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Original Article

Adrenomedullin as an Immunomodulator of CD14$^+$MerTK$^+$ Circulating Monocytes in Liver Failure Syndromes

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Abstract

Background and Aims: Liver failure syndromes are characterised by a dysregulated immune response leading to immune paralysis. Adrenomedullin (ADM) is a potent vasodilator and immunoregulator. This study aimed to explore the role of ADM in liver failure, hypothesising that there is a detrimental imbalance between ADM and adrenomedullin binding protein (AMBP)1 that promotes a switch of monocytes/macrophages towards a pro-restorative phenotype and function. ***Methods:*** Consecutive patients with acute liver failure (ALF), acute-on-chronic liver failure (ACLF), and decompensated cirrhosis, as well as healthy controls (HC) were included between April 2020 and June 2024. Peripheral blood mononuclear cells/monocytes were isolated and used for RNA sequencing and cell culture. ADM and AMBP1 were measured by enzyme-linked immunosorbent assay. ***Results:*** Fifty-four patients with ALF, 25 with ACLF, 9 with decompensated cirrhosis, and 16 with HC were included. ADM expression in isolated monocytes was increased in ALF (log fold change = 5.88, p = 0.000216413) and ACLF (log fold change = 4.62, p = 0.00057122) compared to HC. Plasma ADM concentration was higher in ALF (1,684 ± 1,156 pg/mL) vs. ACLF (836.1 ± 765.2 pg/mL) and HC (164.8 ± 62.73 pg/mL). AMBP1 was significantly reduced in ALF (59.27 ± 44 µg/mL) vs. ACLF (126.3 ± 72.23 µg/mL) and HC (252.8 ± 159.7 µg/mL) (p < 0.0001, ALF vs. HC). Treatment with LPS increased ADM concentration in peripheral blood mononuclear cell supernatant (ALF n = 6; 561.4 ± 1,038 pg/mL vs. 259.2 ± 213.7 pg/mL, ACLF n = 4; 3,202 ± 491.2 vs. 1,757 ± 1,689 pg/mL). The percentage of CD14$^+$ cells expressing Mer tyrosine kinase was reduced after culture with LPS (2.077 ± 0.87%); however, co-culture with ADM 100 nM restored the phenotype (3.852 ± 1.063%). ***Conclusions:*** ADM is increased in liver failure, whereas AMBP1 is reduced. ADM affects monocyte function, increasing Mer Tyrosine Kinase and promoting a pro-restorative, anti-inflammatory phenotype.

Keywords: Adrenomedullin; Adrenomedullin binding protein-1; Mer tyrosine kinase; MerTK; Acute liver failure; Acute-on-chronic liver failure; Monocyte.
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Introduction

Acute liver failure (ALF) is a devastating syndrome, characterised by acute impairment of liver synthetic function with coagulopathy, altered level of consciousness from hepatic encephalopathy, and subsequent multi-organ failure in people without underlying chronic liver disease.[1] In the last 50 years, survival has improved from 20% to 70% with intensive care management, without any further increase since the early 2000s.[2] ALF affects three to six cases per million people per year, and if patients do not recover spontaneously, liver transplant is the only curative option.[3]

ALF causes an intense systemic inflammatory response secondary to damage-associated molecular patterns released by the liver.[4] No treatments are currently available, and sepsis and cardiovascular dysfunction with refractory vasoplegia remain common modes of death, thought to be secondary to inflammatory cytokines and vasoactive mediator release.[5] "Acute-on-chronic liver failure" (ACLF), instead, is a distinct syndrome observed among patients with acutely decompensated chronic liver disease and shares some features with ALF, such as intense systemic inflammation, multiple organ dysfunction, and high 28-day mortality.[6,7]

Bacterial infections are the most common precipitant in patients with ACLF, being present at diagnosis in almost half of the cases. Moreover, in 50% of the patients not having infection at diagnosis, infections can also frequently complicate the course of ACLF irrespective of the grade and worsen the prognosis.[8]

Both ALF and ACLF, though clinically distinct, are characterised by innate immune dysfunction, where patients present with acquired immune defects that make them prone to infections. Liver-resident macrophages and circulating monocytes play an essential role in the immune response but also contribute to resolution and healing processes, switching

from a pro- to an anti-inflammatory phenotype with a transcriptional signature typical of tissue repair and regenerative functions, apparently mediated by Mer tyrosine kinase (MerTK).[9,10]

Exploring the monocytic transcriptome in ALF, we found hyperexpression of adrenomedullin (ADM), a potent vasodilator, and in this paper, we investigated this novel pathway potentially linked to monocyte dysfunction in ALF.[4] The human ADM gene encodes the precursor hormone preproadrenomedullin, which undergoes a multi-step cleavage to yield various products, including pro-ADM N-terminal 20 peptide, midregional pro-ADM, adrenotensin, and ADM. These products have been employed as severity markers in cardiovascular diseases and sepsis.[11] ADM regulates vasodilatation, acting on the proliferation and migration of vascular smooth muscle cells and endothelial cells. ADM is also involved in immune cell recruitment and inflammatory response.[12] It significantly inhibited cytokine-induced neutrophil chemoattractant,[13] and similar effects were observed in microglia upon stimulation with LPS, inhibiting both TNFα and interleukin (IL)-6,[14] as well as in macrophages and rat Kupffer cells.[15]

ADM is relatively unstable in the circulation and is degraded by proteases, with a circulating half-life of 22 ± 1.6 m.[16] This is where adrenomedullin binding protein (AMBP) 1, also known as complement factor H and produced by the liver, exerts its pivotal effect. AMBP1 inhibits ADM cleavage, thus enhancing ADM's activity and extending its half-life, potentially facilitating its transition from the interstitial space to the bloodstream.[11] AMBP1, with reported average concentrations ranging from 233 to 400 μg/mL or 1.5–2.6 μM,[17] is 10^5 times more concentrated than ADM and exerts several functions as a regulator of complement activation. Deficiency of AMBP1 also results in increased consumption of C3 and higher susceptibility to recurrent infections.[18] This interaction with AMBP1 holds promise for prolonging the beneficial therapeutic effects of ADM.

We hypothesised that in acute liver failure syndromes there is a detrimental imbalance between decreased AMBP1, secondary to liver impairment, and increased ADM, generated during the inflammatory response. We further propose that this imbalance promotes a dysfunctional monocyte/macrophage phenotype and function.

Methods

Patients

This observational cohort included consecutive patients with acute (defined as the onset of encephalopathy and coagulopathy within 26 weeks of a patient diagnosed with liver disease) and acute-on-chronic (with decompensated cirrhosis and organ failures as per the European Association for the Study of the Liver-Chronic Liver Failure (CLIF) Acute-on-Chronic Liver Failure in Cirrhosis (CANONIC) study[6]) liver failure, recruited at King's College Hospital in London between April 2020 and June 2024 as part of the "Immunometabolism in Sepsis, Inflammation and Liver Failure Syndromes/IMET" study (Research Ethics Committee No.: 19/NW/0750, IRAS No.: 244089). Patients with cancer, pre-existing immunosuppressive conditions (either drug-induced or infective, i.e. HIV), chronic granulomatous diseases, and pregnant women were excluded. Patients, or consultees if they lacked capacity, provided written informed consent within 24 h of admission. Clinical and laboratory data were prospectively collected, and severity scores were calculated, including Child-Pugh, Model for End-Stage Liver Disease, Sequential Organ Failure Assessment (SOFA),[19] CLIF-Consortium Acute Decompensation, CLIF-SOFA,[20] and King's College Criteria.[21] Healthy controls (HC) were recruited from hospital personnel.

