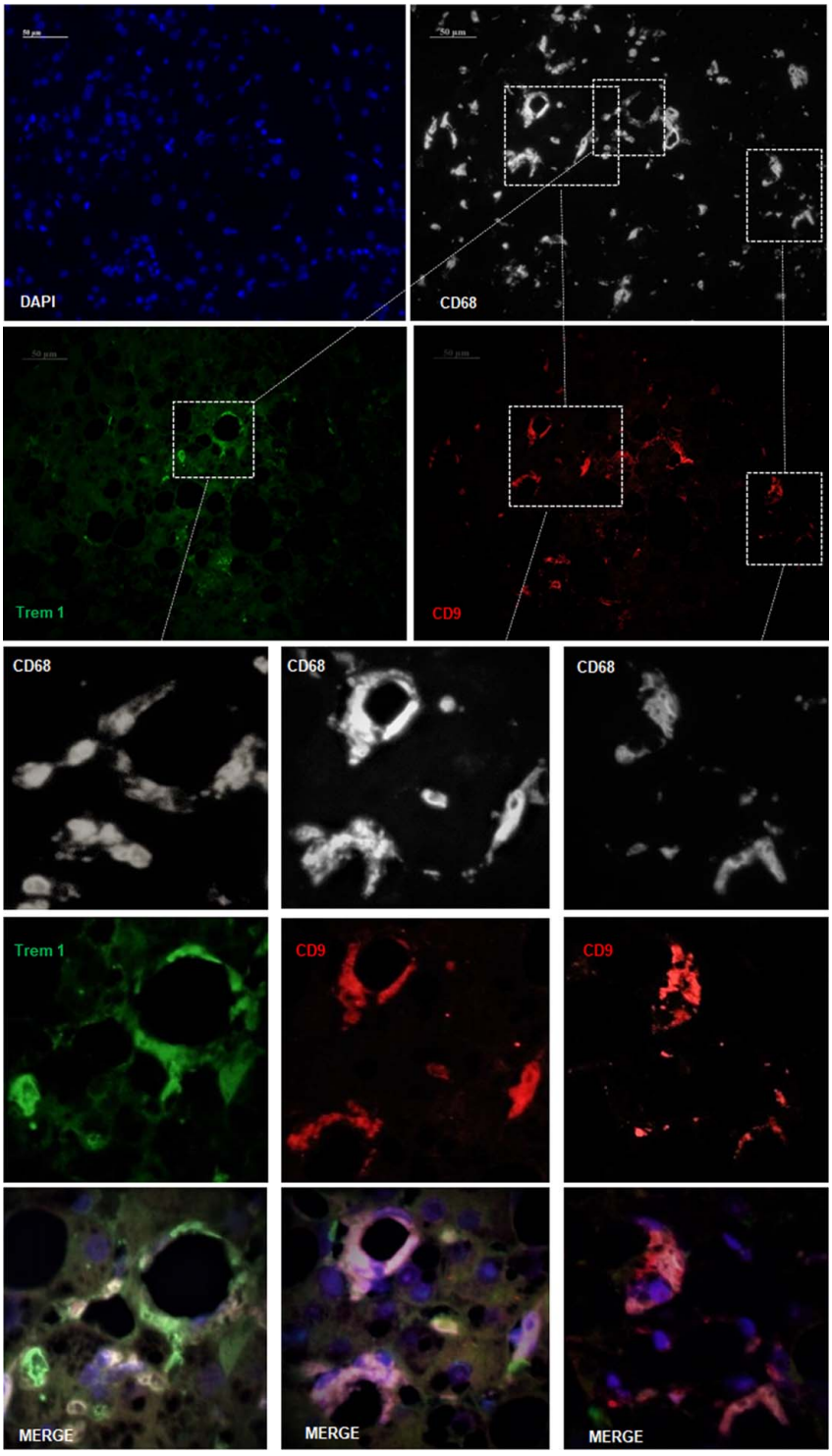

FIGURE 6 Representative immunofluorescence staining in liver biopsies of $CD68^+$ CD9 and TREM1. Boxes highlight at higher magnification (×40) $CD68^+$ macrophages coexpressing either TREM1 or CD9.

monocytes eventually leave the general circulation and infiltrate the liver.

