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Ductular Reaction Cells Display an Inflammatory Profile and Recruit Neutrophils in Alcoholic Hepatitis

Beatriz Aguilar-Bravo[1,*], **Daniel Rodrigo-Torres**[1,*], **Silvia Ariño**[1], **Mar Coll**[1,2], **Elisa Pose**[1,3], **Delia Blaya**[1], **Isabel Graupera**[1,3], **Luis Perea**[1], **Júlia Vallverdú**[1], **Teresa Rubio-Tomás**[1], **Laurent Dubuquoy**[4], **Carolina Armengol**[2,5], **Antonio Lo Nigro**[6], **Peter Stärkel**[7], **Philippe Mathurin**[4], **Ramon Bataller**[8], **Joan Caballería**[1,2,3], **Juan José Lozano**[1,2], **Pere Ginès**[1,2,3], **Pau Sancho-Bru**[1,2]

[1]Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

[2]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona, Spain

[3]Liver Unit, Hospital Clínic, Faculty of Medicine, University of Barcelona, Barcelona, Spain

[4]Lille Service des Maladies de l'Appareil Digestif, Hopital Huriez, Unité INSERM 995, Faculté de médecine, Lille, France

[5]Childhood Liver Oncology group (c-LOG), Program of Predictive and Personalized Medicine of Cancer (PMPPC), Health Sciences Institute Germans Trias i Pujol (IGTP), Campus Can Ruti, Badalona, Spain

[6]Ri. Med Foundation, Department of Laboratory Medicine and Advanced Biotechnologies, IRCCS-ISMETT (Istituto Mediterraneo per i Trapianti e Terapie ad Alta Specializzazione), Palermo, Italy

[7]Department of Hepato-Gastroenterology, Cliniques Universitaires Saint-Luc, Brussels, Belgium

[8]Pittsburgh Liver Research Center, University of Pittsburgh Medical Center, Pittsburgh, PA.

Abstract

Chronic liver diseases are characterized by the expansion of ductular reaction (DR) cells and the expression of liver progenitor cell (LPC) markers. In alcoholic hepatitis (AH), the degree of DR expansion correlates with disease progression and short-term survival. However, little is known about the biological properties of DR cells, their impact on the pathogenesis of human liver disease, and their contribution to tissue repair. In this study, we have evaluated the transcriptomic profile of DR cells by laser capture microdissection in patients with AH and assessed its association with disease progression. The transcriptome analysis of cytokeratin

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO: Pau Sancho-Bru, Ph.D., Institut d'Investigacions, Biomèdiques August Pi i Sunyer, (IDIBAPS)C/Rosselló, 149-153, third floor, 08036 Barcelona, Spain, psancho@clinic.cat, Tel.: +1-34-932275400 Extension 3371.

*These authors contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30472/suppinfo.

7-positive ($KRT7^+$) DR cells uncovered intrinsic gene pathways expressed in DR and genes associated with alcoholic liver disease progression. Importantly, DR presented a proinflammatory profile with expression of neutrophil recruiting C-X-C motif chemokine ligand (CXC) and C-C motif chemokine ligand chemokines. Moreover, LPC markers correlated with liver expression and circulating levels of inflammatory mediators such as CXCL5. Histologically, DR was associated with neutrophil infiltration at the periportal area. In order to model the DR and to assess its functional role, we generated LPC organoids derived from patients with cirrhosis. Liver organoids mimicked the transcriptomic and proinflammatory profile of DR cells. Conditioned medium from organoids induced neutrophil migration and enhanced cytokine expression in neutrophils. Likewise, neutrophils promoted the proinflammatory profile and the expression of chemokines of liver organoids.

Conclusion: Transcriptomic and functional analysis of $KRT7^+$ cells indicate that DR has a proinflammatory profile and promote neutrophil recruitment. These results indicate that DR may be involved in the liver inflammatory response in AH, and suggest that therapeutic strategies targeting DR cells may be useful to mitigate the inflammatory cell recruitment in AH.

Alcoholic hepatitis (AH) is the most severe form of alcoholic liver disease (ALD), and it is characterized by jaundice, hepatic failure, and short-term mortality.[1] Histologically, AH is characterized by the presence of steatosis, hepatocellular damage, bilirrubinostasis, fibrosis, neutrophil infiltration, and a prominent ductular reaction.[2,3]

Ductular reaction (DR) emerges and expands in most chronic liver diseases or after massive liver damage, and is associated with poor regenerative capacity of hepatocytes.[4] Several studies have investigated the presence of DR in liver disease.[5–9] In patients with AH, it correlates with the degree of inflammation, fibrosis, and with short-term mortality.[2,10,11] The role of DR has been mainly studied in biliary diseases showing the expression of profibrogenic modulators such as platelet-derived growth factor, transforming growth factor (TGF)-β1, vascular endothelial growth factor, insulin-like growth factor 1, and nerve growth factor and proinflammatory mediators such as interleukin (IL)-1, IL-6, IL-8, interferon-γ, TGF-β, monocyte chemoattractant protein 1, and endothelin-1.[12–14] In nonalcoholic fatty liver disease, ductular reaction expansion results from an adaptive response to oxidative stress, leading to periportal fibrosis.[15–17] The relationship between hepatic fibrosis and ductular reaction in patients with hepatitis C virus has also been reported.[8,18]

DR contains a heterogeneous population of cells comprising reactive biliary cells, intermediate hepatobiliary cells, and more immature facultative liver progenitor cells (LPCs). DR is generally accompanied by extracellular matrix and inflammatory cells, which participate in DR expansion.[6,19] Although DR has been shown to produce profibrogenic mediators,[20] to what extent DR cells participate in the generation of this rich milieu is poorly understood.

The presence of facultative LPCs has been demonstrated *in vivo* by lineage tracing studies and by *in vitro* systems such as liver organoid cultures, which allow culturing leucine-rich repeat-containing G protein-coupled receptor 5 $(LGR5)^+$ LPCs with the potential to differentiate to hepatocytes and cholangiocytes.[21] Nevertheless, the origin and fate of LPCs in chronic liver diseases is not completely understood. By genetic labeling of

biliary epithelium cells and hepatocytes, animal studies suggest that both biliary cells and hepatocytes may be able to generate facultative LPCs.[22–24] Yet, the contribution of LPCs to hepatocyte generation and liver regeneration is still controversial. Although most animal models of acute or chronic liver injury do not show the contribution of LPCs to newly generated hepatocytes,[25,26] animal models with extended hepatocyte replicative arrest have shown that the biliary compartment might be able to give rise to LPCs that differentiate to mature hepatocytes.[27,28] These reports suggest that the origin of facultative LPCs and their contribution to liver regeneration in animal models is dependent on the type and extent of injury.

Overall, the biological properties, impact, and contribution of DR and LPCs in human chronic liver diseases is not completely understood; thus, this study was undertaken to fill this knowledge gap.

LPCs are strongly influenced by their niche. Ligands released by cell types conforming the LPC niche, such as hepatic stellate cells or inflammatory cells, are known to determine DR expansion and LPC proliferation and differentiation.[6,29,30] Yet, their effect on the LPC gene expression profile during liver disease progression has not been assessed. In addition, mediators secreted by LPCs and their effects in disease are largely unknown. Therefore, the global changes in LPCs gene expression profile in liver disease and the influence of DR cells on other cell populations remain elusive.

