SUPPLEMENTARY MATERIALS AND METHODS

Histological assessment of polymorphonuclear cells in human biopsies

Liver specimens from patients with AH were formalin-fixed and paraffin-embedded, and slides of 3 μm were stained with hematoxylin-eosin and Masson's trichrome. Regarding polymorphonuclear cells (PMN) assessment, an expert liver pathologist blinded reviewed all biopsies and considered as "mild" PMN infiltration the presence of isolated or a row of few PMNs around one or around a small cluster of 3-4 hepatocytes. (Usually the number of PMNs is less than 15 per focus, and they are difficult to find at low magnification). We considered as "marked" PMN infiltration the presence of PMNs when they were easily recognized at low magnification (x200), and when we observed numerous PMN around damaged hepatocytes (recognized for the presence of ballooning or Mallory-Denk bodies).

RNA isolation and PCR analysis

RNA was isolated from cells and liver tissues using Trizol and following manufacturer's manual instructions (Invitrogen, Carlsbad, CA, USA). RNA from cell-sorted samples was extracted using the QIAGEN RNeasy MICROKit (QIAGEN GmbH, Hilden, Germany). After reverse transcription, mRNA levels were determined by quantitative real-time PCR on an ABI 7900HT cycler (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) using commercial primer-probe pairs (Applied Biosystems) for CCL20, RANTES, COL1A1, F4/80, ICAM1, MCP1, NOS2 and TGFβ. Murine data were normalized to GAPDH and human data were normalized to 18s. The gene expression values were calculated based on the ΔΔCt method. The results were expressed as $2^{-\Delta\Delta Ct}$.

Cell migration assay

Migration assays were performed as previously described using a Boyden Chamber (Neuro Probe, Gaithersburg, MD, USA).[1] Briefly, polycarbonate filters of 8 μm pores (Poretics Corp., Livermore, CA, USA) were coated with 1% gelatin (Sigma-Aldrich, St. Louis, MO, USA). The lower chamber was filled with serum free medium containing 250ng/ml of CCL20 (R&D Systems, Minneapolis, MN, USA) or 20ng/ml of PDGF (R&D Systems) as positive control. As negative control we filled the lower chamber only with serum free medium plus vehicle (PBS containing 0.1% bovine serum albumin). Overnight serum-starved HSCs were trypsinized and placed in the upper chamber ($2x10^4$ cells). After 6 hours of incubation at 37ºC in a 5% CO_2 humidified incubator, cells were fixed with methanol and migrated cells were stained with Giemsa and counted (at x400 magnification). In some experiments cells were pre-incubated with 10μM of U0126, a specific MEK1/2 inhibitor (Cell Signaling Technologies, Danvers, MA, USA) one hour before the incubation with 250ng/ml of CCL20 or with 20ng/ml of PDGF. Each experiment was performed in triplicate, and migration was expressed as fold change respect to control.

Western blot

We performed electrophoresis of protein extracts and subsequent blotting as previously described.[2] Membranes were incubated with primary antibodies against phospho-ERK and total ERK (Cell Signaling Technologies), ICAM1 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), CCL20 (Abcam, Cambridge, UK), NOS2 (Abcam), Caspase-8 and Caspase-3 (Cell Signaling Technologies), GAPDH (Abcam) and with horseradish peroxidase conjugated secondary antibody. GAPDH was used as endogenous control. Proteins were detected by enhanced chemiluminescence (Biological Industries, Beit-Haemek, Israel) and were visualized using Las 4000 Imaging system (GE Healthcare Life Sciences, Piscataway, NJ). The quantification of the proteins was performed by

densitometric analysis using Image GE ImageQuant TL analysis software (GE Healthcare).

