Research Article

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Cirrhosis

Absent in melanoma 2 triggers a heightened inflammasome response in ascitic fluid macrophages of patients with cirrhosis

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Background & Aims: Inflammation is a common event in the pathogenesis of liver cirrhosis. The inflammasome pathway has acquired significant relevance in the pathogenesis of inflammation, but its role in the inflammatory response in patients with decompensated cirrhosis remains unexplored.

Methods: We performed a prospective study in which 44 patients with decompensated cirrhosis and 12 healthy volunteers were included. We isolated macrophages from blood and ascitic fluid and assessed the expression and activation of the inflammasome, its response to priming by bacterial products, and its association with the degree of liver disease.

Results: Macrophages from sterile ascitic fluids showed constitutive activation of caspase-1 and a marked increase in the expression of IL-1β, IL-18, and absent in melanoma 2 (AIM2) when compared to blood macrophages. Pre-stimulation of blood-derived macrophages from cirrhotic patients with bacterial DNA increased the expression of AIM2 and induced a higher AIM2-mediated inflammasome response than priming with other bacterial products such as lipopolysaccharide. By contrast, activation of the AIM2 inflammasome did not require a priming signal in ascitic fluid-derived macrophages, demonstrating the preactivated state of the inflammasome in these cells. Last, higher IL-1β and IL-18 production by ascitic fluid macrophages correlated with a more advanced Child-Pugh score.

Conclusions: The inflammasome is highly activated in the ascitic fluid of cirrhotic patients, which may explain the exacerbated inflammatory response observed in these patients under non-infected conditions. Clinically, activation of the inflammasome is associated with a higher degree of liver disease.

Keywords: Inflammasome; Absent in melanoma 2; Ascitic fluid.

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Abbreviations: AF, ascitic fluid; AIM2, absent in melanoma 2; EcDNA, genomic bacterial DNA from E. coli; Poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid sodium salt; SBP, spontaneous bacterial peritonitis; PRRs, pattern recognition receptors.

Introduction

A key innate immune response that is often associated with sterile inflammation is the rapid activation of cytosolic protein complexes termed inflammasomes [1]. Full activation of the inflammasome requires two different signals. The first signal is provided by the engagement of pattern recognition receptors (PRRs), mainly Toll-like receptors (TLRs), resulting in the transcriptional upregulation and accumulation of inactive pro-IL-1β and pro-IL-18 inside the cell. The second signal can be provided by a plethora of molecules, including microbial motifs known as pathogen-associated molecular patterns (PAMPs), as well as host-derived signals known as damage-associated molecular patterns (DAMPs) that are released as a result of tissue damage or cellular stress. These molecules activate cytosolic receptors, including the nucleotide-binding domain leucine-rich repeat (NLR) proteins NLRP1, NLRP3 or NLRC4. Upon activation these sensors recruit pro-caspase-1, which in turn becomes active and cleaves pro-IL-1β and pro-IL-18 into biologically active cytokines [1,2]. In addition, the HIN-200 domain-containing protein absent in melanoma 2 (AIM2) has been recently described as the first non-NLR receptor that induces inflammasome activation [3,4]. Each of these sensors is activated by different danger signals. NLRP3, for example, is activated by a wide variety of PAMPs and DAMPs (e.g., pore-forming toxins, ATP, uric acid, silica crystals) [2,5,6], whereas AIM2 is activated only by double-stranded DNA (dsDNA) independently of its source [3,4].

Emerging evidence suggests that the inflammasome plays a crucial role in chronic liver disease [7]. Activation of the inflammasome has been documented in response to HCV infection [8], in drug-induced liver injury [9], or in the transformation of non-alcoholic fatty liver disease to non-alcoholic steatohepatitis and liver fibrosis in mice [10]. Patients with decompensated cirrhosis frequently develop episodes of bacterial translocation from the intestinal lumen to extra-intestinal sites such as the ascitic fluid (AF). In

addition to these infectious events, we have previously shown that the presence of molecules of microbial origin (e.g., bacterial DNA) is sufficient to mount a sterile inflammatory response in the AF of these patients, which is associated with severe complications and poor prognosis and survival [11–13]. However, the impact of the inflammasome in these sterile inflammatory complications remains unexplored. The purpose of this study was to uncover the role of the inflammasome in sterile inflammatory responses occurring in cirrhotic patients, as well as its association with the clinical stage of the disease.

Patients and methods

Patient samples

A prospective series of 44 patients with decompensated cirrhosis, admitted at the Liver Unit of the University Hospital of Alicante (HGUA), and 12 healthy volunteers were included in this study. The protocol was supervised and approved by the Clinical Research Ethics Committee of the HGUA and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent to participate in the study was obtained from each patient and healthy volunteer. Inclusion criteria for patients were age between 25 and 65 years, diagnosis of cirrhosis with presence of AF, and negative bacterial culture of blood and AF. Inclusion criteria for controls were age between 25 and 65 years, absence of cirrhosis or excessive alcohol consumption (more than 2 drinks per day or 14 drinks per week), and negative blood cultures. Cirrhosis was diagnosed by clinical, biological, ultrasonographical, and/or histological criteria. Exclusion criteria were the presence of culture-positive blood or AF, upper gastrointestinal bleeding, hepatocellular carcinoma beyond Milan criteria [14], portal thrombosis, previous liver transplantation, transjugular intrahepatic portosystemic shunt (TIPS), prescription of vasoactive drugs, presence of neutrocytic ascites (PMN >250 cells/mm^3), bacterial infection, treatment with antibiotics in the preceding two weeks, and refusal to participate in the study. Five patients had a previous history of spontaneous bacterial peritonitis (SBP), which occurred more than 24 months before their inclusion in the study, and these patients were noncompliant with the continuous norfloxacin treatment.