The aim of this study was to identify the gene expression profile of DR cells. Moreover, we aimed to determine the functional role of the pathways identified. By using a unique set of samples and laser microdissection, we obtained a global transcriptomic analysis of human DR cells and identified signaling pathways and genes expressed during liver disease progression. Importantly, our results identified DR cells as a potential source of chemokines and neutrophil recruitment, which strongly suggests that they might be involved in liver inflammation and inflammatory cell recruitment during the progression of AH.

Materials and Methods

PATIENT INFORMATION

Samples for the microdissection procedure were obtained from patients with clinical, analytical, and histological features of AH admitted to the Hôpital Claude Huriez of Lille (France) (Table 1). Patient information was described.[10] Informed consent was provided by all the patients (QuickTrans [NCT01756794] and TargetOH [CPP 14/67] studies).

All other samples were selected from patients admitted to the Liver Unit of the Hospital Clinic of Barcelona. Informed consent was obtained from all the patients and the study was approved by the Ethics Committee of the hospital. DR genes and their correlation with disease progression were evaluated in 22 patients with ALD: patients with precirrhosis (n = 4), patients with cirrhosis without AH (n = 7), and patients with AH (n = 11). The clinical and biochemical parameters of these patients are shown in Supporting Table S1. Liver and serum samples from patients with AH were selected based on RNA availability and quality, and were used to perform RNA gene expression analysis and cytokine determination.[31]

Liver organoids were generated from nontumor liver tissue obtained from liver resections and liver explants from patients with cirrhosis with alcohol-induced cirrhosis, nonalcoholic steatohepatitis, and hepatitis C virus. Organoid transcriptomic data was compared with the gene expression profile of whole liver tissue from 4 patients with compensated cirrhosis with mixed etiologies.

LASER CAPTURE MICRODISSECTION OF PARAFFIN-EMBEDDED LIVER SAMPLES

Eight-μm sections were cut from formalin-fixed, paraffin-embedded liver samples from patients with AH (n = 6) in RNase-free conditions, and mounted on RNase-free Glass foiled PEN slides (Leica, Wetzlar, Germany; NO 11505158, 2.0 mm). Slides were placed at 55°C for 2 hours before deparaffinization with Xylene (3 times, 20 seconds each), Ethanol 100% (twice, 30 seconds each), Ethanol 95% (twice, 30 seconds each), Ethanol 70% (twice, 30 seconds each), ddH20 30 seconds, and sterile Dulbecco's phosphate-buffered saline (DPBS; Lonza) 30 seconds in RNase-free conditions. Sections were then incubated with mouse anti-human cytokeratin 7 (KRT7) antibody (Dako, Glostrup, Denmark; 1:50, M7018) for 10 minutes at room temperature in a humidified chamber. After a short wash with sterile DPBS, the samples were incubated for 15 minutes with EnVision peroxidase-conjugated mouse secondary antibody (Dako, K4007). As chromogen, 3,3′-diaminobenzidine solution (Dako, K1011) was used for 2 minutes and rinsed with DEPC-treated water (ThermoFisher Scientific, Waltham, MA). Once the samples were dry, the slides were kept at −80°C until the laser capture microdissection was performed.

Microdissection was performed using a Leica LMD6000 Microscope (Leica). For each sample, three different fractions were dissected: KRT7-positive ($KRT7^+$) DR cells, KRT7-negative ($KRT7^-$) cells comprising small areas of hepatocytes and areas of fibrosis, and a total fraction composed by random selection of KRT7-positive and -negative areas (n = 4). A minimum area of 1.7 mm^2 was used for RNA isolation and sequencing.

GENERATION OF HUMAN LIVER ORGANOIDS FROM CIRRHOTIC LIVER TISSUE

Liver cells were isolated and cultured as described[32] with minor modifications. Briefly, collagenase XI (Sigma, St. Louis, MO) and dispase II (ThermoFisher) digestion was performed. Digested material was washed and pelleted at 300 g for 5 minutes at 8°C. The pellet obtained was mixed with basement matrix extract (BME; Amsbio, Abingdon, United Kingdom)[33] and 50,000–100,000 cells were seeded per well in a 24-well plate. After the BME had solidified, organoid expansion medium was added (Advanced DMEM/F12; Thermofisher), 1% Glutamax (Thermofisher), 1% Hepes (Life Technologies, Carlsbad, CA), 1% Penicilin-Streptomicin (Lonza, Basilea, Switzerland), 1% N2 and 1% B7 without vitamin A (both from Life Technologies), 1.25 mM N-Acetylcysteine, 10 mM Nicotinamide and 10 nM Gastrin (Sigma), 50 ng/mL epidermal growth factor, 100 ng/mL fibroblast growth factor 10, 25 ng/mL hepatocyte growth factor, 25 ng/mL Noggin and 500 ng/mL Rspo1 (Peprotech, London, United Kingdom), 5 uM A8301, 0.5 uM CHIR99021, 10 uM Forskolin, and 10 uM of Y27632 (Axon Medchem, Groningen, the Netherlands). Y27632 was added only the first 3 days after isolation. At a confluence of 70%, organoid expansion medium was replaced by organoid basal medium overnight. Organoids were then stimulated with 50 ng/mL tumor necrosis factor-alpha (TNF-α; R&D Systems, Minneapolis, MN),

10 ng/mL IL-1β (Sigma), and 100 ng/mL lipopolysaccharide (LPS; Sigma) for 6 hours. Organoids were also incubated with 10 μM nuclear factor kappa B (NF-κB) inhibitor (BAY 11–7082, Sigma) for 1 hour before stimulation with IL-1P for 6 hours.

RAW DATA INFORMATION

Microdissection raw sequence data were deposited to the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the accession number PRJNA393451, and microarray data from organoids have been deposited in NCBI's Gene Expression Omnibus with accession number GSE100901.

SUPPORTING INFORMATION

Detailed information on RNA processing and sequencing, genome-wide transcriptome analysis, functional analysis, immunohistochemistry and immunofluorescence, enzyme-linked immunosorbent assay, and neutrophil studies can be found in Supporting Information.

Results

WHOLE-TRANSCRIPTOME ANALYSIS OF DUCTULAR REACTION CELLS REVEALS A PROINFLAMMATORY PROFILE

In order to perform a transcriptome analysis of DR cells, paraffin-embedded liver sections from 6 patients with AH were stained for KRT7 and laser capture microdissected. Areas with $KRT7^+$ staining, negative fraction, and total liver were obtained and analyzed by whole-transcriptome sequencing (Fig. 1A). As expected, principal component analysis showed $KRT7^+$ fraction to be distant from the $KRT7^-$ fraction and at an intermediate distance from the total fraction (Fig. 1B). $KRT7^+$ cells showed 2,321 up-regulated genes and 2,031 down-regulated genes compared with the $KRT7^-$ fraction. As shown in Fig. 1C, the top 50 up-regulated genes contained well-known LPC markers, such as KRT7 and prominin 1 (PROM1), as well as genes not previously identified to be expressed in DR and LPCs. Expression of LPC markers in DR was confirmed by immunohistochemistry in liver sections of patients with AH (Fig. 1D). Importantly, expression of cell markers from other liver cell types was low in $KRT7^+$ compared with $KRT7^-$ fraction: Neutrophils (myeloperoxidase [MPO]; Fold change [FC] = −4.91 ± 7.44), Kupffer cells (cluster of differentiation 68 [CD68]; FC = −3.9 ± 1.34), hepatic stellate cells (platelet-derived growth factor receptor beta; FC = −2.43 ± 1.64), endothelial cells (von Willebrand factor; FC = −8.27 ± 2.21), and infiltrating inflammatory cells (cluster of differentiation 45; FC = −5.34 ± 2.49) showing the purity of the microdissected fraction.