PBMC isolation

The protocol has already been published elsewhere.[4] Briefly, blood was collected in lithium heparin tubes and subsequently diluted 1:1 with phosphate-buffered saline (Gibco™, Thermo Fisher, USA), and PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, UK) at 1:3 v/v. The buffy coat was collected and, after centrifugation, the cell pellet was resuspended in the appropriate volume according to future use (culture or storage at −80°C).

Isolation of CD14$^+$ monocytes using CD14 MicroBeads (Miltenyi Biotec): As previously described,[4] we used MACS BSA Stock Solution (#130-091-376) diluted 1:20 with autoMACS Rinsing Solution (#130-091-222) (i.e. 50 mL for two isolations: 47.5 mL of rinsing solution + 2.5 mL of BSA stock solution). PBMCs were first passed through a 30 μm nylon mesh and, after centrifugation, resuspended in buffer with the addition (1:5) of CD14 MicroBeads (#130-050-201). LS columns (#130-042-401) were used to separate CD14$^+$ cells as per the manufacturer's instructions.

RNA sequencing

As previously described,[4] the RNAqueous™-Micro Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used according to the manufacturer's instructions. We used the NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, USA) for library preparation, as previously described.[4] A 250–300 bp insert strand-specific library was built using the rRNA removal method with TruSeq Stranded Total RNA Library Prep and the Novogene NGS Stranded RNA Library Prep Set (PT044), as follows:

The rRNA removal kit was used to remove the ribosomal RNA, and rRNA-free residue was cleaned up by ethanol precipitation. The rRNA-depleted RNA was used to generate sequencing libraries. The insert size of the library was validated on a Bioanalyzer 2100 (Agilent, Santa Clara, USA) and quantified by PCR. The Illumina NovaSeq 6000 S4 flow cell (Illumina, San Diego, USA) was used to sequence the library with a target sequencing depth of 40 million read pairs per sample, using 150 bp paired-end reads. FastQC v0.11.9 was used to evaluate sequence data for quality control issues. Adaptors were trimmed using Trim Galore v0.6.5. Reads were aligned to the reference genome GRCh38[22] and the corresponding Ensembl genebuild release 104 using the STAR 2.7.9a aligner.[23] Transcript quantification was performed using the RSEM 1.3.3 algorithm.[24] Raw count data were analysed in R v4.0.4 (R Statistical Foundation, Vienna, Austria). The limma-voom pipeline (limma v3.46.0)[25] was used for differential expression analyses. Lowly expressed genes were filtered; data were normalised and transformed. The associated precision weights were generated using the voom function. The design model incorporated clinical group, blocked on batch and without an intercept; differential gene expression was estimated for contrasts of interest. Pathway analysis was conducted using Generally Applicable Gene-set Enrichment for Pathway Analysis (GAGE, gage v2.40.2)[26] and human KEGG pathways (release 103.0) annotated as "signalling" or "metabolism". As the aim of the study was exploratory in nature, unadjusted p-values were used with $\alpha = 0.05$ as the threshold for significance.

Enzyme-linked immunosorbent assay (ELISA)

Plasma and serum were collected after spinning the blood for 10 m at RCF 800×g (20°C) with the brake on. Supernatants were transferred into 2 mL tubes and stored at −80 °C.

Adrenomedullin: The Elabscience® Human ADM (Adrenomedullin) ELISA Kit was used according to the manufacturer's instructions. Briefly, samples were diluted 1:2 and loaded onto the pre-coated plate, then incubated for 90 m at 37°C. After decanting the sample (without washing), Biotinylated Detection Antibody working solution was added and the plate was incubated for 1 h at 37°C. After washing, the HRP Conjugate working solution was added to each well. After a 30-m incubation at 37°C and a washing step, Substrate Reagent was added and incubated for about 15 m at 37°C. After adding the Stop Solution to each well, the optical density was determined with a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) set to 450 nm.

AMBP1: The Human Complement Factor H ELISA Kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, samples were diluted 2000-fold and loaded onto the pre-coated plate, then incubated for 2.5 h at room temperature with gentle shaking. All the following steps were alternated with washing. The biotin conjugate (incubated for 1 h at room temperature), the Streptavidin-HRP solution (incubated for 45 m at room temperature), and the TMB (incubated for 30 m in the dark) were sequentially loaded into the wells. After stopping the reaction, the plate was read and the optical density determined with a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) set to 450 nm.

IL-10 and TNFα in cell culture supernatants: We used the *Human IL-10 and TNFα DuoSet ELISA kits (R&D Systems)* according to the manufacturer's instructions. The test was performed on cell culture supernatants (please see below for details on culture). Plates were pre-coated with 100 µL of Capture Antibody and incubated overnight. The following day, after washing, 300 µL of reagent diluent was added to each well. After one hour of incubation and washing, 100 µL of standard or sample were loaded into each well. After 2 h of incubation at room temperature with gentle shaking (500 ± 50 rpm) and subsequent washing, 100 µL of Detection Antibody working solution was loaded into each well, and the plates were incubated for 2 h at room temperature with gentle shaking. After washing, plates were incubated for 20 m with 100 µL of Streptavidin-HRP working solution, protected from light. After further washing, plates were incubated for 20 m with 100 µL of Substrate Solution. Then, 50 µL of Stop Solution was added to stop the reaction. The FLUOstar® Omega microplate reader (BMG Labtech Ltd, UK) was used to assess optical density (450 nm with 540 nm wavelength correction). The average of the duplicate readings was analysed after subtraction of the average zero standard optical density. A third-order polynomial standard curve was used for quantification.

V-PLEX Proinflammatory Panel 1 Human Kit from Meso Scale Discovery: A coated multi-spot plate by Meso Scale Diagnostics (Rockville, USA), including *IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13,* and *TNFα,* was used for cytokine quantification. Lithium heparin plasma samples were twofold diluted and processed according to the manufacturer's instructions. The results were read with an MSD instrument. Averages of the duplicate readings for each standard and sample were analysed, and analyte concentrations were established by fitting a 4-parameter logistic calibration curve.

Public microarray datasets

Public microarray datasets from the Gene Expression Omnibus (GEO) — GSE61298, GSE80751, and GSE168049 series — were interrogated to measure the gene of interest and analyzed with GEO2R, available on the same platform.

Cell culture

PBMCs were cultured at 37°C with 5% CO_2 in a 24-well plate (0.5×10^6 cells per well) for 24 h in 500 µL of complete medium, including RPMI without HEPES, 10% Fetal Bovine Serum (FBS, Thermo Fisher), and 1% PenStrep (Penicillin-Streptomycin 10,000 U/mL, Gibco, Thermo Fisher, USA), as per our standard protocol previously described.[4] Different treatments including LPS, ADM 3, 100, and 200 nM (Cambridge Bioscience), AMBP1 65 nM (Complement factor H from human plasma; Sigma-Aldrich), and ADM 22–52 10 µM (Cambridge Bioscience) were added after 1 h of culture and incubated overnight. The following morning, LPS 100 ng/mL was added for the last 6 h or 8.5 h of culture. A late boost of ADM at different concentrations was added 30 m before terminating the experiment, based on the hypothesis that its short half-life could have affected the results. Cells were then washed with phosphate-buffered saline and stained for phenotyping as follows. Supernatant was used for cytokine assays. For intracellular staining, the protein transport inhibitor 100× working solution (eBioscience cat: 00-4980) was added (5 µL in 495 µL of media) 3 h before the end of the experiment.

