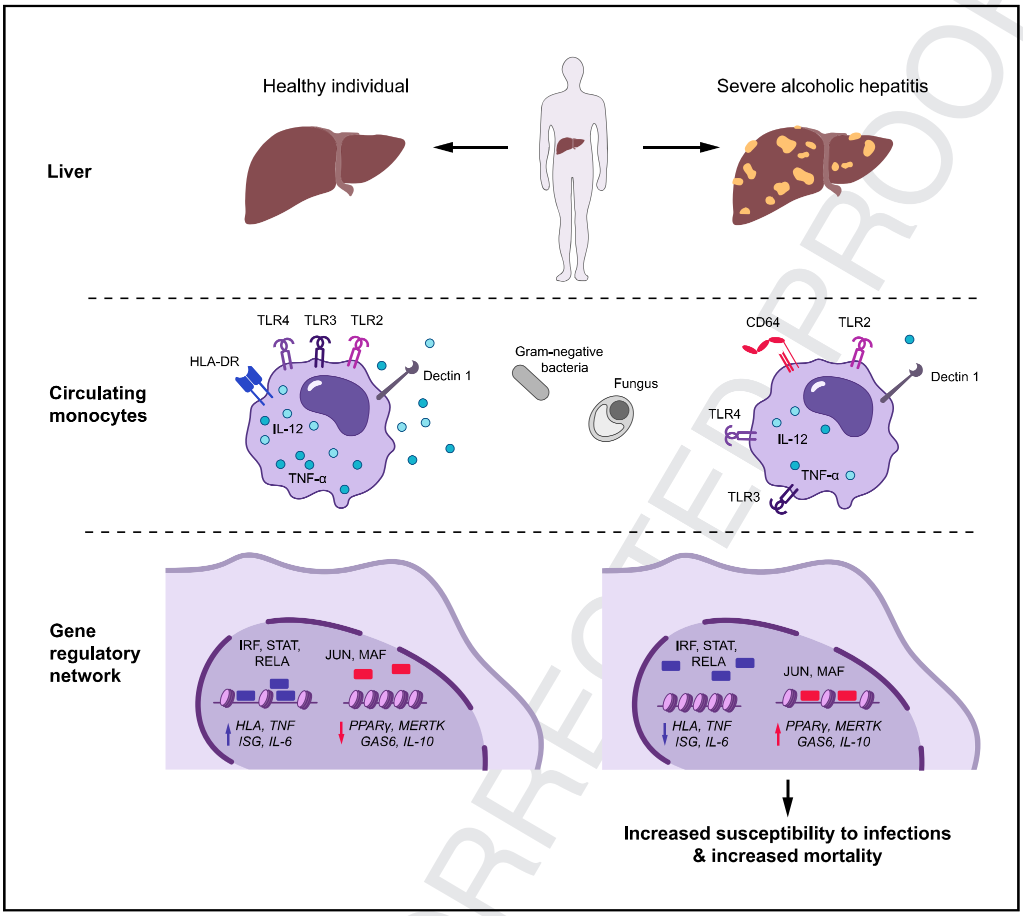
Research Article
Cirrhosis and Liver Failure

JOURNAL OF HEPATOLOGY

Epigenetic basis for monocyte dysfunction in patients with severe alcoholic hepatitis

Graphical abstract



Authors

Laura Weichselbaum, Abdulkader Azouz, Kinga K. Smolen, …, Frederick Libert, Thierry Gustot, Stanislas Goriely

Correspondence

thierry.gustot@erasme.ulb.ac.be (T. Gustot), stgoriel@ulb.ac.be (S. Goriely).

Lay summary

Patients with severe alcoholic hepatitis are at increased risk of infections, which contribute to the poor prognosis associated with the disease. Herein, we show that epigenetic determinants underly the immune cell dysfunction and inappropriate responses to pathogens that are associated with severe alcoholic hepatitis.

Highlights

- Monocytes and dendritic cells are impaired in severe alcoholic hepatitis.
- This immune dysfunction is associated with higher risk of infection and mortality.
- The presence of ACLF does not enhance monocyte and dendritic alterations.
- The altered transcriptomic program of monocytes has strong epigenetic determinants.

https://doi.org/10.1016/j.jhep.2020.02.017
 J. Hepatol. 2020, ■, 1–12

FLA 5.6.0 DTD ■ JHEPAT7631_grabs ■ 17 April 2020 ■ 9:33 pm ■ ce

Research Article
Cirrhosis and Liver Failure

JOURNAL OF HEPATOLOGY

Epigenetic basis for monocyte dysfunction in patients with severe alcoholic hepatitis Q1

Q9 Laura Weichselbaum[1,6], Abdulkader Azouz[1,†], Kinga K. Smolen[1,†], Jishnu Das[2], Marion Splittgerber[1], Antonia Lepida[3], Christophe Moreno[3,6], Jonas Schreiber[3], Thomas Sersté[3,4], Eric Trepo[3,6], Frederick Libert[5], Thierry Gustot[3,6,7,*,‡], Stanislas Goriely[1,*,‡]

[1]Université Libre de Bruxelles, Institute for Medical Immunology (IMI), Gosselies, Belgium; [2]Ragon Institute of MGH, MIT and Harvard; Department of Biological Engineering, MIT; [3]Department of Gastroenterology and Hepato-Pancreatology, C.U.B. Erasme Hospital, Brussels, Belgium; [4]Department of Hepato-gastroenterology, C.U.B. Saint Pierre, Brussels, Belgium; [5]Université Libre de Bruxelles, BRIGHTcore ULB-VUB and Institute of Interdisciplinary Research in Human and Molecular Biology (IRIBHM), Brussels, Belgium; [6]Université Libre de Bruxelles, Laboratory of Experimental Gastroenterology; [7]Inserm Unité 1149, Centre de Recherche sur l'inflammation (CRI), Paris, France

Background & Aims: Severe forms of alcohol-related liver disease are associated with increased susceptibility to infections which are associated with poor prognosis. The cellular and molecular mechanisms responsible for this altered host defense are incompletely understood.

Methods: We performed whole blood phenotypic analysis and *ex vivo* stimulation with various pathogen-associated molecular patterns (PAMPs). We included 34 patients with alcohol-related cirrhosis (18 of whom had biopsy-proven severe alcoholic hepatitis [sAH]), 12 healthy controls and 11 patients with chronic alcohol consumption without significant liver disease. We also evaluated the transcriptomic (RNA-seq) and chromatin accessibility (ATAC-seq) profiles of CD14$^+$ monocytes from a subset of patients.

Results: Circulating monocytes and conventional dendritic cells (DCs) from patients with sAH displayed complex alterations characterized by increased expression of both activating and inhibitory surface markers and an impaired pro-inflammatory response upon stimulation with PAMPs representative of gram-negative bacteria (lipopolysaccharide, Pam3CSK4) or fungal pathogens (Zymosan). Their decreased ability to produce more than 1 cytokine (polyfunctionality) upon PAMP stimulation correlated with the risk of developing infection at 28 days or mortality at 90 days. The presence of acute-on-chronic liver failure in patients with sAH did not significantly modify the immune profile of monocytes and DCs. Moreover, CD14$^+$ monocytes of patients with sAH displayed altered transcriptional and epigenomic profiles characterized by downregulation of key innate immune and metabolic pathways and upregulation of important immunomodulatory factors.

Conclusions: In patients with sAH, the altered transcriptional program and functional properties of monocytes that contribute to patients' susceptibility to infection have strong epigenetic determinants.

Lay summary: Patients with severe alcoholic hepatitis are at Q3 increased risk of infections, which contribute to the poor prognosis associated with the disease. Herein, we show that epigenetic determinants underly the immune cell dysfunction and inappropriate responses to pathogens that are associated with severe alcoholic hepatitis.

Introduction

Increased susceptibility to infections is a hallmark of severe forms of alcohol-related liver disease (ALD), like severe alcoholic hepatitis (sAH) and acute-on-chronic liver failure (ACLF), and majorly contributes to their poor prognosis.[1] This susceptibility is not limited to bacterial infections but also observed for viral and fungal infections.[2,3] The main clinical challenge remains their early diagnosis and prompt/adequate management.

The causative mechanisms of this susceptibility remain elusive. sAH and/or alcohol-related ACLF is associated with an immune dysfunction characterized by the coexistence of systemic inflammation[4] and impaired response of immune cells to pathogens and their products.[5] Several studies demonstrated a reduced pro-inflammatory cytokine production by lipopolysaccharide (LPS)-stimulated monocytes and a decrease in their capacities to kill microbes. The mechanisms responsible for this monocyte dysfunction are supposed to be multifactorial: reduced expression of HLA-DR,[6] increased expression of MER tyrosine kinase (MERTK),[7] increased exposure to prostaglandin-E2[8] and alterations in monocyte glutamine metabolism.[9] The function of other mononuclear phagocytes, namely conventional and plasmacytoid dendritic cells (DCs), that play a major role in the orchestration of immune responses, remains unexplored in ALD.