CD9 is highly expressed on monocytes as well as on monocyte-derived macrophages involved in tissue scaring,[25,26] Activated monocytes, especially those secreting IL-8 and MCP-1, do express triggering receptor expressed on myeloid cells 1 (TREM1) which is upregulated by bacterial products such as LPS.[27,28] Furthermore, TREM1 expressing monocyte-derived macrophages in the liver augment inflammation and injury.[29] We used those 2 markers to test the presence of monocyte-derived macrophages in ALD livers. Staining in liver biopsies from AUD patients showed the presence of 2 different subpopulations of $CD68^+$ macrophages: one

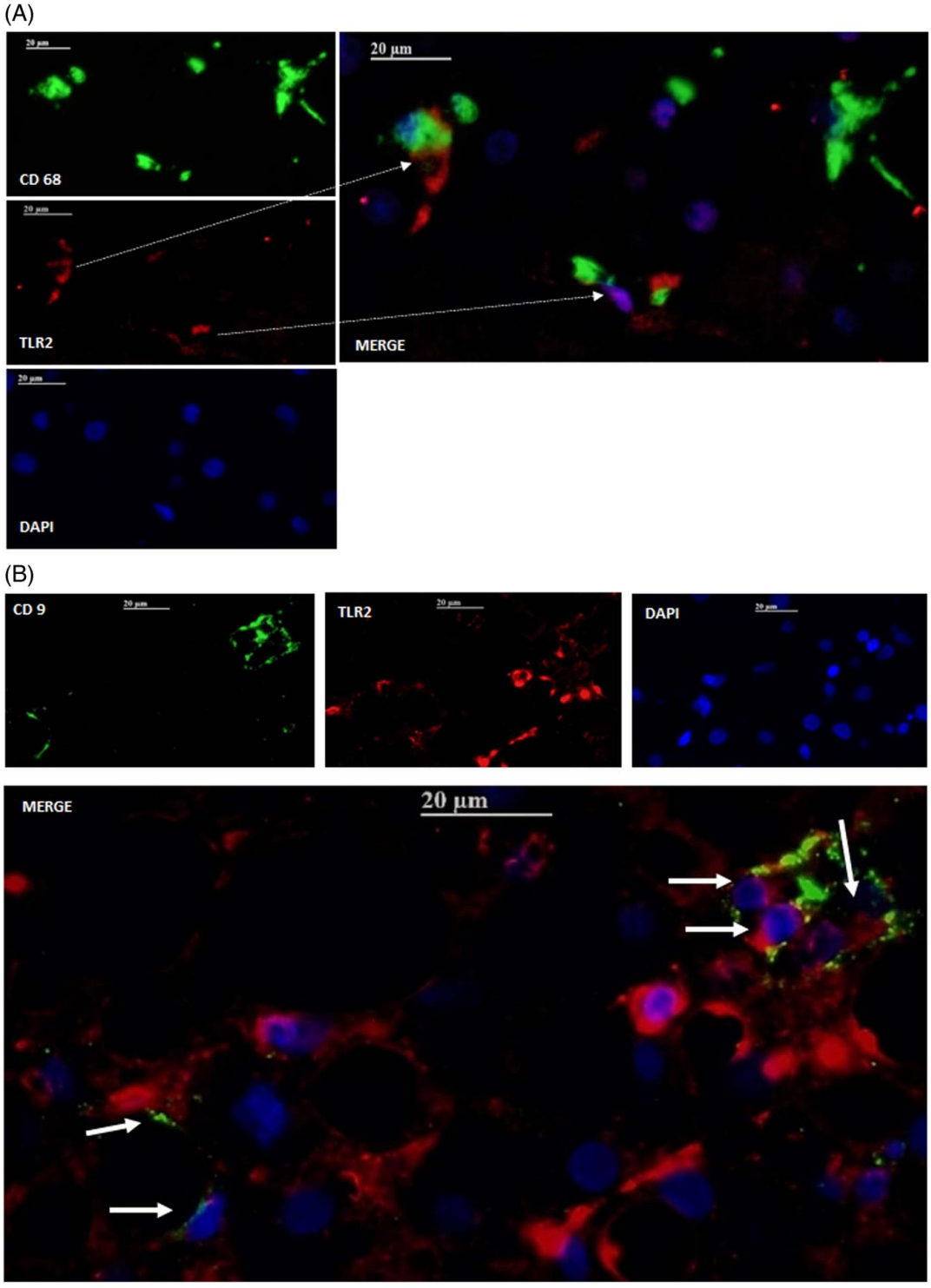

FIGURE 7 Representative immunofluorescence staining in liver biopsies showing CD68 and TLR2 (A). Arrows are indicating cells coexpressing C68 and TLR2. (B) CD9 and TLR2. Arrows indicate clusters of cells coexpressing CD9 and TLR2$^+$.

