IMMUNOSUPPRESSION IN ACUTELY DECOMPENSATED CIRRHOSIS IS MEDIATED BY PROSTAGLANDIN E_2

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Abbreviations: AD – Acute Decompensation; ESLD – end-stage liver disease; AoCLF – acute-on-chronic liver failure; PG – prostaglandin; CCL4 – carbon tetrachloride; BDL – bile duct ligation.

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Liver disease is one of the leading causes of death worldwide[1]. Cirrhosis patients display an increased predisposition to and mortality from infection due to multimodal defects in the innate immune system[2-4], however the causative mechanism has remained elusive. We present evidence that the cyclooxygenase (COX)-derived eicosanoid prostaglandin E_2 (PGE_2) drives cirrhosis-associated immunosuppression. Elevated circulating concentrations (>7x healthy volunteers) of PGE_2 were observed in acutely decompensated (AD) patients. Plasma from these and end-stage liver disease (ESLD) patients suppressed macrophage pro-inflammatory cytokine secretion and bacterial killing *in-vitro* in a PGE_2-E prostanoid receptor 2 (EP2) dependent manner, effects not replicated with plasma from stable cirrhotics (Child score A). Albumin, which reduces PGE_2 bioavailability, was decreased in the AD/ESLD population (<30mg/dL) and appears to play a key role in modulating PGE_2-mediated immune dysfunction. *In vivo* administration of human albumin solution (median 200ml of 20% HAS) to these patients improved the immunosuppressive burden *in vitro*. Two murine models of liver injury (bile duct ligation [BDL] and carbon tetrachloride [CCL_4]) also exhibited elevated PGE_2, reduced circulating albumin concentration and EP-2 mediated immunosuppression. Treatment with COX inhibitors or albumin restored immune competence and survival following infection. PGE_2 suppression via stratified, targeted HAS infusions may represent a new immune restorative treatment paradigm for AD and ESLD inpatients.

The prevalence of liver disease is predicted to rise dramatically secondary to the global trends of obesity, higher alcohol consumption and rising hepatitis C virus transmission[1]. Infection represents a key cause of early death in this population, precipitating 50% of hospital admissions, with a further 15-35% developing nosocomial infections as inpatients[5]. Secondary sepsis and requirement for multi-organ support in intensive care is associated with 65-90% mortality[6]. The dysfunction of both neutrophils and monocytes in cirrhosis has long been recognised to underlie this and the presence of an inhibitory humoral mediator speculated upon, yet not definitively identified[7-9].

Cirrhosis patients are markedly heterogeneous, with critical variance in aetiology and disease stage. Susceptibility to infection, reflecting immune dysfunction is highest in AD patients and lowest in stable cirrhosis[10]. Decompensated cirrhosis patients present with hepatic encephalopathy, ascites, variceal bleeding, alcoholic hepatitis and hyperbilirubinaemia. AD is defined by the acute development of one or more of these complications and includes acute on chronic liver failure (AoCLF[4]). Immunomodulatory therapy to both prevent and treat infections in the most vulnerable groups could reduce early mortality and thus reduce the health-economic burden of liver cirrhosis in the twenty-first century.

Inflammation-driven COX-derived lipid mediators exert diverse effects on the innate immune system that could explain increased infective susceptibility in cirrhosis patients[11-14]. In particular PGE_2 has widespread immunomodulatory roles dependent on its site of action and formation; being shown to be a key mediator of myeloid-derived cell dysfunction inhibiting NADPH oxidase-mediated bacterial killing[15,16] via up-regulation of cAMP and inhibition of FcγR-mediated phagocytosis[17,18].

Using electrospray ionization liquid chromatography tandem mass spectrometry (ESI/LC-MS) to analyze AD patient plasma (day 1-2 of hospital admission) we demonstrated significantly elevated PGE_2, $PGF_2\alpha$, 5- and 15-HETE compared to healthy volunteer (HV) plasma but not TxB2, 9-HODE, 12-HETE and 13-HODE (**Fig. 1a and Supplementary Fig. 1a-g**). Of these, only PGE_2 dampened TNFα release from LPS-stimulated human monocyte-derived macrophages (MDM) at the concentrations observed (0.1ng/ml) (**Fig. 1b**). LPS-stimulated MDM TNFα release was significantly decreased by incubation with culture media supplemented with 25% (vol./vol.) plasma from AD patients (**Supplementary Table 1** for clinical characteristics) as opposed to HV plasma, an effect reversed by the addition of the EP 1-3/D-prostanoid (DP) 1 receptor antagonist AH6809 (50μM (**Fig. 1c**). Macrophages were also incubated with *Escherichia coli* (clinical isolate). Compared to macrophages treated with HV plasma, those incubated with AD plasma exhibited reduced bacterial killing, an effect again reversed by pretreatment with AH6809 (50μM) (**Fig. 1d**). AH6809 had no direct bactericidal effect while cell viability was unaffected by AD/HV plasma.

Plasma from AD patients showed persistent PGE_2-mediated immunosuppression up to day six post-admission, able to reduce MDM TNFα and elevate IL-10 production (**Fig. 1e,f**). Importantly, although ~60% of day 1 AD plasma samples induced this immunosuppressive cytokine pattern (**Fig. 1c**), all patients' plasma sampled from days 2-6 did in a PGE_2-dependent manner (**Fig. 1e,f**). PGE_2 also mediated immunosuppression in ESLD patients with plasma from ESLD patients taken 24 weeks apart significantly impairing macrophage TNFα synthesis, an effect blocked by AH6809 (**Fig. 2a**). In contrast, samples from Child score A cirrhosis or non-cirrhotic liver disease outpatients had no effect on macrophage

function (**Fig. 2b**). PGE_2 levels were twice healthy volunteer levels in Child score A as opposed to 7-fold higher in AD (**Fig. 1a and Supplementary Table 2**).

rtPCR revealed increased expression of COX 2 ($p<0.00001$), not COX 1 in peripheral blood mononuclear cells of AD patients compared to healthy volunteers (n=5/group, **Fig. 2c**). Immunohistochemical analysis of all organs from a murine model of cirrhosis (CCL_4, which induces elevated PGE_2, **Fig. 2d**) demonstrated up-regulation of COX 2 in Kupffer cells and alveolar macrophages compared to shams but not other organs (**Fig. 2e and f**) suggesting these three cell types as the likely sources of PGE_2.

We discovered that inhibiting PGE_2 increased bacterial killing and restored survival in two animal models of liver injury. Although an imperfect experimental model, BDL mice do exhibit certain similarities to AD patients (e.g. high bilirubin and low albumin, (**Supplementary Table 3**) and were found to have >5-fold elevated PGE_2 concentrations (**Fig. 2d**) but not other eicosanoids (**Supplementary Fig. 2a-p**). As in humans, BDL plasma suppressed macrophage TNFα and increased IL-10 (**Fig. 3a and Supplementary Fig. 3a,b**) without affecting macrophage viability. This was prevented by pretreatment of the mice with the non-selective COX inhibitor indomethacin but not baicalein (12-lipoxygenase inhibitor) or SKF525A (p450 inhibitor) or if BDL plasma was heat-treated (protein denatured). Again, as in humans, immunosuppression was demonstrated to be mediated via a PGE_2-E prostanoid receptor 2 (EP2) dependent mechanism **(Supplementary Fig. 3c-i).** Experiments using CCL_4 treated mice showed similar findings with significantly elevated PGE_2 (**Fig. 3b**) and PGD_2 but not other eicosanoids (**Supplementary Fig. 4a-p**). BDL mice were injected with live Group B streptococcus (GBS, NCTC10/84, serotype V). Consistent with

immunosuppression, bacteria were significantly elevated in BDL blood compared to shams (**Fig. 3c,d**). Treating BDL mice with indomethacin intraperitonealy (i.p.) or intravenously (inhibiting PGE_2 concentration by >80%) 1h before GBS administration restored bacterial killing to sham levels (**Fig. 3c,d**). Indomethacin also improved survival following GBS challenge in BDL mice to that of shams (**Fig. 3e**). In the lung PGE_2 protects against fibrosis[19] and therefore reducing levels may worsen liver fibrosis. However histological analysis with H&E and Masson trichrom staining showed no difference in livers from celecoxib (selective COX 2 inhibitor) and non-celecoxib treated CCL_4 mice (**Fig. 3f,g).**

Correlating levels of TNFα from macrophages incubated in the presence of AD plasma with corresponding plasma PGE_2 levels surprisingly resulted in a modest r^2 of only 0.41 (p=0.085). Albumin, hepatically synthesized and frequently reduced in liver disease, is known to bind A- and E-type PGs at the ligand-binding site I in subdomain 2A[20]. We postulated that immunosuppression might be a consequence of the bioavailability of PGE_2 determined by blood albumin concentration. Expressing AD plasma PGE_2 levels according to corresponding albumin levels and then correlating these values with TNFα synthesis resulted in a superior r^2 value, 0.72 (p=0.0076) (**Fig. 4a**). Adding albumin to AD plasma in order to restore levels to 40mg/dl (equivalent to HV) reversed AD plasma induced suppression of TNFα generation (**Fig. 4b**). Furthermore supplementing culture media with 40mg/dl of albumin impaired PGE_2 inhibition of TNFα release (**Fig. 4c**).

To further define this relationship AD plasma samples that dampened TNFα below the lowest TNFα exerted by healthy plasma (**Fig. 4a**) were arbitrarily grouped into AD^{MIS} (AD plasma from ***m***ost ***i***mmuno***s***uppressed) and the remainder AD^{LIS} (AD plasma from ***l****east*

immuno***s***uppressed) (**Fig. 4d**). Albumin and AH6809 reversed AD^{MIS} immunosuppression, whereas AD^{LIS} were refractory, (**Fig. 4d and Supplementary Fig. 5 a-c**). This differential effect of AH6809 on LIS/MIS samples was also observed in ESLD patients (**Supplementary Fig. 5d**). Analysis of AD patient data (**Supplementary Table 1**) demonstrated that albumin was the only feature that discriminated between MIS and LIS plasma samples (*P*=0.0012). Surprisingly the conventional markers of advanced liver disease, MELD score, Bilirubin or INR were unable to differentiate between MIS and LIS (**Supplementary Table 1**). Receiver Operating Characteristic (ROC) analysis of this cohort of 35 patients revealed that a cut off level of albumin of <30mg/dl predicted immunosuppression with a sensitivity of 70% (CI 47-87) and a specificity 67% (CI 35-90).

To examine the therapeutic potential of albumin BDL mice were treated with 20% HAS or 0.9% saline (0.5ml, i.p.) 2h prior to GBS challenge. In albumin treated mice the mean albumin levels rose from 24mg/dl to 32mg/dl. Albumin treatment resulted in significantly lower levels of blood bacteria 3h after GBS challenge compared to saline (**Fig. 4e**). The plasma PGE_2 concentration was slightly reduced in albumin treated mice (**Fig. 4f**) but as PGE_2 measured in plasma reflects both free and albumin bound PGE_2, the effect of albumin on the immune system may be unrelated to total plasma PGE_2 concentration. As albumin has no direct bactericidal effect we suggest that it improves immune competence in BDL mice via reducing PGE_2's bioavailability.

20% HAS given to patients admitted to hospital with AD (median 200 ml) with albumin <30 mg/dl raised albumin levels from 23.7±1.7 to 30.1±3.1 mg/ml and reduced *in-vitro* evidence of immunosuppression (**Fig. 4g**), *P*<0.05, n=6). Matched samples from patients not given

albumin demonstrated persistent immunosuppression. In patients admitted with acute hepatic encephalopathy we observed that plasma induced immunosuppression persisted for up to 60 days following discharge (P<0.001, **Fig. 4h**) and that administration of 20% HAS on days 1 & 3 had no effect on long term immunosuppression (samples 8-60 days after albumin). Saline had no effect (**Supplementary Fig. 5e**).

Taken together these findings implicate increased bioavailability of PGE_2 as a primary cause of innate immune dysfunction and thus vulnerability to infection in cirrhotic patients presenting with AD and in ESLD. We accept that other mediators are highly likely to also be responsible for cirrhosis-induced immunosuppression such as circulating endotoxin secondary to bacterial translocation[21]. However we suggest that elevated PGE_2 is likely to represent the primary driver and offers a potential target for immunomodulatory therapy. Our studies reveal the likely source of the PGE_2 to be peripheral blood mononuclear cells, liver Kupffer cells and alveolar macrophages. Increased PGE_2 production may be triggered by a combination of liver-injury related inflammatory cell infiltrate and gut-bacterial translocation resulting in elevated COX activity in these cell types[22]. Critically, the immunosuppressive effect of PGE_2 was increased by reduced serum albumin concentration in AD patients. Albumin is known to both bind and catalyse E-series prostaglandins and its reduction may be expected to result in elevated PGE_2 bioavailability[20]. This relationship may explain why plasma from stable cirrhosis patients had no immunosuppressive effects despite elevated PGE_2 (twice control values); this population being protected by normal albumin concentrations. Despite the apparent anti-fibrotic properties of PGE_2[23] 5 days of COX 2 inhibition did not worsen liver fibrosis.

PGE_2 mediated immunosuppression was observed in >75 patients with either AD or ESLD recruited from 5 sites in two countries and in almost all AD patients from day 2 of their admission till at least 60 days following discharge. In this population a serum albumin concentration <30mg/dl may be predictive of immunosuppression (reflecting an inverse correlation with PGE2 bioavailability). Crucially we have demonstrated that 20% HAS infusions given *in vivo* to achieve a concentration above this level restores (albeit temporarily) immune competency, reversing AD-plasma induced impairment of cytokine production *ex-vivo* and both bacterial killing and survival in *in vivo* mouse models to HV/sham levels respectively.

Although effective experimentally, non-steroidal anti-inflammatory drugs (NSAIDS) are contraindicated in advanced cirrhosis patients due to increased incidence of renal impairment and gastrointestinal bleeding[24]. Albumin, already safely used to treat cirrhosis-associated complications such as spontaneous bacterial peritonitis (SBP)[25], appears to selectively antagonize PGE_2's effects on the immune system without compromising its protective role in other tissues. Recently suggested to exert multimodal beneficial effects in the cirrhotic population[26,27], we provide mechanistic insight into one of these: its immune restorative properties. Stratified, targeted HAS therapy to achieve and maintain serum concentrations at near normal concentrations may thus represent an elegant, cheap, and rapidly implementable management paradigm to ameliorate PGE_2-induced innate immune impairment both preventing and treating infection in patients presenting with AD. HAS may also represent a therapeutic strategy in ESLD patients[28]. These management paradigms would clearly require validation by large-scale clinical trials.