Blood was obtained for routine haematologic, biochemical, and coagulation tests. AF samples were obtained in aseptic conditions from large volume paracentesis performed as part of the patient's clinical treatment. Both blood and AF samples were inoculated at bedside in aerobic and anaerobic culture bottles. CD14+ macrophage isolation for *in vitro* stimulation studies was performed in a randomly selected subset of patients (n = 18) and in all controls (n = 12). Cytokine measurements were performed in serum and AF from all the patients (n = 44) and in serum from all healthy controls (n = 12). The clinical and analytical characteristics of patients and controls are shown in Supplementary Table 1.

Isolation of RNA and quantitative RT-PCR

Isolation of RNA was carried out using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After isolation, RNA was treated with DNase I (Life Technologies, Carlsbad, CA) to digest contaminating DNA. 10 ng of RNA sample was then used for one-step qRT-PCR using QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) for gene expression analysis. GAPDH expression was used as internal reference in all experiments. RT-PCR primers for specific target genes were designed based on their reported sequence (Supplementary Table 2).

Quantification of cytokine levels

Enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of IL-1β and IL-18 levels as representative cytokines of inflammasome activation were performed using Ready-SET-Go ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions. All samples were tested in triplicate. The lower limits of detection for all cytokine assays were 20–25 pg/ml. Standard curves were generated for each plate, and the average optical density of the zero standard was subtracted from the rest of the standards and samples to obtain a corrected concentration for all cytokines.

Culture and stimulation of AF macrophages

Advanced RPMI 1640 medium (Life Technologies, Carlsbad, CA), supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin was used throughout the experiments. CD14+ cells were isolated by gradient centrifugation using Biocoll solution (Biochrom, Berlin, Germany) and then CD14+ immunomagnetic selection using the CD14+ human isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched CD14+ population from AF was then cultured overnight in complete RPMI medium at a concentration of 10^6 CD14+ cells/ml in 24-well plates. Purity of the enriched population was assessed by flow cytometry and was greater than 95% in all experiments (Supplementary Fig. 1). For pre-stimulation experiments, different concentrations of bacterial DNA and ultrapure LPS were initially titrated (Supplementary Fig. 2). Optimal stimulation was achieved with either 100 ng/ml of ultrapure LPS from *S. minnesota* (InvivoGen, San Diego, CA) or 100 ng/ml of genomic bacterial DNA isolated from *E. coli* (EcDNA). EcDNA was treated with 50 μg/ml of polymixin B (InvivoGen, San Diego, CA) to eliminate the effects of endotoxin contamination.

After 3 h of pre-stimulation with LPS or EcDNA, cells were washed with sterile PBS and fresh media was added to each well. Then, AIM2 stimulation was triggered by transfecting 1.5 μg/ml of the synthetic dsDNA poly(dA:dT) (InvivoGen, San Diego, CA) to the cytosol of the cultured macrophages using lipofectamine 2000 Transfection Reagent (Life Technologies, Carlsbad, CA). NLRP3 was activated with 5 mM of ATP (InvivoGen, San Diego, CA) after LPS pre-stimulation.

Culture and stimulation of blood macrophages

CD14+ cells were isolated from peripheral blood mononuclear cells (PBMCs) by immunomagnetic cell sorting as indicated above. Cells were then cultured at 10^6 cells per ml in 24-well plates in the presence of 25 ng/ml of recombinant human macrophage-colony stimulating factor (M-CSF) (Sigma-Aldrich, St. Louis, MO) to stimulate the formation of macrophage colonies. Fresh media containing M-CSF was added at day 3 after initial seeding. Cells were stimulated at day 5 following the protocols described above for AF-derived macrophages. For some experiments, cells were treated with 10 ng/ml of an AIM2 inhibitor oligodeoxynucleotide (ODN) containing suppressive TTAGGG motifs (InvivoGen, San Diego, CA) 3 h before poly(dA:dT) transfection.

Immunoblot

For Western blot analysis, cells were collected and total cell lysates were obtained in lysis buffer containing 0.15 M NaCl, 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM NaF, 1 mM Na3VO4, 10 mM KCl, 0.5% NP-40, and protease inhibitor cocktail (10%, vol/vol) (Sigma-Aldrich, St. Louis, MO). Proteins (20 μg/lane) were then boiled at 95 °C in the presence of LDS sample buffer and 2-mercaptoethanol (Life Technologies, Carlsbad, CA), subjected to SDS PAGE and then transferred to Immun-Blot PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 30 min in 3% BSA and 0.05% Tween 20 in PBS and incubated overnight with either rabbit anti-caspase-1 (Cell Signaling, Danvers, MA), mouse anti-AIM2 (Adipogen, San Diego, CA), mouse anti-NLRP3 (Adipogen), or mouse anti-β-actin specific antibodies (Sigma-Aldrich, St. Louis, MO), and then for 1 h at room temperature with the correspondent anti-mouse or anti-rabbit IgG-HRP secondary antibody (Jackson Immunoresearch, West Grove, PA). The activity of membrane-bound peroxidase was detected using the ECL system from Thermo Scientific (Waltham, MA) and scanned in a ChemiDoc XRS+ system (Bio-Rad, Hercules, CA). Quantification of protein bands was made using ImageJ software (http://imagej.nih.gov/ij/). The intensity of each protein band was normalized to the intensity of its corresponding β-actin band from the same blot.

Detection of bacterial DNA fragments

The detection of bacterial DNA (bactDNA) in AF was carried out by conventional broad-range polymerase chain reaction (PCR) of the bacterial 16S rRNA gene as previously described [15]. Amplification of the gene was performed using the following universal primers: 5′-AGAGTTTGATCATGGCTCAG-3′ and 5′-ACCGCGACTGCTGCTGGCAC-3′.