expressing the activation marker TREM1 and one expressing CD9 (Figure 6), a marker of activated monocytes primed to infiltration into the tissues.[30] These results indicate infiltration of the liver with different subpopulations of macrophages possibly coming from the circulation. Finally, we assessed whether macrophages express TLR2 in the liver. We found CD68$^+$ macrophages expressing TLR2 (Figure 7A). Among those macrophages, we observed clusters of cells coexpressing both CD9 and TLR2 (Figure 7B), indicating the presence of TLR2$^+$ monocyte-derived macrophages in ALD livers. Overall, these observations suggest that the hepatic recruitment of monocytes and transformation into proinflammatory macrophages could contribute to ALD.

DISCUSSION

Our study demonstrates a higher activation of the TLR2/inflammasome/AP-1 pathway in response to PGN in circulating monocytes that release key inflammatory mediators such as IL-1β and IL-8 contributing to systemic inflammation. Furthermore, those activated monocytes retain the capacity to infiltrate the liver and transform into monocyte-derived macrophages that likely contribute to an inflammatory response already at early stages of ALD which might ultimately lead to the progression of ALD.

We show an increased number of circulating monocytes in AUD patients compared with healthy subjects. Under pathological conditions, it has been proposed that bone marrow stem cells could produce myeloid cells to counteract the increased translocation of microbial products and other inflammatory stimuli.[31] The increased production of monocytes may be caused by different cytokines and growth factors as well as by alcohol-associated endotoxemia. Activation of pattern-recognition receptors, like TLRs, in myeloid progenitors by bacterial antigens could induce signaling pathways involved in cell proliferation, differentiation, and migration to enhance the immune response against different insults.[32] We found higher activation of TLR2 in response to PGN in monocytes and monocyte-derived macrophages linked to enhanced secretion of IL-1β and IL-8. This cascade might also be activated in progenitor cells and then lead to enhanced production of monocytes with their subsequent release into the blood circulation. Classical monocytes are the first subset released into the blood circulation from the bone marrow and a small proportion of them further mature into intermediate monocytes, which finally convert to nonclassical monocytes before homing into the tissues.[33] We found a reduced proportion of classical monocytes accompanied by an increased proportion of both intermediate and nonclassical monocytes in actively drinking AUD patients. These results suggest an increased transition of classical to intermediate and nonclassical monocytes. The expansion of intermediate and/or nonclassical subsets has been well described in many different types of infectious and inflammatory diseases where they were associated with an increased release of proinflammatory cytokines.[34] The mechanisms of this process are still not known but appear to be related to microbial stimuli and/or inflammatory conditions, which characterize also subjects with an alcohol use disorder.

Serum surrogate markers of microbial translocation such as PGRPs and sCD14 were elevated in AUD patients. Levels of Gram-positive translocation marker PGRPs were correlated with the proportion of intermediate monocytes. Those monocytes express high levels of MHC class II processing and presentation genes,[35] this may reflect a link between the translocation of bacterial products such as PGN, recognition, and presentation of antigens to T cells with subsequent activation of adaptive immune responses. Phagocytosis represents a defense mechanism used by innate cells like monocytes to deal with bacterial translocation.[36] Interestingly, bacterial phagocytic capacity was maintained in culture in monocytes from AUD patients compared with monocytes from healthy controls, indicating no defects in bacterial phagocytosis in monocytes at early ALD stages. Chronic alcohol consumption is also associated with gut fungal overgrowth with overrepresentation of *C. albicans*.[4] The human intestine can become the source for systemic fungal products or fungal infection when the gut barrier is disrupted.[37] We here revealed an intriguing downregulation of CARD9, an intracellular sensor playing a pivotal role in fungal immune surveillance. In accordance with this result, stimulation of monocytes with *C. albicans* showed a reduced transcription of inflammatory mediators IL-1β and TNFα in monocytes from AUD patients compared with controls. Looking at IL-1β and TNFα mRNA after 24 hours might not always be optimal. Therefore, we cannot formally exclude that differences may exist at earlier time points.