ONLINE METHODS

Human Models

Samples were obtained from several sources representing the spectrum of cirrhosis patients that vary in vulnerability to infection. Non-cirrhotic liver disease patients were used as a control. Informed consent was obtained from all subjects. Consecutively sampled unselected plasma samples from these cohorts were used in a blinded fashion for the assays of immune function. We were able to gain anonymised patient data for the patients from the DASIMAR study for correlation with our laboratory results following the experiments.

We obtained plasma samples from the following patient cohorts with acutely decompensated cirrhosis. Patients with Acute-on-Chronic-Liver-Failure (AoCLF) samples from the Predictive utility of DASIMAR as a prognostic biomarker in AoCLF study (ClinicalTrials.gov: NCT01071746). Patients with acute encephalopathy from the ALFAE study (Efficacy of albumin for acute encephalopathy in patients with cirrhosis, Hospital Clinic of Barcelona; ClinicalTrials.gov: NCT00886925). Patients with an acute presentation of decompensated liver cirrhosis secondary to alcoholic liver disease from University College London Hospital (UCLH). Patients on the liver transplant waiting list, representing end stage liver disease (ESLD) were followed up for 24 weeks under the MACHT study (Effect of midodrine and albumin in the prevention of complications in cirrhotic patients awaiting liver transplantation, Hospital Clinic of Barcelona; ClinicalTrials.gov: NCT00839358). Patients with stable cirrhosis (Child's Pugh A) from The Royal London Hospital outpatient clinic and Patients withnon-cirrhotic liver disease (Non-Alcoholic Fatty Liver Disease) from The Royal London Hospital outpatient clinic.

Ethical approval was granted for all studies. AoCLF samples were obtained from 'Predictive utility of DASIMAR as a prognostic biomarker in AoCLF' (ClinicalTrials.gov identifier: NCT01071746; UCLH NHS Research Ethics Committee number: 08/H0714/8) along with age/gender matched healthy volunteer controls. Samples were obtained from patients admitted to the Royal Free Hospital on days 1, 2, 5, 6 following admission. Samples from patients awaiting transplant originated from MACHT (Effect of midodrine and albumin in the prevention of complications in cirrhotic patients awaiting liver transplantation; ClinicalTrials.gov identifier: NCT00839358). Patients enrolled to MACHT received 200g albumin/placebo on admission with blood sampling at baseline, 4 and 12weeks time-points post-intervention. ALFAE (Efficacy of albumin for acute encephalopathy in patients with cirrhosis; ClinicalTrials.gov identifier: NCT00886925) provided samples from patients with non-terminal cirrhosis (early-phase) randomized to receive either 200g albumin (1L, 20% human albumin solution) over 48hours or an equivalent volume of 0.9% saline. Samples were drawn prior to intervention and 8-10 days and 30-60 days post. Samples were also acquired through on-going local research from University College Hospitals NHS Trust (UCLH) and the Royal London Hospitals NHS Trust (Monocyte and macrophage phenotype and function in liver failure; Harrow NHS Research Ethics Committee no. 12/LO/0167) were utilised to explore its effect on short-term immune function. At UCLH albumin was administered in response to clinical indication(s), and samples drawn pre and 24 hours post infusion.

Extraction and analysis of lipid mediators

Lipid mediators in mice/human plasma were analyzed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) based on protocols published previously[16]. Briefly, samples were collected and stored immediately at −80 °C. Plasma Samples (250-500µl for mice, 500–1,000µL for humans) were defrosted on ice and

adjusted to 15% (v/v) methanol: water (final volume 4mL). Internal standards, PGB_2-*d*4 (40 ng) and 12-HETE-*d*8 (40ng) (Cayman Chemical Company, Ann Arbor, USA) were added and the pH of resulting solutions adjusted to 3.0 (1M HCL). Acidified samples were immediately applied to preconditioned solid-phase cartridges (C18-E, Phenomenex, Macclesfield, UK) and lipid mediators eluted with methyl formate. LC/ESI-MS/MS analysis was performed on a HPLC pump (Waters Alliance 2695) coupled to an electrospray ionization triple quadruple mass spectrometer (Quattro Ultima, Waters, UK). Chromatographic separation was performed on a C18 Luna column (5µm, 150 x 2.0mm, Phenomenex) for eicosanoids and a C18 Kinetex column (2.6µm, 100 x 2.1mm, Phenomenex) for hydroxy- fatty acids. Analytes were monitored on multiple reaction monitoring mode as reported with the following additions: 15-hydroxyeicosatrienoic acid (HETrE) *m/z* 321>221, 10-hydroxydocosahexaenoic acid (HDHA) *m/z* 343>153, 14-HDHA *m/z* 343>161, 13-HDHA *m/z* 343>193 and 17-HDHA *m/z* 343>201.

Human blood analysis and macrophage isolation/culture

To prepare human monocyte-derived macrophages (MDM) , healthy volunteer mononuclear cells were isolated by differential centrifugation (900g, 30min, 20°C) over Lymphoprep™ (Axis-Shield) and washed with sterile PBS (Invitrogen™, Life Technologies™)[17]. Cells were resuspended in 10ml RPMI-1640 medium (Invitrogen™, Life Technologies™)) supplemented with 100 $U.ml^{-1}$ of penicillin/100µg.ml^{-1} streptomycin (Invitrogen™, Life Technologies™)) and plated at a density of ~5×10^6 cells/ml in 8cm^2 Nunclon Surface tissue culture dishes (Nunc®). After 2h incubation at 37°C (5% CO2), non-adherent cells were discarded and 10ml of fresh RPMI supplemented with 10% FBS added (Sigma-Aldrich®) (with antibiotics as above). After culture for 5 days at 37°C (5% CO2), with addition of a further 10ml of fresh 10% FBS/RPMI after 24h (total 20mls), adherent cells were scraped on

day 5 and replated in 96-well culture plates (Nunc®) at equal densities (10^5/well) in X-Vivo-15 serum free 0.2% HAS medium (Lonza). Resulting primary MDM, validated by morphological inspection, were incubated overnight at 37°C (5% CO_2) to adhere prior to stimulation with 200ng/ml LPS (*Salmonella abortus equi*, Enzo Biochem, Inc). To examine the effect of plasma/serum on macrophages, these were added to cell culture 30min prior to LPS to give a final volume of 25% plasma/serum. Similarly eicosanoids of interest were also added to cell culture 30min prior to LPS. AH6809 (50-300μM) was dissolved in X-vivo and added to the macrophages 45min prior to plasma. Macrophage supernatants were collected after 24h stimulation with LPS and stored at -80 °C for cytokine analysis by ELISA as described. Cell viability was ascertained with calcein AM cell viability assay kit (Biotium, Inc). Human serum albumin was obtained from Sigma-Aldrich >99% (agarose gel electrophoresis), essentially fatty acid (0.005%) and globulin free. For bacterial killing, *Escherichia Coli* (clinical isolate, provided by Dr Vanya Gant, Clinical Director for Infection, Consultant Microbiologist, UCLH) was added to healthy volunteer macrophages (ratio 100 *E.coli*: 1 macrophage) in the presence/absence of plasma from patient/healthy volunteer plasma ± AH6809 (50-300μM, Sigma Aldrich Dorset, UK) or albumin (15g/l) as above for 1h in media without antibiotics. Supernatants were plated on blood agar overnight and CFUs counted.

RNA isolation and gene expression studies

Peripheral blood mononuclear cells (PBMC) were isolated from total blood with the Ficoll method (Ficoll-Paque^{TM} PLUS, GE-Healthcare, Cat. No:17-1440-03) from four patients (3 males/1female) and six healthy controls (3 males/3 females). The PBMC cell pellet was immediately frozen at -80^{o}C until cell lysis and total RNA extraction with the RNeasy Mini Kit (Qiagen, Cat. No: 74104). RNA concentration was measured spectrophotometrically at

260 nm (Nanodrop8000, Thermo Fishcer), and RNA purity was determined by the *A*260/*A*280, *A*230/260 ranges and spectral pattern. 500ng of total RNA were reverse transcribed to cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase, RNase H Minus, Point Mutant (M-MLV RT (H–) (Promega, Cat No: M368A) and Promega oligodTs, random primers and M-MLV X5 RT buffer (Promega). cDNA was then subjected to quantitative real-time PCR using an 7900HT ABI thermal cycler (Applied Biosystems), SYBGR (Applied Biosystems, Cat. No: 4367659) and in-house designed and optimised primers for the genes of interest and internal controls. Primer sequences were: *PTGS1*, forward primer: *TACCAGGTGCTGGATGGAGA*, reverse primer: *CCTTCAGCAGGTCACACAC*; *PTGS2*, forward primer: *GTTTTGACATGGGTGGGAAC*, reverse primer: *CCCTCAGACAGCAAAGCCTA*, *CYPH*: forward primer: *GCCGAGGAAAACCGTGTACT*, reverse primer: *ACCTTGTCTGCAAACAGCTCA; RPL27* (ribosomal protein 27), forward primer: *TCCAAGGGGATATCCACAGA*, reverse primer: *CATGGGCAAGAAGAAGATCG*, *RSP20 (ribosomal protein 20)*, forward primer: *GGAAACGATCCCACGTCTTA*, reverse primer: *AGAGGCGCAAAAGAAAAGAA*. Dissociation curve analysis was performed at 40 cycles to verify the identity of PCR product. For data analysis, the comparative threshold cycle values for constitutively expressed *CYPH* were used to normalize loading variations and data are expressed as arbitrary units (AU). Gene expression data analysis was also undertaken with the use of *RPL27* and *RPS20* as endogenous controls.

Animal models

Mice were maintained in a 12h/12h light/dark cycle at 22 ± 1°C and given food and tap water ad libitum in accordance with UK Home Office regulations. Studies were performed in male C57Bl6/J mice (20-25g) from Charles River UK, Margate, UK. Two models of liver injury

were used: Bile duct ligation (BDL, 2 weeks) was performed[29] or Carbon Tetrachloride (CCL_4, Merck, Darmstadt, Germany, 8 weeks) given s.c. (1 ml/kg) twice weekly and 300mg/L phenobarbital in water[30]. We used these to investigate whether PGE_2 inhibitors affected bacterial killing and survival in infection models in vivo.

Bile duct ligation/sham procedures were carried out under anesthesia (isoflurane 1.5%) as described previously[18]. Carbon tetrachloride (CCL_4, Merck, Darmstadt, Germany) was given subcutaneously (s.c., 1:1 dissolved in olive oil; 1 ml/kg) twice weekly and 300mg/L phenobarbital added to drinking water[19]. Sham mice received s.c. injections of olive oil. After 2 weeks for BDL mice or 8 weeks for CCL_4 mice, interventional models were undertaken or blood and liver were taken and prepared for histology or further experimental use. Experiments were performed on CCL_4 mice on the same day as the final injection.

Interventional models: peritonitis and intra-venous bacterial inoculation

Group B Streptococcus (GBS) (NCTC10/84, serotype V) was grown in Todd Hewitt broth without agitation at 37°C to an OD600 of 0.4, equivalent to 10^8 Colony Forming Units (CFU)/mL, centrifuged/washed with sterile PBS and injected intraperitoneally (i.p.) at $30x10^6$ colony forming units (CFUs) in 300µl sterile PBS or for bacterial killing assays. A clinical isolate of GBS was provided by Dr Vanya Gant, Clinical Director for Infection, Consultant Microbiologist, UCLH and $1x10^7$CFUs in 300µl sterile PBS i.p. was used for mouse survival studies; $6x10^7$ CFUs for i.p saline vs albumin infusion studies and $4x10^7$ in 200µl sterile PBS for intravenous (i.v.) studies. For bacterial killing assays in mice with/without the non-selective COX-inhibitor indomethacin ($3mg.kg^{-1}$ p.o. 1h prior to bacterial challenge, Sigma-Aldrich®. or equivalent volume of vehicle), heparinized blood was collected 3h after GBS injection, centrifuged (10,000g, 4°C, 10min), plated on agar overnight and CFUs counted the

following day. For the survival experiments, mice were observed every 6h for up to 80h. In accordance with Home Office guidance, mice were humanely culled if they had a "sickness score" of 4 or 3 for greater than 24h (**Supplementary Table 4**).

Cytokine analysis

Murine cytokine expression profiles were determined using Th1/Th2 10-plex FlowCytomix Mouse Kits (Bender MedSystems). Fluorescence was measured on a flow cytometer (BD FACSCanto II™, BD bioscience) and data analyzed using manufacturer's software. The expression profile of a cytokine panel in human macrophage supernatants was measured using the MSD® Bio-Plex human cytokine assay (Merck, Sharp & Dohme Ltd.). Our assay was customized to detect/quantify IL-1, IL-6, IL-8, IL-10 and TNFα. Key cytokine changes were confirmed by dedicated ELISA (TNFα and IL10 - mouse eBioscience or human Duoset®, R&D systems). All samples were run in duplicate.

Mouse blood analysis, macrophage isolation/culture

Blood was collected by intracardiac puncture into heparin and centrifuged (10,000g, 4^{O}C, 10min). Plasma was analyzed for liver and renal function tests using the COBAS® INTEGRA 400 plus multianalyser with appropriate diagnostic kits (Roche - Diagnostics) or stored at -80°C. Peritoneal macrophages from healthy animals were isolated as described previously[20] and incubated with/without LPS (*Salmonella Typhosa*, 0.1μg/mL for 24h; Sigma-Aldrich®) in the presence of plasma (50μl) from naïve, sham, BDL or CCL_4 mice in cell culture media (complete DMEM; Life Technologies™) to give a final concentration of 25% by volume. In other experiments plasma was added to cell culture, as above, from BDL mice given indomethacin (3mg.kg^{-1} po 6h prior to sampling); the cytochrome P450 inhibitor

SKF525A (Enzo® Life Sciences; 50mg.kg^{-1} s.c. 1h prior to sampling); or the 12/15 lipoxygenase (LOX) inhibitor Bacalein (Sigma-Aldrich®; 100mg.kg^{-1} s.c. 90min prior to sampling). In experiments examining the effect of the DP1/EP1-3 receptor antagonist AH6809 (300μM; Sigma-Aldrich®), this compound was dissolved in DMEM and added to macrophages 45min prior to addition of plasma. Cell viability was ascertained with the calcein AM cell viability assay kit (Biotium, Inc) in accordance with manufacturer's instructions. For heat inactivation, plasma was placed at 56°C in a water bath for 1h and samples stored overnight at 4°C and used for cell culture the following day.