Functional analysis performed by ingenuity pathway analysis (IPA) and gene ontologies (GO) showed that genes up-regulated (FC >2) in $KRT7^+$ cells were involved in pathways related to inflammation such as "agranulocyte adhesion" and "granulocyte adhesion and diapedesis," "NIK/NF-kappaB signaling" or "neutrophil chemotaxis" as well as key signaling pathways such as "Wnt signaling" or "aphrin A signaling" (Table 2). Interestingly, a number of chemokine C-X-C motif chemokine ligand (CXC) and C-C motif chemokine ligand (CCL) family members included in the granulocyte adhesion and diapedesis pathway

such as *CCL28, CXCL1, CXCL2, CXCL5, CXCL6,* and *CXCL8* were highly expressed in the KRT7$^+$ fraction (Supporting Table S2).

Next, we evaluated the protein interaction network of genes highly expressed in the DR by functional protein association networks (STRING). As shown in Supporting Fig. S1, DR showed genes related to claudin family, which are expressed in tight junctions in developing bile ducts; bile acid metabolism and ion transport pathways; and LPC markers such as epithelial cell adhesion molecule *(EpCAM), KRT7,* cytokeratin 19 *(KRT19),* and *PROM1,* together with mucins family members.

DUCTULAR REACTION GENES CORRELATE WITH ALCOHOLIC LIVER DISEASE PROGRESSION AND INFLAMMATION

In order to assess the expression of DR genes along alcoholic liver disease progression, we analyzed the expression of the KRT7$^+$ profile in a cohort of patients with different stages of ALD.

KRT7+ gene expression profile was evaluated in an ALD data set (n = 28) including the whole liver transcriptome from healthy controls (n = 6), patients with precirrhosis (n = 4), patients with cirrhosis (n = 7), and patients with AH (n = 11), together with the clinical data associated. As shown in Fig. 2A, 23 genes up-regulated in KRT7$^+$ fraction versus KRT7$^-$ fraction (FC >3; $P < 0.05$) were found to positively correlate with Model for End-Stage Liver Disease (MELD) or Child-Pugh score. Two groups of genes were identified according to the pattern of expression along the ALD progression: a first group of genes showing an increase along disease progression but with similar expression level between liver tissue from patients with cirrhosis and those with AH (Supporting Table S3); and a second group of genes showing a steady increase in gene expression along ALD progression and differentially expressed between cirrhotic and AH liver tissue (Supporting Table S4). This last group of genes contains LPC markers such as *KRT23* and tumor-associated calcium signal transducer 2 *(TACSTD2)* as well as CXC family members such as *CXCL5, CXCL6,* and *CXCL8* (Fig. 2A; Supporting Table S4), indicating the association of LPC markers and inflammatory mediators with advanced ALD and particularly AH. To confirm this association, whole-tissue transcriptome analysis was evaluated, revealing a strong correlation of LPC markers with proinflammatory CXC and CCL family members (Fig. 2B). Whereas markers expressed in ductular reaction cells such as *KRT7, KRT19,* SRY (sex determining region Y)-box 9 *(SOX9), HNF1B, SPP1, CFTR,* and *SCTR* showed a strong correlation with proinflammatory chemokines, cholangiocyte transporters *(SLC10A2, SLC4A2, SLC9A1, SLC2A1, SLC51A, SLC8A1, SLC5A1, ABCB4, ATP2B1, KCNN2, KCNN4)* showed lower correlation with inflammatory chemokines (Supporting Fig. S2A), suggesting that LPC may be an important source of inflammatory mediators.

Correlation of proinflammatory chemokines with LPC markers was confirmed by real-time PCR in AH liver tissue samples. Gene expression analysis of whole liver samples from patients with AH showed that *KRT7* expression positively correlated with *CXCL1, CXCL3, CXCL5, CXCL6,* and *CXCL8* (Fig. 3A). Altogether, these results indicate that the presence of DR is strongly associated with the expression of proinflammatory chemokines in the liver.

In order to evaluate if the proinflammatory profile of DR cells may have an effect systemically in AH, we evaluated plasma levels of TROP2 (encoded by TACSTD2 [tumor-associated calcium signal transducer 2]), a well-known marker of LPCs, and CXCL5, a chemokine associated with poor prognosis in patients with AH[31] and highly expressed in $KRT7^+$ cells. As shown in Fig. 3B, TROP2 serum levels were increased in patients with AH compared with healthy controls. Furthermore, TROP2 levels correlated with CXCL5 serum levels (Fig. 3C). Moreover, liver expression of CXCL5 and TROP2 in DR cells was confirmed by immunohistochemistry in liver tissue from patients with AH (Fig. 3D). These results suggest that besides the potential intrahepatic effect, proinflammatory mediators expressed by DR cells may also affect systemic inflammation.

LIVER ORGANOIDS FROM PATIENTS WITH CIRRHOSIS MIMIC DUCTULAR REACTION AND HAVE A PROINFLAMMATORY PROFILE

In order to study the DR *in vitro*, we explored if liver organoids mimicked DR and if they could therefore be used as a model to study DR.

In biliary diseases, reactive cholangiocytes[12] are known to show a proinflammatory profile. In order to determine if LPCs with a more immature phenotype also express inflammatory mediators, liver organoids were generated from human cirrhotic liver tissues. As shown in Fig. 4A, human liver organoids expressed KRT7 and EpCAM as assessed by immunofluorescence. Interestingly, transcriptomic analysis of liver organoids showed a high enrichment in genes expressed by the DR as assessed by gene set enrichment analysis, thus indicating a high similarity between organoids and DR (Fig. 4B). These results suggest that liver organoids could be used as a model to mimic DR. Of note, as observed in DR cells, transcriptome analysis of liver organoids showed expression of CXC and CCL chemokines (Fig. 4C). As shown in Table 3, functional analysis performed by IPA and Kyoto Encyclopedia of Genes and Genomes showed that genes up-regulated in organoids compared with whole liver tissue from healthy patients were also involved in inflammation pathways such as “agranulocyte adhesion,” “granulocyte adhesion and diapedesis,” “chemokine signaling,” or “TNF signaling pathway” but also in other relevant pathways such as “ErbB signaling pathway,” “Wnt/β-catenin signaling,” or “extracellular signal-regulated kinase/mitogen-activated protein kinase signaling.”

Real-time quantitative PCR analysis confirmed the expression of *KRT7, SOX9, EpCAM,* and *KRT19* LPC markers as well as the proinflammatory mediators *CXCL1, CXCL2, CXCL5, CXCL6, CXCL8,* and *CCL28* in liver organoids (Fig. 4D). Moreover, liver organoids produced CXCL5 (11.5 ± 5.6 ng/mL), as detected in the culture supernatant when cultured for 24 hours.