Immunophenotyping

Monocyte phenotype of fresh/cultured PBMCs was determined by flow cytometry using monoclonal antibodies against CD14, CD16, CD163, IL-6, CD206 (BioLegend, USA), Human Leukocyte Antigen - DR isotype (eBioscience, San Diego, USA), Mer-Tyrosine Kinase (R&D Systems, USA), IL-10, TNFα (BD), and Fixable Viability Dye eFluor 506 (Invitrogen, USA). (Antibody details can be found in Supplementary Table 1). After washing, the surface marker antibody mix was added to 100 µL of cell resuspension. Cells were incubated in the dark at 4°C for 20 m. Then, 100 µL of IC Fixation Buffer (eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, Invitrogen, Massachusetts, USA) was added, and cells were incubated for 20 m at room temperature, protected from light. Cells were washed twice with 2 mL of Permeabilization Buffer and centrifuged at 1,800 rpm for 5 m at room temperature. The cell pellet was resuspended in 100 µL of 1× Permeabilization Buffer, and the intracellular antibody mix was added. Cells were incubated for 20 m at room temperature, protected from light. After washing and resuspension in FACS buffer, they were analyzed. The analysis was performed with a BD LSRFortessa™ cell analyzer (BD Biosciences). Flow cytometry data analysis was performed using FlowJo™ v10 (Becton Dickinson & Company). The gating strategy is shown in the supplementary material (Supplementary Figs. 1 and 2). Fluorescence minus one samples were used to apply the gates accurately. Results are expressed as percentages (%) and/or mean fluorescence intensity.

Statistical analysis

T-test/Wilcoxon test, one-way ANOVA/Kruskal-Wallis, and Pearson/Spearman's correlations were used respectively for parametric and non-parametric data. Analysis was performed with GraphPad Prism v10 (GraphPad Software, San Diego, CA). Missing data were addressed by casewise deletion. An a priori power analysis was conducted using G*Power version 3.1.9.7 for sample size estimation, based on data from

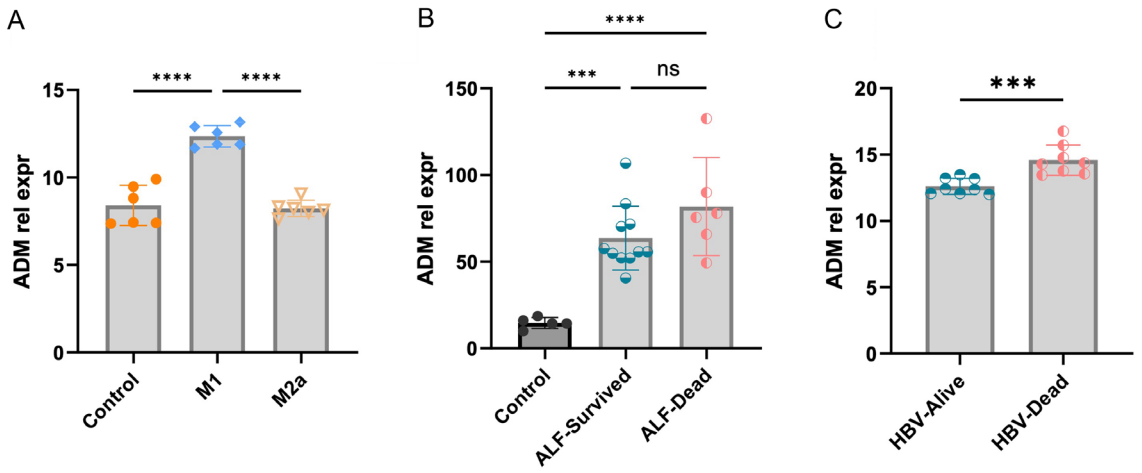

Fig. 1. Analysis from datasets publicly available on GEO. (A) Data from "Transcriptional profiling of human monocyte-derived macrophages" GSE61298. The study contains data from healthy donors' isolated monocytes. They were differentiated with either GM-CSF for seven days and then either mock-activated with culture medium only (control; condition 1), or activated with LPS + IFNγ (condition 2), or IL-4 (condition 3) for 48 h. LPS plus interferon-γ was used to induce macrophage polarisation towards the M1 type, or IL-4 to induce macrophage polarisation towards the M2a type. M1 macrophages showed increased expression of ADM compared to the other groups. One-way ANOVA, $p < 0.0001$. (B) Data from "Monocyte phenotype indicates a poor prognosis in paracetamol (acetaminophen)-induced acute liver failure but not in non-ae hepatitis: a prospective observational cohort study" GSE80751. The study contains data from 22 human peripheral monocyte samples, comprising five from controls and 17 from patients with acute ALF. Of the liver failure group, six subsequently died or received a liver transplant. Patients with ALF had increased ADM expression, independently of their outcome. One-way ANOVA, $p < 0.0001$. (C) Data from "Prognosis-associated mRNA and microRNA in PBMCs from HBV-ACLF" GSE168049. The study contains data from 16 human PBMCs from HBV-ACLF: eight survival cases and eight deceased controls within 28 days. Deceased patients had increased ADM expression compared to survivors. Unpaired T test, $p = 0.0007$. ****$p < 0.0001$, ***$p < 0.001$. GEO, Gene Expression Omnibus; ADM, adrenomedullin; LPS, lipopolysaccharide; IFN, interferon; IL, interleukin; ALF, acute liver failure; PBMCs, peripheral blood mononuclear cells; HBV-ACLF, hepatitis B virus-related acute-on-chronic liver failure.

Hirata *et al*.,[27] which compared healthy controls and patients with sepsis. Results indicated that the required sample size to achieve 80% power for detecting a medium effect, at a significance criterion of α = 0.05, was N = 36 (18 in each group) for comparison of means. Thus, the obtained sample size is adequate to test the study hypothesis.

Results

Patients

We included 54 patients with ALF and 25 with ACLF. Our controls were 9 patients with decompensated cirrhosis (DC) and 16 with HC.

In the ALF group, the most frequent aetiology was drug-induced liver injury (77.7%, n = 42); among them, 30 patients had a proven paracetamol overdose. Mortality at 30, 90 days, and one year was 24%, 25.9%, and 25.9%, respectively. Although 46.3% of the patients met the King's College Criteria,[21] only 25.9% were transplanted (most were excluded due to extensive psychosocial background history after evaluation by a multidisciplinary team). Additionally, 42.6% of the patients developed an infection during the first 10 days of admission.

In the ACLF group, the most frequent aetiology was alcohol-related liver disease in 87.5% of the patients (n = 21), and the principal precipitant was infection (n = 20). Five patients also presented with gastrointestinal bleeding. Mortality at 30, 90 days, and one year was 33.3%, 45.8%, and 58.3%, respectively. A total of 83.3% of the patients developed an infection during the first 10 days of admission.

ADM expression is increased in ALF and ACLF monocytes transcriptome

Monocyte RNA from seven ACLF, five DC, four ALF, and three HC samples was used for this experiment. ADM expression in isolated monocytes was higher in ALF (log fold change = 5.8812121, $p = 0.000216413$) and ACLF (log fold change = 4.61940976, $p = 0.00057122$) compared to HC (Supplementary Fig. 3). No difference in ADM expression was found between ALF/ACLF and DC.