Immunohistochemistry

For COX 1 and COX 2 expression, immunohistochemistry was carried out on formalin-fixed, wax-embedded mouse tissues using COX 1 polyclonal antibody (Cayman Chemical, catalogue number 160109) and COX-2 polyclonal antibody **(**Cayman Chemical, catalogue number 160106) using rabbit anti-mouse as secondary.

Randomisation

Cages of animals (n=5/cage) were randomly allocation to surgical or chemical-induced liver injury. Thereafter, groups of mice were allocated to receive drug intervention, infectious stimuli or both. In addition, groups of liver disease mice and sham controls were chosen for plasma extraction for *ex vivo* bioassays, where appropriate. For human studies, randomisation was not possible as human monocyte-derive macrophages received plasma from either healthy volunteers or from liver disease patients.

Blinding

For all mouse and human cell culture and bacteria experiments and mouse survival experiments, the investigator was blinded to the sample source during both the experiment and analysis of the data.

Statistical analysis

For calculation of group sizes, from experiments with murine peritonitis, cellular profiles, inflammatory protein expression and lipid mediator production is extremely reproducible. We found with random allocation of animals to each group that intra-animal replicate variability is much less than inter-animal biological variability. An effect size of ~40% of parameter mean is considered biologically relevant. Using this and population statistics, to enable statistical determination at a $P<0.05$ in a primary ANOVA screen followed by *post-hoc* Bonferroni corrected T-test at 90% power, a group size of 5 animals is necessary with a maximum of 5 groups per experiment. Applying this approach to humans using human cirrhotic plasma on humans cells with bacterial killing and TNFα as a readout, a minimum of n=10/group was required in order to discern significant changes in immune function. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software). For comparisons between multiple groups, 1-way ANOVA with repeated measures was performed followed by Bonferroni post-test. Comparisons between 2 groups were made by 2-tailed (un)paired t test. Differences between time-response curves were assessed by 2-way ANOVA. Correlations between variables were calculated using linear regression with Pearson statistic. For data not normally distributed (clinical data presented in **Supplementary Table 1**) the Mann-Whitney test was used. $P < 0.05$ was considered statistically significant. No data, either rodent or human, were excluded from analysis.

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We declare that the authors have no competing interests as defined by Nature Publishing Group, or other interests that might be perceived to influence the results and/or discussion reported in this article.

AUTHOR CONTRIBUTIONS

DWG/AO'B conceived the idea, AO'B carried out the work. DWG and AO'B co-wrote the paper and JNF edited. JNF/GS/JN/SJ/EK/GA carried out biochemical assays while WA/RG supplied clinical samples. KM/AN carried out electrospray ionization tandem mass spectrometry analysis, AW carried out histological analysis.

FIGURE LEGENDS

Figure 1. Elevated PGE_2 in plasma of patients admitted to hospital with acute decompensation (AD) is immunosuppressive (**a**) LC/ESI-MS/MS analysis of PGE_2 in plasma of AD patients (day 1 or 2 of admission) and healthy volunteers. (**b**) TNFα release from human monocyte-derived macrophages stimulated with LPS in the presence of PGF2a, PGE2, 5-HETE, 12-HETE and 15-HETE (experiments on cells from n=7 healthy volunteers in duplicate). (**c**) TNFα release from human monocyte-derived macrophages stimulated with LPS in the presence of plasma from AD patients (day 1 or 2 of admission) and healthy volunteers with or without the PGE_2 EP1-3 antagonist receptor antagonist AH6809, 50μM/ 300μM (plasma from n=35 patients used with experiments in duplicate). (**d**) Human monocyte-derived macrophage bacterial killing in the presence of plasma from AD patients (day 1 or 2 of admission) or healthy volunteers with or without AH6809, 50μM (n=8-10). TNFα (**e**) and IL-10 (**f**) release from human monocyte-derived macrophages stimulated with LPS in the presence of plasma from AD patients (day 2 to 6 of admission) and healthy volunteers with or without AH6809, 50μM (n= 3-15 patients). Data are represented as mean ± SEM. * $P<0.05$ ** $P<0.01$ *** $P<0.001$, ANOVA.

Figure 2. Plasma from End Stage Liver Disease patients but not stable cirrhosis or non-cirrhotic liver disease demonstrates PGE_2 mediated immunosuppression. Elevated PGE_2 is derived from circulating monocytes, kuppfer cells and alveolar macrophages.

(**a**) TNFα release from human monocyte-derived macrophages stimulated with LPS in the presence of plasma from patients awaiting liver transplant sampled 24 weeks apart and healthy volunteers with and without AH6809 (n=13 patients). (**b**) TNFα release from human monocyte-derived macrophages stimulated with LPS in the presence of plasma from stable

cirrhosis (Child score A, n=16) or non-cirrhotic liver disease outpatients (n=5) and healthy volunteers with and without AH6809. (**c**) rtPCR of COX-2 and COX-1 in peripheral blood mononuclear cells isolated from AD patients and healthy volunteers (n=5 per group). (**d**) Plasma PGE_2 concentrations in naïve, sham, bile duct ligation (BDL) and carbon tetrachloride (CCL_4) liver injury mice (n≥6 mice/group). (**e**) Immunohistochemical staining for COX 2 in liver Kupffer cells and alveolar macrophages, scale bar 50μM. Data are represented as mean ± SEM. * P<0.05 ** P<0.01 ***P<0.001, ANOVA.

Figure 3. Inhibiting PGE_2 reversed impairs bacterial killing and restores survival following bacterial infection in mice models of liver injury. (**a**) TNFα release from peritoneal macrophages from naïve mice stimulated with LPS in the presence of plasma from healthy (naïve) mice or BDL mice with or without administration of indomethacin, SKF525A, LNAME or baicalein prior to blood sampling (n≥6 mice/group). (**b**) TNFα release from peritoneal macrophages from naïve mice stimulated with LPS in the presence of plasma from healthy (naïve) mice or CCL_4 mice with or without indomethacin prior to blood sampling or AH6809 (300μM) added in vitro to cell culture. Blood bacterial counts 3 hours following either (**c**) intraperitoneal or (**d**) intravenous Group B *streptococcus* administration to sham or BDL mice (with or without indomethacin pretreatment) (n≥8 mice/group). (**e**) Kaplan-Meier survival curves of sham and BDL mice (with or without indomethacin pretreatment) following intraperitoneal Group B *streptococcus* administration. Haematoxylin and eosin stain of liver from CCL_4 liver mice treated with (**f**) or without (**g**5 days of celecoxib, scale bar 50 μM. Data are represented as mean ± SEM. * P<0.05 ** P<0.01 ***P<0.001, ANOVA.

Figure 4. PGE_2-mediated immunosuppression by AD plasma is improved by albumin. (**a**) Correlation graph of LPS stimulated human monocyte-derived macrophage TNFαα synthesis in the presence of AD plasma expressed by that plasma samples' PGE_2 concentration divided by its albumin concentration. (**b**) LPS stimulated monocyte-derived macrophage TNFαα synthesis in the presence of AD plasma samples compared with the same AD samples that have had >99% purified human serum albumin added in vitro to restore mean levels to 40 g/l (n=19 patients, experiments in duplicate). (**c**) LPS stimulated monocyte-derived macrophage TNFα synthesis in the presence of increasing concentrations of PGE_2 with or without addition of 40mg/dl of >99% purified human serum albumin. (**d**) LPS stimulated monocyte-derived macrophage TNFα synthesis in the presence of AD plasma samples from the ***m***ost ***i***mmuno***s***uppressed (AD^{MIS}) and the ***l****east* ***i***mmuno***s***uppressed (AD^{LIS}) (AD plasma from) compared with the same AD samples that have had >99% purified human serum albumin added in vitro to restore mean levels to 40 g/l. (**e**) 3 hour blood bacterial counts from BDL mice given 0.5ml of 20% Human Albumin Solution (HAS) subcutaneously compared to normal saline (0.9%) prior to intraperitoneal *Group B streptococcus* administration(n=10/group). (**f**) PGE_2 plasma concentrations 3 hours after intraperitoneal *Group B streptococcus* administration from BDL mice given either 20% HAS or normal saline prior to bacterial administration (n=5). (**g**) LPS-stimulated human monocyte-derived macrophages TNFα synthesis in the presence of AD plasma samples from patients pre and post infusion of 20% HAS (median 200ml) (n=6) or plasma samples taken 1 day apart in patients not treated with 20& HAS. (**h**) LPS-stimulated human monocyte-derived macrophages TNFα synthesis in the presence of healthy volunteer or AD plasma samples in patients administered 20% HAS on days 1(400-600ml) & 3 (200-400ml) of admission with samples obtained during admission and at 10-20 or 30-60 days post discharge (n=10). Data are represented as mean ± SEM. * $P<0.05$ ** $P<0.01$ ***$P<0.001$, ANOVA.

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Figure 4

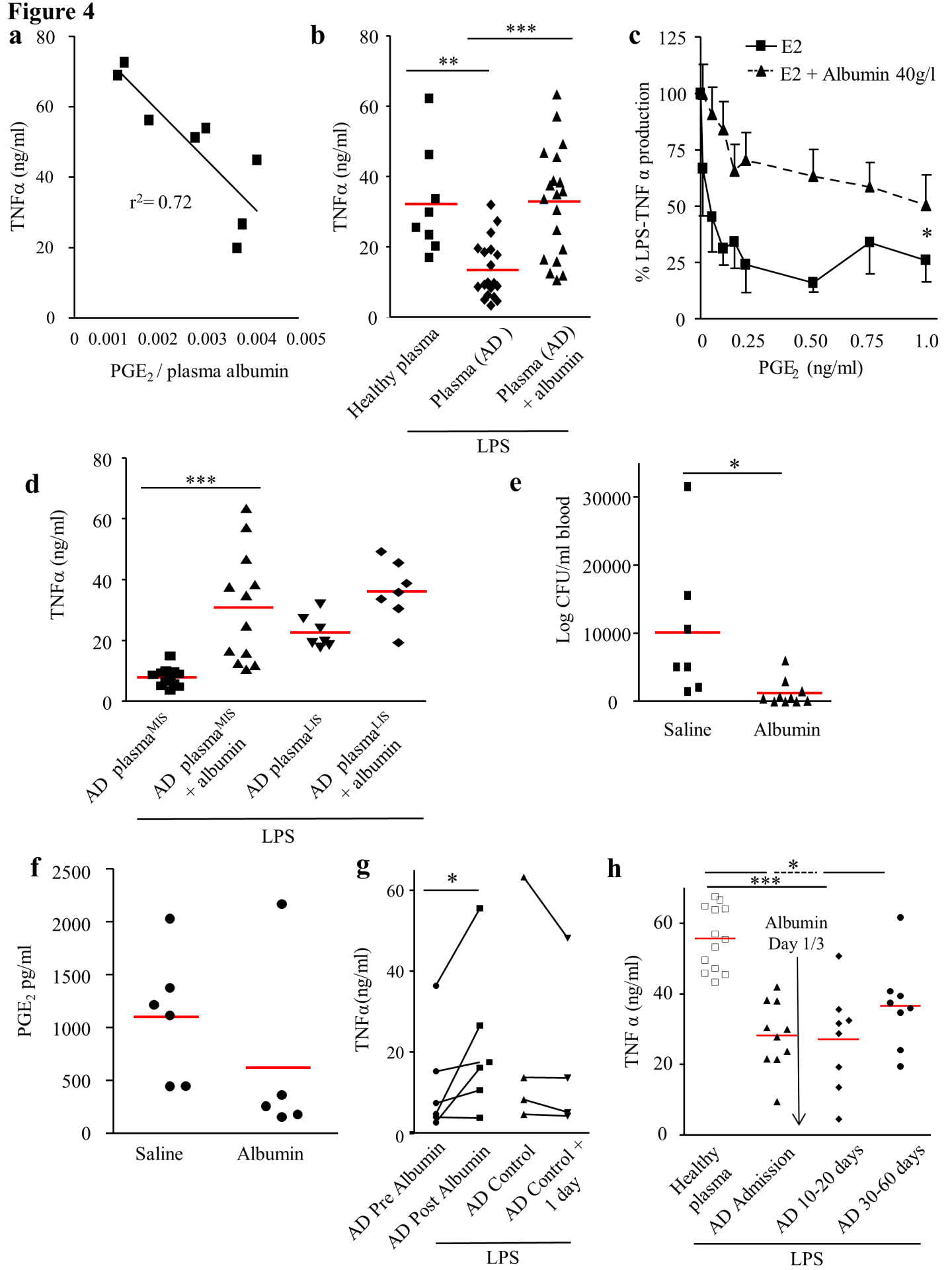

a
TNFα (ng/ml)
r²= 0.72
PGE2 / plasma albumin
b
TNFα (ng/ml)
**
***
Healthy plasma
Plasma (AD)
Plasma (AD) + albumin
LPS
c
E2
E2 + Albumin 40g/l
% LPS-TNF α production
PGE2 (ng/ml)
d
TNFα (ng/ml)
***
AD plasma MIS
AD plasma MIS + albumin
AD plasma LIS
AD plasma LIS + albumin
LPS
e
Log CFU/ml blood
*
Saline
Albumin
f
PGE2 pg/ml
Saline
Albumin
g
TNFα(ng/ml)
*
AD Pre Albumin
AD Post Albumin
AD Control
AD Control + 1 day
LPS
h
TNF α (ng/ml)
*
***
Albumin Day 1/3
Healthy plasma
AD Admission
AD 10-20 days
AD 30-60 days
LPS