During the different stages of alcohol-associated liver disease, monocytes influx the liver where they transform into monocyte-derived macrophages that are likely contributing to the ALD, as shown in animal models of chronic alcohol exposure.[10] We found that expression of hepatic inflammatory mediators, such as the proinflammatory cytokine IL-1β and especially the chemokine IL-8, increased progressively in more severe forms of liver disease as compared with simple fatty liver. Moreover, both factors strongly correlated with markers of hepatocyte damage and apoptosis, pointing to their contribution to the early development of ALD in humans. Recruitment of monocytes appears to play an important role, since both chemokines MCP-1 and CX3CL1 correlated with IL-1β and IL-8 and were associated with a more advanced disease state such as the presence of significant fibrosis. Markers of macrophage activation (eg, iNOS and CD163) increased in livers with steatohepatitis and fibrosis compared with simple hepatic steatosis, further supporting the concept of the pivotal role of macrophage activation in ALD. Increased release of IL-1β and IL-8 by circulating monocytes was triggered by TLR2 in response to PGN. The limited material available precluded us from testing the functional role of TLR2 in liver monocyte-derived macrophages, especially in those expressing CD9 and TREM1. It is possible that those cells share similar mechanisms and this aspect clearly deserves future investigations It has been believed for many years that activation of TLR4 signaling is crucial in the pathogenesis of alcohol-induced liver injury.[38] Deficiency in TLR2 had no

protective effect on alcohol-induced liver injury in a chronic ethanol-feeding mouse model.[39] However, differences in the characteristics of murine and human circulating monocytes/tissue macrophages exist and this may influence the pathophysiological divergences of ALD present in mice and humans.

Here, we investigated the potential contribution of monocyte-derived macrophages in ALD. However, liver macrophages are a highly heterogenous population composed by monocyte-derived and resident macrophages (eg, KCs).[40] Both cell types exhibit tremendous plasticity, adapting their function in response to tissue-specific signals within their microenvironment.[41] In disease states where resident macrophages are depleted or reduced by injury, infiltrating monocytes can transform into macrophages with similar characteristics of KCs.[42] We identified 2 subpopulations of macrophages expressing either TREM1 or CD9. Especially CD9 is not expected to be expressed by liver resident macrophages.[43] Therefore, it is conceivable that these $CD9^+$ damage-associated macrophages are derived from circulating monocytes. However, using only these markers to identify activated monocyte-derived macrophages might be too restrictive considering the high heterogeneity of liver macrophages. Future studies combining flow cytometry analysis with single-cell approaches are needed to reveal specific inflammatory subsets contributing to ALD pathology.

A limitation of this study is that data are in part descriptive and/or based on correlations, which does not prove a formal cause-effect relationship. Furthermore, since suspicion of significant liver disease was a prerequisite for liver biopsy, more severe stages might be overrepresented in the histology cohort. Given the limited amount of material available, several analyses have only been performed on the gene expression level, which is an additional limitation of our study. Posttranscriptional activation controls especially activation of transcription factors such as NF-κB and AP-1. Therefore, future studies would be required to confirm posttranscriptional changes related to the TLR2-AP-1 axis in monocytes from AUD patients. However, we have previously demonstrated in PBMCs that the changes at the gene level also correspond to changes in protein expression in a similar cohort of AUD patients.[5] Since monocytes belong to PBMCs, it is plausible to assume that those protein changes are also found in the monocyte fraction.

Given the difficulties of accessing tissue from an early disease stage in humans, we believe our investigation contributes to a better comprehension of the role of circulating monocytes in the pathogenesis of ALD. The main strength of our study is the unique human cohort of a high number of heavily, actively drinking AUD subjects undergoing elective alcohol withdrawal in whom liver biopsies and blood collections have been performed in a strict, highly standardized clinical program.

In summary, the current study highlights the contribution of TLR2 activation in monocytes and monocyte-derived macrophages in the development of systemic inflammation and potentially ALD. A deeper understanding of the connections between this pathway and other inflammatory mediators involved in human ALD would provide us with the identification of innovative therapeutic targets in early ALD stages.