Recently, it has been described that myeloid cells from the innate immune system can be trained through epigenetic and metabolic programming by diverse mediators resulting in

Keywords: Alcoholic liver disease; Alcoholic hepatitis; Acute-on-chronic liver failure; Immune dysfunction; Infection; Epigenetic.
Received 9 August 2019; received in revised form 14 February 2020; accepted 17 February 2020; available online xxx

* Corresponding authors. Addresses: Liver Transplant Unit, Department Gastroenterology and Hepato-Pancreatology, C.U.B. Erasme Hospital, Brussels, Belgium; Laboratory of Experimental Gastroenterology, Université Libre de Bruxelles, Brussels, Belgium (T. Gustot), or Institute for Medical Immunology, 8 rue Adrienne Bolland, B-6041, Charleroi-Gosselies, Belgium. Tel: 32-2-650-9586; Fax: 32-2-650-9563 (S. Goriely).
E-mail addresses: thierry.gustot@erasme.ulb.ac.be (T. Gustot), stgoriel@ulb.ac.be (S. Goriely).
† These authors contributed equally to this work.
‡ These authors jointly supervised this work.

https://doi.org/10.1016/j.jhep.2020.02.017

hyper- or hypo-responsiveness upon re-stimulation.[10] In sepsis, the current paradigm suggests that pathogen-associated molecular patterns (PAMPs) induce innate immune cell tolerance through epigenetic silencing of several host defense genes, thus contributing to the higher risk of secondary infections.[11]

In this study, we explored phenotypic profiles of the main subsets of the mononuclear phagocyte system in different stages of ALD. We also assessed their responses to diverse PAMPs and their ability to produce pro-inflammatory cytokines. We compared these immunological parameters with clinical outcomes (mortality and risk of infection). Finally, we studied transcriptional and chromatin accessibility profiles of CD14$^+$ monocytes.

Materials and methods

Patient recruitment

For this study, we prospectively recruited 34 patients with alcohol-related cirrhosis diagnosed based on liver biopsy or classical clinical, biochemical and radiological criteria, 11 chronic alcohol abusers without signs of liver fibrosis (Fibroscan <7 kPa and Fibrotest <0.3), and 12 healthy control participants at C.U.B. Erasme Hospital between May 2016 and October 2017. Patients with cirrhosis were divided into 2 groups based on histology and severity scores: alcohol-related cirrhosis alone or severe (modified discriminant function higher than 32[12]) biopsy-proven AH. The sAH group was further divided into patients with or without ACLF according to the EASL-CLIF definition[13] for subgroup analyses. The inclusion and exclusion criteria used are detailed in the Supplementary Methods.

Whole blood assays

Ex vivo phenotypic analysis, whole blood stimulation, cytokine measurements and preparation of the samples for flow cytometric analysis was performed as described previously[14] and is detailed in the Supplementary Methods and Supplementary CTAT Table.

RNA-Seq and ATAC-seq on CD14$^+$ monocytes

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences) and were cryopreserved. CD14$^+$ monocytes were isolated by FACS on a BD FACS Aria III (>90% purity) and processed for RNA isolation or ATAC-seq as previously described[15] and detailed in the Supplementary Methods.

For further details regarding the materials used, please refer to the CTAT Table and Supplementary Information.

Results

Characteristics of the enrolled individuals

Based on clinical, histological and biochemical parameters (Table S1), we defined 4 groups: healthy controls (HCs), chronic alcohol abusers (CAAs) without signs of hepatic disease, patients with alcohol-related cirrhosis and patients with biopsy-proven severe alcoholic hepatitis (sAH). Overall, most patients were male, while cirrhotic patients tended to be older than other groups. Seven of the 18 patients with sAH had ACLF according to the EASL-CLIF definition (Table S2).[13] Occurrence of infection, liver transplant or death was monitored for up to 90 days after recruitment (Table S1).

Skewed pattern of PAMP-elicited cytokine production in whole blood cells from patients with sAH

In order to gain further insight into the basic features of innate immune cells in ALD, we used a highly standardized and controlled protocol of *ex vivo* whole blood culture assay.[16] We assessed the production of cytokines in culture supernatants with medium alone or upon stimulation with representative PAMPs: bacterial LPS, a Toll-like receptor (TLR)4 ligand; Zymosan, a component of the cell wall from yeast that signals through TLR2 and Dectin-1 and R848, a synthetic ligand for TLR7/8. For almost all the conditions tested, no significant changes in cytokine levels in the supernatants were observed between HCs, CAAs and cirrhotic patients. In response to LPS (and to a lesser extent to Zymosan), we noted a general trend for reduced production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β and IL-12/23p40 in patients with sAH compared to the other groups (Fig. 1A). IL-10 levels in this group were also decreased compared to the cirrhotic group. However, this was not associated with global hypo-responsiveness in these patients since, in response to R848, production of these cytokines was maintained or even increased in the case of IL-6. Remarkably, we observed hyperproduction of IL-8 in patients with sAH compared to the other groups, irrespective of the stimulus. Conversely, induction of the interferon (IFN)-dependent chemokine CXCL10 was consistently decreased in these patients. Complementing this finding, levels of Th1 supporting cytokines IL-12p70 and IFNγ elicited by R848 were also drastically reduced in sAH samples. In stark contrast, production of R848-induced type I IFNs (mostly produced by plasmacytoid DCs) was comparable among the groups (Fig. 1B). These results indicate that innate immune circulating cells from patients with sAH exhibit a distinct profile of cytokine production characterized by a strongly compromised IL-12p70/IFNγ/CXCL10 axis. The capacity to produce TNFα, IL-6 and IL-1β is also reduced but in response to certain PAMPs only. In contrast, production of other cytokines such as IFNα was maintained or even increased in the case of IL-8.

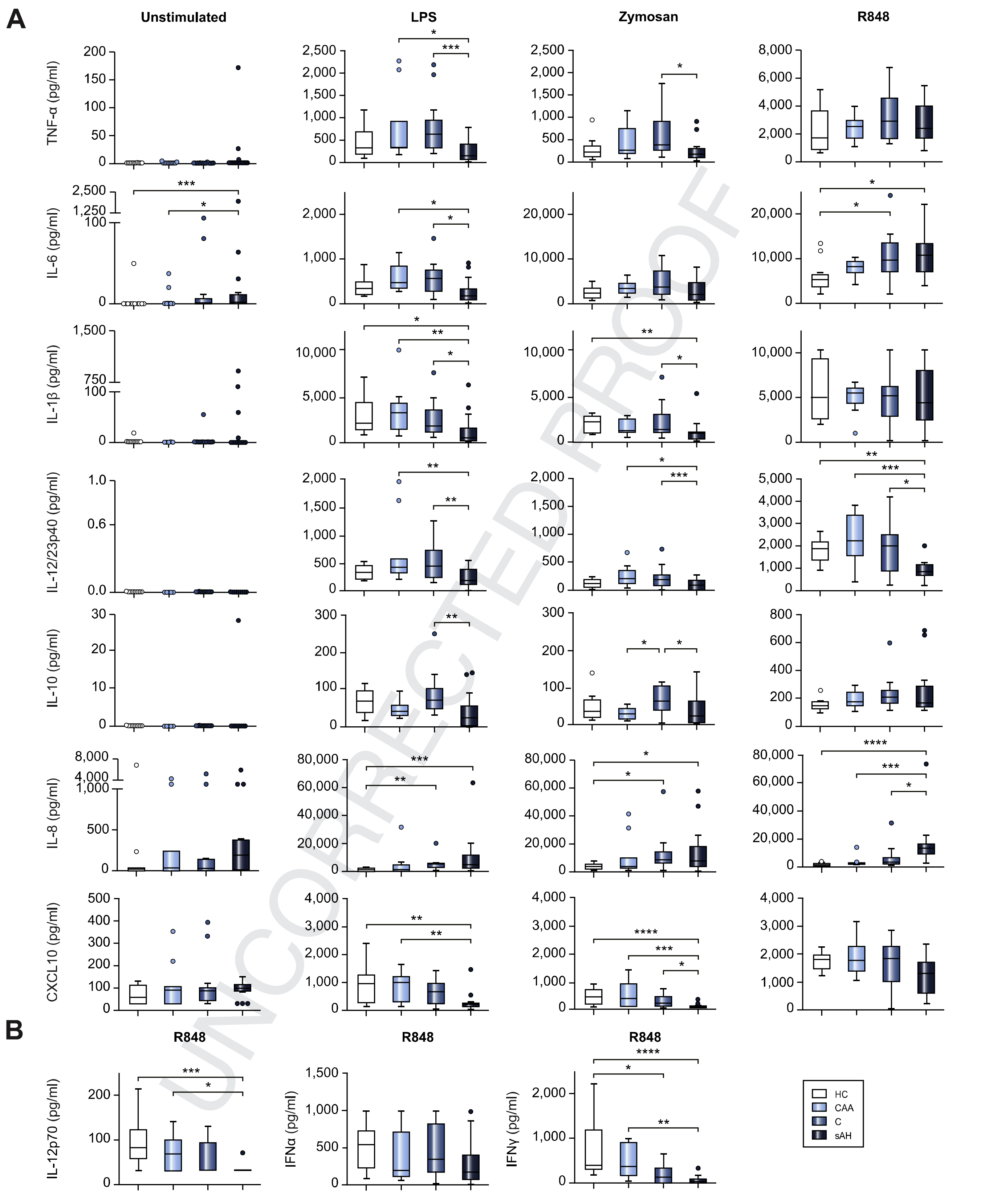
Increased severity of ALD is accompanied by alterations in the frequency and phenotype of circulating mononuclear phagocytes

In order to establish the cellular basis for altered pathogen-recognition receptor responsiveness in patients with sAH, we analyzed the main circulating immune cell populations by multicolor flow cytometry. We did not observe major changes in lymphocytes, natural killer cells or granulocytes although there was a trend toward a decrease in the absolute count of these cells with the severity of the disease, except for neutrophils (Fig. 2A). Next, we evaluated the 3 main subsets of monocytes based on CD14 and CD16 expression (Fig. 2B,C). Intermediate (CD14^{hi}CD16$^+$) and to a lesser extent classical (CD14^{hi}CD16$^-$) monocytes were increased in patients with sAH compared to CAAs. This was associated with a clear decrease in the non-classical (CD14^{lo}CD16$^+$) monocyte subset. In addition, we observed that patients with sAH displayed reduced counts of conventional DC (cDCs) and plasmacytoid DC (pDCs) subsets (Fig. 2B,C and Fig. S1A). These data indicate that increased severity of ALD is associated with important changes in the relative proportions of circulating mononuclear phagocytes.