Figure 3

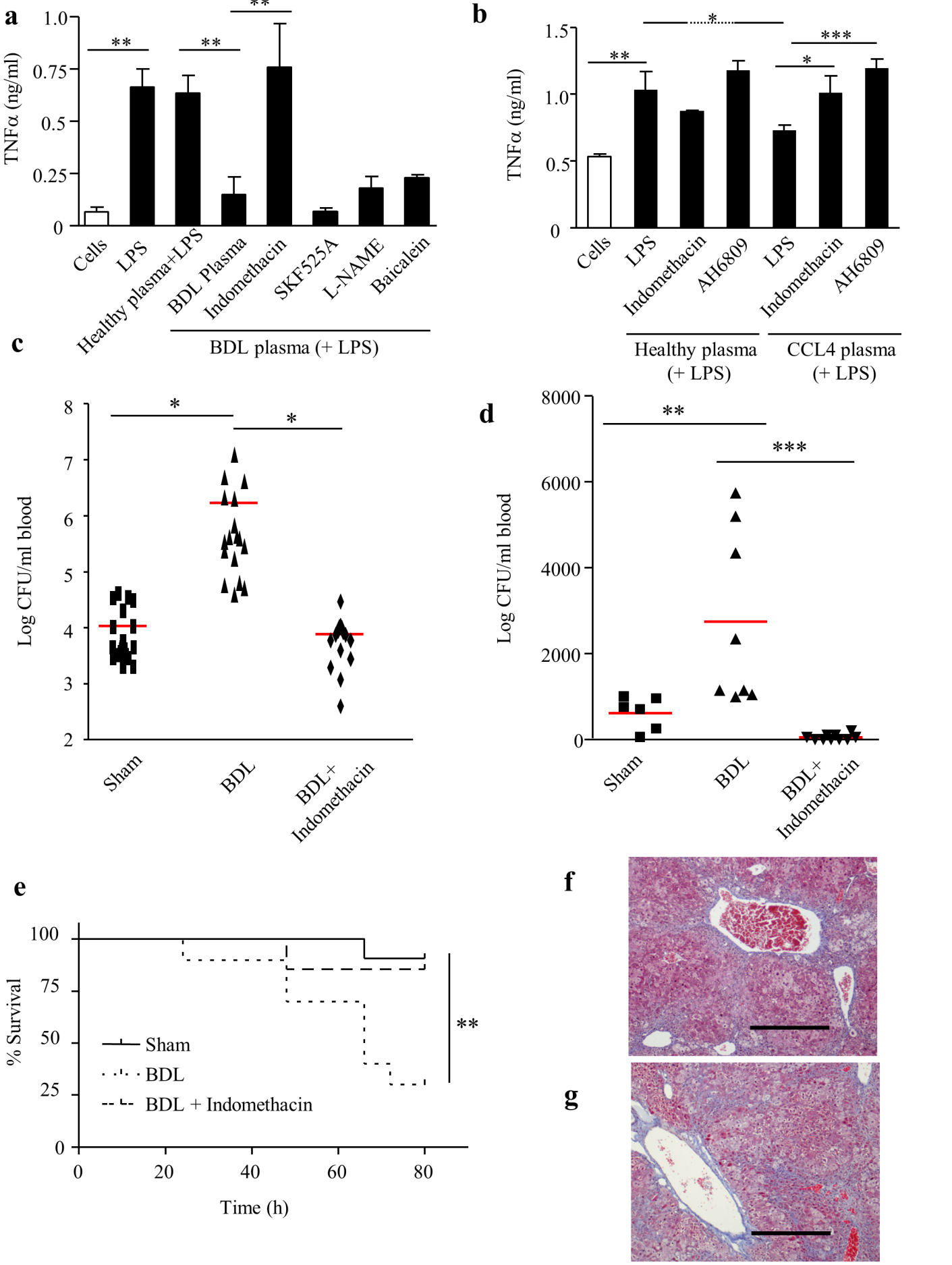

a
TNFα (ng/ml)
1.0
0.75
0.5
0.25
0
**
**
**
Cells
LPS
Healthy plasma+LPS
BDL Plasma
Indomethacin
SKF525A
L-NAME
Baicalein
BDL plasma (+ LPS)
b
TNFα (ng/ml)
1.5
1.0
0.5
0
**
*
*
***
Cells
LPS
Indomethacin
AH6809
LPS
Indomethacin
AH6809
Healthy plasma (+ LPS)
CCL4 plasma (+ LPS)
c
Log CFU/ml blood
8
7
6
5
4
3
2
*
*
Sham
BDL
BDL+ Indomethacin
d
Log CFU/ml blood
8000
6000
4000
2000
0
**
***
Sham
BDL
BDL+ Indomethacin
e
% Survival
100
75
50
25
0
Sham
BDL
BDL + Indomethacin
**
0
20
40
60
80
Time (h)
f
g

Figure 2

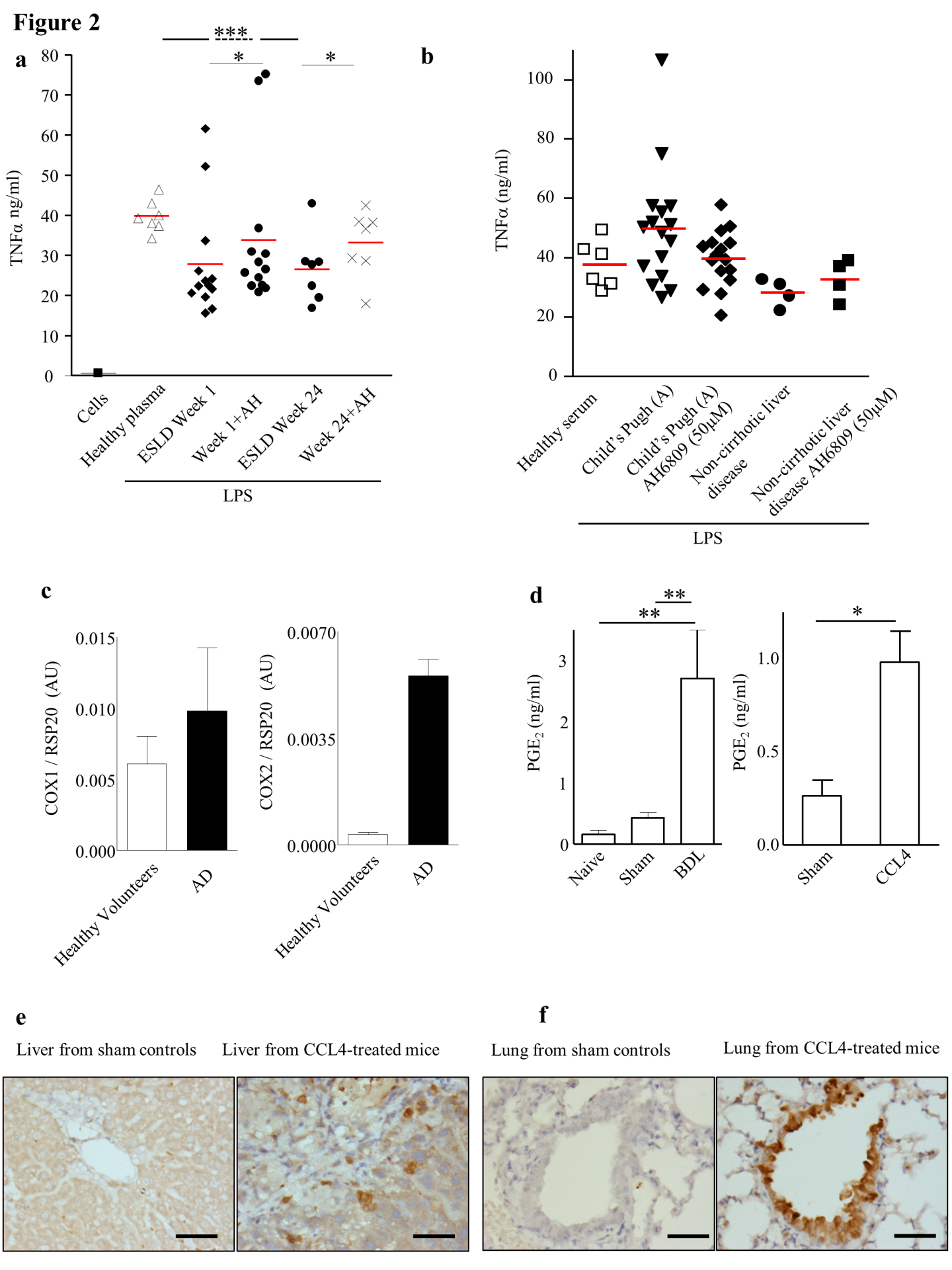
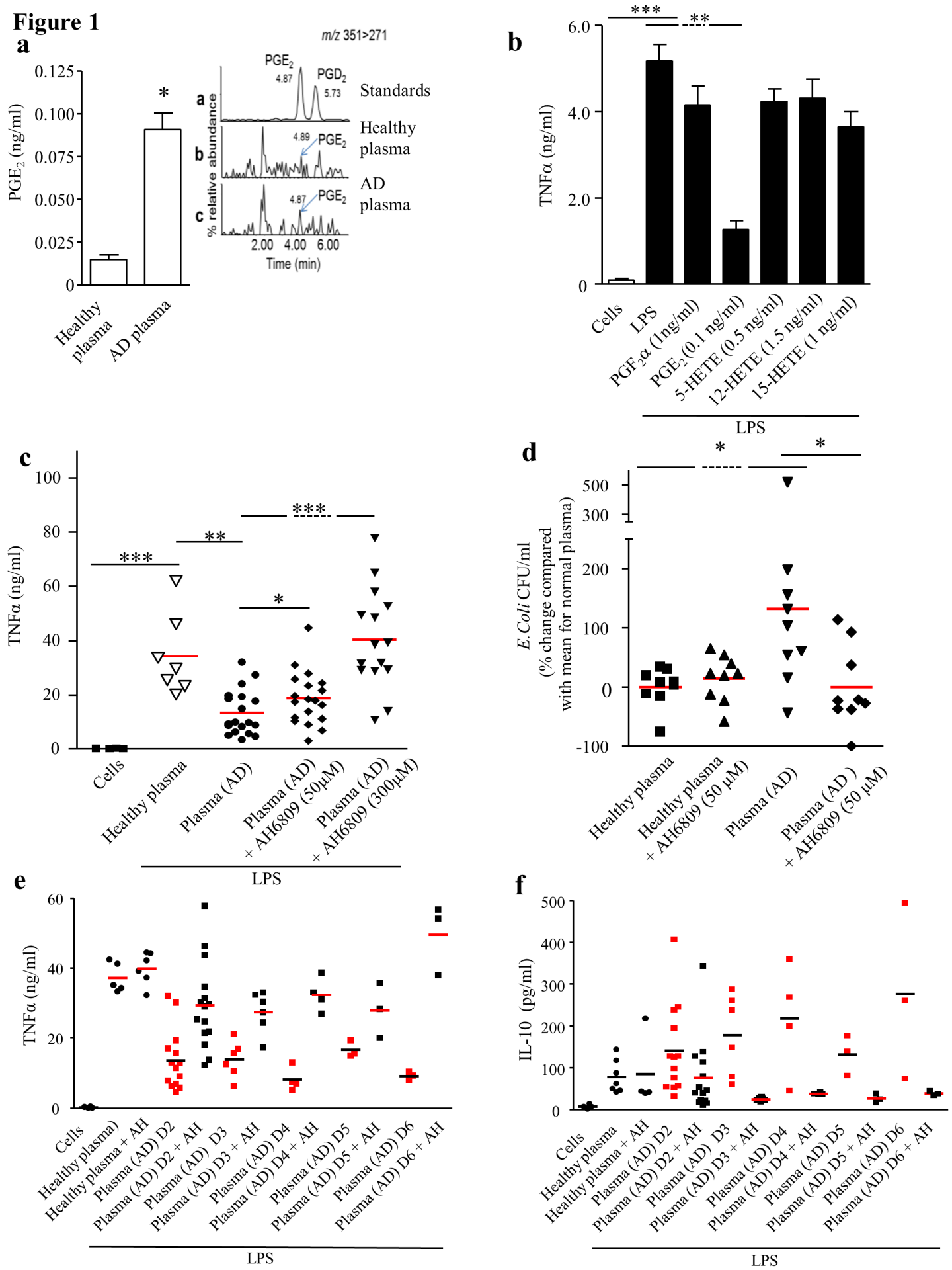
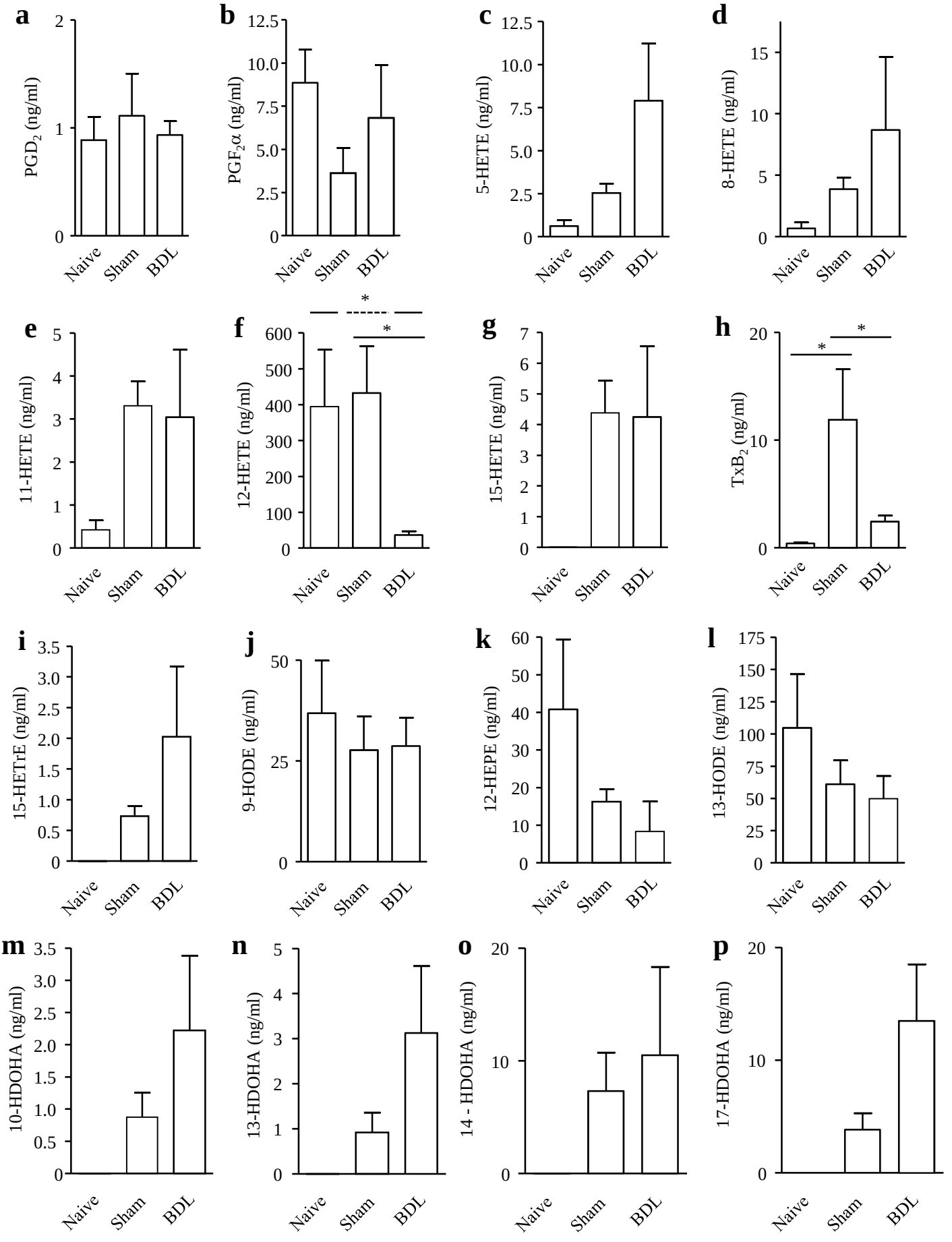

a
***
*
*
TNFα ng/ml)
80
70
60
50
40
30
20
10
0
Cells
Healthy plasma
ESLD Week 1
Week 1+AH
ESLD Week 24
Week 24+AH
LPS
b
TNFα (ng/ml)
100
80
60
40
20
0
Healthy serum
Child's Pugh (A)
Child's Pugh (A) AH6809 (50μM)
Non-cirrhotic liver disease
Non-cirrhotic liver disease AH6809 (50μM)
LPS
c
COX1 / RSP20 (AU)
0.015
0.010
0.005
0.000
Healthy Volunteers
AD
COX2 / RSP20 (AU)
0.0070
0.0035
0.0000
Healthy Volunteers
AD
d
**
**
PGE2 (ng/ml)
3
2
1
0
Naive
Sham
BDL
*
PGE2 (ng/ml)
1.0
0.5
0.0
Sham
CCL4
e
Liver from sham controls
Liver from CCL4-treated mice
f
Lung from sham controls
Lung from CCL4-treated mice