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CONFLICT OF INTEREST

Bernd Schnabl has been consulting for Ferring Research Institute, HOST Therabiomics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals, and Takeda. Bernd Schnabl's institution UC San Diego has received research support from Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals, Prodigy Biotech, and Synlogic Operating Company. Bernd Schnabl is the founder of Nterica Bio. UC San Diego has filed several patents with Bernd Schnabl as an inventor related to this work. Peter Stärkel received grant support from Gilead Sciences Belgium. The remaining authors have no conflicts to report.

ORCID

Luca Maccioni https://orcid.org/0000–0003–3727–0664
Sophie Leclercq https://orcid.org/0000–0002–2894–5220
Géraldine Laloux https://orcid.org/0000–0003–3230–1679
Yves Horsmans https://orcid.org/0000–0002–5779–2454
Isabelle Leclercq https://orcid.org/0000–0001–7934–6693

Bernd Schnabl https://orcid.org/0000–0002–6281–825X
Peter Stärkel https://orcid.org/0000–0003–2938–4442

REFERENCES

1. Lackner C, Stauber RE, Davies S, Denk H, Dienes HP, Gnemmi V, et al. Development and prognostic relevance of a histologic grading and staging system for alcohol-related liver disease. J Hepatol. 2021;75:810–9.
2. Hammer JH, Parent MC, Spiker DA, World Health Organization. Global Status Report on Alcohol and Health, Vol 65; 2018.
3. Maccioni L, Gao B, Leclercq S, Pirlot B, Horsmans Y, De Timary P, et al. Intestinal permeability, microbial translocation, changes in duodenal and fecal microbiota, and their associations with alcoholic liver disease progression in humans. Gut Microbes. 2020;12:1782157.
4. Yang AM, Inamine T, Hochrath K, Chen P, Wang L, Llorente C, et al. Intestinal fungi contribute to development of alcoholic liver disease. J Clin Invest. 2017;127:2829–41.
5. Leclercq S, De Saeger C, Delzenne N, De Timary P, Stärkel P. Role of inflammatory pathways, blood mononuclear cells, and gut-derived bacterial products in alcohol dependenc. Biol Psychiatry. 2014;76:725–33.
6. Leclercq S, Cani PD, Neyrinck AM, Stärkel P, Jamar F, Mikolajczak M, et al. Role of intestinal permeability and inflammation in the biological and behavioral control of alcohol-dependent subjects. Brain Behav Immun. 2012;26:911–8.
7. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol. 2014;5(Oct):1–12.
8. Gao B, Ahmad MF, Nagy LE, Tsukamoto H. Inflammatory pathways in alcoholic steatohepatitis. J Hepatol. 2019;70:249–59.
9. Tacke F. Targeting hepatic macrophages to treat liver diseases. J Hepatol. 2017;66:1300–12.
10. Wang M, You Q, Lor K, Chen F, Gao B, Ju C. Chronic alcohol ingestion modulates hepatic macrophage populations and functions in mice. J Leukoc Biol. 2014;96:657–65.
11. Gao B, Xu MJ, Bertola A, Wang H, Zhou Z, Liangpunsakul S. Animal models of alcoholic liver disease: pathogenesis and clinical relevance. Gene Expr. 2017;17:173–86.
12. Ju C, Mandrekar P. Macrophages and alcohol-related liver inflammation. Alcohol Res Curr Rev. 2015;37:251–62.
13. Suraweera DB. Alcoholic hepatitis: the pivotal role of Kupffer cells. World J Gastrointest Pathophysiol. 2015;6:90.
14. Zheng M, Karki R, Williams EP, Yang D, Fitzpatrick E, Vogel P, et al. TLR2 senses the SARS-CoV-2 envelope protein to produce inflammatory cytokines. Nat Immunol. 2021;22:829–38.
15. Boero E, Brinkman I, Juliet T, van Yperen E, van Strijp JAG, Rooijakkers SHM, et al. Use of flow cytometry to evaluate phagocytosis of staphylococcus aureus by human neutrophils. Front Immunol. 2021;12(February):1–15.
16. Chinenov Y, Kerppola TK. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. Oncogene. 2001;20:2438–52.
17. Kelley N, Jeltema D, Duan Y, He Y. The NLRP3 inflammasome: an overview of mechanisms of activation and regulation. Int J Mol Sci. 2019;20:1–24.
18. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med. 2006;12:1365–71.