To gain further insight into the activation status of these cells, we analyzed the expression of several markers at the surface of CD14$^+$ monocytes, cDCs and pDCs. As previously demonstrated in ACLF,[6,7] CD14$^+$ monocytes from patients with sAH displayed

FLA 5.6.0 DTD ■ JHEPAT7631_proof ■ 17 April 2020 ■ 9:31 pm ■ ce

JOURNAL OF HEPATOLOGY



print & web 4C/FPO

Fig. 1. Skewed pattern of PAMP-elicited cytokine production in whole blood cells from patients with sAH. (A) Dosage of cytokines and chemokines in the supernatant of whole blood culture after stimulation for 24 h in HCs (n = 12), CAAs (n = 11), patients with cirrhosis (n = 16) and those with sAH (n = 18). (B) Dosage of Th1-related cytokines after stimulation of whole blood with R848 for 24 h. Tukey box and whiskers. **p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001. Kruskal-Wallis test was performed to examine the statistical differences of each cytokine or chemokine per group, followed by Dunn's correction for multiple testing. CAAs, chronic alcohol abusers; HCs, healthy controls; PAMP, pathogen-associated molecular pattern; sAH, severe alcoholic hepatitis. Q2

FLA 5.6.0 DTD ■ JHEPAT7631_proof ■ 17 April 2020 ■ 9:31 pm ■ ce

Q7 Q8

Table 1. Clinical characteristics of patients.

	Healthy controls (n = 12)	Chronic alcohol abusers (n = 11)	Cirrhosis (n = 16)	Severe alcoholic hepatitis (n = 18)	*p* value
Age (years)	47.5 (41–52)	50 (39–62.5)	62 (59.5–65)	54.5 (43.25–61.75)	0.013
Sex, male (%)	7 (58)	8 (72)	13 (81)	11 (61)	0.508
Total bilirubin (mg/dl)	–	–	1.55 (1.1–2.9)	11.25 (7.3–18.4)	0.0001
INR	–	–	1.48 (1.2–1.6)	1.76 (1.56–1.98)	0.0025
Creatinine	–	–	0.85 (0.8–1.2)	0.95 (0.6–1.2)	0.71
MELD score	–	–	14.5 (11.3–17.3)	24 (22–33)	0.0001
Child-Pugh score	–	–	B8 (A6–B9)	C10 (C10–C11)	0.0016
HVPG (mmHg)	–	–	16 (13–18)	17 (14–20.5)	0.62
Norfloxacin	–	–	2 (12%)	0	
mDF	–	–	–	61 (48–70)	
ACLF (%)—grades (1,2,3)	–	–	–	7 (39%)–(3,3,1)	
CLIF-C ACLF score*	–	–	–	49 (45–50)	
Corticosteroids (28 days after recruitment)**	–	–	0	8 (44.4%)	
Infection (28 days)	–	–	3 (19%)	9 (50%)	0.057
Transplant-free mortality (28 days)	–	–	1 (6.7%)	3 (18.8%)	0.31
Infection (90 days)	–	–	5 (31.3%)	11 (61.1%)	0.08
Transplant-free mortality (90 days)	–	–	3 (20%)	6 (37.5%)	0.28
Liver transplant (90 days)	–	–	1 (6.3%)	2 (11.1%)	0.11

Data are presented as median (IQR) or number of patients (%).
ACLF, acute-on-chronic liver failure; HVPG, hepatic venous pressure gradient; INR, international normalized ratio; mDF, modified Maddrey's discriminant function; MELD, model for end-stage liver disease.
*Score only calculated for the subgroup of patients with sAH who have ACLF (n = 7).
**Corticosteroid use remains the same at 90 days.

reduced HLA-DR expression (Fig. 3), mainly driven by reduced expression on classical monocytes (data not shown). HLA-DR expression by pDCs was significantly reduced in ALD compared to HCs, even in CAA. Although there was a trend for decreased HLA-DR expression on cDCs, it did not reach statistical significance due to high heterogeneity. Steady-state expression of CD86, a costimulatory protein, and CD69, an early activation marker, was stable across the groups. In contrast, for the 3 cellular populations, we observed a decrease of the maturation marker CD83 in stable cirrhotic patients. In comparison to this latter group, expression of CD83 was significantly upregulated in patients with sAH. A similar profile was observed for the inhibitory protein, programmed death ligand 1 (or PD-L1). Finally, expression of the FcγRI receptor CD64 by monocytes, generally associated with systemic inflammation, was progressively enhanced with the severity of the disease (Fig. 3). Of note, the phenotype of monocytes from patients with sAH was not modified by the presence of ACLF with the exception of lower CD64 expression (Fig. S2A). Taken together, these data indicate complex and dynamic alterations of the proportion and phenotype of the different subsets of the mononuclear phagocyte system during ALD. Chronic alcohol abuse in itself induces slight changes in the phenotype of mononuclear phagocytes with increased expression of CD64 on monocytes and decreased absolute numbers and HLA-DR expression of pDCs. Cirrhotic patients display decreased expression of both CD83 and PD-L1 across all mononuclear phagocytes. Yet, the expression of these markers increases on the cells of patients with sAH. Thus, these patients have the unique feature of increased markers of both activation and inhibition compared to cirrhotic patients.

Global alteration of the capacity of monocytes of patients with sAH to produce cytokines

We identified distinctive patterns of whole blood cytokine production and changes in the frequency and phenotype of circulating mononuclear phagocytes, which led us to assess cytokine production at the single-cell level by flow cytometry in response to various stimuli in the same experimental settings.[16] We evaluated the expression of IL-6, IL-1β, TNF-α and IL-12/23p40 by CD14$^+$ monocytes in response to PAMPs. We observed that for LPS, Pam3CSK4 and Zymosan, the proportion of monocytes expressing each of these cytokines was consistently decreased in patients with sAH compared to the other groups. For R848, the proportions of IL-12/23p40$^+$ and to a lesser extent of TNF-α$^+$ and IL-6$^+$ cells were decreased but their capacity to produce IL-1β was maintained. For peptidoglycan (PGN), we did not observe any statistical differences between the groups (Fig. 4A). There were no major differences in the ability of monocytes to respond to PAMP stimulation between patients with sAH, with or without ACLF (Fig. S2B). Furthermore, cDCs had a similar response profile to monocytes (Fig. S4A). These experiments demonstrate that the ability of monocytes and cDCs to mount an appropriate pro-inflammatory response is severely impaired in patients with sAH upon stimulation with PAMPs associated with gram-negative bacteria (LPS, Pam3CSK4) or fungal pathogens (Zymosan). Yet, these cells are still able to either partially or globally respond to, respectively, endosomal (R848) or other bacterial (PGN via NOD2) PAMPs.

To visualize how all the different immune parameters globally vary across the clinical groups we used t-distributed stochastic neighbour embedding, an analytical method that compares biological samples without considering sample classification. This analysis shows an approximate 3-way separation between the CAAs/HCs (cluster A), cirrhotic patients (cluster B) and the sAH group (cluster C), thus showing that immunological features of patients with sAH are sufficient to distinguish them from other groups in an unsupervised manner (Fig. S3A). Next, using 2 different metrics, we identified the top 10 immunological parameters that can discriminate these 3 groups (Fig. S3B). They encompass predominantly the capacity of monocytes and cDCs to produce cytokines.