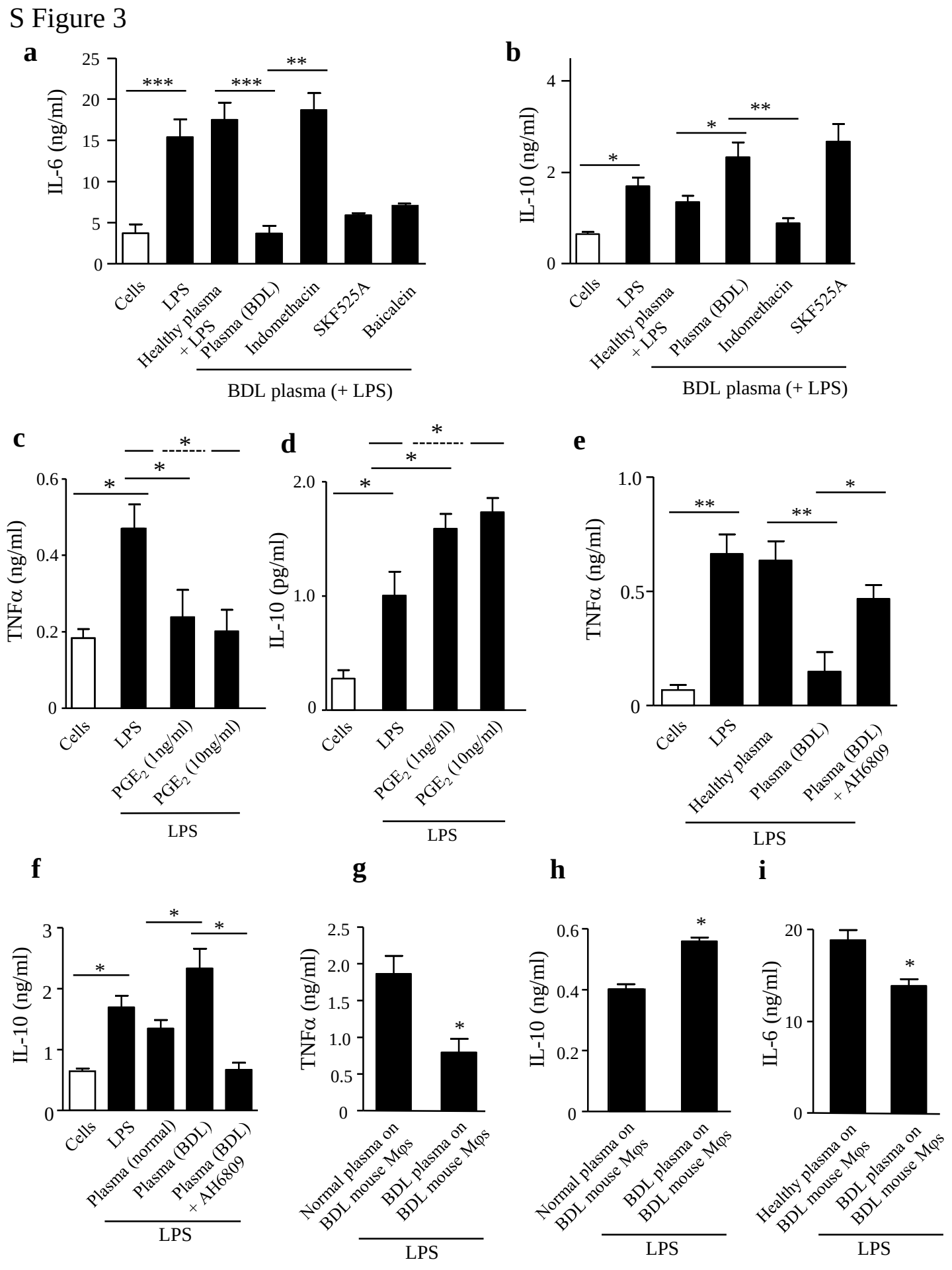
Figure 1


a
m/z 351>271
PGE2
4.87
PGD2
5.73
Standards
Healthy plasma
AD plasma
4.89
4.87
% relative abundance
2.00 4.00 6.00
Time (min)
PGE2 (ng/ml)
0.125
0.100
0.075
0.050
0.025
0
*
Healthy plasma
AD plasma
b
***
**
6.0
4.0
2.0
0
TNFα (ng/ml)
Cells
LPS
PGF2α (1ng/ml)
PGE2 (0.1 ng/ml)
5-HETE (0.5 ng/ml)
12-HETE (1.5 ng/ml)
15-HETE (1 ng/ml)
LPS
c
100
80
60
40
20
0
TNFα (ng/ml)
***
**
*
***
Cells
Healthy plasma
Plasma (AD)
Plasma (AD) + AH6809 (50μM)
Plasma (AD) + AH6809 (300μM)
LPS
d
*
*
500
300
200
100
0
-100
E. Coli CFU/ml (% change compared with mean for normal plasma)
Healthy plasma
Healthy plasma + AH6809 (50 μM)
Plasma (AD)
Plasma (AD) + AH6809 (50 μM)
e
60
40
20
0
TNFα (ng/ml)
Cells
Healthy plasma)
Healthy plasma + AH
Plasma (AD) D2
Plasma (AD) D2 + AH
Plasma (AD) D3
Plasma (AD) D3 + AH
Plasma (AD) D4
Plasma (AD) D4 + AH
Plasma (AD) D5
Plasma (AD) D5 + AH
Plasma (AD) D6
Plasma (AD) D6 + AH
LPS
f
500
400
300
200
100
0
IL-10 (pg/ml)
Cells
Healthy plasma
Healthy plasma + AH
Plasma (AD) D2
Plasma (AD) D2 + AH
Plasma (AD) D3
Plasma (AD) D3 + AH
Plasma (AD) D4
Plasma (AD) D4 + AH
Plasma (AD) D5
Plasma (AD) D5 + AH
Plasma (AD) D6
Plasma (AD) D6 + AH
LPS

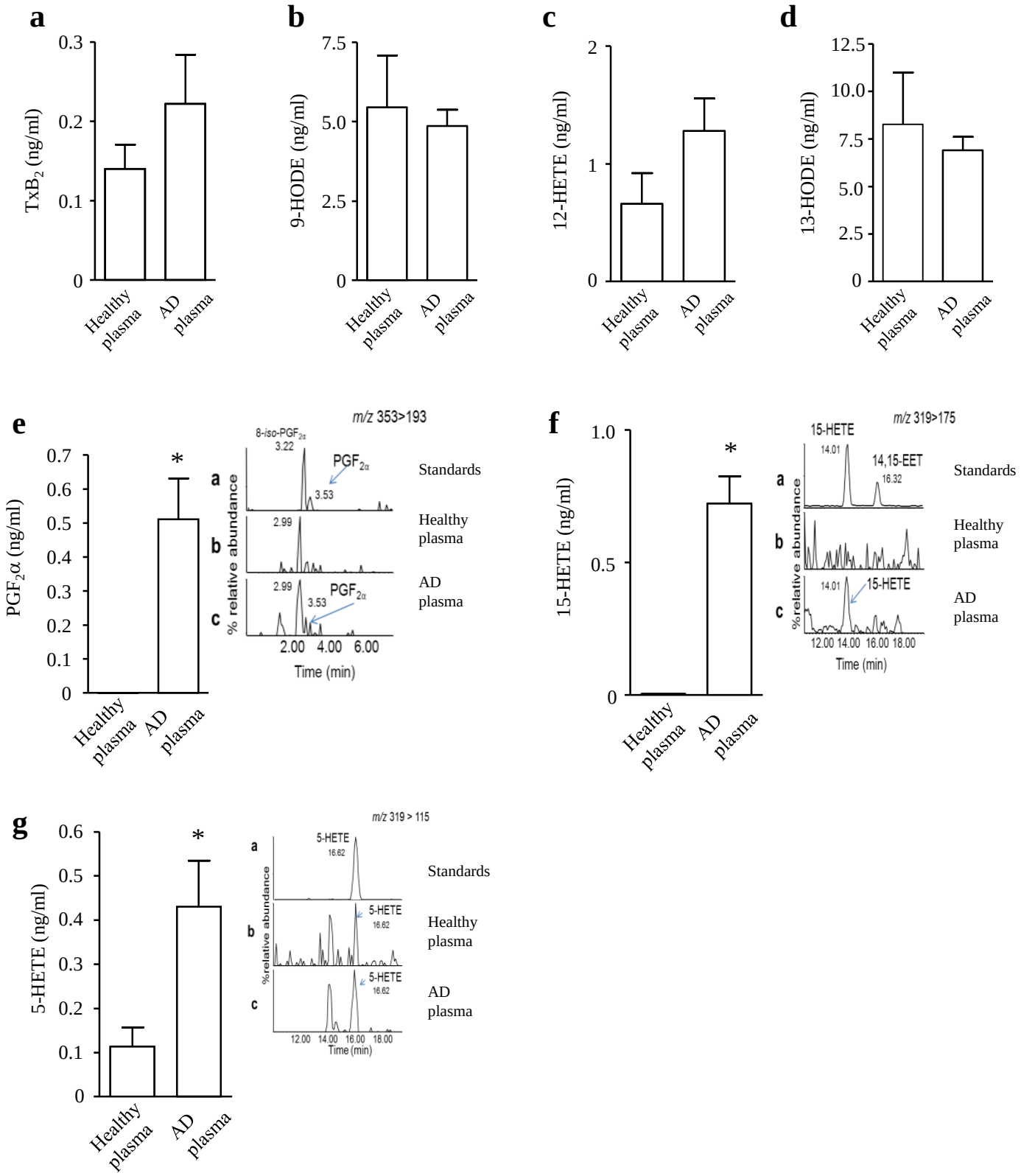
S Figure 3


a
PGD2 (ng/ml)
b
PGF2α (ng/ml)
c
5-HETE (ng/ml)
d
8-HETE (ng/ml)
e
11-HETE (ng/ml)
f
12-HETE (ng/ml)
*
*
g
15-HETE (ng/ml)
h
TxB2 (ng/ml)
*
*
i
15-HETrE (ng/ml)
j
9-HODE (ng/ml)
k
12-HEPE (ng/ml)
l
13-HODE (ng/ml)
m
10-HDOHA (ng/ml)
n
13-HDOHA (ng/ml)
o
14 - HDOHA (ng/ml)
p
17-HDOHA (ng/ml)
Naive
Sham
BDL