In order to evaluate if organoids show cholangiocyte features, key markers and transporters were evaluated. As shown in Supporting Table S5 and Supporting Fig. S2B, most cholangiocyte transporters were not expressed in organoids. Moreover, although organoids express ABCB1 (multidrug resistance protein 1 [MDR1]) and were able to secrete rodhamine 123 (Supporting Fig. S2C) into the lumen, secretion was not blocked by incubation with verapamil, an inhibitor of MRD1 transporter, suggesting that rodhamine

secretion was not MDR1 dependent. These results are in agreement with previous studies suggesting the immature/stem phenotype of cultured organoids.[21]

Next, to evaluate if inflammatory response may drive the expression of inflammatory mediators in LPCs, organoids were treated with IL-1β, TNF-α, and LPS. Whereas stimulation of liver organoids with IL-1β and TNF-α increased the expression of *CXCL1, CXCL5, CXCL6,* and *CXCL8* as assessed by real-time quantitative PCR (Fig. 4E; Supporting Fig. S3), LPS treatment did not induce any effect on proinflammatory gene expression (Supporting Fig. S3). As shown by GO analysis, NF-κB pathway was enriched in DR genes (Table 2); nevertheless, blocking of the NF-κB canonical pathway with NF-κB inhibitor did not show any effect on the basal proinflammatory profile of organoids. On the contrary, treatment with NF-κB inhibitor reduced the expression of *CXCL1* induced by IL-1β and showed a nonsignificant reduction of *CXCL5, CXCL6, CXCL8,* and *CCL20* expression (Fig. 4E). Altogether, these results indicate that liver organoids may be a good tool to study DR, and suggest that LPCs present a proinflammatory profile in injured livers.

DUCTULAR REACTION CELLS RECRUIT NEUTROPHILS AT THE PERIPORTAL AREA

CXCL1, CXCL3, and CXCL5 are strong chemoattractants of neutrophils, the main inflammatory cell type in AH; thus, we next investigated if DR and LPCs recruit neutrophils to the periportal area. Indeed, double staining of KRT7 and MPO showed that neutrophils locate close to the DR (Fig. 5A), suggesting a potential cross-talk between the two cell types. In order to evaluate if macrophages may be also recruited to DR, we performed double immunofluorescence of EpCAM and CD68. Liver macrophages were distributed uniformly throughout the tissue and not close to the DR (Supporting Fig. S4).

In order to determine if LPCs could promote neutrophil recruitment, we analyzed *in vitro* the effect of liver organoids on neutrophils. Conditioned medium from liver organoids induced an increase of neutrophils migration as compared with the induction with basal medium (Fig. 5B). Moreover, a change in the shape of neutrophils was observed as it is shown in the forward versus side scatter plot (Fig. 5B). In order to analyze if the cross-talk between LPCs and neutrophils induced a proinflammatory profile, conditioned medium from both populations was used. Neutrophils cultured for 18 hours with organoid-conditioned medium increased the expression of *CXCL1, CXCL2, TNF-α,* and *IL-1β* (Fig. 5C). On the other hand, neutrophil-conditioned medium increased the expression of *CXCL1, CXCL5, CXCL6, CXCL8,* and *CCL20* in liver organoids when compared with vehicle-treated organoids (Fig. 5D). Interestingly, when neutrophils were stimulated for 6 hours with LPS, conditioned medium from LPS-stimulated neutrophils further increased the expression of inflammatory mediators in organoids (Fig. 5D). Given that no direct effect of LPS on organoids was observed (Supporting Fig. S3), mediators secreted by neutrophils may be enhancing the inflammatory profile in organoids. These results suggest the potential cross-talk between DR cells and neutrophils, which may result in neutrophil migration at the site of DR and an enhanced inflammatory response.

Discussion

In this study, we describe the transcriptome profile of DR and identify genes and pathways associated with ALD progression. Moreover, we show that DR cells as well as LPCs have a proinflammatory profile with expression of CXC and CCL chemokines and are associated with neutrophil infiltration. In addition, we show that liver organoids mimic liver DR, thus being a good *in vitro* model to study DR cells, and promote neutrophil migration and inflammation. Overall, this study provides an understanding of the main pathways involved in DR expansion and suggests that it may play a role in ALD by promoting intrahepatic inflammation.

Several studies have shown the important expansion of DR cells in AH as well as the association of LPC markers with disease.[2,10,11] However, the contribution of DR to tissue repair or its potential pathophysiologic role in AH has not been specifically investigated. Up to now, human studies have relied almost exclusively on histological assessment to understand the role of DR in chronic liver diseases.[2,30] One of the main limitations of DR characterization has been the limited availability of liver tissue from patients with AH. To overcome this limitation, we performed microdissection in the unique set of samples from Lille University of patients undergoing early liver transplantation for AH[10,34] within the context of the clinical trials QuickTrans (NCT01756794) and TargetOH (CPP 14/67). Here we describe a whole transcriptome of DR cells, which may allow the discovery of pathways involved in LPC expansion and differentiation. Our results are in accordance with previous reports exploring the gene expression profile of DR cells in human diseases in which Notch and Wnt pathways were also described.[30] Nevertheless, our study identifies the involvement of pathways such as “ephrin A signaling,” “erb B signaling,” or “leukocyte recruitment,” and pathways such as “NF-kappaB signaling” or “response to hypoxia pathway,” previously described in ductular reaction from other liver diseases.[35,36] These pathways may deserve further analysis to evaluate their suitability as targets to enhance the contribution of LPCs to liver tissue repair. Interestingly, key genes expressed in DR correlated with MELD and Child-Pugh. Because of the limited number of patients included in this analysis, results should be confirmed in a bigger cohort of patients to better assess the correlation of DR with disease progression.

Hepatic expression of CXC chemokines has been shown to predict portal hypertension and survival in patients with AH.[31] The present study suggests that an important source of CXC and CCL chemokines may be the DR and LPCs, which is in agreement with other reports showing that biliary epithelial cells express and secrete chemokines such as IL-6, IL-8, and CCL28.[13‘37‘38] The finding that DR and LPCs have a proinflammatory profile opens important questions regarding its actual contribution to tissue repair and to intrahepatic inflammatory response in liver diseases and in particular in AH. Previous studies have shown the association of inflammatory cells with DR in chronic liver diseases.[6,15] In this study, we show that although DR express a number of cytokines involved in neutrophil as well as macrophage recruitment, only neutrophils were localized at the periductular region. Our results show that DR may be an important player in the intrahepatic inflammatory response in AH, producing cytokines that might participate in the inflammatory cell recruitment to the liver. The results obtained in the context of AH are in agreement with

other reports describing the expression of profibrogenic and proinflammatory mediators by ductular reaction cells in other liver diseases.[12,13,15,38] Moreover, the fact that CXCL5, one of the main inflammatory mediators expressed by DR cells, can be detected in serum and correlates with DR expansion suggests that inflammatory mediators expressed in the DR may have both a paracrine and potentially a systemic effect.

The contribution of LPCs to liver regeneration is a controversial topic and is still only partially understood in human diseases. In this regard, the impact of the inflammatory profile of LPCs in their regenerative potential is not known, and whether targeting the inflammatory profile of LPCs might enhance their contribution to liver regeneration deserves further investigation.

AH is characterized by neutrophil infiltration in necrotic inflammatory foci in the liver parenchyma, which correlates with good prognosis in AH.[3] Nonetheless, no studies have clearly investigated zonal recruitment of neutrophils and the association of neutrophils with the DR.[39] It is important to note that mechanisms driving neutrophil recruitment may be diverse, and those mediating parenchymal recruitment (mainly hepatocyte necrosis) may be different from those promoting recruitment to the periportal area that may be mediated by chemokine production, among other factors. Although we show neutrophil recruitment to the DR area, interaction between LPC and neutrophils is not formally proved, and the tissue context, the microenvironment, and other cell types might also be important in regulating neutrophil recruitment. Although it is well known that neutrophils play an important role in sterile and nonsterile acute liver injury, their role in liver tissue repair in chronic diseases is not well understood.[40,41] Moreover, neutrophil function has been reported to be impaired in acute-on-chronic liver failure, thus suggesting that both recruitment and function of neutrophils may be altered in the context of AH.[42–44] Whether the recruitment of neutrophils to the DR plays a deleterious role in the progression of the diseases or helps to orchestrate inflammatory response as a result of an acute-on-chronic injury is unknown.