Patients with ALD and poor polyfunctionality are at higher risk of infection and death

Based on the impaired ability of circulating phagocytes to respond to stimulation by various PAMPs, we hypothesized that the ability of a single cell to produce more than 1 cytokine

FLA 5.6.0 DTD ■ JHEPAT7631_proof ■ 17 April 2020 ■ 9:31 pm ■ ce

JOURNAL OF HEPATOLOGY

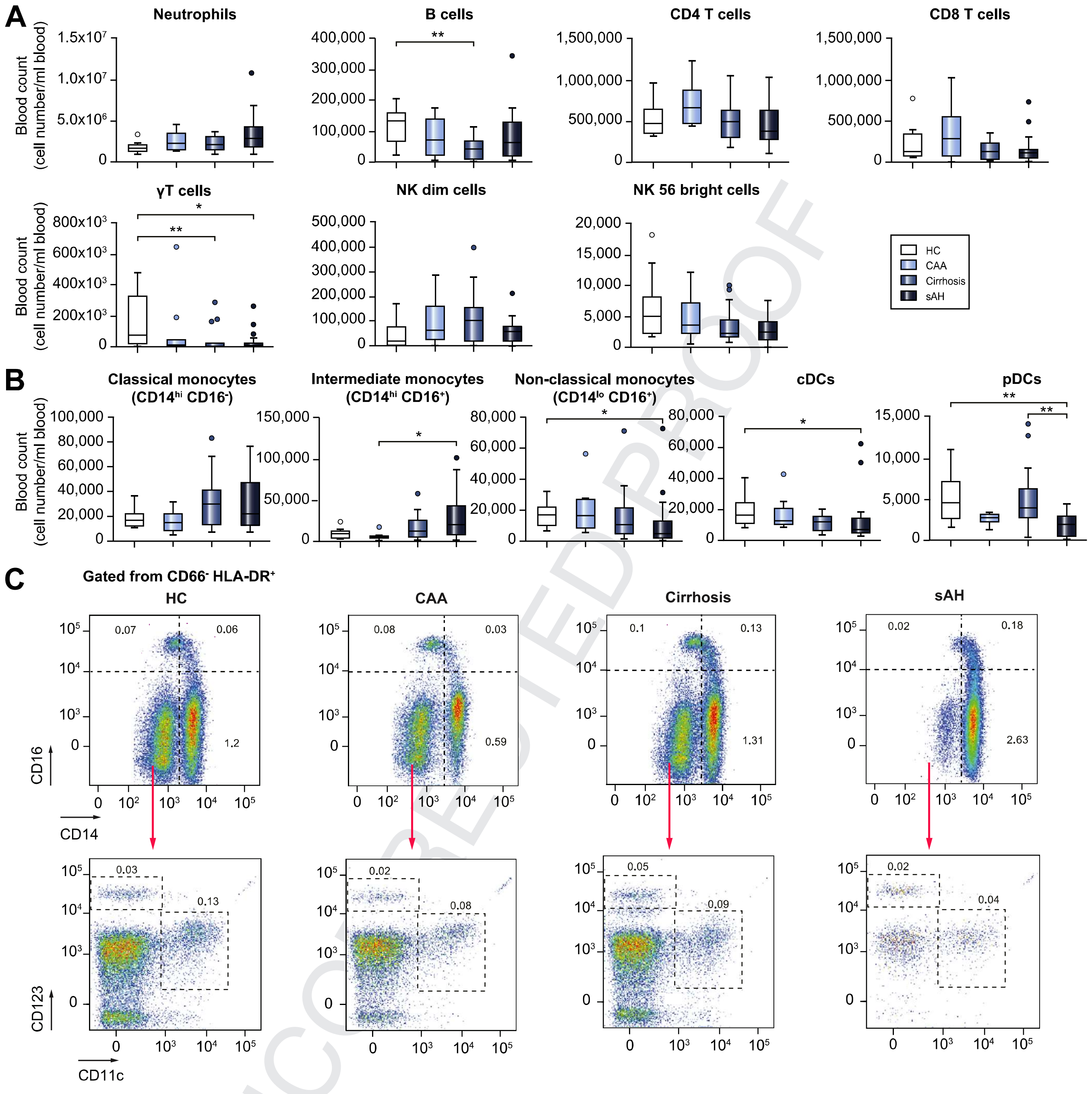
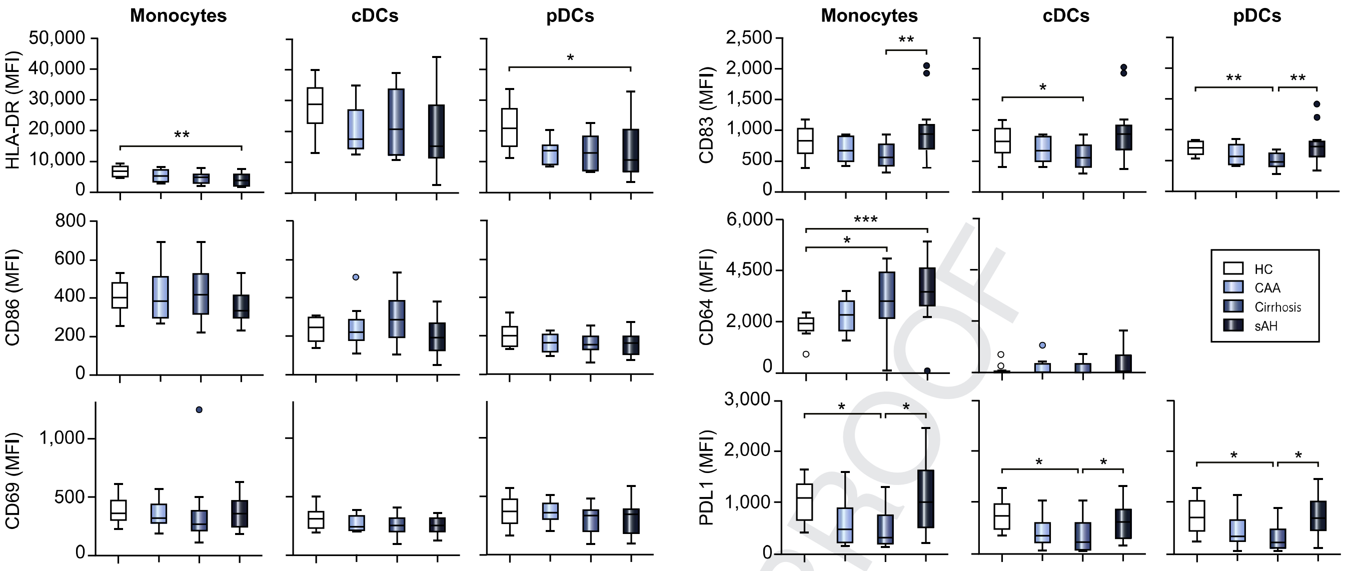


Fig. 2. Alterations of the frequency of circulating mononuclear phagocytes in advanced ALD. (A) Cellular composition of whole blood in HCs (n = 12), CAAs (n = 11), patients with cirrhosis (n = 16) and those with sAH (n = 18). (B) Cellular composition of monocytes and DCs in whole blood of HCs (n = 12), CAAs (n = 11), patients with cirrhosis (n = 16) and those with sAH (n = 18). (C) Gating strategy and representative plots of monocytes and DCs for each group. Tukey box and whiskers. **p* <0.05, ***p* <0.01. Kruskal-Wallis test was performed to examine the statistical differences of each cytokine or chemokine per group, followed by Dunn's correction for multiple testing. CAAs, chronic alcohol abusers; DCs, dendritic cells; HCs, healthy controls; NK, natural killer; PAMP, pathogen-associated molecular pattern; sAH, severe alcoholic hepatitis.

simultaneously (*i.e.* polyfunctionality) could represent an important parameter to define the immune status of patients with ALD. This approach revealed that monocytes from patients with sAH were deficient in their capacity to produce 3 or 4 cytokines (Fig. 4B). We also performed these analyses on cDCs and reached similar conclusion (Fig. S4B). Based on these data for our 34 cirrhotic patients, we used an unsupervised approach to define whether this polyfunctionality parameter was correlated with clinical outcomes. Using a 2-dimensional reduction, we identified a cluster of patients at a higher risk of infection (Fig. 4C) or mortality (Fig. 4F) within 28 or 90 days after sampling, respectively. We generated a radar plot of the most discriminatory variables for the infectious and mortality outcome. As shown in Fig. 4D,G, a low degree of

FLA 5.6.0 DTD ■ JHEPAT7631_proof ■ 17 April 2020 ■ 9:31 pm ■ ce



print & web 4C/FPO

Fig. 3. Alterations of the phenotype of circulating mononuclear phagocytes in advanced ALD. Median fluorescence intensity of surface markers on CD14+ monocytes, cDCs or pDCs in unstimulated whole blood of HCs (n = 12), CAAs (n = 11), patients with cirrhosis (n = 16) and those with sAH (n = 18). Tukey box and whiskers. **p* <0.05, ***p* <0.01, ****p* <0.001. Kruskal-Wallis test was performed to examine the statistical differences of each cytokine or chemokine per group, followed by Dunn's correction for multiple testing. ALD, alcohol-related liver disease; CAAs, chronic alcohol abusers; cDCs, conventional DCs; DCs, dendritic cells; HCs, healthy controls; pDCs, plasmacytoid DCs; sAH, severe alcoholic hepatitis.

polyfunctionality is significantly associated with a higher risk of infection in the next 28 days or death in the next 90 days. Of note, the infection rate in the follow-up was not associated with the administration of corticosteroids (Table S1) and the ability to predict the occurrence of an infection was better, although not significant, using polyfunctionality than the most discriminative immunological parameters (Fig. 4E). In conclusion, low polyfunctionality of monocytes and cDCs can be used as a predictor of higher risk of infection or mortality during follow-up in patients with ALD.