Publicly available datasets confirmed transcriptional ADM expression in ALF and ACLF PBMCs

We next explored whether PBMC and isolated monocyte expression was also upregulated in publicly available datasets. Data from GEO showed that in human monocyte-derived macrophages from healthy donors (GSE61298), macrophage polarization toward the M1 type increased ADM expression compared to control or IL-4-induced M2a-type polarization (one-way ANOVA, $p < 0.0001$) (Fig. 1A).

In monocytes from paracetamol-induced ALF patients (GSE80751), ADM relative expression in survivors was 64.68 ± 18.44, while in those who died it was 81.86 ± 28.33. Both were increased compared to controls (14.63 ± 3.18) (one-way ANOVA, $p < 0.0001$) (Fig. 1B).

In PBMCs from patients with ACLF secondary to hepatitis B chronic infection (GSE168049), the eight cases that survived showed reduced relative expression of ADM (12.6 ± 0.6) compared to eight patients who died within 28 days (14.59 ± 1.14) (T-test, $p = 0.0007$) (Fig. 1C).

Plasma ADM concentration was increased in ALF, and a reciprocal AMBP1 decrease was observed

ALF patients showed increased plasma ADM concentration (1,684 ± 1,156 pg/mL) vs. ACLF (836.1 ± 765.2 pg/mL) and HC (164.8 ± 62.73 pg/mL) (Kruskal-Wallis, $p < 0.0001$ for ALF vs. HC) (Fig. 2A).

ADM concentration was reduced at day 7 of admission compared to baseline in six ALF patients (Day 1: 1,718 ± 988.6 pg/mL vs. Day 7: 567.1 ± 186 pg/mL, Wilcoxon test,

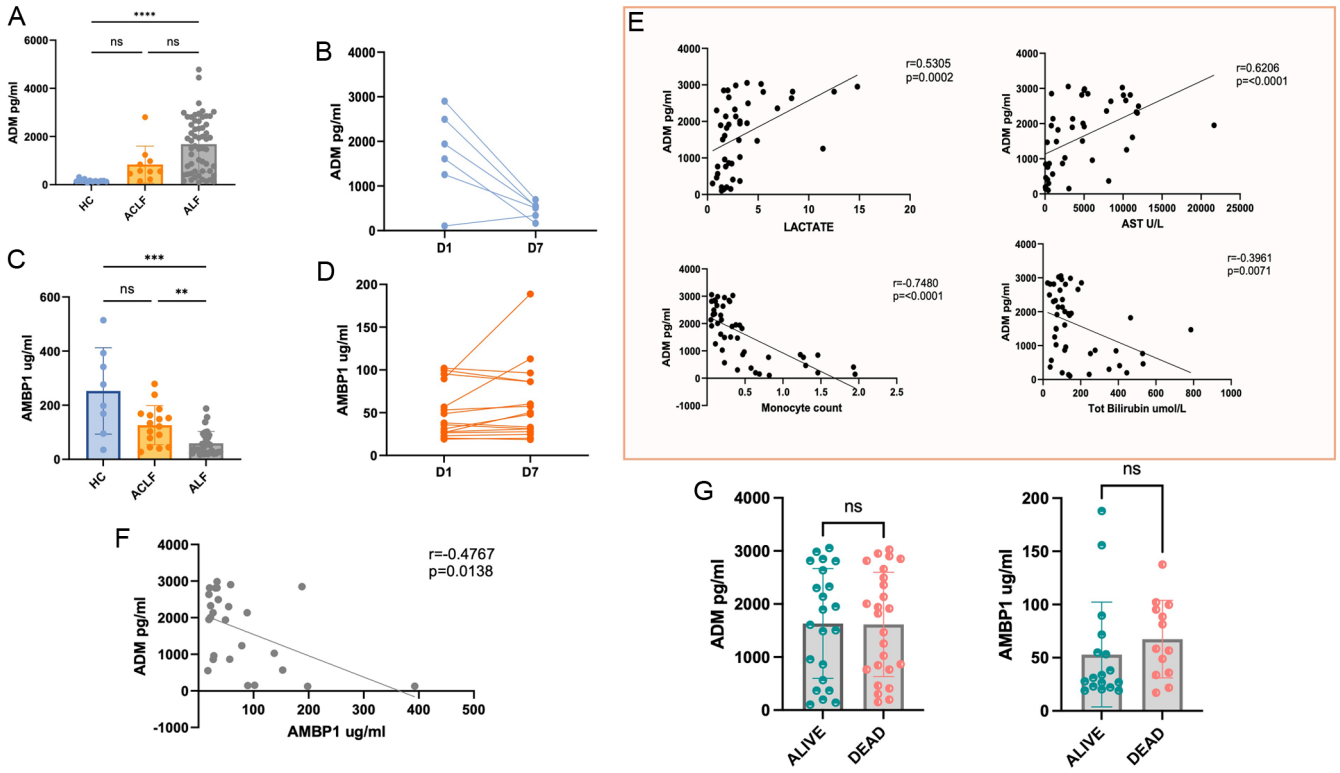

Fig. 2. ADM and AMBP1 plasmatic concentrations. (A) ADM was increased in ALF (n = 57; 1,684 ± 1,156 pg/mL) vs. ACLF (n = 10; 836.1 ± 765.2 pg/mL) and HC (n = 12; 164.8 ± 62.73 pg/mL), Kruskal-Wallis, $p < 0.0001$ (ALF vs. HC). (B) ADM concentration was reduced at day 7 of admission in six ALF patients (D1 1,718 ± 988.6 pg/mL vs. D7 567.1 ± 186 pg/mL), Wilcoxon test, $p = 0.0625$. (C) AMBP1 was decreased in ALF (n = 30; 59.27 ± 44 µg/mL) vs. ACLF (n = 16; 126.3 ± 72.23 µg/mL) and HC (n = 8; 252.8 ± 159.7 µg/mL), Kruskal-Wallis, $p < 0.0001$ (ALF vs. HC). (D) Despite a trend, AMBP1 concentration was unchanged at day 7 of admission in 16 ALF patients (D1 49.63 ± 30.25 µg/mL vs. D7 60.84 ± 44.96 µg/mL), Wilcoxon test, $p = 0.3484$. (E) In ALF, ADM was directly correlated with lactate and inversely with monocyte count and bilirubin concentrations (Spearman Correlation). (F) ADM and AMBP1 were inversely correlated (Pearson test). (G) No difference in ADM and AMBP1 concentrations was found between ALF patients who spontaneously survived and those who died or were transplanted (Mann-Whitney test, ADM $p = 0.9703$; AMBP1 $p = 0.1253$). ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$. ADM, adrenomedullin; AMBP, adrenomedullin binding protein; ALF, acute liver failure; ACLF, acute-on-chronic liver failure; HC, healthy controls.

$p = 0.0625$) (Fig. 2B).

AMBP1 was decreased in ALF (59.27 ± 44 µg/mL) vs. ACLF (126.3 ± 72.23 µg/mL) and HC (252.8 ± 159.7 µg/mL) (Kruskal-Wallis, $p < 0.0001$ for ALF vs. HC) (Fig. 2C).

AMBP1 concentration was unchanged at day 7 of admission in 16 ALF patients (Day 1: 49.63 ± 30.25 µg/mL vs. Day 7: 60.84 ± 44.96 µg/mL, Wilcoxon test, $p = 0.3484$) (Fig. 2D).