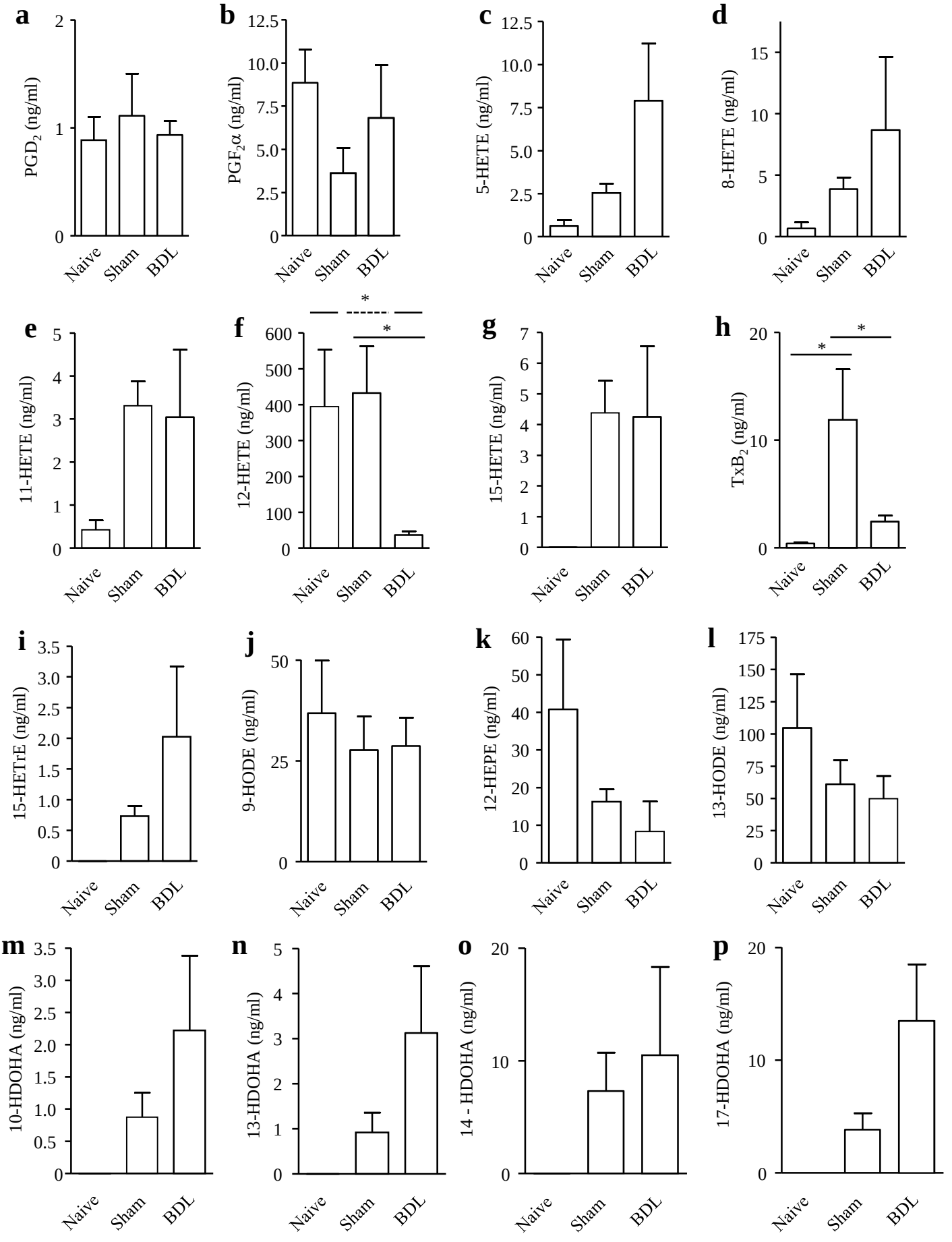
S Figure 3


a
IL-6 (ng/ml)
25
20
15
10
5
0
***
***
**
Cells
LPS
Healthy plasma + LPS
Plasma (BDL)
Indomethacin
SKF525A
Baicalein
BDL plasma (+ LPS)
b
IL-10 (ng/ml)
4
2
0
*
*
**
Cells
LPS
Healthy plasma + LPS
Plasma (BDL)
Indomethacin
SKF525A
BDL plasma (+ LPS)
c
TNFα (ng/ml)
0.6
0.4
0.2
0
*
*
*
Cells
LPS
PGE2 (1ng/ml)
PGE2 (10ng/ml)
LPS
d
IL-10 (pg/ml)
2.0
1.0
0
*
*
*
Cells
LPS
PGE2 (1ng/ml)
PGE2 (10ng/ml)
LPS
e
TNFα (ng/ml)
1.0
0.5
0
**
**
*
Cells
LPS
Healthy plasma
Plasma (BDL)
Plasma (BDL) + AH6809
LPS
f
IL-10 (ng/ml)
3
2
1
0
*
*
*
Cells
LPS
Plasma (normal)
Plasma (BDL)
Plasma (BDL) + AH6809
LPS
g
TNFα (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
*
Normal plasma on BDL mouse Mφs
BDL plasma on BDL mouse Mφs
LPS
h
IL-10 (ng/ml)
0.6
0.4
0.2
0
*
Normal plasma on BDL mouse Mφs
BDL plasma on BDL mouse Mφs
LPS
i
IL-6 (ng/ml)
20
10
0
*
Healthy plasma on BDL mouse Mφs
BDL plasma on BDL mouse Mφs
LPS

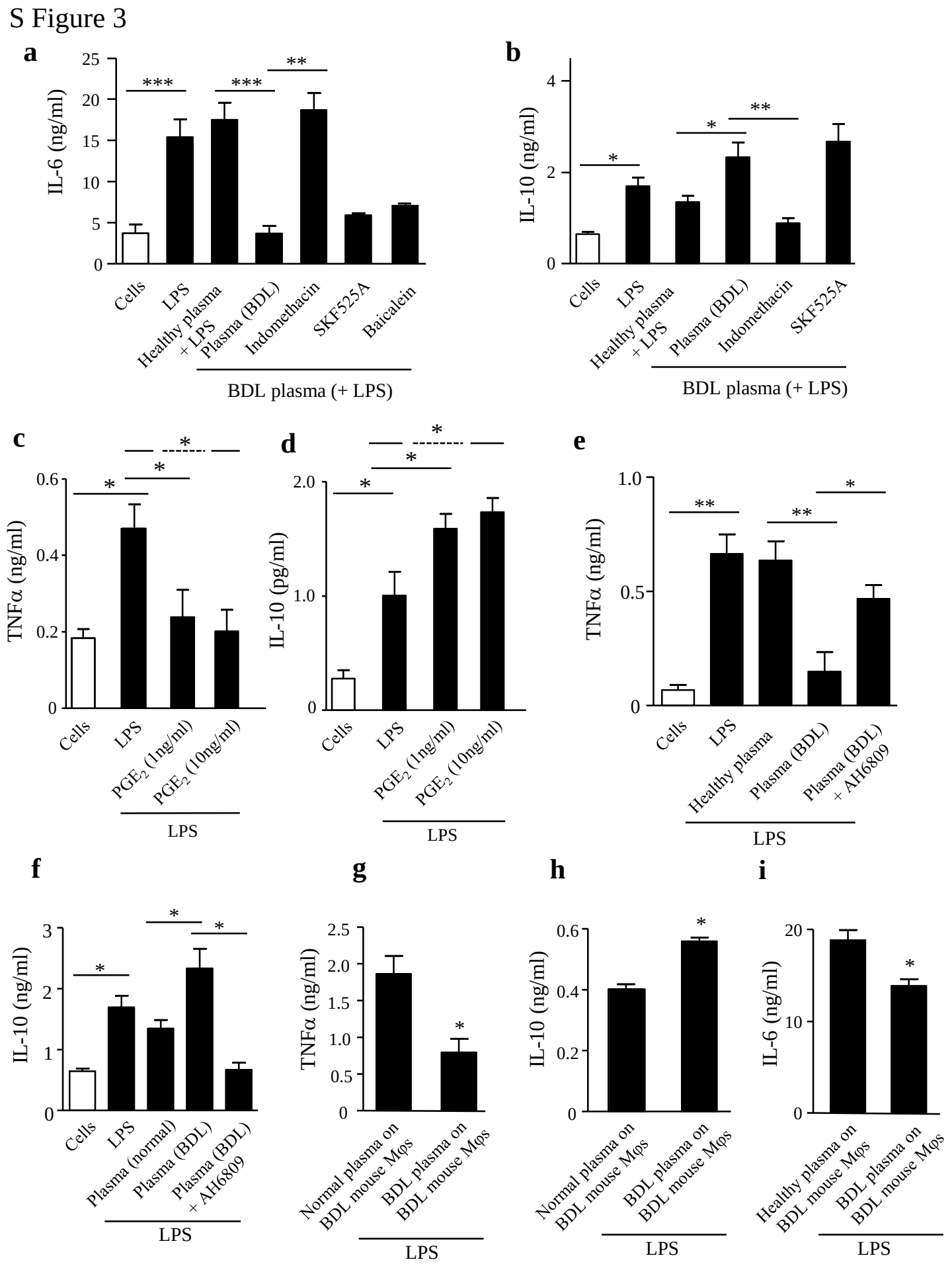
S Figure 1


a
TxB2 (ng/ml)
0.3
0.2
0.1
0
Healthy plasma
AD plasma
b
9-HODE (ng/ml)
7.5
5.0
2.5
0
c
12-HETE (ng/ml)
2
1
0
d
13-HODE (ng/ml)
12.5
10.0
7.5
5.0
2.5
0
e
PGF2α (ng/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
m/z 353>193
8-iso-PGF2α
3.22
PGF2α
3.53
2.99
% relative abundance
a
b
c
Standards
Healthy plasma
AD plasma
2.00 4.00 6.00
Time (min)
f
15-HETE (ng/ml)
1.0
0.5
0
*
m/z 319>175
15-HETE
14.01
14,15-EET
16.32
%relative abundance
12.00 14.00 16.00 18.00
Time (min)
g
5-HETE (ng/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
m/z 319 > 115
5-HETE
16.62
%relative abundance
12.00 14.00 16.00 18.00
Time (min)

S Figure 3


a
PGD2 (ng/ml)
b
PGF2α (ng/ml)
c
5-HETE (ng/ml)
d
8-HETE (ng/ml)
e
11-HETE (ng/ml)
f
12-HETE (ng/ml)
*
*
g
15-HETE (ng/ml)
h
TxB2 (ng/ml)
*
*
i
15-HETrE (ng/ml)
j
9-HODE (ng/ml)
k
12-HEPE (ng/ml)
l
13-HODE (ng/ml)
m
10-HDOHA (ng/ml)
n
13-HDOHA (ng/ml)
o
14 - HDOHA (ng/ml)
p
17-HDOHA (ng/ml)
Naive
Sham
BDL

S Figure 3


a
IL-6 (ng/ml)
25
20
15
10
5
0
***
***
**
Cells
LPS
Healthy plasma + LPS
Plasma (BDL)
Indomethacin
SKF525A
Baicalein
BDL plasma (+ LPS)
b
IL-10 (ng/ml)
4
2
0
*
*
**
Cells
LPS
Healthy plasma + LPS
Plasma (BDL)
Indomethacin
SKF525A
BDL plasma (+ LPS)
c
TNFα (ng/ml)
0.6
0.4
0.2
0
*
*
*
Cells
LPS
PGE2 (1ng/ml)
PGE2 (10ng/ml)
LPS
d
IL-10 (pg/ml)
2.0
1.0
0
*
*
*
Cells
LPS
PGE2 (1ng/ml)
PGE2 (10ng/ml)
LPS
e
TNFα (ng/ml)
1.0
0.5
0
**
**
*
Cells
LPS
Healthy plasma
Plasma (BDL)
Plasma (BDL) + AH6809
LPS
f
IL-10 (ng/ml)
3
2
1
0
*
*
*
Cells
LPS
Plasma (normal)
Plasma (BDL)
Plasma (BDL) + AH6809
LPS
g
TNFα (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
*
Normal plasma on BDL mouse Mφs
BDL plasma on BDL mouse Mφs
LPS
h
IL-10 (ng/ml)
0.6
0.4
0.2
0
*
Normal plasma on BDL mouse Mφs
BDL plasma on BDL mouse Mφs
LPS
i
IL-6 (ng/ml)
20
10
0
*
Healthy plasma on BDL mouse Mφs
BDL plasma on BDL mouse Mφs
LPS