Monocytes from patients with sAH display an altered transcriptomic profile characterized by immunosuppressive features

To gain further insight into the molecular features of CD14+ monocytes in sAH, we performed global transcriptional profiling on a subset of patients. We observed a clear separation between samples from patients with sAH and HCs upon principal component analysis (PCA) (Fig. 5A) and identified statistically differentially expressed genes (610 up- and 111 downregulated genes compared to HCs, with a fold change >2 and a false discovery rate <0.05, Fig. 5B). Consistent with their altered phenotype and functional response, we observed decreased expression of genes related to key immune pathways such as innate immune responses, cytokines, response to IFNs and antigenic presentation in monocytes from patients with sAH (Fig. 5C,D). Gene set enrichment analysis (GSEA) also revealed dysregulated expression of genes involved in key metabolic processes, including lipid metabolism, cellular respiration and translation (Fig. 5C). This was accompanied by increased expression of genes involved in cell homeostasis and ion transport (Fig. 5E). Furthermore, we confirmed previous results showing increased expression of *MERTK*.[7] Its ligand *GAS6*, was also strongly upregulated in patients. High expression of *THBS1* (encoding Thrombospondin 1), a multifunctional extracellular matrix protein, could contribute to immune-suppression and liver fibrosis as it controls latent transforming growth factor-β activation.[17] Expression of several semaphorins (*SEMA6B, SEMA4C, SEMA3F*) was also elevated in patients. These proteins play multiple roles in the control of cell migration, inflammation and angiogenesis.[18] Furthermore, important intracellular immunomodulatory proteins were also upregulated such as *JAK3* and genes encoding the transcription factors *PPARG* and *MAF* (Fig. 5F). JAK3 was shown to dampen inflammatory cytokine production by human monocytes upon TLR stimulation.[19] Along the same line, treatment with a peroxisome proliferator-activated receptor (PPAR)-γ agonist reduces TLR-dependent stimulation of DCs[20] and c-Maf is an important immunoregulatory factor that promotes IL-10 expression by macrophages.[21]

Recently, Korf *et al.* also provided transcriptomic data for monocytes from patients with ACLF.[9] We observed that genes that were up- or downregulated in patients with ACLF in their study were enriched in monocytes from sAH or HC individuals, respectively (Fig. 5G). This indicates that a core molecular signature could be identified in independent cohorts. We also analysed publicly available gene sets from blood monocytes isolated during gram-negative sepsis or its resolution.[22] Inflammatory genes that were upregulated during sepsis tended to be downregulated in patients with sAH compared to HCs, indicating that during ALD monocytes do not display similarities with those in the acute phase of sepsis. However, genes that were modulated in the recovery phase (*i.e.* several weeks after sepsis) were significantly up- or downregulated in our patients, indicating potential common underlying mechanisms between these 2 clinical situations associated with heightened sensitivity to infections. Next, we evaluated the potential role of different circulating mediators in inducing the transcriptomic changes observed in our patients. Monocytes exposed to LPS display altered capacity to respond to rechallenge (the classical model of endotoxin tolerance).[23] A similar process occurs when cells are primed by TNFα.[24] We observed that genes that were downregulated 24 h after LPS or TNFα stimulation were significantly decreased in monocytes from individuals with sAH. However, genes induced in these conditions were not enriched in patients, thus showing that LPS or TNFα, alone, cannot recapitulate the transcriptomic signature observed in patients with sAH. No enrichment was observed for gene sets from β-glucan-trained monocytes; a situation that leads to increased responsiveness upon rechallenge.[23] IFNγ priming also leads to functional reprogramming of monocytes.[25] Here we observed that IFNγ-induced genes were significantly decreased in our patients. Immunosuppressive mediators such as ATP, PGE2 and IL-10 have a major impact on myeloid cell functional state.[26,27] Although PGE2 was shown to be increased in these patients,[26] the gene signature it induces in myeloid cells was not significantly modified in our dataset. We reached similar conclusions regarding ATP-responsive genes. Yet, genes that are up- or downregulated upon IL-10 treatment were significantly modulated in sAH samples.

Altogether, our data indicate that $CD14^+$ monocytes from patients with sAH display an altered profile with downregulation of key innate immune and metabolic pathways and upregulation of important immunomodulatory factors that could account for their altered responsiveness to PAMPs. This unique profile displays striking similarities with monocytes from patients that recovered from sepsis and is compatible with the effect of immunosuppressive factors such as IL-10.

Monocytes from patients with sAH display altered patterns of chromatin accessibility

The functional status of monocytes is highly plastic in response to environmental cues. In the context of endotoxin tolerance, trained immunity or cytokine priming, epigenetic reprogramming has emerged as a critical determinant.[23,24] To further determine whether similar underlying molecular processes were at play in the context of sAH, we analyzed the epigenomic landscapes of these monocytes by ATAC-seq. We observed extensive modifications in monocytes from patients with sAH (Fig. 6A). We focused on the 4,316 differentially accessible regions that allow us to segregate sAH *vs.* HC samples in PCA (Fig. 6B). We then scanned for binding motifs at the center of ATAC peaks located in these enhancer regions. Analysis of putative transcription factor site enrichment in sAH *vs.* HC-specific enhancers indicated a significant enrichment for distinct motifs in both groups. For example, there was a clear overrepresentation of AP1 (Jun/Fos), CEBP and MAF binding sites in the sAH group (Fig. 6C,D). In contrast, NF-κB, STAT or IRF motifs were preferentially identified in enhancer regions that were less accessible in this group (Fig. 6C,E). Regulatory regions that were more or less accessible were clearly associated with genes that were up or downregulated in monocytes from sAH, respectively (Fig. 6F,G). This observation strongly suggests that epigenetic imprinting is responsible for sAH transcriptional signature. This approach also allowed us to infer genes that are potentially directly regulated by these differentially active elements (Fig. 6H). For example, less accessible regions were found in the loci of HLA genes, while more accessible regions were associated with important immunoregulatory genes such as *MERTK*, *GAS6* or *PPARG.* (Fig. 6I).

Consistent with our transcriptomic data, we observed that regulatory regions that are less accessible in monocytes from patients with sAH were associated with genes involved in antigen presentation, innate immune response and cytokine secretion (Fig. 6J, Fig. S5A). Regions that were more accessible in this group were associated with genes that were found to be non-tolerizeable upon repeated exposure to LPS. This suggests that, as shown in the context of endotoxin tolerance, some LPS-responsive genes are epigenetically silenced while others are preserved or potentiated. Remarkably, these regulatory regions were also significantly associated with genes encoding components of alcohol, phospholipid and sterol metabolic pathways (Fig. 6K, Fig. S5B). Taken together, these data support the notion that the altered transcriptional program and functional properties of monocytes in this pathological context have strong epigenetic determinants.