19. Royet J, Gupta D, Dziarski R. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. Nat Rev Immunol. 2011;11:837–51.
20. Maccioni L, Leclercq IA, Schnabl B, Stärkel P. Host factors in dysregulation of the gut barrier function during alcohol-associated liver disease. Int J Mol Sci. 2021;22:12687.
21. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140:805–20.
22. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020;20:375–88.
23. Chu H, Duan Y, Lang S, Jiang L, Wang Y, Llorente C, et al. The *Candida albicans* exotoxin candidalysin promotes alcohol-associated liver disease. J Hepatol. 2020;72:391–400.
24. Drummond RA, Franco LM, Lionakis MS. Human CARD9: a critical molecule of fungal immune surveillance. Front Immunol. 2018;9(Aug):1–7.
25. Ramachandran P, Dobie R, Wilson-Kanamori JR, et al. Resolving the fibrotic niche of human liver cirrhosis at single-cell level. Nature. 2019;575:512–8.
26. Tohami T, Drucker L, Radnay J, Shapira H, Lishner M. Expression of tetraspanins in peripheral blood leukocytes: a comparison between normal and infectious conditions. Tissue Antigens. 2004;64:235–42.
27. Carrasco K, Boufenzer A, Jolly L, Le Cordier H, Wang G, Heck AJ, et al. TREM-1 multimerization is essential for its activation on monocytes and neutrophils. Cell Mol Immunol. 2019;16:460–72.
28. Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. J Immunol. 2000;164:4991–5.
29. Sun H, Feng J, Tang L. Function of TREM1 and TREM2 in liver-related diseases. Cells. 2020;9:1–17.
30. Saviano A, Henderson NC, Baumert TF. Single-cell genomics and spatial transcriptomics: discovery of novel cell states and cellular interactions in liver physiology and disease biology. J Hepatol. 2020;73:1219–30.
31. Takizawa H, Boettcher S, Manz MG. Demand-adapted regulation of early hematopoiesis in infection and inflammation. Blood. 2012;119:2991–3002.
32. Takeda K, Akira S. Toll-like receptors. Curr Protoc Immunol. 2015;109(April):14.12.1–10.
33. Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, et al. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. J Exp Med. 2017;214:1913–23.
34. Wong KL, Yeap WH, Tai JJY, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. Immunol Res. 2012;53:41–57.
35. Wong KL, Tai JJY, Wong WC, Han H, Sem X, Yeap WH, et al. Gene expression profiling reveals the defining features of the classical, intermediate,and nonclassical human monocyte subsets. Blood. 2011;118:e16–31.
36. Sivaloganathan DM, Brynildsen MP. Phagosome-bacteria interactions from the bottom up. Annu Rev Chem Biomol Eng. 2021;12:309–31.
37. Lang S, Duan Y, Liu J, Torralba MG, Kuelbs C, Ventura-Cots M, et al. Intestinal fungal dysbiosis and systemic immune response to fungi in patients with alcoholic hepatitis. Hepatology. 2020;71:522–38.
38. Roh YS, Seki E. Toll-like receptors in alcoholic liver disease, non-alcoholic steatohepatitis and carcinogenesis. J Gastroenterol Hepatol. 2013;28(suppl 1):38–42.
39. Hritz I, Mandrekar P, Velayudham A, Catalano D, Dolganiuc A, Kodys K, et al. The critical role of toll-like receptor (TLR) 4 in

alcoholic liver disease is independent of the common TLR adapter MyD88. Hepatology. 2008;48:1224–31.
40. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity. 2013;38:79–91.
41. Bonnardel J, T'Jonck W, Gaublomme D, Browaeys R, Scott CL, Martens L, et al. Stellate cells, hepatocytes, and endothelial cells imprint the Kupffer cell identity on monocytes colonizing the liver macrophage niche. Immunity. 2019;51:638–654.e9.
42. Guilliams M, Mildner A, Yona S. Developmental and functional heterogeneity of monocytes. Immunity. 2018;49:595–613.
43. Zwicker C, Bujko A, Scott CL. Hepatic macrophage responses in inflammation, a function of plasticity, heterogeneity or both? Front Immunol. 2021;12(June):1–15.

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