There are no good markers to specifically identify facultative LPCs within the DR in the human liver. Therefore, liver organoids were generated in order to evaluate the transcriptome and the inflammatory profile of LPCs. Tissue organoids generated from stem/progenitor cells have shown great potential as disease models and as a source of cells for regenerative medicine.[21] Liver organoid growth is dependent on Wnt signaling by Wnt family member 3a and R-spondin, the ligand for LGR5,[45,46] a receptor present in most adult stem cells. Liver organoid technology has recently been described, and therefore, organoids generated from liver tissue are poorly characterized. Using this technology, we provide evidence that the gene expression profile of liver organoids from cirrhotic livers closely resembles DR from patients, showing a high gene expression profile overlap and a proinflammatory profile. Moreover, organoids respond to inflammatory mediators, enhancing chemokine expression. Therefore, these results suggest that liver organoids may be a good *in vitro* model to study DR in chronic liver diseases.

In conclusion, AH is characterized by impaired liver function, reduced regenerative potential, and important local and systemic inflammatory response. Several studies have suggested a role for LPCs as potential cellular targets to promote liver regeneration in

chronic liver diseases. The present study suggests that DR and LPCs may also play a role in liver inflammation, and therefore, targeting DR may represent a strategy to modulate intrahepatic inflammatory response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AH	alcoholic hepatitis
ALD	alcoholic liver disease
CCL	C-C motif chemokine ligand
CXC	C-X-C motif chemokine ligand
DR	ductular reaction
EpCAM	epithelial cell adhesion molecule
FC	Fold change
GO	gene ontologies
IL	interleukin
KRT7	cytokeratin 7
KRT19	cytokeratin 19
LPCs	liver progenitor cells
LPS	lipopolysaccharide

NF-κB	nuclear factor kappa B
TNF-α	tumor necrosis factor-alpha

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Author names in bold designate shared co-first authorship.

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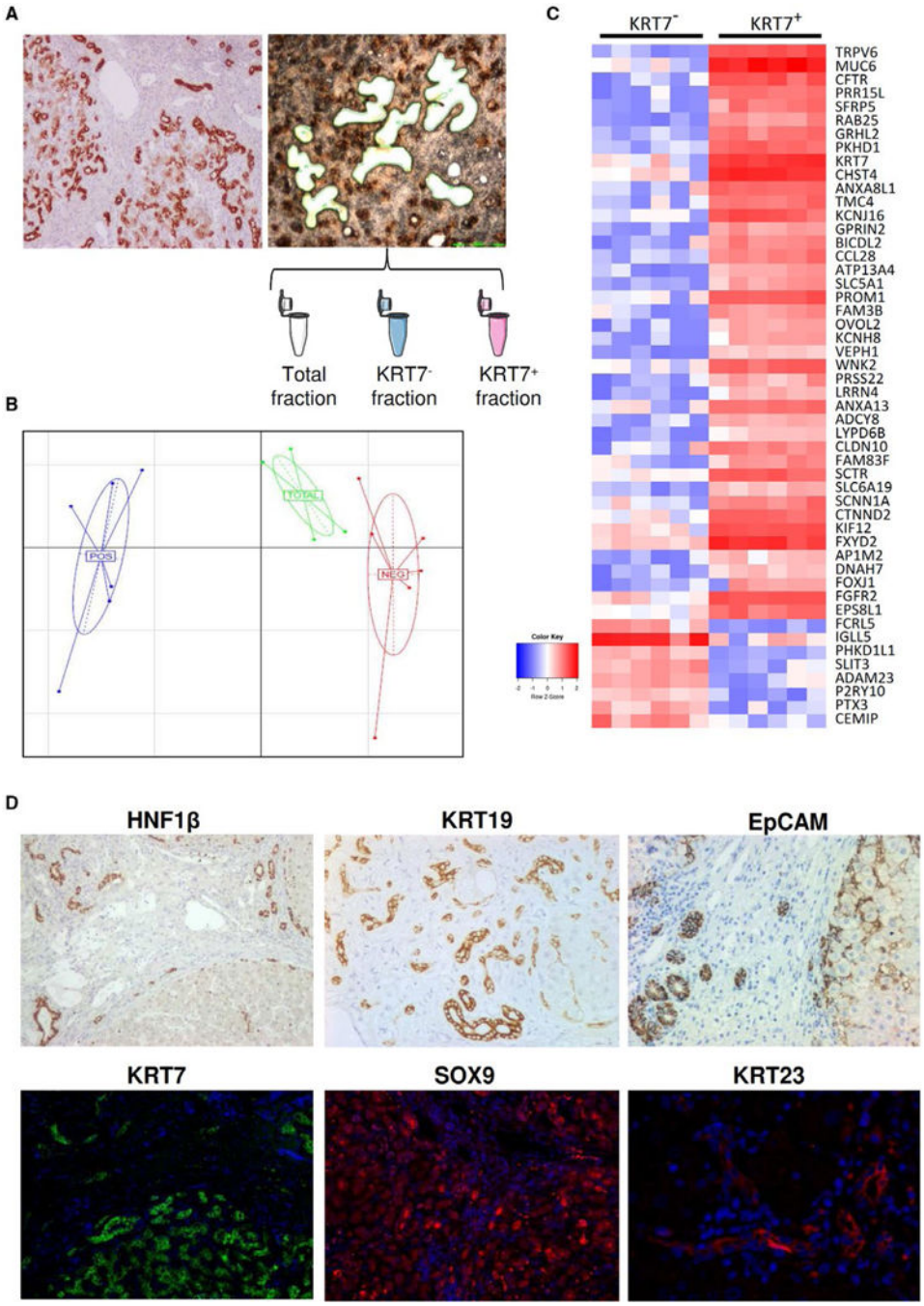

FIG. 1.

Gene expression profile of ductular reaction in AH. (A) KRT7 staining with and without counterstaining of paraffin-embedded liver sections of patients with AH. Scheme of populations selected by laser capture microdissection: KRT7-positive (n = 6), -negative (n = 6), and total (n = 4) areas. (B) Principal component analysis showing the three different fractions that underwent RNA sequencing. Each sample is placed in the two-dimensional space according to its RNA expression. The KRT7-positive fraction (POS) is represented in blue, the KRT7-negative fraction (NEG) in red, and the total fraction in green. (C) Heat

map of the top 50 differentially expressed genes in KRT7-positive fraction compared with the negative fraction. The red color indicates an enrichment of gene expression, whereas the blue color shows decreased gene expression. (D) Immunohistochemistry of liver progenitor cell markers HNF1β, KRT19, EpCAM, KRT7, SOX9, and KRT23 in hepatic sections of patients with AH. Abbreviation: HNF1β, hepatocyte nuclear factor 1β.