Follow-up of patients

Clinical and analytical data of all patients were recorded on inclusion in the study together with previous clinical history. Data from successive hospitalizations including reasons for admission, therapy, and diagnosis at discharge were recorded during the follow-up period. Management of patients was as usual according to the patient's clinical requirements. Follow-up was finalized if the patient was submitted to TIPS or liver transplantation. The causes of death were recorded for final analysis.

Statistical analysis

Continuous variables are displayed as mean ± standard deviation, and categorical variables as frequencies or percentages. Statistical differences between groups

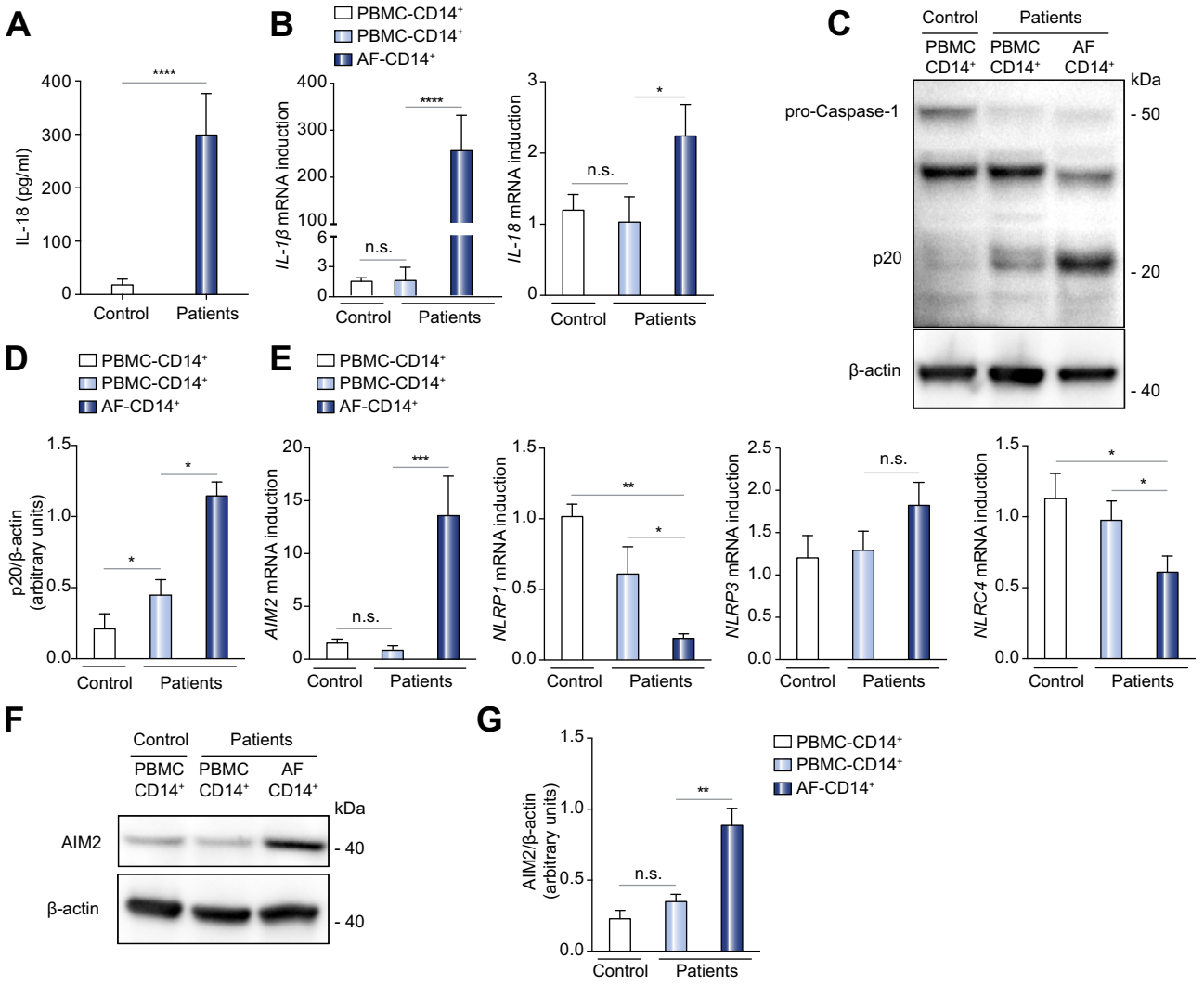


Fig. 1. Constitutive activation of the inflammasome in AF macrophages. (A) IL-18 level in serum from healthy controls and cirrhotic patients measured by ELISA. (B) Quantitative PCR analysis of unstimulated CD14+ macrophages from peripheral blood mononuclear cells (PBMCs) of healthy controls and of PBMCs and ascitic fluid (AF) from cirrhotic patients. (C) Representative immunoblot of caspase-1 p20 (active subunit) in CD14+ cells from healthy controls and cirrhotic patients. (D) Quantification of p20 protein bands in each group by densitometry analysis. The intensity of the bands was normalized to the intensity of β-actin. Data (C and D) represent results from six independent experiments. (E) Quantitative PCR analysis of inflammasome receptors in unstimulated CD14+ macrophages from PBMCs of healthy controls and from PBMCs and AF of cirrhotic patients. (F) Representative immunoblot of AIM2 protein in CD14+ cells from healthy controls and cirrhotic patients. (G) Quantification of AIM2 protein bands in each group by densitometry analysis. The intensity of the bands was normalized to the intensity of β-actin. Data (F and G) represent results from three independent experiments. Error bars represent mean ± SD. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001, $^{****}p$ <0.0001. n.s., not significant.

were analysed using the nonparametric Mann-Whitney *U* test for quantitative data and χ^2 test for categorical data. Bivariate correlations between continuous variables were calculated using the Spearman's rho test. Covariance analysis (ANCOVA) was used to study interaction effects of categorical variables, such as gender or Child-Pugh class, on the IL-18 levels after controlling for the effects of continuous variables. Logistic regression analysis was performed to determine the association between clinical variables and SBP occurrence. Results of logistic regression are reported as odds ratio and 95% confidence interval (CI). All *p* values were 2-tailed, and *p* values lower than 0.05 were considered significant. All calculations were performed using GraphPad Prism 6.0 or SPSS 16.0 software.