Considering the whole cohort, ADM was correlated to the SOFA score (Spearman r = 0.4468, $p = 0.0002$). In ALF, ADM plasma concentration was directly correlated with several clinical parameters of deterioration, including respiratory rate (Spearman r = 0.47, $p = 0.0003$), heart rate (r = 0.273, $p = 0.0437$), lactate, Aspartate Aminotransferase (AST) (r = 0.6323, $p \leq 0.0001$), INR (r = 0.352, $p = 0.0084$), and serum sodium (r = 0.3934, $p = 0.003$), and inversely correlated with monocyte count (r = −0.726, $p \leq 0.001$), C-reactive protein (r = −0.4665, $p = 0.0003$), and bilirubin (r = −0.298, $p = 0.027$). (Fig. 2E and Supplementary Fig. 4). In ACLF, ADM was directly correlated with disease severity as expressed by the CLIF-acute decompensation score (Spearman r = −0.758, $p = 0.015$), but not with the CLIF-SOFA score (Spearman r = 0.247, $p = 0.518$). As expected, ADM and AMBP1 concentrations were inversely correlated (Pearson r = −0.4767) (Fig. 2F).

No differences in ADM and AMBP1 concentrations were found between ALF patients who survived spontaneously and those who died or were transplanted (Mann-Whitney test: ADM $p = 0.9703$; AMBP1 $p = 0.1253$) (Fig. 2G). We next explored potential inflammatory cues to modulate ADM expression in PBMCs.

LPS stimulated PBMCs to produce ADM in liver failure patients but not in HC

In the supernatant of PBMCs from patients, after stimulation with LPS, we found increased ADM concentration (ALF n = 6; 561.4 ± 1,038 pg/mL vs. 259.2 ± 213.7 pg/mL, ACLF n = 4; 3,202 ± 491.2 vs. 1,757 ± 1,689 pg/mL). This was not confirmed in HC (HC n = 7; 1,920 ± 1,184 vs. 2,299 ± 1,188 pg/mL) (two-way ANOVA with Tukey's multiple comparisons test) (Fig. 3A).

When we compared prolonged HC PBMC culture with LPS (8.5 h) to the standard 6-h culture, we found an overall increase in ADM concentration in the supernatant, but no significant difference compared to unstimulated cells (two-way ANOVA with Šidák's multiple comparisons test) (Fig. 3B).

Serial increasing concentrations of ADM [3 nM (data not shown), 100 nM, and 200 nM (data not shown)] did not affect cytokine (IL-10 and TNFα) concentrations in ALF PBMC cultures, while after LPS stimulation, there was a trend toward IL-10 reduction with ADM 100 nM and no change in TNFα concentration (Kruskal-Wallis test, Dunn's multiple comparisons test) (Fig 3C, D).

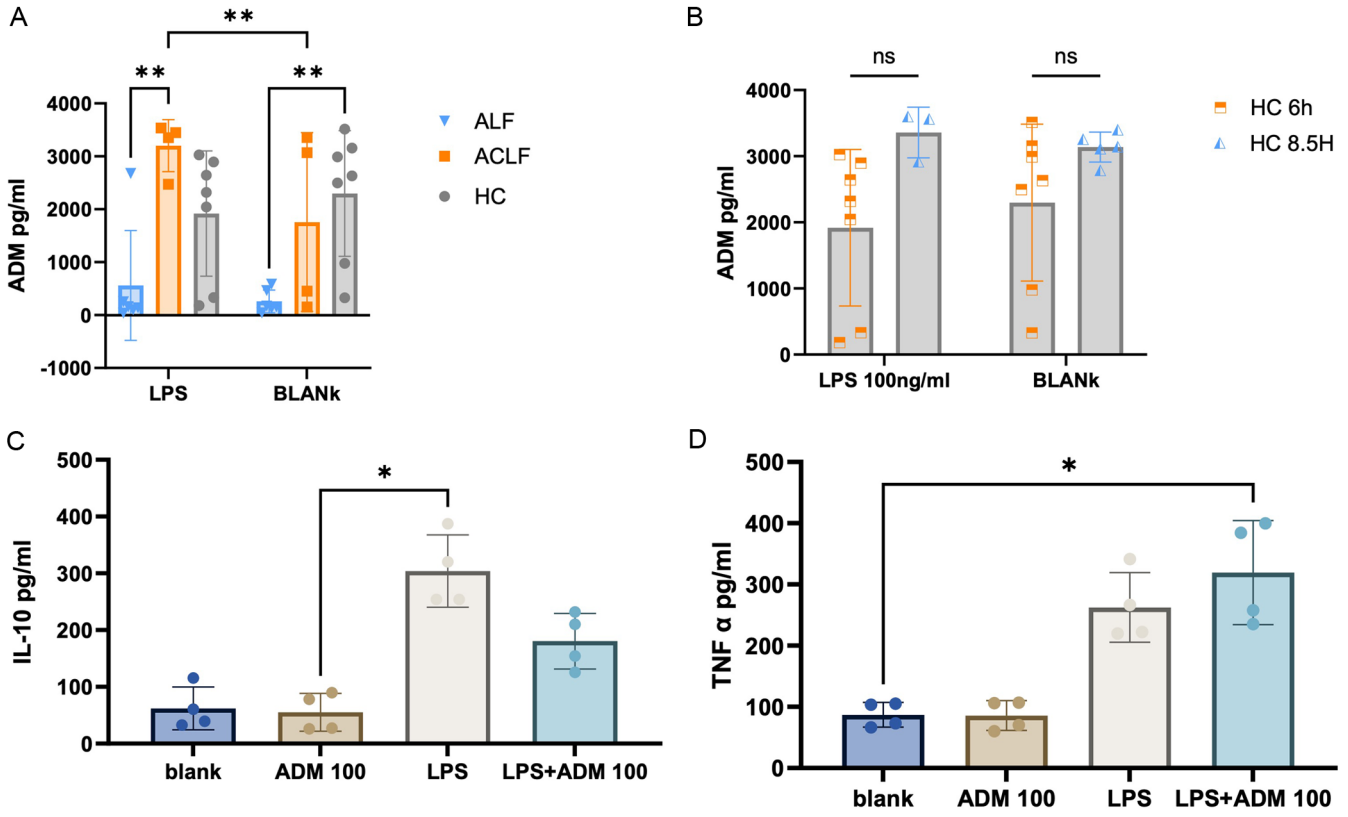

Fig. 3. PBMC supernatant analysis. (A) In patients, LPS treatment increased ADM concentration in PBMCs supernatant (ALF n = 6; ACLF = 4; HC = 7); however, in ALF, ADM concentration was reduced compared to controls and ACLF, both in treated and untreated cells (two-way ANOVA with Tukey's multiple comparisons test). (B) In HC, prolonged culture (8.5 h) increased ADM concentration compared to standard (6 h), without reaching statistical significance (two-way ANOVA with Šidák's multiple comparisons test). (C) In ALF, IL-10 concentration in supernatant remained unchanged after ADM stimulation in untreated cells, while in LPS-treated cells, there was a trend of IL-10 reduction with ADM 100 nM (Kruskal-Wallis test, Dunn's multiple comparisons test). (D) In ALF, TNFα concentration in supernatant remained unchanged after ADM stimulation (both in untreated and LPS-treated cells) (Kruskal-Wallis test, Dunn's multiple comparisons test). $^{**}p < 0.01$, $^{*}p < 0.05$. PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; ADM, adrenomedullin; ALF, acute liver failure; ACLF, acute-on-chronic liver failure; HC, healthy controls; IL, interleukin; TNF, tumour necrosis factor.