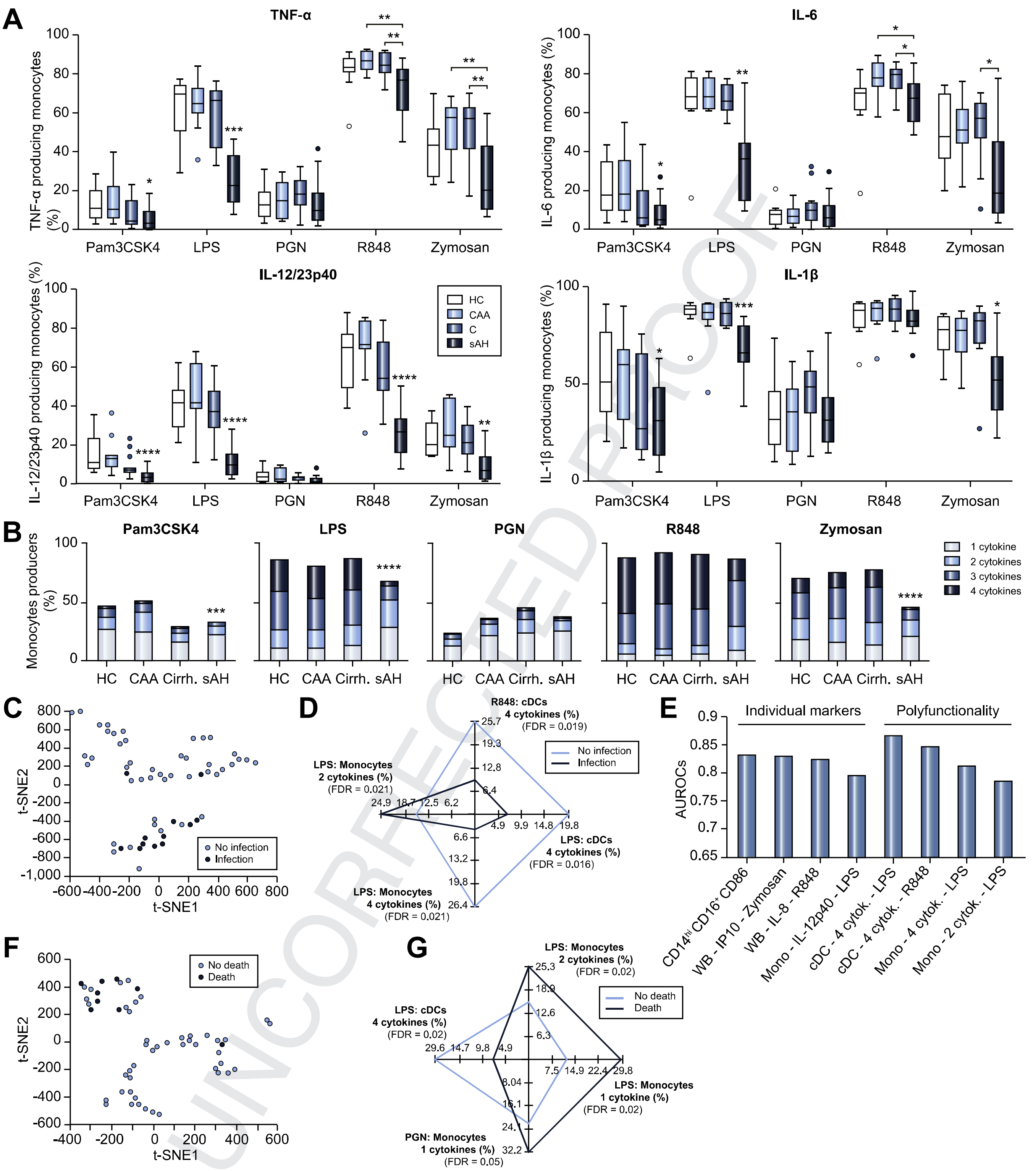
Discussion

In recent years, interest in exploring immune dysfunction in advanced ALD has increased, as bacterial infection is the principal event leading to acute decompensation of cirrhosis, ACLF and death. The current paradigm suggests that this susceptibility to infections is driven by the dysfunction of immune circulating cells. We observed that the ability of monocytes and cDCs from patients with sAH to produce inflammatory cytokines was severely impaired upon stimulation with PAMPs associated with gram-negative bacteria or fungal pathogens . Of note, IL-8 levels in whole blood cultures were actually increased in patients with sAH and stimulation with R848 or PGN was less affected than with other ligands, indicating complex functional changes. Nevertheless, we observed a strong impairment of the IL-12p70/IFNγ/CXCL10 pathway, together with a downregulation of IFN-related genes at the transcriptional level in these patients. Polyfunctionality is recognized as a parameter to assess the quality of T cell responses.[28] Here, we showed that this parameter might also represent a useful marker for the functional status of innate immune cells as low polyfunctionality of monocytes and cDCs in patients with ALD was associated with a higher risk of infection and mortality.

In our group of patients with sAH, the presence of ACLF did not significantly modify the phenotype and PAMP-elicited cytokine production of circulating mononuclear phagocytes, suggesting that observed immune alterations are related to the presence of sAH and not ACLF. We did not explore the function of circulating mononuclear phagocytes of patients with ACLF without sAH.

Multiple molecular mechanisms may account for the unique phenotypic and functional features of monocytes and DCs in patients with sAH. We confirmed previous findings showing that they display increased expression of the inhibitory receptor MERTK.[7] In addition, we observed important modifications in the expression of genes involved in cellular respiration and metabolic pathways. This is in line with a previous report indicating that in patients with advanced cirrhosis, altered plasma amino acids levels interfered with the mitochondrial tricarboxylic acid cycle and ATP levels in DCs.[29] Furthermore, pharmacological inhibition of glutamine synthetase could partially restore the function of these cells.[9] In parallel, we observed that this distinct transcriptional status was associated with important changes in chromatin accessibility within enhancer regions, indicating that these cells are also reprogrammed at the

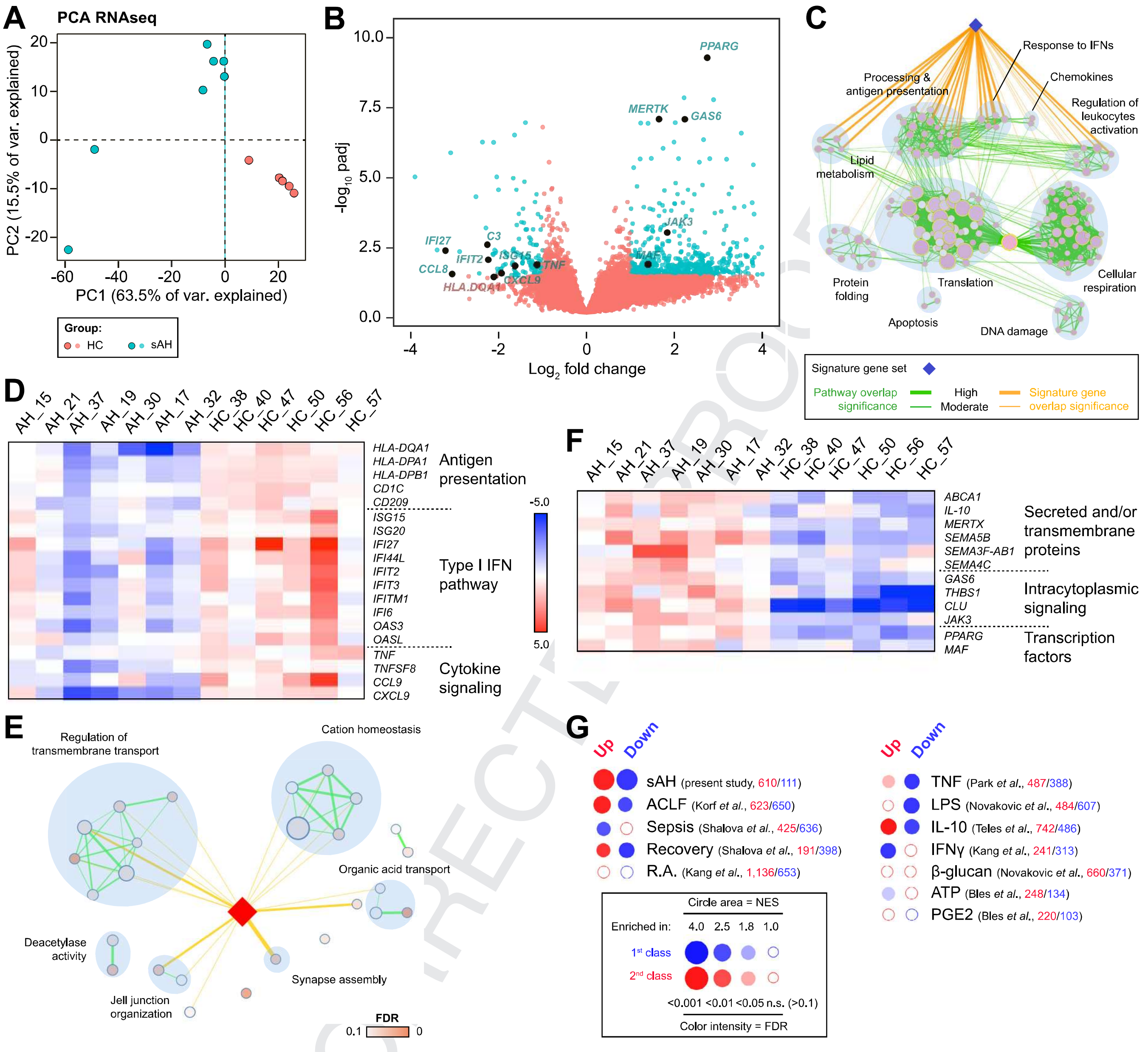
FLA 5.6.0 DTD ■ JHEPAT7631_proof ■ 17 April 2020 ■ 9:31 pm ■ ce



print & web 4C/FPO

Fig. 4. Cytokine production by CD14+ monocytes is impaired in response to various PAMPs in patients with sAH. (A) Flow cytometry analysis of intracellular production of cytokines by CD14+ monocytes upon 6 h stimulation of whole blood from HCs (n = 12), CAAs (n = 11), patients with cirrhosis (n = 16) and those with sAH (n = 18). Kruskal-Wallis test was performed to examine the statistical differences of each cytokine/monocyte per group, followed by Dunn's correction for multiple testing. (B) Measure of the ability of monocytes to produce up to 4 cytokines among TNF-α, IL-6, IL-12p40 and IL-1β simultaneously (polyfunctionality) upon stimulation with various ligands. Kruskal-Wallis test was performed to examine the statistical differences in the ability to produce 3 or 4 cytokines simultaneously per group, followed by Dunn's correction for multiple testing. (C) t-SNE plot of polyfunctionality profiles in patients with cirrhosis (n = 16) and sAH (n = 18). Occurrence of infection within 28 days of follow-up (green = no infection, red = infection). (D) Radar plot showing the most discriminatory features stratifying patients according to their infection status at 28 days. Axes show the frequency (%) of monocytes or cDCs producing the indicated number of cytokines upon stimulation with LPS or R848. (E) Histogram with AUROC values (*i.e.* area under ROC curves) of the 4 most predictive individual and polyfunctionality

JOURNAL
OF HEPATOLOGY



print & web 4C/FPO

Fig. 5. Transcriptomic profile of monocytes from patients with sAH is characterized by immunosuppressive features. (A) PCA plot representing the clustering of CD14^{+} monocytes from HCs (n = 6) and sAH (n = 7) based on their transcriptional profile. (B) Volcano plot showing gene expression changes in monocytes of HCs *vs.* patients with sAH (in green: down- or upregulated genes with a fold change >2 and FDR <0.05). (C) Enrichment maps displaying gene sets significantly downregulated in monocytes of patients with sAH compared to HCs. (D) Expression of genes involved in innate immune responses (E) Enrichment maps displaying gene sets significantly upregulated in monocytes of patients with sAH compared to HCs. (F) Expression of immunomodulatory genes in monocytes of HCs and patients with sAH. (G) BubbleGUM GSEA map established from available gene sets in monocytes or macrophages during various inflammatory settings or from monocytes as defined in B. For each gene set, origin of the data set and number of up- (red) and down- (blue) regulated genes are indicated. The panel summarizes the NES and FDR parameters. FDR, false discovery rate; GSEA, gene set enrichment analysis; HCs, healthy controls; NES, normalized enrichment score; PCA, principal component analysis; sAH, severe alcoholic hepatitis.