Results

The inflammasome is highly activated in AF macrophages

To investigate the potential role of the inflammasome in the inflammatory processes that take place in patients with decompensated cirrhosis, we first measured the levels of inflammasome-related cytokines in serum samples from patients and healthy controls. IL-1β levels in serum were below the limit of detection in all the controls and in more than 80% of the patients (data not shown). By contrast, biologically active IL-18 was consistently detectable in the serum from patients and showed significantly higher levels than those detected in serum from healthy controls (Fig. 1A). Quantitative PCR analysis of highly pure CD14+ macrophages (Supplementary Fig. 1) isolated from AF of patients and from PBMCs of controls and patients revealed that macrophages from AF express high levels of pro-IL-1β and pro-IL-18 mRNA at baseline (Fig. 1B), suggesting that these cells are primed to mount an inflammasome response. To better define the activation state of the inflammasome we analysed the amount of active caspase-1 (caspase-1 p20 subunit) by immunoblot. Caspase-1 p20 was absent in PBMC-derived CD14+

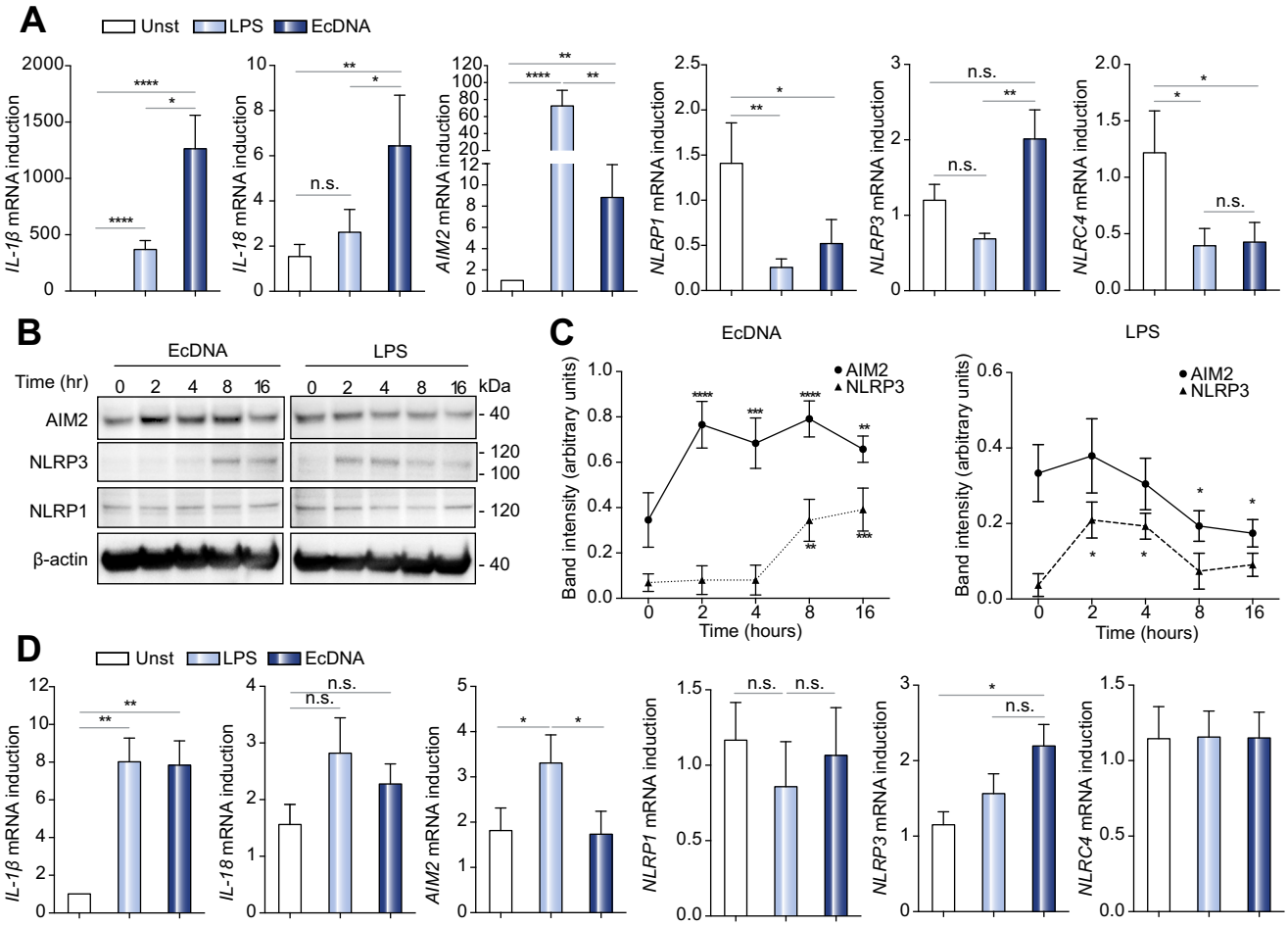


Fig. 2. Bacterial DNA increases the expression of AIM2 in cirrhotic patients. (A) Quantitative PCR analysis of inflammasome components in PBMC-derived CD14+ macrophages isolated from cirrhotic patients and stimulated with 100 ng/ml of bacterial DNA from *E. coli* (EcDNA) or ultrapure LPS for 3 h. (B) Representative immunoblot of different inflammasome receptors in PBMC-derived CD14+ cells, isolated from cirrhotic patients and stimulated with EcDNA or LPS for the indicated time points. (C) Quantification of the AIM2 and NLRP3 protein bands by densitometry analysis. Changes in AIM2 and NLRP3 protein expression after EcDNA or LPS stimulation were compared with the unstimulated control (0 h). The intensity of bands was normalized to the intensity of β-actin. Data (B and C) represent results from three independent experiments. (D) Quantitative PCR analysis in AF-derived macrophages stimulated with 100 ng/ml of EcDNA or LPS for 3 h. Error bars represent mean ± SD. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{****}p$ <0.0001. n.s., not significant.

macrophages from healthy controls and only modestly present in PBMC-derived CD14+ cells from patients. By contrast, the basal expression of active caspase-1 p20 was markedly augmented in cells from AF of the same patients (Fig. 1C and D). Since several inflammasome-associated receptors may be responsible for the activation of caspase-1, we measured the expression of *NLRP1*, *NLRP3*, *NLRC4* and *AIM2*. AF macrophages showed high mRNA levels of *AIM2*, whereas the transcriptional level of the other receptors was either unaltered or reduced (Fig. 1E). In accordance with the mRNA data, the protein level of AIM2 was also significantly increased in AF macrophages of cirrhotic patients (Fig. 1F and G). Moreover, the transcriptional level of pro-IL-1β in AF macrophages positively correlated with the mRNA levels of *AIM2* ($r = 0.747$, $p = 0.004$), but not with the other inflammasome receptors (Supplementary Fig. 3). Together, these results indicate that the AF environment favours the expression of AIM2 and the constitutive activation of caspase-1, which subsequently activates the inflammasome response in innate immune cells.

Microbial products predominantly activate AIM2 expression