Experimental mouse models

To investigate the role of CCL20 in the pathogenesis of liver damage, we performed different animal models. Male Balb/c mice 8 weeks aged were purchased from Charles River (Charles River, l'Arbresle, France). To test the effects of ethanol in CCL20 induction, mice (n=8) were fasted for 8 hours with free access to water and treated with a single dose of 50% ethanol (5g/kg body weight) or water by gavage. Animals were sacrificed 8 hours after gavage as previously described [2]. Next, in order to investigate the effects of LPS in CCL20 induction, mice (n=6) were injected intravenously with lipopolysaccharide (LPS, Sigma-Aldrich) 10mg/kg body weight or saline as control (n=3) and were sacrificed 4 hours after the injection. A chronic liver injury model was performed by injecting mice (n=6) with carbon tetrachloride (CCl_4) intraperitoneally (Sigma-Aldrich; diluted 1:4 in corn oil) at dose of 0.5ml/kg body weight twice per week for a total of 5 injections. Control mice (n=3) were given vehicle (corn oil, Sigma-Aldrich). To mimic the effects of endotoxemia in the context of chronic liver disease we performed a model of acute-on-chronic liver injury. Mice (n=12) were injected with CCl_4 as described in the chronic model and additionally, two days after the last CCl_4 injection, animals were administrated intravenously LPS 10mg/kg body weight and sacrificed 4 hours later. The effects of CCL20 *in vivo* were evaluated in male C57BL/6 mice 8-10 weeks aged. Mice were injected intraperitoneally with 50µg of control shRNA (scrambled sequence) (n=6) or shRNA specific for CCL20 (n=6) complexed with *in vivo* jet-PEI® (Polyplus, Illkirch, France) in a final volume of 400µl. 24 hours later mice were injected intravenously with LPS or saline at dose 2,5mg/kg body weight and immediately after received a second intraperitoneal injection of 50µg of control shRNA

or CCL20 specific shRNA. Mice were sacrificed 24 hours after the last injection. In all animal models, livers were excised and collected.

All animal procedures were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and by the Columbia University Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Assessment of inflammatory injury and fibrosis

Paraffin-embedded liver sections were incubated with primary F4/80 (1:200, Serotec, Oxford, UK), CCL20 (1:200 Santa Cruz Biotechnologies), i NOS (1:150, Abcam), MPO (1:50, Abcam) antibodies overnight at 4°C. After washing, sections were incubated with secondary antibodies (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Finally, the sections were stained with 3,3'-diaminobenzidine (DAB, Dako) and counterstained with hematoxilin. In order to quantify macrophages and neutrophils, sections were visualized at magnification x200 and F4/80-positive and myeloperoxidase (MPO)-positive staining were quantified in 9 different fields for each section by histomorphometry. Results were expressed as % of F4/80-positive and MPO-positive area. To assess the presence of liver fibrosis, liver specimens were stained with Sirius Red (Gurr-BDH Lab Supplies; Poole, England).

Murine hepatic cells isolation, flow cytometry analysis and cell sorting

Hepatic cell populations including HSCs and macrophages were isolated by a two-step collagenase-pronase perfusion of livers followed by 17% Nycodenz (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) two-layer discontinuous

density gradient centrifugation as previously described.[3] Cells from liver of mice treated with CCl_4 plus LPS were stained with F4/80-Alexa Fluor 647 (Serotec), CD3-Alexa Fluor 700 and Ly6G-APC (eBioscience, Affymetrix, San Diego, CA, USA). HSCs were purified by vitamin A-based on FACS sorting as previously described.[4, 5] All samples were purified by high speed sorting using a FACSAria cell sorter (Becton, Dickinson and Company, BD, New Jersey, NJ, USA) and immediately lysed in RNA lysis buffer. We also isolated total HSCs as previously described using pronase-collagenase perfusion followed by 9,7% Nycodenz gradient centrifugation.[5, 6]