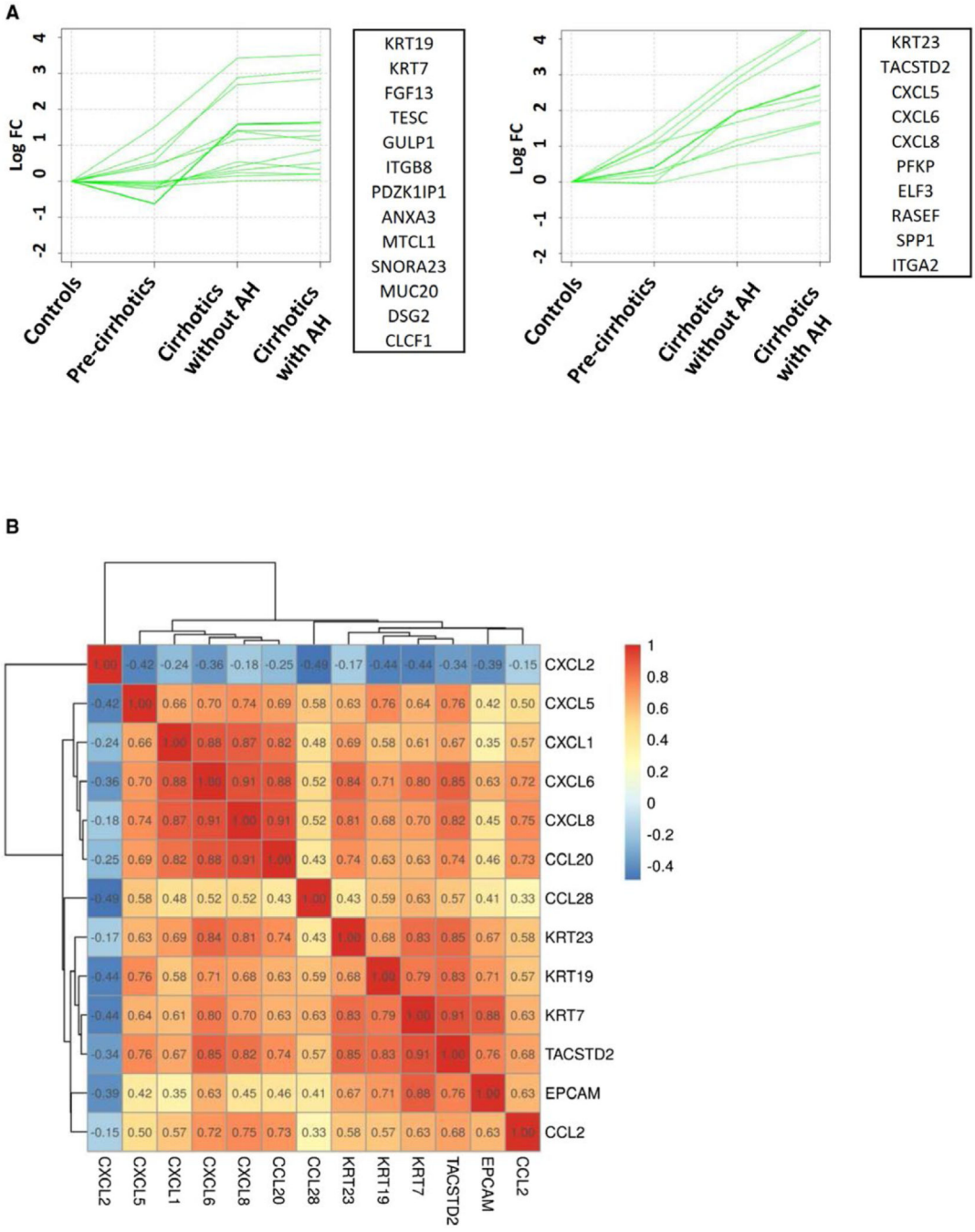

FIG. 2.

Expression of DR genes in ALD progression. (A) Expression of DR genes that positively correlate with MELD and Child-Pugh score along ALD progression (data set containing healthy controls [n = 6], patients with precirrhosis [n = 4], and patients with cirrhosis without AH [n = 7] and with AH [n = 11]). Two differentiated group of genes are represented according to the pattern of expression along ALD progression. (B) Correlation between expression of LPC markers and proinflammatory mediators in the ALD data set.

Correlations are shown as a heat map. Patient cohort characteristics are shown in Supporting Table S1. Abbreviation: FC, fold change.

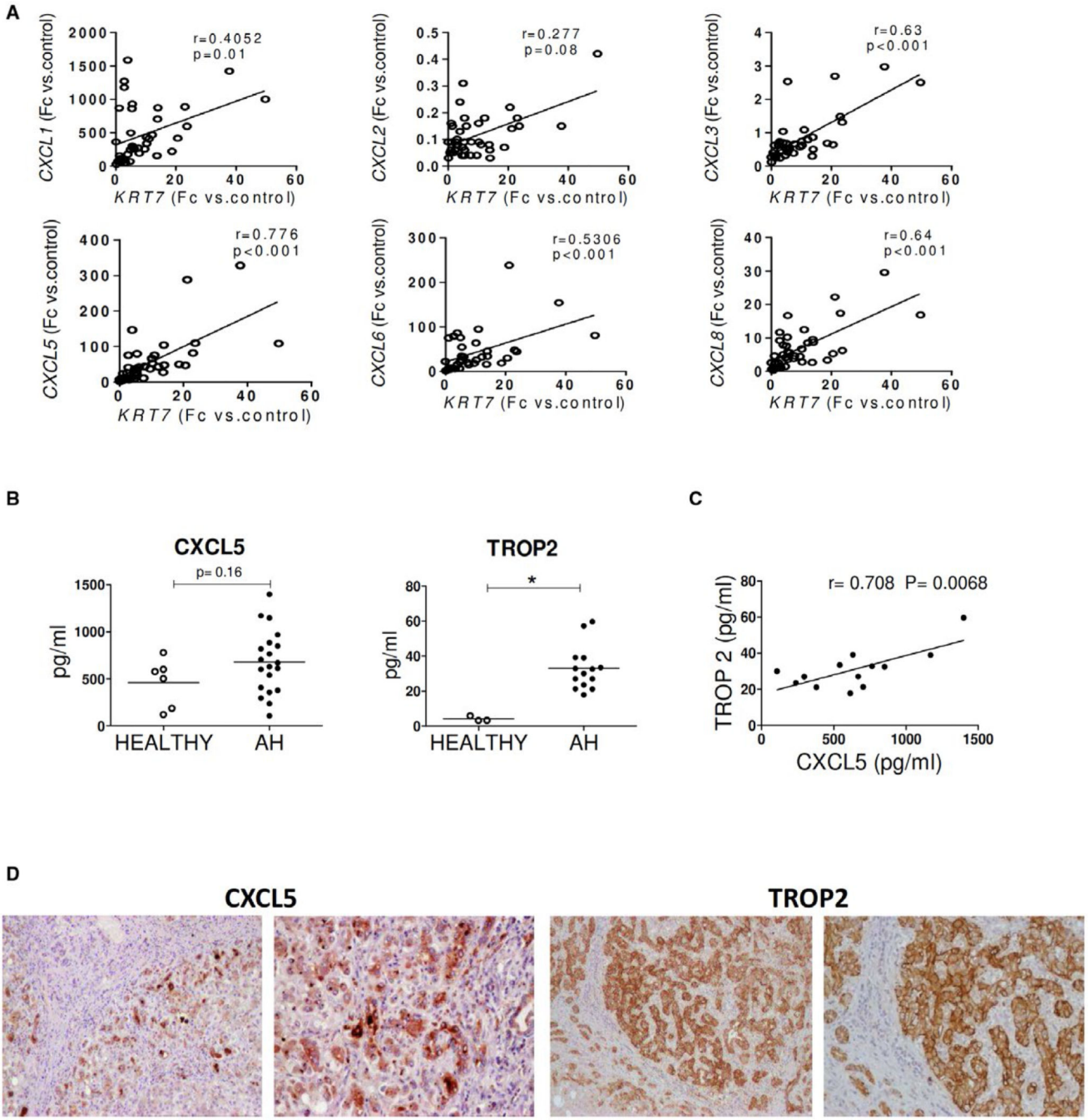

FIG. 3.