ADM restored MerTK expression after LPS stimulation

After LPS culture (HC n = 6 and ALF n = 6), immunophenotyping showed a reduced percentage of MerTK+CD14+ cells (2.077 ± 0.87%) compared to untreated cells (4.637 ± 2.029%). However, co-culture with ADM 100 nM restored a similar phenotype in both HC and ALF (3.852 ± 1.063%). No effect was seen with ADM 3 nM (data not shown). There was also a trend in ADM effect on the scavenger receptor CD163 expression (LPS 9.603 ± 5.115% vs. LPS+ADM 11.87 ± 7.02%) without reaching statistical significance. No effect was observed on the mannose scavenging receptor CD206 (LPS 35.58 ± 12.45% vs. LPS+ADM 34.5 ± 12.2%) (Fig. 4).

ADM Antagonism (ADM 22-52) and binding to AMBP1 did not affect monocyte phenotype

To further assess the effect of ADM on MerTK expression, we attempted to modulate the ADM signal through either receptor antagonism or the adjunct of AMBP1. In PBMCs from HCs and patients with decompensated cirrhosis, treated with LPS 100 ng/mL and ADM 100 nM, the adjunct of ADM 22–52 (10 μM) or AMBP1 (65 nM) did not affect MerTK, CD206, or CD163. However, ADM seemed to reduce human leukocyte antigen (HLA- DR expression compared to other treatments, without reaching statistical significance (LPS 63.84 ± 18.2%, LPS+ADM 60.27 ± 17.4%, LPS+ADM+AMBP1 61.54 ± 17.4%, LPS+ADM+ADM22–52 63.34 ± 17.9%) (Fig. 5). Intracellular staining, including IL-1, TNFα, and IL-10, did not show any differences between treatments (Fig 6).

Discussion

In patients with ALF and ACLF, we found increased plasma concentrations of ADM and reduced AMBP1 compared to controls. ADM was progressively increased in the most severe cases according to the SOFA score, and its concentration correlated with liver function tests and lactate. However, no correlation with cardiovascular parameters, except for heart rate, was seen. By day 7 of admission, ADM concentrations dropped below 1,000 pg/mL, while AMBP1 concentration remained low, the probable cause being persistent liver dysfunction, as hepatocytes are the main source of AMBP1.[28]

ADM concentration was instead directly correlated with monocyte count, and this was intriguing since in our previous research, RNA sequencing of isolated monocytes showed hyperexpression of ADM in ALF compared to controls.[4] We further explored this finding in our ACLF and DC cohort, as well as in publicly available datasets from GEO, and confirmed an increase of ADM expression in M1 macrophages, monocytes from ALF patients, and PBMCs from ACLF patients, includ-

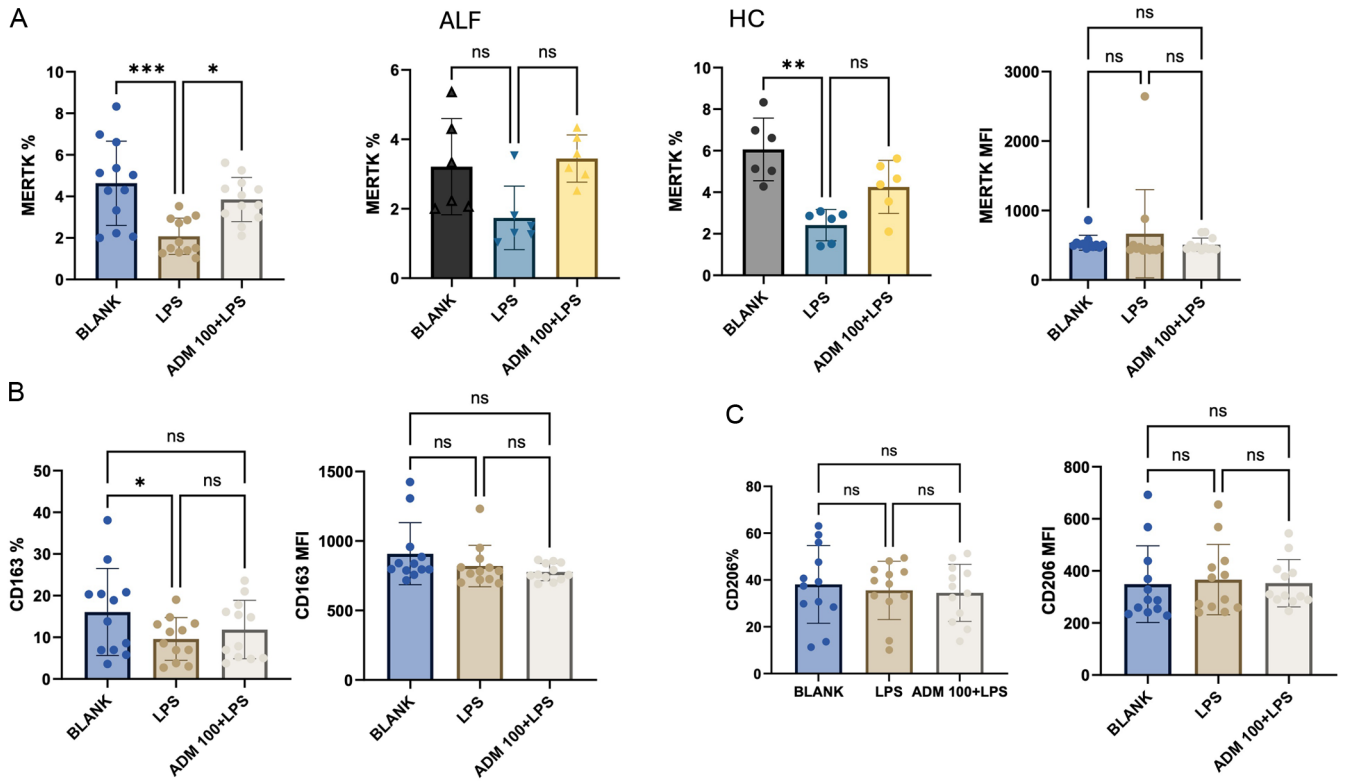

Fig. 4. Immunophenotyping of CD14+ve cells from PBMCs culture: Six HC and six ALF patients. (A) The percentage of cells expressing MerTK was reduced after LPS treatment but restored by co-culture with ADM 100 nM; the finding was similar in both HC and ALF. (B) The percentage of cells expressing CD163 was reduced after LPS treatment, without any statistical difference with cells co-cultured with ADM 100 nM. (C) The percentage of cells expressing CD206 did not change after treatment with LPS, nor with co-culture with ADM 100 nM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. PBMCs, peripheral blood mononuclear cells; ALF, acute liver failure; HC, healthy controls; LPS, lipopolysaccharide; ADM, adrenomedullin.

ing higher expression in patients with poor prognosis. The fact that M1-polarised monocytes increase ADM expression confirms that ADM is upregulated during the inflammatory response, as are other pro-inflammatory and anti-inflammatory cytokines. However, its role is more complex and controversial.[29]

We then stimulated cultured PBMCs with different concentrations of ADM: 3 nM, which was 10 times higher than that found in ALF; 100 nM, the most frequently used dose reported in the literature; and 200 nM. We found that, *per se,* ADM was not causing cytokine production, despite the fact that, in our cohort, ADM concentration was directly correlated with TNFα, IL-10, and IL-6 plasma concentrations (Supplementary Fig. 4).