S Figure 4

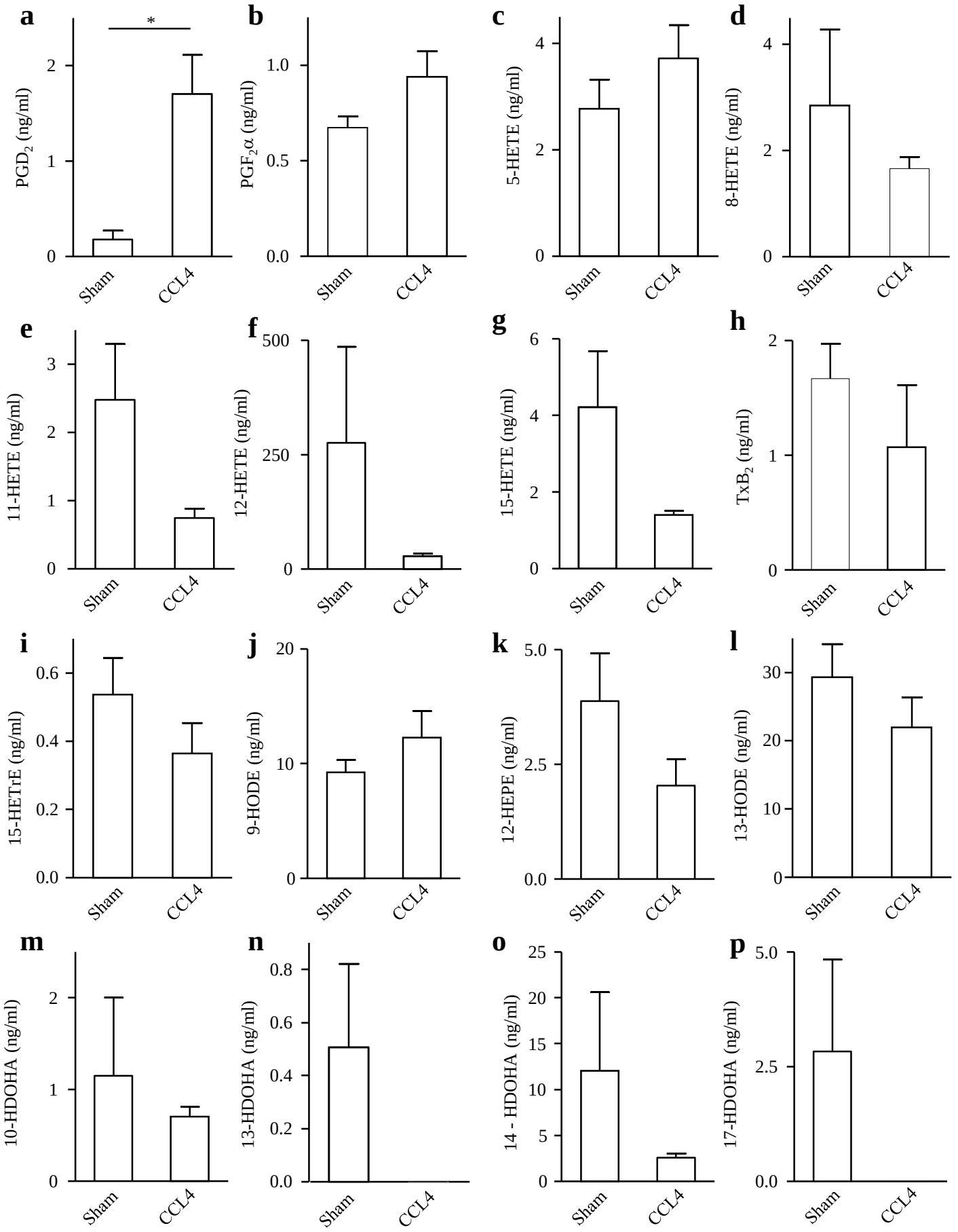
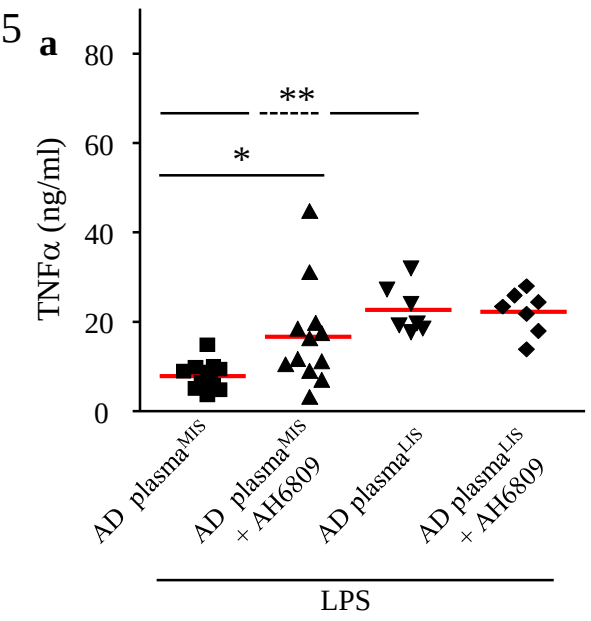
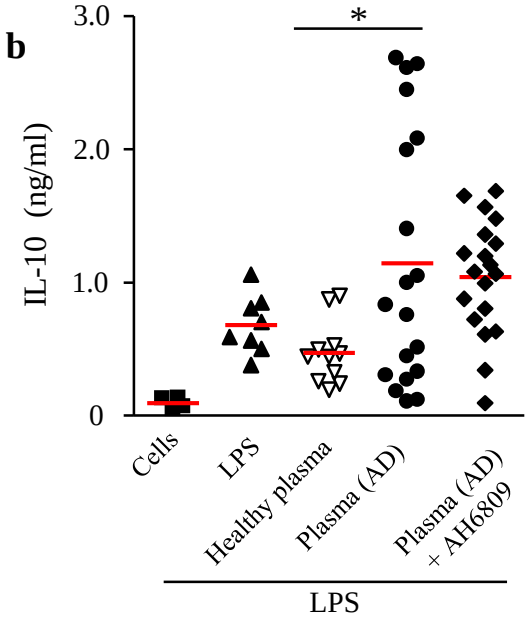
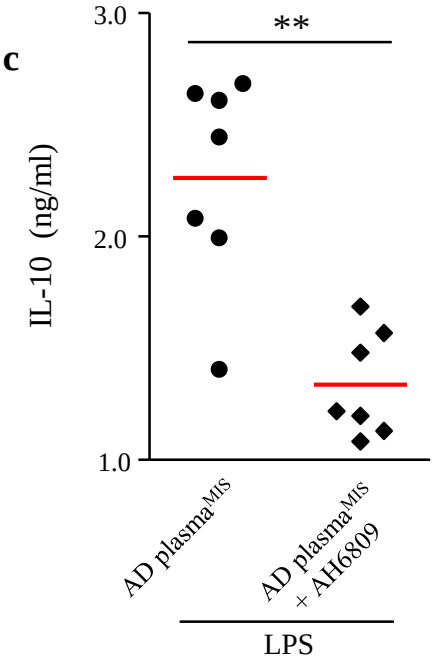
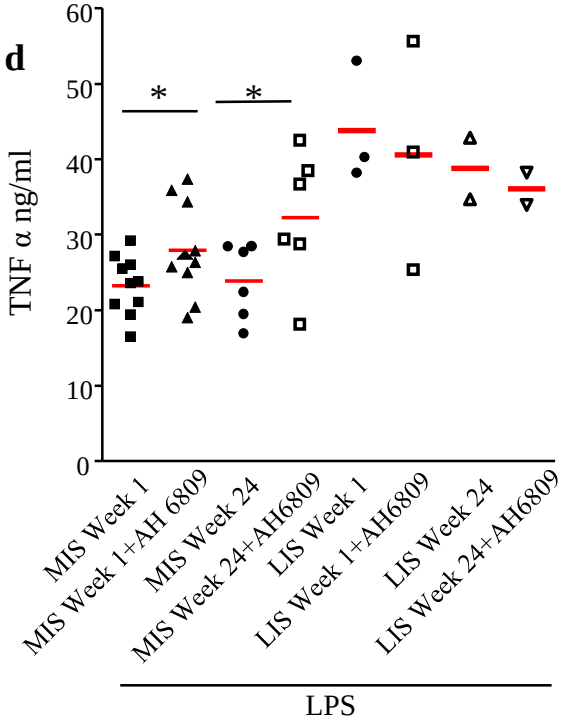

a
*
PGD2 (ng/ml)
0
1
2
Sham
CCL4
b
PGF2α (ng/ml)
0.0
0.5
1.0
Sham
CCL4
c
5-HETE (ng/ml)
0
2
4
Sham
CCL4
d
8-HETE (ng/ml)
0
2
4
Sham
CCL4
e
11-HETE (ng/ml)
0
1
2
3
Sham
CCL4
f
12-HETE (ng/ml)
0
250
500
Sham
CCL4
g
15-HETE (ng/ml)
0
2
4
6
Sham
CCL4
h
TxB2 (ng/ml)
0
1
2
Sham
CCL4
i
15-HETrE (ng/ml)
0.0
0.2
0.4
0.6
Sham
CCL4
j
9-HODE (ng/ml)
0
10
20
Sham
CCL4
k
12-HEPE (ng/ml)
0.0
2.5
5.0
Sham
CCL4
l
13-HODE (ng/ml)
0
10
20
30
Sham
CCL4
m
10-HDOHA (ng/ml)
0
1
2
Sham
CCL4
n
13-HDOHA (ng/ml)
0.0
0.2
0.4
0.6
0.8
Sham
CCL4
o
14 - HDOHA (ng/ml)
0
5
10
15
20
25
Sham
CCL4
p
17-HDOHA (ng/ml)
0.0
2.5
5.0
Sham
CCL4

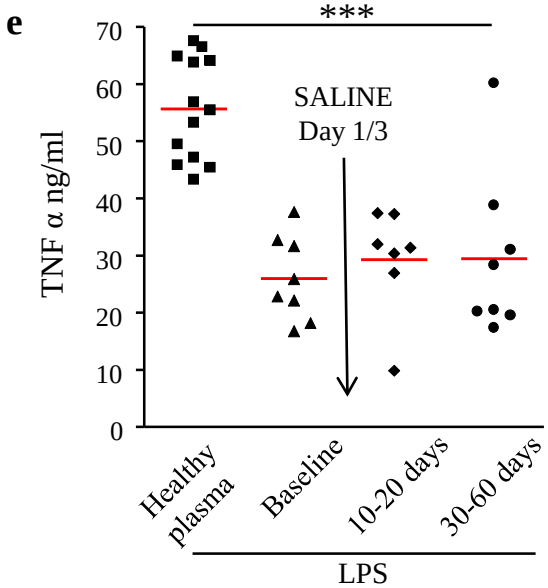
Figure S5


a
TNFα (ng/ml)
80
60
40
20
0
**
*
AD plasma MIS
AD plasma MIS + AH6809
AD plasma LIS
AD plasma LIS + AH6809
LPS


b
IL-10 (ng/ml)
3.0
2.0
1.0
0
*
Cells
LPS
Healthy plasma
Plasma (AD)
Plasma (AD) + AH6809
LPS


c
IL-10 (ng/ml)
3.0
2.0
1.0
**
AD plasma MIS
AD plasma MIS + AH6809
LPS


d
TNF α ng/ml
60
50
40
30
20
10
0
*
*
MIS Week 1
MIS Week 1+AH 6809
MIS Week 24
MIS Week 24+AH6809
LIS Week 1
LIS Week 1+AH6809
LIS Week 24
LIS Week 24+AH6809
LPS


e
TNF α ng/ml
70
60
50
40
30
20
10
0
***
SALINE
Day 1/3
Healthy plasma
Baseline
10-20 days
30-60 days
LPS

Supplementary Table 1. Patient Clinical Characteristics from DASIMAR study

	AD^{MIS} n=22 (median, IQR)	**AD^{LIS} n =13 (median, IQR)**	***P* value**	**Overall n=35 (median, IQR)**
%	63	27		
Age	49.5 (46.5-64.5)	54 (48-70)	0.56	52 (47-65)
Sex (males)	16 (73%)	6 (46%)		22 (63%)
Albumin	28 (24-30)	30 (28-31.5)	0.0012	29 (27-31)
INR	1.7 (1.4-2.04)	1.6 (1.23-2)	0.93	1.7 (1.4-2)
Creatinine	62 (47-109)	62 (47.5-122)	0.54	62 (49-108)
MELD score	19.4 (15.6-21.8)	18.39 (12.1-21.4)	0.93	19 (14.2-23)
Positive microbiology	6	3		9
Died during admission	2	1		3
Died within 6 months	7	4		11
Length of stay (days)	11.5 (8-28)	10 (5.5-19)	0.22	11 (7-25)
CRP	28 (115-50.5)	23 (5-43.5)	0.24	26 (13-49)
Bilirubin	78 (34-119)	53 (19-73)	0.87	67.5 (24-104)
Cause of cirrhosis	15 Alcohol (68%) 3 NASH 1 PBC 1 Sarcoid 1 Congenital hepatic fibrosis 1 HCV	6 Alcohol (46%) 3 NASH 1 PBC 1 Cryptogenic 1 HBV 1 HCV		21 Alcohol (60%) 6 NASH (17%) 2 PBC 2 HCV 1 HBV 1 Congenital hepatic fibrosis
PGE_2	0.12 (0.09-0.16); n=7	0.065 (0.035-0.085); n=6	0.005	0.09 (0.065-0.13); n=13
PGE_2/albumin	0.0041 (0.0036-0.0058); n=7	0.002 (0.001-0.0028); n=6	0.001	0.003571 (0.0022-0.0049); n=13
NH3	208 (128-260.5); n=5	151; n=1		204 (133-236); n=6
Ischemia modified albumin ratio (IMAR)	0.025 (0.014-0.06); n=10	0.011 (0.005-0.02); n=5	0.075	0.017(0.01-0.04); n=15

Supplementary Table 2. Lipid levels in human plasma

	Healthy Volunteers (n=8) (ng/ml)	**AD Patients (n=14) (ng/ml)**
PGE2	0.015(0.003)	0.091(0.01)***
PGD2	0	0
PGF2A	0	0.8(0.08)***
PGE1	0	0
PGD1	0	0
TXB2	0.31(0.1)	0.77(0.11)
9-HODE	5.45(1.63)	4.87(0.51)
13-HODE	8.27(2.7)	6.91(0.69)
5-HETE	0.11(0.044)	0.43(0.1)*
8-HETE	0	0.15(0.02)
11-HETE	0	0.06(0.02)
15-HETE	0	0.72(0.1)***
12-HETE	0.66(0.26)	1.28(0.28)
15-HETrE	0	0.39(0.03)***

* P<0.05 ** P<0.01 ***P<0.001, t-test cirrhosis vs healthy volunteers

Supplementary Table 3. Mouse clinical and biochemical data

	Naïve mouse	**Sham mouse**	**BDL mouse**	**CCL_4 mouse**
Albumin	29(3)	29(1)	24(2)**	28.5(1)
ALT	20(9)	26(2.4)	340(33)***	539(158)**
AST	68(11)	94(18)	496(55)	
Total Protein	41(3)	43(2)	32(2.7)**	
Glucose	15(0.2)	12(1)	6(1)	
Bilirubin	12(4)	17(2.9)	380(35)***	
Creatinine	9(0.7)	11(0.3)	27(2.6)**	
Urea	9(0.7)	8(0.4)	9(0.9)	
TNFα ρg.ml^{-1}	4(2.6)	53(16)	60(25)	
Cardiac output	28.1(0.9)	26.7(1.5)	16(1)***	
Mean arterial pressure (mmHg)	-	112(4)	99(4)*	
Core Temperature	37.8(0.2)	37.6 (0.1)	35.1(0.2)**	37.8(0.2)
Age at surgery	11 weeks	11 weeks	11 weeks	
Weight (g)	27.8(0.6)	27(0.4)	19.4(0.3)	28(0.4)

* P<0.05 ** P<0.01 ***P<0.001, t-test for BDL or CCL_4 vs naïve or sham mice

Supplementary Table 4. Murine Sickness score following bacterial peritonitis. Mice culled if score of 4 at any point or score of 3 for greater than 24h

Clinical Feature	Score
Piloerection	1
Slow movement	1
Hunched posture	1
Pus in eye(s)	3
Failure to move	4
Weight loss >25%	4

Supplementary Methods Section

Extraction and analysis of lipid mediators

Lipid mediators in mice/human plasma were analyzed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) based on protocols published previously[16]. Briefly, samples were collected and stored immediately at −80 °C. Plasma Samples (250-500µl for mice, 500–1,000µL for humans) were defrosted on ice and adjusted to 15% (v/v) methanol: water (final volume 4mL). Internal standards, PGB_2-*d*4 (40 ng) and 12-HETE-*d*8 (40ng) (Cayman Chemical Company, Ann Arbor, USA) were added and the pH of resulting solutions adjusted to 3.0 (1M HCL). Acidified samples were immediately applied to preconditioned solid-phase cartridges (C18-E, Phenomenex, Macclesfield, UK) and lipid mediators eluted with methyl formate. LC/ESI-MS/MS analysis was performed on a HPLC pump (Waters Alliance 2695) coupled to an electrospray ionization triple quadruple mass spectrometer (Quattro Ultima, Waters, UK). Chromatographic separation was performed on a C18 Luna column (5µm, 150 x 2.0mm, Phenomenex) for eicosanoids and a C18 Kinetex column (2.6µm, 100 x 2.1mm, Phenomenex) for hydroxy- fatty acids. Analytes were monitored on multiple reaction monitoring mode as reported with the following additions: 15-hydroxyeicosatrienoic acid (HETrE) *m/z* 321>221, 10-hydroxydocosahexaenoic acid (HDHA) *m/z* 343>153, 14-HDHA *m/z* 343>161, 13-HDHA *m/z* 343>193 and 17-HDHA *m/z* 343>201.

Human samples

Samples were obtained from several sources representing the spectrum of cirrhosis patients which vary in vulnerability to infection. Non-cirrhotic liver disease patients were used as a control.

(i) Acutely decompensated patients:

a. AoCLF samples - Predictive utility of DASIMAR as a prognostic biomarker in AoCLF (ClinicalTrials.gov: NCT01071746).

b. Acute encephalopathy - ALFAE (Efficacy of albumin for acute encephalopathy in patients with cirrhosis, Hospital Clinic of Barcelona; ClinicalTrials.gov: NCT00886925).

c. First presentation with decompensation secondary to alcoholic liver disease from University College Hospital (UCLH).