SUPPLEMENTARY REFERENCES

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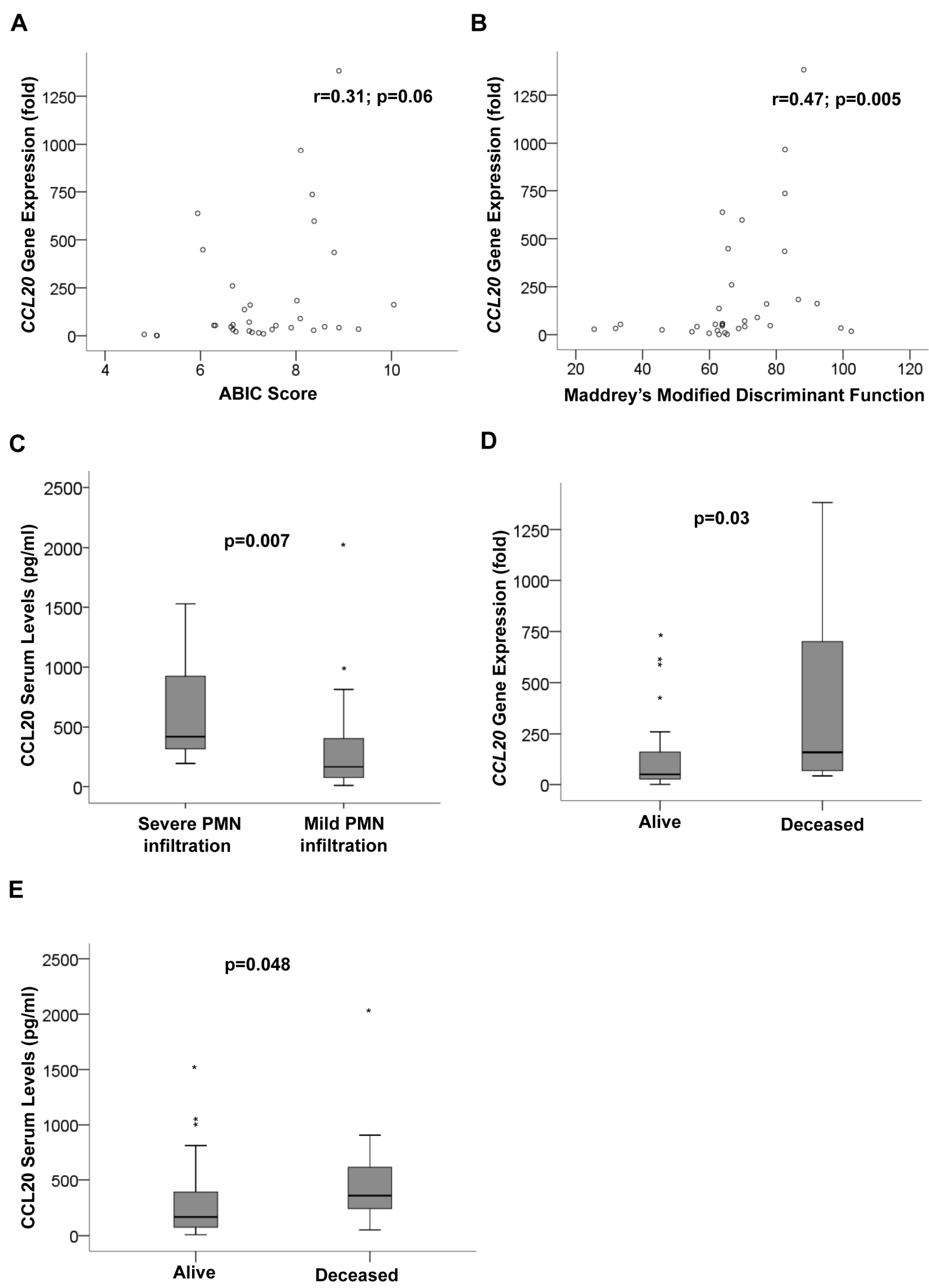
SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure 1. *CCL20* expression and correlations with clinical scores and mortality in patients with AH. (A) Correlation between *CCL20* hepatic gene expression and ABIC score in patients with AH (n=32) (p=0.06); (B) Correlation between *CCL20* hepatic gene expression and Maddrey's modified discriminant function in patients with AH (n=32) (p=0.005); (C) Correlation between CCL20 serum levels and hepatic PMN infiltration (p=0.007); (D,E) Correlation between *CCL20* hepatic gene expression and CCL20 serum levels with mortality at 90-day in patients with AH (p=0.03 [alive n=24, deceased n=8] and p=0.048 [alive n=34, deceased n=15] respectively).



Supplementary Figure 1