The first step in the activation of the inflammasome involves the transcriptional upregulation of different molecules in response to an inflammatory stimulus via the activation of innate receptors [16]. To determine the transcriptional profile associated with the inflammasome in response to pro-inflammatory stimuli in cirrhotic patients, we stimulated PBMC-derived CD14+ macrophages *in vitro* with either genomic bacterial DNA from *E. coli* (EcDNA) or ultrapure LPS. Both stimulations resulted in a striking increase in the transcriptional levels of pro-IL-1β, whereas pro-IL-18 expression was significantly increased only by EcDNA but not by LPS (Fig. 2A). *AIM2* mRNA expression was also highly inducible in response to both LPS and EcDNA, while the expression of other inflammasome receptors was unaffected or reduced (Fig. 2A), emphasizing the differential impact of these priming signals in the expression of each inflammasome. PBMC-derived CD14+ cells from healthy controls showed very similar expression patterns upon stimulation (Supplementary Fig. 4). When we analysed

the expression of these receptors at the protein level, we observed that EcDNA stimulation also resulted in a relatively fast (2 h) increase in the amount of total AIM2 protein but in a more delayed increase in the amount of total NRLP3 protein (8 h) (Fig. 2B and C). By contrast, LPS stimulation induced a fast increase in NLRP3 protein (2 h) but this was accompanied by a reduction in the expression of AIM2 protein during the tested time points (Fig. 2B and C). Both stimulations were unable to increment the protein expression of NLRP1. These results suggest that there is a differential regulation of inflammasome-related receptors by microbial ligands and therefore different pro-inflammatory stimuli may have different impact in the activation of each inflammasome.

In AF-derived macrophages, by contrast, *in vitro* stimulation with EcDNA or LPS was unable to further increase the expression of pro-IL-18 and resulted only in a weak upregulation of pro-*IL-1β* and *AIM2* mRNA (Fig. 2D) when compared to the induction obtained in PBMC-derived macrophages from the same patients (Fig. 2A). These data, together with the data shown in Fig. 1, suggest that the machinery of the inflammasome is already activated in AF macrophages of cirrhotic patients at baseline.

Priming with bacterial DNA induces a strong AIM2-mediated response in blood macrophages

The data presented above led us to hypothesize that the AIM2 inflammasome may play an important role in the sterile inflammatory processes that take place in cirrhotic patients [11,12]. We therefore analysed the function of the AIM2 inflammasome by transfecting poly(dA:dT), a synthetic dsDNA, to the cytosol of cultured macrophages and then measuring the secretion of active IL-1β and IL-18 as readout. As a benchmark of inflammasome activation, we used the prototypical stimuli of the NLRP3 inflammasome, i.e., LPS pre-stimulation plus ATP.

Transfection of poly(dA:dT) alone in control macrophages did not generate secretion of IL-1β or IL-18 (Fig. 3A), indicating that a priming signal is required in these cells. Of note, priming cells with EcDNA induced a very strong AIM2-mediated inflammasome response, which was higher than the AIM2 response obtained after priming with LPS and the NLRP3-mediated response triggered by LPS and extracellular ATP (Fig. 3A). To confirm that this response is mediated by AIM2, we used a suppressive oligodeoxynucleotide (ODN) that functions as a competitive inhibitor of AIM2 [17]. As expected, pretreatment of PBMC-derived CD14+ cells with the AIM2 inhibitor abrogated the secretion of mature IL-1β and IL-18 after LPS and poly(dA:dT) stimulation, but not after LPS and ATP stimulation (Fig. 3B). These data are consistent with previous reports showing that AIM2 is strictly required for the production of IL-1β and IL-18 in response to poly(dA:dT) in murine cells [18].