Correlation of ductular reaction with inflammatory mediators in patients with AH. (A) Correlation of real-time quantitative PCR gene expression (Fc vs. healthy individuals) of *KRT7* with inflammatory cytokines *(CXCL1, CXCL2, CXCL3, CXCL5, CXCL6,* and *CXCL8*) in patients with AH (n = 40). The regression coefficient (*r*) and *P* value of each correlation are indicated. (B) Serum levels (pg/mL) of CXCL5 and TROP2 in patients with AH (n = 20 and n = 14, respectively) compared with healthy individuals (n = 6 and n = 3, respectively); *, $P < 0.05$ compared with healthy individuals. (C) Correlation of CXCL5

and TROP2 serum levels (pg/mL) in patients with AH (n = 14). *r* value and *P* value is indicated. (D) Representative immunohistochemistry of CXCL5 and TROP2 in liver sections of patients with AH. Abbreviation: Fc, fold change.

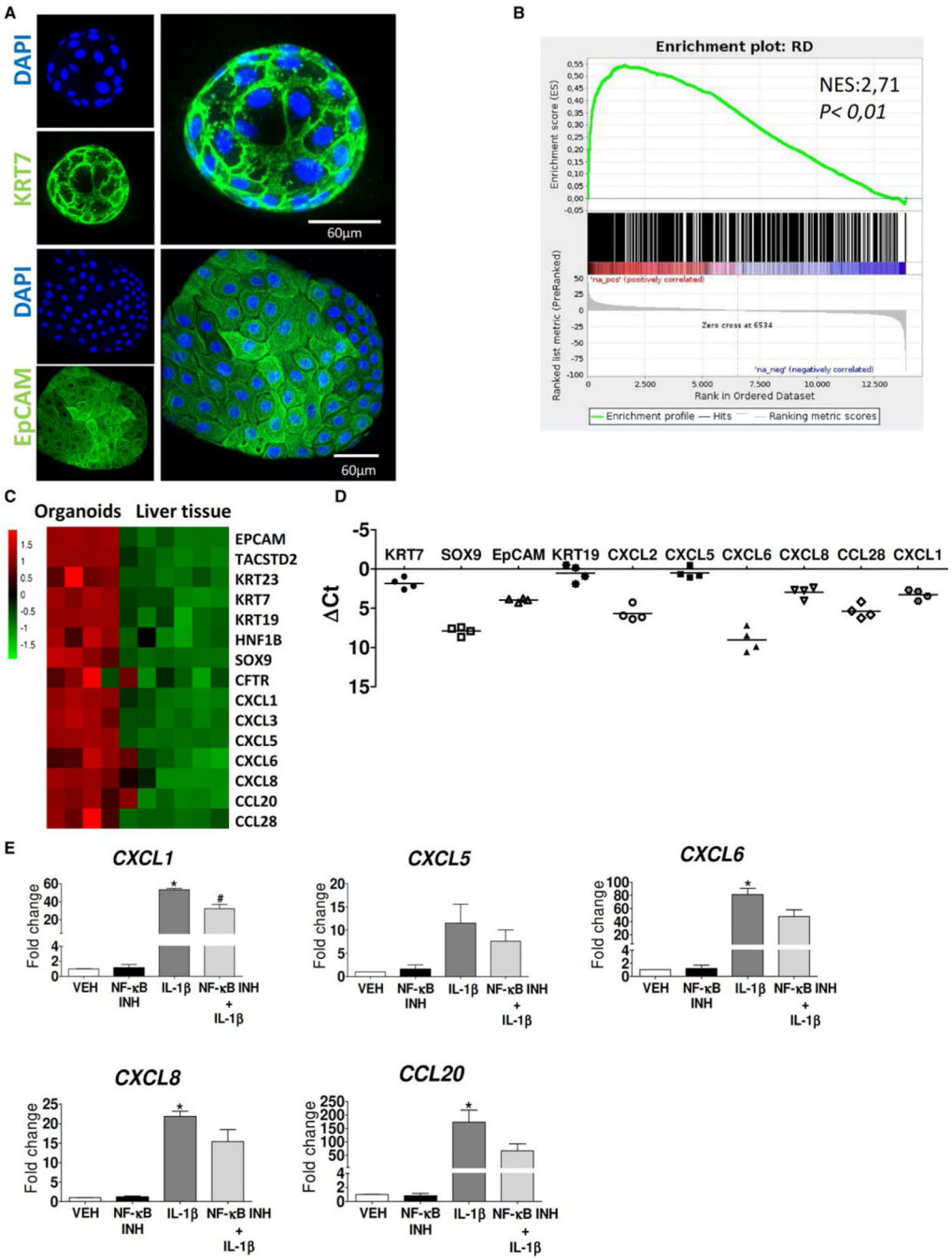

FIG. 4.

Transcriptomic and functional analysis of liver organoids from patients with cirrhosis. (A) Immunofluorescence of LPC markers, EpCAM and KRT7, in liver organoids. (B) Gene set enrichment analysis between liver organoid transcriptome and genes expressed in KRT7^{+} microdissected fraction. The gene expression profile of human organoids (n = 4) versus liver tissue from healthy patients (n = 4) was used as a data set. NES and significance is shown. (C) Heat map illustrating gene expression of LPC markers and inflammatory cytokines in liver organoids compared with liver tissue from healthy patients.

(D) Real-time PCR gene expression of LPC markers *(KRT7, KRT19, EpCAM,* and *SOX9)* and inflammatory mediators *(CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, and CCL28)* in human organoids. mRNA levels are shown as ΔCt values compared with glyceraldehyde 3-phosphate dehydrogenase Ct values (n = 4). (E) Expression of inflammatory cytokines in organoids after IL-1β stimulation and NF-κB inhibition. Organoids were incubated with VEH or NF-κB INH and stimulated with IL-1β for 6 hours. Organoid gene expression of *CXCL1, CXCL5, CXCL6, and CXCL8* is shown as fold change versus VEH. n = 3; *, $P < 0.05$ compared with VEH; #, $P < 0.05$ compared with IL-1β. Abbreviations: NE S, normalized enrichment score; NF-κB INH, NF-κB inhibitior; VEH, vehicle.