epigenetic level. Exposure to IL-10 could contribute to this finding as this cytokine was shown to mediate epigenetic reprogramming, leading to the repression of pro-inflammatory gene expression in intestinal macrophages.[30] Several reports indicate increased circulating levels of IL-10 in patients with sAH.[31,32] Our results suggest that the transcriptional status of

markers of occurrence of infection within 28 days after recruitment. (F) t-SNE plot of polyfunctionality profiles in cirrhosis (n = 16) and sAH (n = 18). Occurrence of death within 90 days of follow-up (green = no death, red = death). (G) Radar plot showing the most discriminatory features stratifying patients according to their mortality status at 90 days. Axes show the frequency (%) of monocytes or cDCs producing the indicated number of cytokines upon stimulation with LPS or PGN. Boxplots are Tukey box and whiskers. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 compared to HCs unless specified otherwise. CAAs, chronic alcohol abusers; cDCs, conventional DCs; DCs, dendritic cells; HCs, healthy controls; LPS, lipopolysaccharide; PAMPs, pathogen-associated molecular patterns; PGN, peptidoglycan; t-SNE, t-distributed stochastic neighbour embedding.

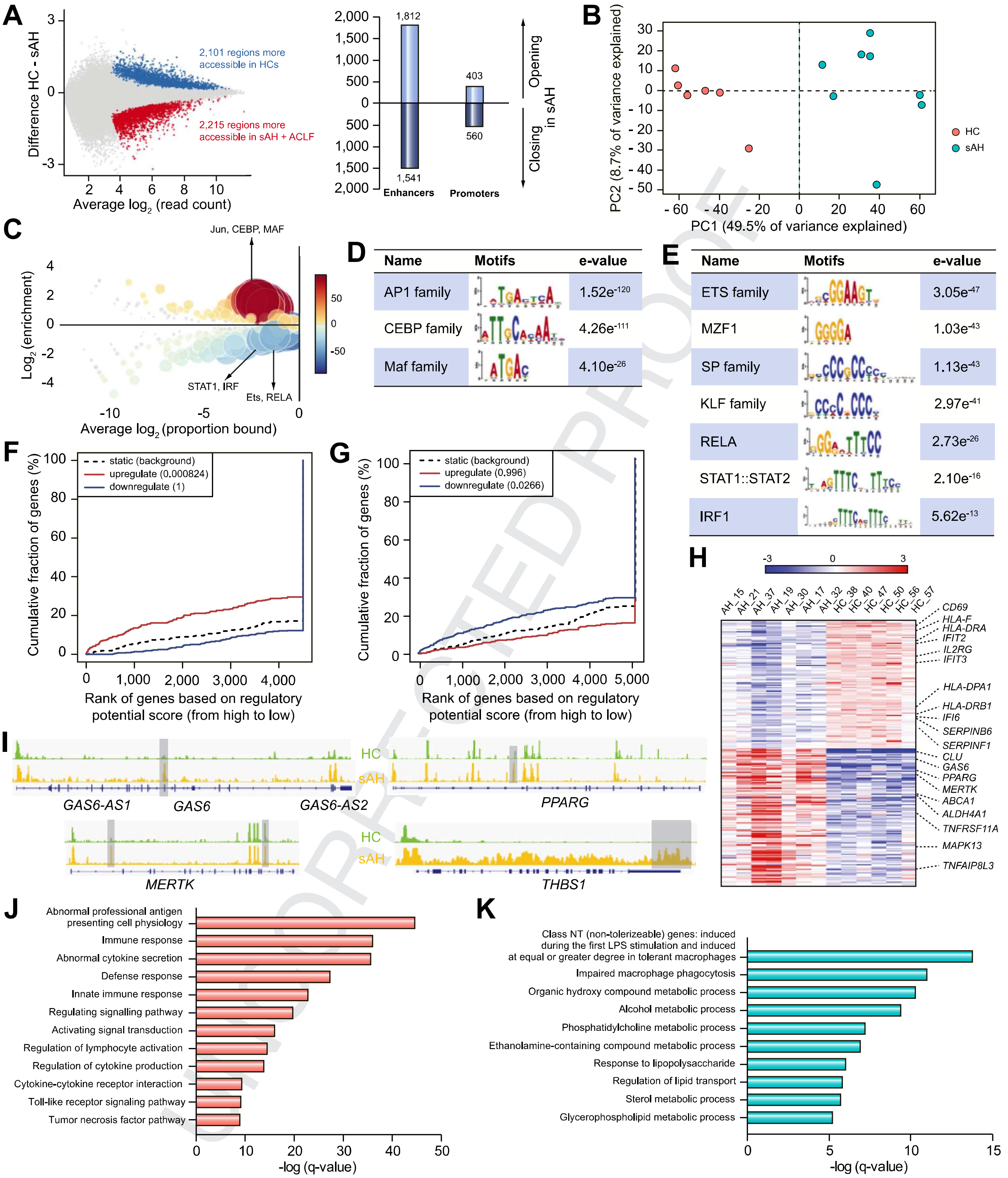


Fig. 6. Altered patterns of chromatin accessibility displayed in monocytes from patients with sAH. (A) MA plot of mean ATAC-seq counts per peaks showing the DOR of CD14$^+$ monocytes of HCs (blue) and patients with sAH (red), with the indicated number of regions. Histograms indicate the number of opening or closing regions in CD14$^+$ monocytes of patients with sAH (n = 8) compared to HCs (n = 6) at promoters and enhancers. (B) PCA plot representing the clustering of monocytes of HCs and patients with sAH based on DORs. (C) CiiiDER analysis for putative transcription factors motifs from DOR at enhancers. Transcription factors colored according to their gene coverage *p* value and whether they are over (red) or under (blue) represented. The size of each point is proportional to the Log10 *p* value. (D,E) Motif enrichment analysis in more (D) or less (E) accessible enhancer regions in monocytes of patients with sAH using AME. (F,G) Cumulative distribution plot generated by BETA algorithm showing the predicted activating/repressive function of more (F) or less (G) accessible enhancer regions in

FLA 5.6.0 DTD ■ JHEPAT7631_proof ■ 17 April 2020 ■ 9:31 pm ■ ce

monocytes in patients with sAH is distinct from the immune paralysis/tolerance observed during acute sepsis. In contrast, we observed a strong enrichment for genes that are modulated in monocytes isolated several weeks/months after the septic event. This is quite striking as patients that experienced sepsis display a higher susceptibility to secondary infections that can persist for years.[33] It is therefore tempting to speculate that common mechanisms may account for long-term innate immune dysfunction post-sepsis and during ALD progression.

ALD is associated with dysbiosis,[34] increased bacterial translocation and release of damage-associated molecular patterns (DAMPs) by dying liver cells. These circulating factors lead to systemic inflammation, which is highest in patients with ACLF.[35] Thus, it is likely that chronic exposure to circulating bacterial products, PAMPs and DAMPs, plays a major role in the rewiring of monocytes and cDCs, preventing them from further responding to a new stimulus. Indeed, antibiotic prophylaxis has been shown to reduce the incidence of infections in patients with ACLF.[36]

In conclusion, we showed a profound epigenetic and transcriptomic reprogramming of circulating monocytes in patients with sAH. This altered profile was associated with impaired response to PAMPs and a decrease of polyfunctionality. The presence of ACLF did not modify the immune profile of patients with sAH. These immune features were linked to a higher risk of infections and mortality during follow-up. We provide new insights into the molecular basis of immune dysfunction observed in advanced ALD.

Abbreviations

ACLF, acute-on-chronic liver failure; ALD, alcohol-related liver disease; AME, analysis of motif elements; CAAs, chronic alcohol abusers; cDCs, conventional DCs; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; DOR, differentially open regions; FDR, false discovery rate; GSEA, gene set enrichment analysis; HC, healthy controls; HVPG, hepatic venous pressure gradient; IFN, interferon; IL, interleukin; INR, international normalized ratio; LPS, lipopolysaccharide; mDF, modified Maddrey's discriminant function; MELD, model for end-stage liver disease; MERTK, MER tyrosine kinase; NES, normalized enrichment score; NK, natural killer; PAMPs, pathogen-associated molecular patterns; PCA, principal component analysis; pDCs, plasmacytoid DCs; PPAR, peroxisome proliferator-activated receptor; sAH, severe alcoholic hepatitis; TLR, Toll-like receptor; TNFα, tumor necrosis factor-α; t-SNE, t-distributed stochastic neighbour embedding.

Financial support

Q4 This study was supported by the Fonds National de la Recherche Scientifique (FRS-FNRS, Belgium), the European Regional Development Fund (ERDF) of the Walloon Region (Wallonia-Biomed portfolio, 411132-957270), the Wallonie-Bruxelles International (WBI) and the Fonds ERASME. LW is a research fellow of the FRS-FNRS. SG is a senior research associate of the FRS-FNRS.