In contrast to healthy control macrophages, PBMC-derived macrophages from cirrhotic patients secreted detectable amounts of IL-1β and IL-18 after transfection of poly(dA:dT) alone, although these cells also required a priming signal to mount a robust AIM2-mediated inflammasome response (Fig. 3C). Notably, EcDNA pre-stimulation also induced a superior AIM2-mediated response in these cells than LPS pre-stimulation (Fig. 3C).

Activation of the AIM2 inflammasome does not require a priming signal in the AF

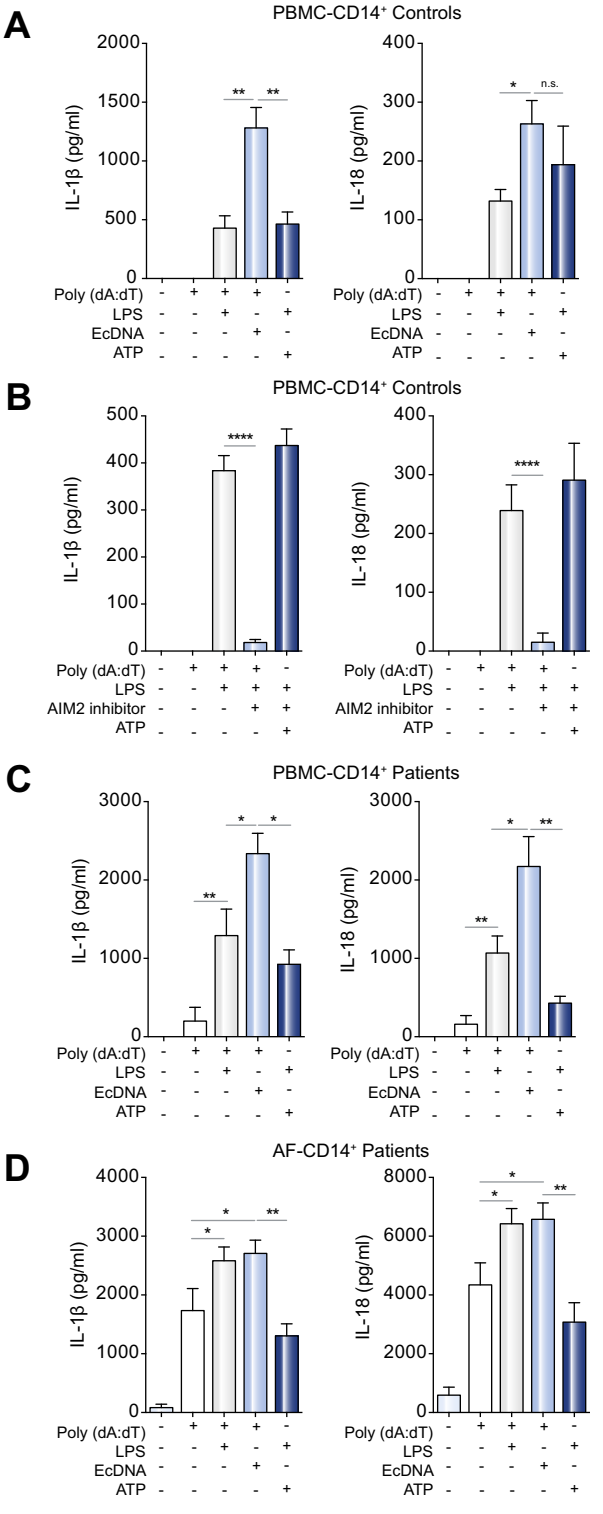
In striking contrast to PBMC-derived macrophages, poly(dA:dT) transfection alone generated the secretion of large quantities of IL-1β and IL-18 in AF macrophages without the need for a priming signal (Fig. 3D). This response by AF macrophages was markedly higher than the response by PBMC-derived macrophages from the same patients, stimulated only with poly(dA:dT) (IL-1β, $p = 0.0001$; IL-18, $p = 0.0003$). To confirm the relationship between the transcriptional expression of AIM2 in AF cells and their ability to secrete biologically active IL-1β and IL-18 without priming, we performed a Spearman's rho test. As expected, the level of *AIM2* mRNA showed a high positive correlation coefficient with the secretion of IL-1β ($r = 0.62$; $p = 0.028$) and IL-18 ($r = 0.72$; $p = 0.005$), whereas the mRNA levels of *NLRP3*, *NLRP1*, or *NLRC4* showed either no correlation or a negative correlation coefficient (Supplementary Fig. 5A and B). Moreover, priming of AF macrophages with LPS or EcDNA induced only a modest increase on the subsequent AIM2 activation by poly(dA:dT) transfection (Fig. 3D), further reflecting the pre-activated state of the AIM2 inflammasome in these cells. Notably, the total amount of IL-18, secreted after priming with microbial ligands, was significantly higher by AF-derived macrophages than by PBMC-derived macrophages (LPS, $p = 0.00019$; EcDNA, $p = 0.00013$), whereas the total secretion of IL-1β was higher only after LPS priming ($p = 0.0018$), but not after EcDNA priming ($p = 0.26$), probably due to the strong priming effect that EcDNA exerted on PBMC-derived cells. Of interest, full activation of the AIM2 inflammasome by EcDNA and poly(dA:dT) always resulted in a more potent secretion of IL-1β and IL-18 than the activation of the NLRP3 inflammasome by LPS and ATP.