Conversely, after treatment with LPS, ALF, and ACLF, PBMCs increased ADM production; this behaviour was not seen in healthy controls, even after prolongation of the culture duration. Over time, there was an increase in ADM production but no difference between LPS-treated and untreated cells.

When ADM was added to the LPS culture, it did not affect cytokine concentration in the supernatant at either 100 or 200 nM, nor the intracellular cytokine staining. This was in disagreement with current literature, in which ADM is reported to inhibit proinflammatory cytokine production.[15]

However, immunophenotyping showed that ADM affects MerTK, a marker of the compensatory anti-inflammatory response in liver failure. The MerTK$^+$ macrophage phenotype that evolves during the resolution phase is hepatoprotective and represents a novel immunotherapeutic target to promote the healing response following liver injury.[10] After LPS stimulation, MerTK was reduced in both HC and ACLF CD14+ cells, while restored by pre-treatment with ADM 100 nM. A similar trend was seen for CD163,[30] while no effect on CD206 was noted; both are also implicated in inflammation resolution.

On one hand, this monocyte phenotype is implicated in the restoration of the anti-inflammatory response, improving liver injury; on the other hand, patients are at increased risk of sepsis, a frequent cause of death in this population.[31]

Other authors reported that plasma ADM concentrations were associated with a reduction in blood pressure and vascular resistance, predicting disease severity and mortality in patients with cardiogenic and septic shock (which shares many pathobiological features with ALF).[29,32,33] ADM's ambivalent nature, inducing both vasodilation and hypotension but able to reinforce the endothelial barrier and improve outcomes in sepsis models, led to the development of ADM-targeted therapies (i.e., the monoclonal antibody adrecizumab),[11,34] currently under scrutiny for their therapeutic potential in sepsis and cardiogenic shock.

Treatment with ADM/AMBP1 decreased tissue injury in an animal model of liver ischemia/reperfusion injury, as evidenced by the reduction in liver enzymes and IL-6 concentration.[35] Similar results were found in rats after caecal ligation and puncture, where the combination attenuated tissue injury, reduced cytokine concentration, ameliorated intestinal barrier dysfunction, and improved survival.[36] However, clinical trials using a monoclonal antibody binding ADM and modulating its function failed to show benefit in patients with

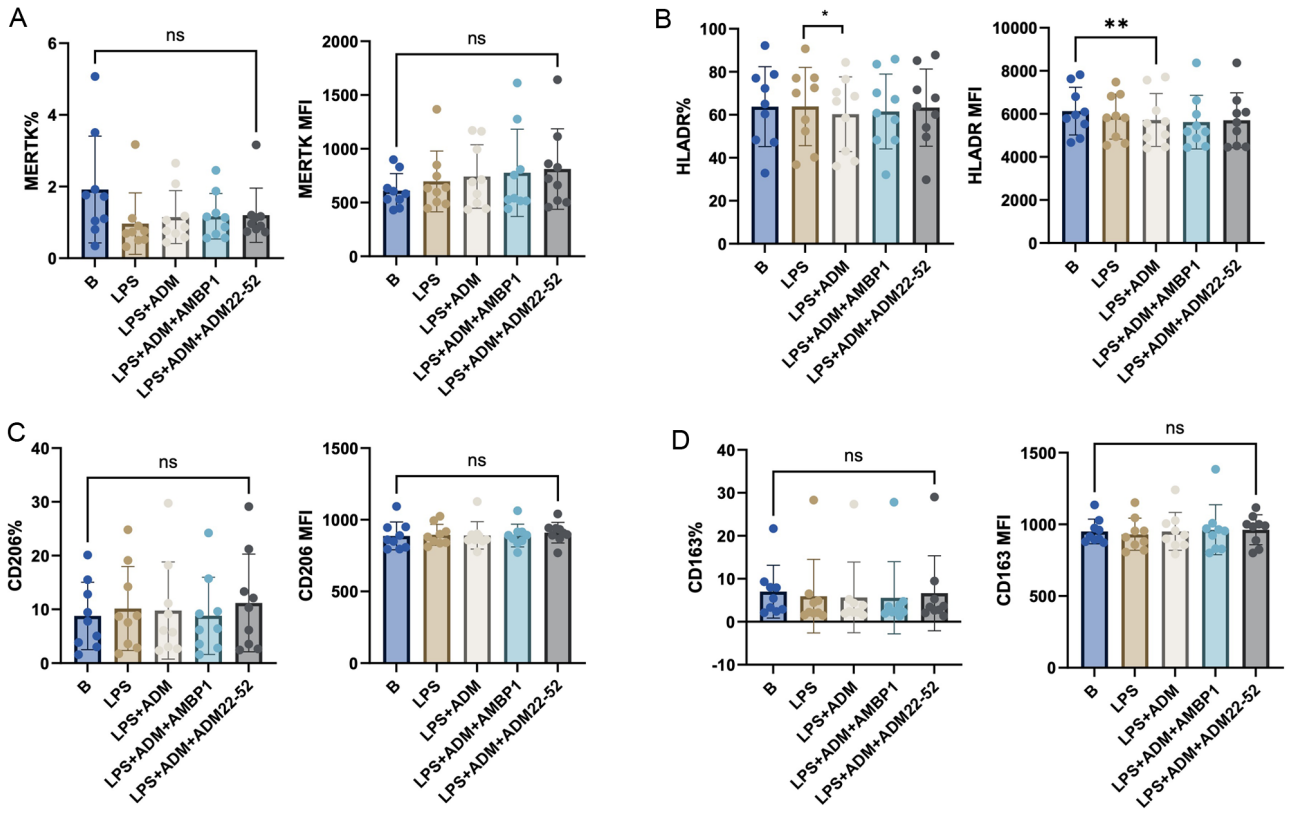

Fig. 5. Immunophenotyping of CD14+ve cells from PBMCs culture (surface staining): Four HC and four patients with decompensated cirrhosis. Treatments: LPS 100 ng/mL, ADM 100 nM, AMBP1 (65 nM), ADM 22-52 (10 μM). (A) The percentage of cells expressing MerTK remained unchanged after treatment with AMBP1 or ADM 22-52 (Friedman test with Dunn's multiple comparisons test; % $p = 0.1395$; MFI $p = 0.9500$). (B) The percentage of cells expressing HLA-DR was reduced by the combination ADM + LPS, without any significant difference compared to the other treatments (% Repeated measures ANOVA, $p = 0.4142$; T-test LPS vs. LPS + ADM $p = 0.0124$; MFI Friedman test with Dunn's multiple comparisons test $p = 0.0067$). (C) The percentage of cells expressing CD206 remained unchanged after treatment with AMBP1 or ADM 22-52 (Friedman test with Dunn's multiple comparisons test; % $p = 0.2667$; MFI $p = 0.6376$). (D) The percentage of cells expressing CD163 remained unchanged after treatment with AMBP1 or ADM 22-52 (Friedman test with Dunn's multiple comparisons test; % $p = 0.1771$; MFI $p = 0.5690$). $^{**}p < 0.01$, $^{*}p < 0.05$. PBMCs, peripheral blood mononuclear cells; HC, healthy controls; LPS, lipopolysaccharide; ADM, adrenomedullin; AMBP, adrenomedullin binding protein; MFI, median fluorescence intensity.

sepsis.[37]

Despite promising animal research, in our culture experiment, the addition of AMBP1 did not make any difference in ADM's effects. This may be attributed to the low dose chosen.