Conflict of interest

TG gives advice to Promethera Biosciences and Martin Pharmaceuticals. CM received grant from Gilead and gives advice to Gilead, Abbvie and Intercept. Other authors have no competing interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

LW conducted most of the experiments. KKS contributed to the design of immunophenotype experiments; AL, CM, JS, TS, ET and TG performed the patient recruitment. AA, MS and FL performed bioinformatics analysis. LW and AA analyzed the data and prepared the figures. JD provided input for data analysis and interpretation. TG and SG supervised the work and wrote the manuscript. All authors were involved in critically revising the manuscript for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

Uncited table

Table 1.

Acknowledgments

We would like to thank Mohammed Amrani , Carole Gilles, Séverine Thomas, Muriel Nguyen and Eric Quertinmont for their invaluable help.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.02.017.

References Q5

[1] Gustot T, Fernandez J, Szabo G, Albillos A, Louvet A, Jalan R, et al. Sepsis in alcohol-related liver disease. J Hepatol 2017;67:1031–1050. Q6
[2] Fernandez J, Acevedo J, Wiest R, Gustot T, Amoros A, Deulofeu C, et al. Bacterial and fungal infections in acute-on-chronic liver failure: prevalence, characteristics and impact on prognosis. Gut 2018;67:1870–1880.
[3] Gustot T, Maillart E, Bocci M, Surin R, Trepo E, Degre D, et al. Invasive aspergillosis in patients with severe alcoholic hepatitis. J Hepatol 2014;60:267–274.
[4] Clària J, Stauber RE, Coenraad MJ, Moreau R, Jalan R, Pavesi M, et al. Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure. Hepatology 2016;64:1249–1264.
[5] Albillos A, Lario M, Alvarez-Mon M. Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance. J Hepatol 2014;61:1385–1396.
[6] Wasmuth HE, Kunz D, Yagmur E, Timmer-Stranghoner A, Vidacek D, Siewert E, et al. Patients with acute on chronic liver failure display "sepsis-like" immune paralysis. J Hepatol 2005;42:195–201.
[7] Bernsmeier C, Pop OT, Singanayagam A, Triantafyllou E, Patel VC, Weston CJ, et al. Patients with acute-on-chronic liver failure have

monocytes of patients with sAH and the indicated *p* values determined by the Kolmogorov-Smirnov test (red: upregulated genes, blue: downregulated genes, dashed line: background). (H) Expression of the genes associated with significant changes in chromatin accessibility as defined through BETA analysis. (I) Representative ATAC-seq tracks of CD14+ monocytes for each patient group at enhancers of indicated genes. Significant different chromatin accessible regions are highlighted in grey. (J,K) Selected Gene Ontology pathways enriched in genes associated with more accessible enhancer regions in CD14+ monocytes of HCs (J) or patients with sAH (K) using GREAT and presented as $-\text{Log}_{10}$ of binomial FDR q-value. AME, analysis of motif elements; DORs, differentially open regions; FDR, false discovery rate; HCs, healthy controls; PCA, principal component analysis; sAH, severe alcoholic hepatitis.

increased numbers of regulatory immune cells expressing the receptor tyrosine kinase MERTK. Gastroenterology 2015;148:603–615.e14.
[8] O'Brien AJ, Fullerton JN, Massey KA, Auld G, Sewell G, James S, et al. Immunosuppression in acutely decompensated cirrhosis is mediated by prostaglandin E2. Nat Med 2014;20:518–523.
[9] Korf H, du Plessis J, van Pelt J, De Groote S, Cassiman D, Verbeke L, et al. Inhibition of glutamine synthetase in monocytes from patients with acute-on-chronic liver failure resuscitates their antibacterial and inflammatory capacity. Gut 2018;68.
[10] Mulder WJM, Ochando J, Joosten LAB, Fayad ZA, Netea MG. Therapeutic targeting of trained immunity. Nat Rev Drug Discov 2019;18:553–566.
[11] Carson WF, Cavassani KA, Dou Y, Kunkel SL. Epigenetic regulation of immune cell functions during post-septic immunosuppression. Epigenetics 2011;6:273–283.
[12] European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu; European Association for the Study of the Liver. EASL clinical practice guidelines: management of alcohol-related liver disease. J Hepatol 2018;69:154–181.
[13] Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. Gastroenterology 2013;144:1426–1437. 1437.e1-9.
[14] Smolen KK, Cai B, Fortuno ESR, Gelinas L, Larsen M, Speert DP, et al. Single-cell analysis of innate cytokine responses to pattern recognition receptor stimulation in children across four continents. J Immunol 2014;193:3003–3012.
[15] Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods 2013;10:1213–1218.
[16] Jansen K, Blimkie D, Furlong J, Hajjar A, Rein-Weston A, Crabtree J, et al. Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation. J Immunol Methods 2008;336:183–192.
[17] Fang LL, Yu HQ, Wu RJ, He C, Li M, Yan H, et al. Thrombospondin 1 modulates monocyte properties to suppress intestinal mucosal inflammation. J Innate Immun 2015;7:601–611.
[18] Nishide M, Kumanogoh A. The role of semaphorins in immune responses and autoimmune rheumatic diseases. Nat Rev Rheumatol 2018;14:19–31.
[19] Wang H, Brown J, Gao S, Liang S, Jotwani R, Zhou H, et al. The role of JAK-3 in regulating TLR-mediated inflammatory cytokine production in innate immune cells. J Immunol 2013;191:1164–1174.
[20] Appel S, Mirakaj V, Bringmann A, Weck MM, Grunebach F, Brossart P. PPAR-gamma agonists inhibit toll-like receptor-mediated activation of dendritic cells via the MAP kinase and NF-kappaB pathways. Blood 2005;106:3888–3894.
[21] Cao S, Liu J, Song L, Ma X. The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. J Immunol 2005;174:3484–3492.
[22] Shalova IN, Lim JY, Chittezhath M, Zinkernagel AS, Beasley F, Hernandez-Jimenez E, et al. Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1alpha. Immunity 2015;42:484–498.
[23] Novakovic B, Habibi E, Wang SY, Arts RJW, Davar R, Megchelenbrink W, et al. β-Glucan reverses the epigenetic state of LPS-induced immunological tolerance. Cell 2016;167:1354–1368.e14.
[24] Park SH, Kang K, Giannopoulou E, Qiao Y, Kang K, Kim G, et al. Type I interferons and the cytokine TNF cooperatively reprogram the macrophage epigenome to promote inflammatory activation. Nat Immunol 2017;18:1104–1116.
[25] Kang K, Park SH, Chen J, Qiao Y, Giannopoulou E, Berg K, et al. Interferon-gamma represses M2 gene expression in human macrophages by disassembling enhancers bound by the transcription factor MAF. Immunity 2017;47:235–250.e4.
[26] Bles N, Horckmans M, Lefort A, Libert F, Macours P, El Housni H, et al. Gene expression profiling defines ATP as a key regulator of human dendritic cell functions. J Immunol 2007;179:3550–3558.
[27] Teles RM, Graeber TG, Krutzik SR, Montoya D, Schenk M, Lee DJ, et al. Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. Science 2013;339:1448–1453.
[28] Almeida JR, Price DA, Papagno L, Arkoub ZA, Sauce D, Bornstein E, et al. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J Exp Med 2007;204:2473–2485.
[29] Kakazu E, Kondo Y, Kogure T, Ninomiya M, Kimura O, Ueno Y, et al. Plasma amino acids imbalance in cirrhotic patients disturbs the tricarboxylic acid cycle of dendritic cell. Sci Rep 2013;3:3459.
[30] Simon JM, Davis JP, Lee SE, Schaner MR, Gipson GR, Weiser M, et al. Alterations to chromatin in intestinal macrophages link IL-10 deficiency to inappropriate inflammatory responses. Eur J Immunol 2016;46:1912–1925.
[31] Li W, Amet T, Xing Y, Yang D, Liangpunsakul S, Puri P, et al. Alcohol abstinence ameliorates the dysregulated immune profiles in patients with alcoholic hepatitis: a prospective observational study. Hepatology 2017;66:575–590.
[32] Naveau S, Balian A, Capron F, Raynard B, Fallik D, Agostini H, et al. Balance between pro and anti-inflammatory cytokines in patients with acute alcoholic hepatitis. Gastroenterol Clin Biol 2005;29:269–274.
[33] Venet F, Monneret G. Advances in the understanding and treatment of sepsis-induced immunosuppression. Nat Rev Nephrol 2018;14:121–137.
[34] Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, et al. Alterations of the human gut microbiome in liver cirrhosis. Nature 2014;513:59–64.
[35] Claria J, Arroyo V, Moreau R. The acute-on-chronic liver failure syndrome, or when the innate immune system goes astray. J Immunol 2016;197:3755–3761.
[36] Moreau R, Elkrief L, Bureau C, Perarnau JM, Thevenot T, Saliba F, et al. Effects of long-term norfloxacin therapy in patients with advanced cirrhosis. Gastroenterology 2018;155:1816–1827.e9.

FLA 5.6.0 DTD ■ JHEPAT7631_proof ■ 17 April 2020 ■ 9:31 pm ■ ce