Inflammasome activity correlates with the degree of liver disease and the presence of bacterial DNA in the AF

It has been proposed that translocation of enteric organisms and inflammation in fully developed cirrhosis increases, depending on the degree of liver damage and the clinical stage of the disease [19]. In turn, the influx of bacteria or bacterial products into the portal circulation or the mesenteric lymph fluid and, from there, into the AF, might lead to the activation of the inflammasome in ascites. Thus, we hypothesized that the severity of liver disease could affect the extent of inflammasome activation in cirrhotic patients. Indeed, patients with Child-Pugh score C showed a significant increase in the mRNA level of pro-*IL-1β* and pro-*IL-18* in isolated AF macrophages (Fig. 4A). Consistent with these data, AF macrophages from Child-Pugh score C patients produced higher amounts of IL-β and IL-18 after *in vitro* stimulation with EcDNA and poly(dA:dT) (Fig. 4B). Furthermore, AF macrophages from these patients also showed an increase in the basal level of mature caspase-1 p20 subunit (Fig. 4C and D), showing that the inflammasome complex is further activated in basal conditions in these patients. These results may explain previous reports, showing increased inflammatory cytokine production in advanced phases of cirrhosis [20,21].

We have previously reported that the presence of bacterial DNA (bactDNA) in AF is associated with exacerbated inflammation and increased risk of mortality in cirrhotic patients [13]. To investigate whether the presence of bactDNA in AF influences the activation of the inflammasome, we collected AF macrophages from a new group of Child-Pugh B patients in whom we detected the presence of bacterial DNA in AF and we compared their activation with AF macrophages from our initial series of Child-Pugh B patients (no presence of bacterial DNA in

AF). Interestingly, the mRNA level of pro-*IL-1β* and pro-*IL-18* was elevated in AF macrophages from patients with bacterial DNA (Supplementary Fig. 6A). In accordance with the mRNA data, AF-derived macrophages from these patients also produced higher amounts of mature IL-1β and IL-18 upon EcDNA



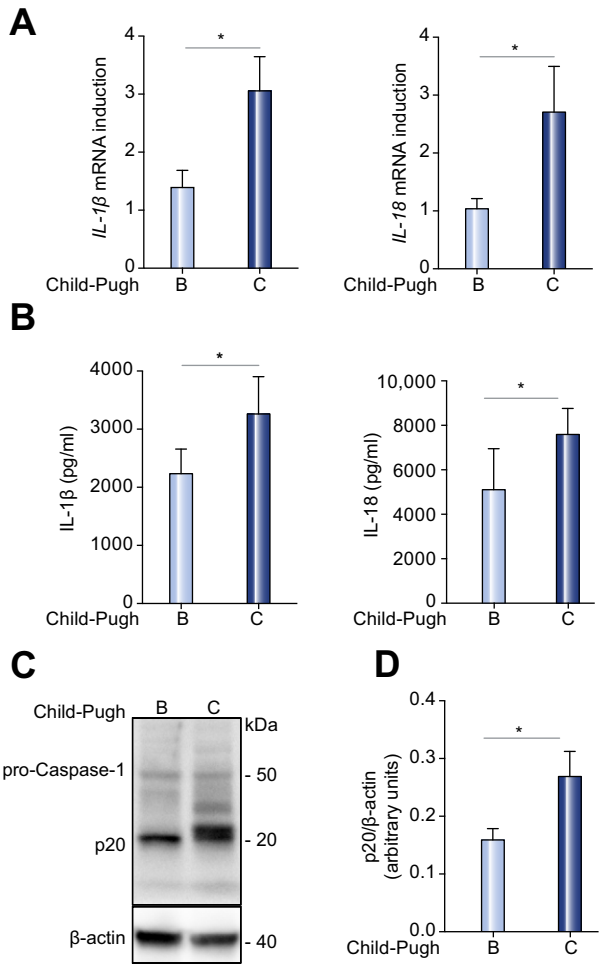


Fig. 4. Increased inflammasome activation in Child-Pugh C patients. (A) qPCR analysis of pro-*IL-1β* and pro-*IL-18* in AF-derived CD14+ macrophages isolated from patients with either Child-Pugh score B (n = 26) or C (n = 18) (B) ELISA analysis of IL-1β and IL-18 protein levels in 24 h culture supernatants of AF-derived CD14+ macrophages, isolated from patients with Child-Pugh score B (n = 10) or C (n = 8). Cells were pre-stimulated with EcDNA and 3 h later transfected with poly(dA:dT). (C) Representative immunoblot of active caspase-1 p20 in AF macrophages isolated from patients with Child-Pugh score B or C. (D) Densitometry analysis of caspase-1 p20 protein level in each group as measured by immunoblot. Protein bands were normalized to the intensity of β-actin. Data (C and D) represent pooled results from five independent experiments. Error bars represent mean ± SD. $^{*}p$ <0.05.

Fig. 3. Inflammasome activation does not require a priming signal in AF macrophages. (A) IL-1β and IL-18 cytokine levels in culture supernatants of PBMC-derived CD14+ cells isolated from healthy controls (n = 12). Cells were pre-stimulated with 100 ng/ml of LPS or EcDNA for 3 h and then transfected with poly(dA:dT) (1.5 μg/ml) to activate AIM2, or stimulated with ATP (5 mM) to activate NLRP3. (B) Cytokine production by PBMC-derived CD14+ cells from healthy controls. Cells were treated with 100 ng/ml of LPS and 10 ng/ml of the AIM2 inhibitor ODN (TTAGGG), and 3 h later transfected with poly(dA:dT) to activate AIM2 or stimulated with ATP to activate NLRP3. (C and D) Cytokine levels in culture supernatants of PBMC-derived (C) and AF-derived (D) macrophages from cirrhotic patients (n = 13) stimulated as described in (A). All cytokines were measured after 24 h of stimulation. Error bars represent mean ± SD. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. n.s., not significant.

and poly(dA:dT) stimulation (Supplementary Fig. 6B). Last, the presence of bactDNA also resulted in higher expression of mature caspase-1 p20 in AF macrophages (Supplementary Fig. 6C and D).