In our cohort, AMBP1 concentration dropped from 252.8 μg/mL in HC to 126.3 μg/mL in ACLF to 59.27 μg/mL in ALF. The concentration we used for the culture experiments was sub-physiological (65 nM), however, similar to those used by other authors.[38]

Comparable negative results were found with receptor antagonism. ADM binds two calcitonin receptor-like receptor/receptor activity-modifying protein (RAMP) (RAMP2 and RAMP3) heterodimers, respectively called AM1 and AM2. We used the only human ADM receptor antagonist available, a truncated form called ADM(22–52), with low affinity and weak selectivity for AM1 over AM2 receptors.[39]

This research has several limitations. In order to reduce bias, consecutive patients were recruited as close to the initiation of liver failure as clinically possible; however, we could not control for antibiotic use prior to admission into our hospital. We did exclude patients with significant pre-existing immunosuppression as per protocol. Also, due to the small number of patients, gender-based analysis was not performed. Similarly, our results may not be generalisable to patients with autoimmune liver disease or chronic infection.

The fact that we did not investigate receptor expression could be considered a limitation of the study, as it would have further elucidated ADM's role in monocyte function. Also, the use of AMBP1 was limited by the high cost of this human product; therefore, testing incremental concentrations was not feasible. Our results need cautious interpretation, and further experiments with higher doses are needed to fully clarify its function.

Conclusions

ADM is increased in acute liver failure syndromes (ALF and ACLF), and AMBP1 is, on the contrary, reduced, mirroring the severity of the disease expressed by the SOFA score. ADM affects monocyte function, increasing MerTK after LPS stimulation and promoting a pro-restorative, anti-inflammatory phenotype. Further studies are needed to fully understand how to modulate this pathway as a possible therapeutic target and restore monocyte function.

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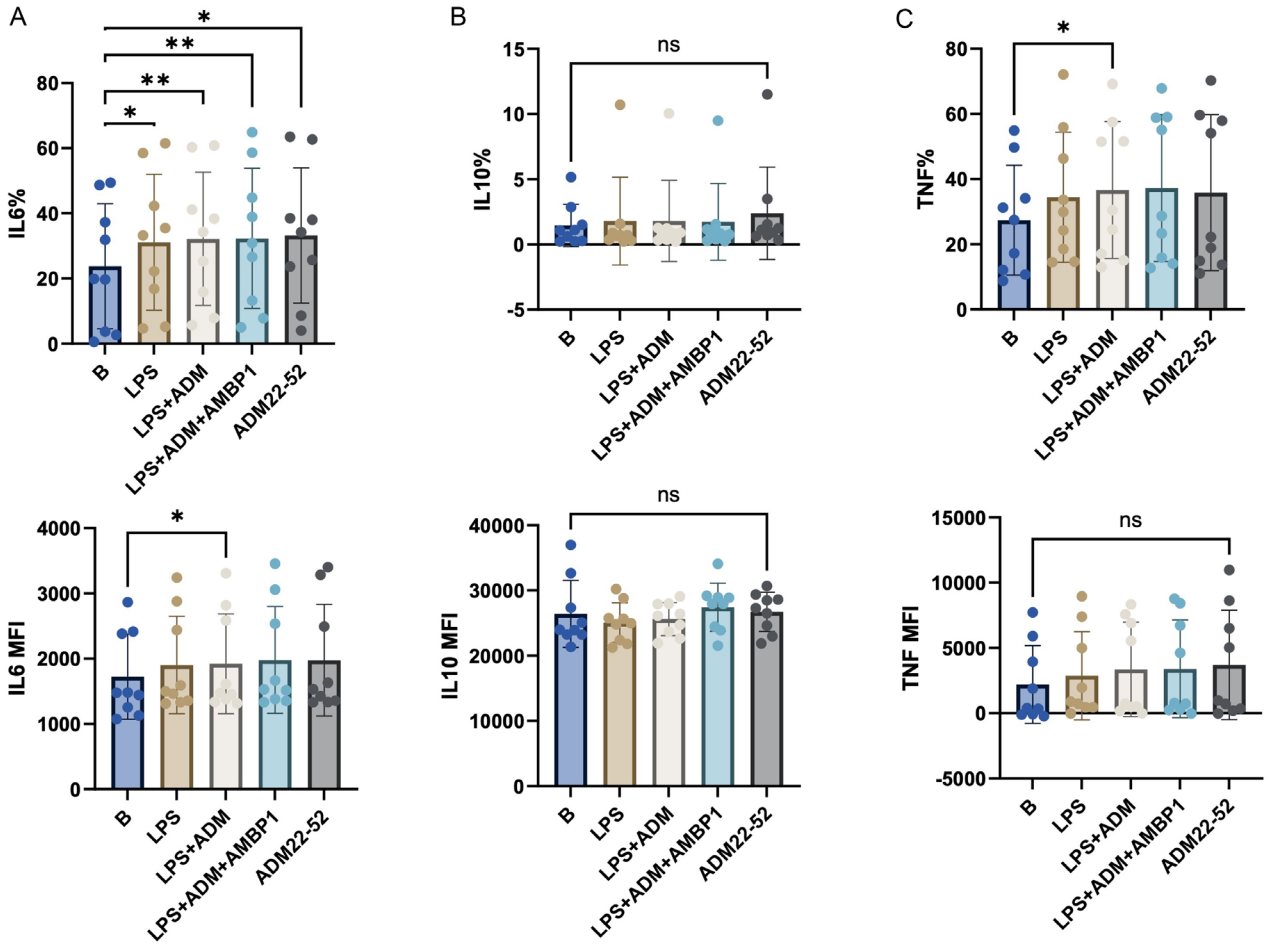

Fig. 6. Immunophenotyping of CD14+ve cells from PBMCs culture (intracellular staining): Four HC and four patients with decompensated cirrhosis. LPS 100 ng/mL, ADM 100 nM, AMBP1 (65 nM), ADM 22-52 (10 μM). (A-C) The percentage of cells expressing IL-6, IL-10, and TNFα remained unchanged after treatment with AMBP1 or ADM 22-52 (Friedman test with Dunn's multiple comparisons test). $^{**}p < 0.01$, $^{*}p < 0.05$. PBMCs, peripheral blood mononuclear cells; HC, healthy controls; LPS, lipopolysaccharide; ADM, adrenomedullin; AMBP, adrenomedullin binding protein; IL, interleukin; TNF, tumour necrosis factor.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceptualization (FMT), methodology (FMT, SA), formal analysis (FMT, SA), investigation (FMT, FA), resources (FMT, FA, RS, RA, JW, AB, JS), data curation (FMT, FA, RS, RA, JW), writing - original draft (FMT), writing - review & editing (FMT, FA, MM), visualization (FMT), project administration (FMT, FA, RS, RA, AB, JS), funding acquisition (FMT, MM), and supervision (MJM). All authors have approved the final version and publication of the manuscript.

Ethical statement

This project was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2024. The study was approved by Research Ethics Committee of King's College Hospital (No.: 19/NW/0750, IRAS No.: 244089). Written informed consent was obtained from authors.

Data sharing statement

The data are available from the corresponding authors upon reasonable request.

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