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