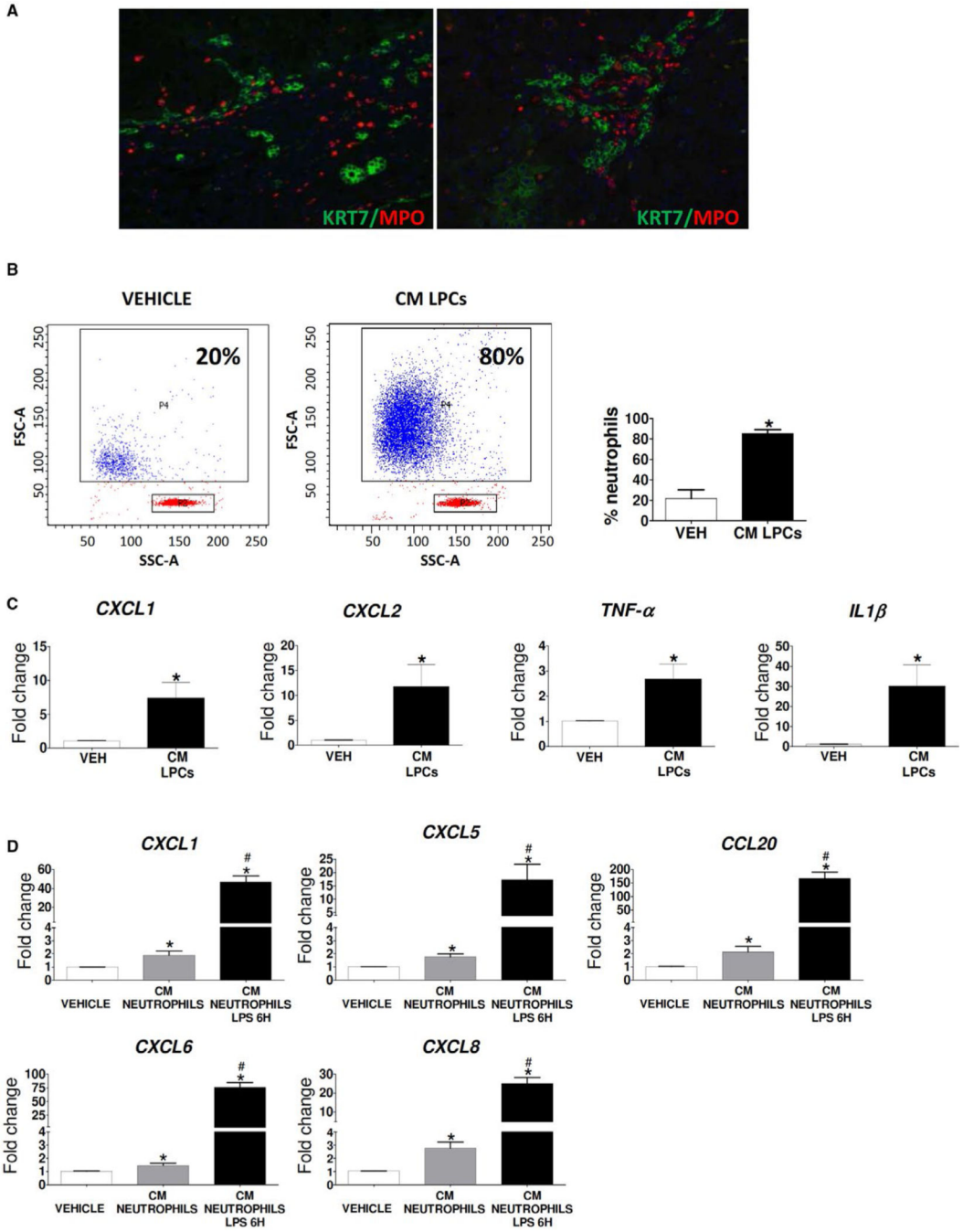

FIG. 5.

DR recruits neutrophils. (A) Representative double staining of KRT7 (green) and MPO (red) in alcohol-induced cirrhotic liver sections from 2 different patients. (B) Neutrophil migration assay in response to CM LPCs. Conditioned medium from three different organoid lines was used in three independent neutrophil isolations. (C) Neutrophil response to organoid-conditioned medium. Freshly isolated human neutrophils were incubated with CM LPCs from 3 different patients with cirrhosis for 18 hours. Gene expression is shown as fold change versus VEH after incubation with CM LPCs. n = 3; *, $P < 0.05$ compared with

VEH. (D) Organoid response to neutrophil-conditioned medium. Conditioned media from neutrophils stimulated with vehicle or LPS for 6 hours was used for organoid stimulation. Results are shown as fold change versus VEH after incubation with CM of neutrophils. n = 5; *, $P < 0.05$ compared with VEH. #, $P < 0.05$ compared with CM. Abbreviations: CM LPCs, liver progenitor cells organoid conditioned medium; VEH, vehicle.

TABLE 1.

Baseline Characteristics of Patients With Alcoholic Hepatitis From Whom Samples Were Obtained for Microdissection

Characteristic	Severe AH (n = 6), Median (25–75 IQR)
Demographic data	
Age, median (IQR)	54 (47–60)
Sex, male, n (%)	3 (50)
Severity scores	
Child-Pugh, median (IQR)	12 (11–13)
MELD, median (IQR)	29 (27–31)
ABIC, median (IQR)	7.9 (6.9–9.0)
HVPG, median (IQR)	nd
SIRS, n (%) *	
Decompensations, n (%)	
Ascites	4 (67)
HE	3(50)
UGIB	4 (67)
AKI	4 (67)
Infections	5 (83)
Analytical parameters, median (IQR)	
Hemoglobin g/dL	9.2 (9.2–11.3)
Leukocyte count ×109/L	12.2 (8.3–20.5)
Platelet count ×109/L	107 (83–120)
AST (IU/L)	177 (146–201)
ALT (IU/L)	58 (44–71)
Albumin (g/dL)	2.4 (2.1–2.6)
Creatinine (mg/dL)	0.8 (0.8–1.5)
Bilirubin (mg/dL)	15.0 (11.0–22.9)
INR	2.1 (1.9–2.3)
GGT (IU/L)	367 (272–720)
ALP (IU/L)	260 (233–304)
Sodium (mEq/L)	129 (127–130)

* SIRS alone or SIRS plus infection.

Abbreviations: ABIC, age-bilirubin-INR-creatinine score; AH, alcoholic hepatitis; AKI, acute kidney injury; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HE, hepatic encephalopathy; HVPG, hepatic venous pressure gradient; INR, international normalized ratio; IQR, interquartile range; MELD, Model for End-Stage Liver Disease; SIRS, systemic inflammatory response syndrome; UGIB, upper gastrointestinal bleeding.

TABLE 2.

Functional Analysis of Up-Regulated Genes in Microdissected KRT7^{+} Fraction

GO Pathway	*P* Value	IPA Pathway	*P* Value
Cell adhesion	4.2E-14	Agranulocyte adhesion and diapedesis	3.34E-03
Inflammatory response	6.8E-10	Granulocyte adhesion and diapedesis	3.39E-03
Wnt signaling pathway	9.0E-07	Ephrin a signaling	9.50E-03
NIK/NF-kappaB signaling	1.3E-06	Leukocyte extravasation signaling	2.10E-02
Cell-cell signaling	4.0E-06	ErbB signaling	4.30E-02
Leukocyte migration	4.2E-06	Tight junction signaling	5.01E-02
Neutrophil chemotaxis	6.4E-06		
Cell-matrix adhesion	8.8E-06		
Chemokine-mediated Signaling pathway	1.1E-05		
Cell chemotaxis	1.3E-05		
Response to hypoxia	3.6E-05		

Abbreviations: ErbB, erythroblastic leukemia viral oncogene homolog; GO, gene ontologies; IPA, ingenuity pathway analysis.

TABLE 3.

Functional Analysis of Up-Regulated Genes in Cirrhotic Organoids Transcriptome

KEGG	*P* Value	IPA Pathways	*P* Value
Hippo signaling pathway	2.16E-06	Wnt/β-catenin signaling	7.76E-05
Tight junction	5.18E-06	ERK/MAPK signaling	8.51E-05
Apoptosis	1.56E-05	PI3K/AKT signaling	1.29E-04
TNF signaling pathway	7.98E-04	IL-8 signaling	1.51E-04
Leukocyte transendothelial migration	2.66E-3	Chemokine signaling	1.70E-04
ErbB signaling pathway	9.92E-3	Granulocyte adhesion and diapedesis	3.33E-2
Wnt signaling pathway	1.33E-2	NF-κB activation by viruses	3.55E-2
TGF-beta signaling pathway	4.71E-2		

Abbreviations: ErbB, erythroblastic leukemia viral oncogene homolog; PI3K, phosphoinositide 3-kinase; TGF, transforming growth factor.