Clinical evolution of patients and development of SBP

To better understand whether the activation of the inflammasome might be associated with the clinical evolution of the disease, we recorded the successive hospitalizations of all patients including reasons for admission, applied therapy, and diagnosis at discharge. The median follow-up period was 36 months (95% CI: 25–47). During that period, 12 patients (27.3%) developed SBP, 9 (20.4%) developed hepatorenal syndrome, 4 (9.1%) developed hepatocellular carcinoma, 13 (29.5%) received liver transplant, and 19 patients (43.2%) died. When comparing baseline characteristics of patients who developed an episode of SBP with patients that did not develop SBP, no significant differences were observed in age ($p = 0.248$), Child-Pugh score ($p = 0.635$), or history of previous SBP ($p = 0.496$). By contrast, patients who developed SBP had a significantly higher production of IL-18 in AF than those without episodes of SBP (680.5 ± 334.7 pg/ml *vs.* 252.6 ± 319.4 pg/ml, $p = 0.011$). We also performed logistic regression using SBP occurrence as dependent variable and age, Child-Pugh score, and IL-18 level in AF as independent variables (age and IL-18 level were dichotomized according to their median values). Interestingly, only IL-18 showed a significant association with SBP occurrence, with an odds ratio of 5.42 (95% CI: 1.03–28.42; $p = 0.046$). These results suggest that increased inflammasome activation might be a marker of increased risk of SBP in these patients.

Discussion

IL-1β and IL-18 are pleiotropic cytokines that affect inflammatory and immune responses and have an important influence in the pathogenesis of many acute and chronic inflammatory diseases [22]. Emerging evidence suggests that inflammasome activation contributes to the pathogenesis of a wide range of liver diseases [16], and its role in fibrogenesis and cirrhosis has been studied in animal models [23,24].

Here, we provide evidence that activation of the inflammasome is an important response in the AF of cirrhotic patients. AF-derived macrophages are in a pre-activated state that allows them to mount a rapid AIM2-mediated response even in the absence of a priming signal, which is required in blood-derived macrophages. Our results also demonstrate that some microbial products may induce more potent inflammasome activation than others, underlining the importance of the differential activation of innate immune cells. The mechanisms by which bacterial DNA exerts a strongest priming signal for inflammasome activation in human macrophages are not totally understood. Human macrophages have a low expression of TLR9 and they do not respond to CpG-DNA sequences [25–27], but they functionally express other DNA receptors, such as DAI (DNA-dependent activator of IFN-regulatory factors), STING (stimulator of interferon genes) or RIG-I (retinoic acid-inducible gene 1), and potentially another so far unidentified cytosolic DNA recognition receptor [28–31]. It is important to note that, although poly(dA:dT) can stimulate some of these receptors, it is unable to directly activate the inflammasome pathway and that AIM2 is strictly required for the activation of the inflammasome in response to cytosolic DNA [18]. However, these receptors may trigger a type I IFN response that might serve as an additional priming signal for inflammasome activation [18]. This is not unlikely since AIM2 is also known to be an IFN-inducible gene, and therefore the induction of type I IFN could explain the difference in induction of AIM2 by LPS and EcDNA.

In cirrhotic patients, complications associated with high mortality are normally triggered by translocation of bacteria or bacterial products, emphasizing the relationship between the microbiota, the immune response and liver pathology [32]. Here, we found higher inflammasome activity in those patients with a higher degree of hepatic damage, showing a different aspect of inflammasome involvement in liver disease. We propose that a two-step mechanism could account for this phenomenon. First, translocation of bacteria as a consequence of severe liver disease [19] would lead to an abnormal influx of TLR ligands and other exogenous PAMPs into the AF, providing the first signal for the activation of the inflammasome. Second, endogenous DAMPs released from the liver as a consequence of severe hepatocyte damage, together with the passage of new PAMPs from the intestinal lumen, would provide the second signal for the activation of the inflammasome in primed AF cells. In support of this mechanism, we found that the presence of bactDNA is indeed associated with a heightened inflammasome response in AF macrophages. This is consistent with previous results showing that translocation of bactDNA into the AF induces a significant inflammatory reaction in patients with decompensated cirrhosis, independently of the presence of bacterial infection [11,33]. Thus, the data shown herein expand this knowledge to include the effects of the inflammasome in this inflammatory response.

In summary, our findings are important for the understanding of the sterile inflammatory reactions in patients with advanced cirrhosis, and could have important translational implications. Several IL-1β blocking agents are currently approved and successfully used in patients, suffering from different inflammatory diseases, including a recombinant form of the soluble IL-1Ra or anti-IL-1β monoclonal antibodies [34,35]. Although the increased risk of lethal infections would strongly argue against using immunosuppressive drugs in some scenarios, such as in SBP, our results could lay the foundation for the development of relevant clinical trials to test the efficacy of these drugs — or the development of new ones — in the treatment or prevention of sterile inflammatory complications in cirrhotic patients.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

BLR acquired and analysed data. VB acquired data. IGM acquired data. PZ provided statistical support and critical revision of the manuscript. IGH acquired data. AM acquired data. PG provided technical support. PB acquired clinical data. RF provided critical revision of the manuscript. JS reviewed the manuscript and analysed the clinical data. JMGN designed the study, interpreted the data and drafted the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.08.027.

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