(ii) Patients on the liver transplant waiting list for a 24 week follow up period representing end stage liver disease - MACHT (Effect of midodrine and albumin in the prevention of complications in cirrhotic patients awaiting liver transplantation, Hospital Clinic of Barcelona; ClinicalTrials.gov: NCT00839358).

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Ethical approval was granted for all studies. AoCLF samples were obtained from 'Predictive utility of DASIMAR as a prognostic biomarker in AoCLF' (ClinicalTrials.gov identifier: NCT01071746; UCLH NHS Research Ethics Committee number: 08/H0714/8) along with age/gender matched healthy volunteer controls. Samples were obtained from patients admitted to the Royal Free Hospital on days 1, 2, 5, 6 following admission. Samples from patients awaiting transplant originated from MACHT (Effect of midodrine and albumin in the prevention of complications in cirrhotic patients awaiting liver transplantation; ClinicalTrials.gov identifier: NCT00839358). Patients enrolled to MACHT received 200g albumin/placebo on admission with blood sampling at baseline, 4 and 12weeks time-points post-intervention. ALFAE (Efficacy of albumin for acute encephalopathy in patients with cirrhosis; ClinicalTrials.gov identifier: NCT00886925) provided samples from patients with non-terminal cirrhosis (early-phase) randomized to receive either 200g albumin (1L, 20%

human albumin solution) over 48hours or an equivalent volume of 0.9% saline. Samples were drawn prior to intervention and 8-10 days and 30-60 days post. Samples were also acquired through on-going local research from University College Hospitals NHS Trust (UCLH) and the Royal London Hospitals NHS Trust (Monocyte and macrophage phenotype and function in liver failure; Harrow NHS Research Ethics Committee no. 12/LO/0167) were utilised to explore its effect on short-term immune function. At UCLH albumin was administered in response to clinical indication(s), and samples drawn pre and 24 hours post infusion.

Human blood analysis and macrophage isolation/culture

To prepare human monocyte-derived macrophages (MDM) , healthy volunteer mononuclear cells were isolated by differential centrifugation (900g, 30min, 20°C) over Lymphoprep™ (Axis-Shield) and washed with sterile PBS (Invitrogen™, Life Technologies™)[17]. Cells were resuspended in 10ml RPMI-1640 medium (Invitrogen™, Life Technologies™)) supplemented with 100 $U.ml^{-1}$ of penicillin/100$\mu g.ml^{-1}$ streptomycin (Invitrogen™, Life Technologies™)) and plated at a density of $\sim 5 \times 10^6$ cells/ml in $8cm^2$ Nunclon Surface tissue culture dishes (Nunc®). After 2h incubation at 37°C (5% CO2), non-adherent cells were discarded and 10ml of fresh RPMI supplemented with 10% FBS added (Sigma-Aldrich®) (with antibiotics as above). After culture for 5 days at 37°C (5% CO2), with addition of a further 10ml of fresh 10% FBS/RPMI after 24h (total 20mls), adherent cells were scraped on day 5 and replated in 96-well culture plates (Nunc®) at equal densities (10^5/well) in X-Vivo-15 serum free 0.2% HAS medium (Lonza). Resulting primary MDM, validated by morphological inspection, were incubated overnight at 37°C (5% CO_2) to adhere prior to stimulation with 200ng/ml LPS (*Salmonella abortus equi*, Enzo Biochem, Inc). To examine the effect of plasma/serum on macrophages, these were added to cell culture 30min prior to LPS to give a final volume of 25% plasma/serum. Similarly eicosanoids of interest were also added to cell culture 30min

prior to LPS. AH6809 (50-300μM) was dissolved in X-vivo and added to the macrophages 45min prior to plasma. Macrophage supernatants were collected after 24h stimulation with LPS and stored at -80 °C for cytokine analysis by ELISA as described. Cell viability was ascertained with calcein AM cell viability assay kit (Biotium, Inc). Human serum albumin was obtained from Sigma-Aldrich >99% (agarose gel electrophoresis), essentially fatty acid (0.005%) and globulin free. For bacterial killing, *Escherichia Coli* (clinical isolate, provided by Dr Vanya Gant, Clinical Director for Infection, Consultant Microbiologist, UCLH) was added to healthy volunteer macrophages (ratio 100 *E.coli*: 1 macrophage) in the presence/absence of plasma from patient/healthy volunteer plasma ± AH6809 (50-300μM, Sigma Aldrich Dorset, UK) or albumin (15g/l) as above for 1h in media without antibiotics. Supernatants were plated on blood agar overnight and CFUs counted.

RNA isolation and gene expression studies

Peripheral blood mononuclear cells (PBMC) were isolated from total blood with the Ficoll method (Ficoll-Paque^{TM} PLUS, GE-Healthcare, Cat. No:17-1440-03) from four patients (3 males/1female) and six healthy controls (3 males/3 females). The PBMC cell pellet was immediately frozen at -80^{o}C until cell lysis and total RNA extraction with the RNeasy Mini Kit (Qiagen, Cat. No: 74104). RNA concentration was measured spectrophotometrically at 260 nm (Nanodrop8000, Thermo Fishcer), and RNA purity was determined by the *A*260/*A*280, *A*230/260 ranges and spectral pattern. 500ng of total RNA were reverse transcribed to cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase, RNase H Minus, Point Mutant (M-MLV RT (H–) (Promega, Cat No: M368A) and Promega oligodTs, random primers and M-MLV X5 RT buffer (Promega). cDNA was then subjected to quantitative real-time PCR using an 7900HT ABI thermal cycler (Applied Biosystems), SYBGR (Applied Biosystems, Cat. No: 4367659) and in-house designed and optimised primers for the genes of interest and

internal controls. Primer sequences were: *PTGS1*, forward primer: *TACCAGGTGCTGGATGGAGA*, reverse primer: *CCTTCAGCAGGTCACACAC*; *PTGS2*, forward primer: *GTTTTGACATGGGTGGGAAC*, reverse primer: *CCCTCAGACAGCAAAGCCTA*, *CYPH*: forward primer: *GCCGAGGAAAACCGTGTACT*, reverse primer: *ACCTTGTCTGCAAACAGCTCA; RPL27* (ribosomal protein 27), forward primer: *TCCAAGGGGATATCCACAGA*, reverse primer: *CATGGGCAAGAAGAAGATCG*, *RSP20 (ribosomal protein 20)*, forward primer: *GGAAACGATCCCACGTCTTA*, reverse primer: *AGAGGCGCAAAAGAAAAGAA*. Dissociation curve analysis was performed at 40 cycles to verify the identity of PCR product. For data analysis, the comparative threshold cycle values for constitutively expressed *CYPH* were used to normalize loading variations and data are expressed as arbitrary units (AU). Gene expression data analysis was also undertaken with the use of *RPL27* and *RPS20* as endogenous controls.

Animal models

Bile duct ligation/sham procedures were carried out under anesthesia (isoflurane 1.5%) as described previously[18]. Carbon tetrachloride (CCL_4, Merck, Darmstadt, Germany) was given subcutaneously (s.c., 1:1 dissolved in olive oil; 1 ml/kg) twice weekly and 300mg/L phenobarbital added to drinking water[19]. Sham mice received s.c. injections of olive oil. After 2 weeks for BDL mice or 8 weeks for CCL_4 mice, interventional models were undertaken or blood and liver were taken and prepared for histology or further experimental use. Experiments were performed on CCL_4 mice on the same day as the final injection.

Interventional models: peritonitis and intra-venous bacterial inoculation

Group B Streptococcus (GBS) (NCTC10/84, serotype V) was grown in Todd Hewitt broth without agitation at 37°C to an OD600 of 0.4, equivalent to 10^8 Colony Forming Units

(CFU)/mL, centrifuged/washed with sterile PBS and injected intraperitoneally (i.p.) at $30x10^6$ colony forming units (CFUs) in 300µl sterile PBS or for bacterial killing assays. A clinical isolate of GBS was provided by Dr Vanya Gant, Clinical Director for Infection, Consultant Microbiologist, UCLH and $1x10^7$CFUs in 300µl sterile PBS i.p. was used for mouse survival studies; $6x10^7$ CFUs for i.p saline vs albumin infusion studies and $4x10^7$ in 200µl sterile PBS for intravenous (i.v.) studies. For bacterial killing assays in mice with/without the non-selective COX-inhibitor indomethacin ($3mg.kg^{-1}$ p.o. 1h prior to bacterial challenge, Sigma-Aldrich®. or equivalent volume of vehicle), heparinized blood was collected 3h after GBS injection, centrifuged (10,000g, 4°C, 10min), plated on agar overnight and CFUs counted the following day. For the survival experiments, mice were observed every 6h for up to 80h. In accordance with Home Office guidance, mice were humanely culled if they had a "sickness score" of 4 or 3 for greater than 24h (Supplementary Table 2).

Cytokine analysis

Murine cytokine expression profiles were determined using Th1/Th2 10-plex FlowCytomix Mouse Kits (Bender MedSystems). Fluorescence was measured on a flow cytometer (BD FACSCanto II™, BD bioscience) and data analyzed using manufacturer's software. The expression profile of a cytokine panel in human macrophage supernatants was measured using the MSD® Bio-Plex human cytokine assay (Merck, Sharp & Dohme Ltd.). Our assay was customized to detect/quantify IL-1, IL-6, IL-8, IL-10 and TNFα. Key cytokine changes were confirmed by dedicated ELISA (TNFα and IL10 - mouse eBioscience or human Duoset®, R&D systems). All samples were run in duplicate.

Mouse blood analysis, macrophage isolation/culture

Blood was collected by intracardiac puncture into heparin and centrifuged (10,000g, 4^{O}C, 10min). Plasma was analyzed for liver and renal function tests using the COBAS® INTEGRA 400 plus multianalyser with appropriate diagnostic kits (Roche - Diagnostics) or stored at -80°C. Peritoneal macrophages from healthy animals were isolated as described previously[20] and incubated with/without LPS (*Salmonella Typhosa*, 0.1μg/mL for 24h; Sigma-Aldrich®) in the presence of plasma (50μl) from naïve, sham, BDL or CCL_4 mice in cell culture media (complete DMEM; Life Technologies™) to give a final concentration of 25% by volume. In other experiments plasma was added to cell culture, as above, from BDL mice given indomethacin (3mg.kg^{-1} po 6h prior to sampling); the cytochrome P450 inhibitor SKF525A (Enzo® Life Sciences; 50mg.kg^{-1} s.c. 1h prior to sampling); or the 12/15 lipoxygenase (LOX) inhibitor Bacalein (Sigma-Aldrich®; 100mg.kg^{-1} s.c. 90min prior to sampling). In experiments examining the effect of the DP1/EP1-3 receptor antagonist AH6809 (300μM; Sigma-Aldrich®), this compound was dissolved in DMEM and added to macrophages 45min prior to addition of plasma. Cell viability was ascertained with the calcein AM cell viability assay kit (Biotium, Inc) in accordance with manufacturer's instructions. For heat inactivation, plasma was placed at 56°C in a water bath for 1h and samples stored overnight at 4°C and used for cell culture the following day.

Immunohistochemistry

For COX 1 and COX 2 expression, immunohistochemistry was carried out on formalin-fixed, wax-embedded mouse tissues using COX 1 polyclonal antibody (Cayman Chemical, catalogue number 160109) and COX-2 polyclonal antibody **(**Cayman Chemical, catalogue number 160106) using rabbit anti-mouse as secondary.

ONLINE METHODS

Human Models

Samples were obtained from several sources representing the spectrum of cirrhosis patients that vary in vulnerability to infection. Non-cirrhotic liver disease patients were used as a control. Informed consent was obtained from all subjects. Consecutively sampled unselected plasma samples from these cohorts were used in a blinded fashion for the assays of immune function. We were able to gain anonymised patient data for the patients from the DASIMAR study for correlation with our laboratory results following the experiments.

Acutely decompensated patients:

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(ii) Patients on the liver transplant waiting list, representing ESLD. These were followed up for 24 weeks - MACHT (Effect of midodrine and albumin in the prevention of complications in cirrhotic patients awaiting liver transplantation, Hospital Clinic of Barcelona; ClinicalTrials.gov: NCT00839358).

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Extraction and analysis of lipid mediators

Lipid mediators in mice/human plasma were analyzed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) based on protocols published previously[16]. Briefly, samples were collected and stored immediately at −80 °C. Plasma Samples (250-500µl for mice, 500–1,000µL for humans) were defrosted on ice and adjusted to 15% (v/v) methanol: water (final volume 4mL). Internal standards, PGB_2-*d*4 (40 ng) and 12-HETE-*d*8 (40ng) (Cayman Chemical Company, Ann Arbor, USA) were added and

the pH of resulting solutions adjusted to 3.0 (1M HCL). Acidified samples were immediately applied to preconditioned solid-phase cartridges (C18-E, Phenomenex, Macclesfield, UK) and lipid mediators eluted with methyl formate. LC/ESI-MS/MS analysis was performed on a HPLC pump (Waters Alliance 2695) coupled to an electrospray ionization triple quadruple mass spectrometer (Quattro Ultima, Waters, UK). Chromatographic separation was performed on a C18 Luna column (5μm, 150 x 2.0mm, Phenomenex) for eicosanoids and a C18 Kinetex column (2.6μm, 100 x 2.1mm, Phenomenex) for hydroxy- fatty acids. Analytes were monitored on multiple reaction monitoring mode as reported with the following additions: 15-hydroxyeicosatrienoic acid (HETrE) *m/z* 321>221, 10-hydroxydocosahexaenoic acid (HDHA) *m/z* 343>153, 14-HDHA *m/z* 343>161, 13-HDHA *m/z* 343>193 and 17-HDHA *m/z* 343>201.

Human blood analysis and macrophage isolation/culture

To prepare human monocyte-derived macrophages (MDM) , healthy volunteer mononuclear cells were isolated by differential centrifugation (900g, 30min, 20°C) over Lymphoprep™ (Axis-Shield) and washed with sterile PBS (Invitrogen™, Life Technologies™)[17]. Cells were resuspended in 10ml RPMI-1640 medium (Invitrogen™, Life Technologies™)) supplemented with 100 $U.ml^{-1}$ of penicillin/100$\mu g.ml^{-1}$ streptomycin (Invitrogen™, Life Technologies™)) and plated at a density of $\sim 5 \times 10^6$ cells/ml in $8cm^2$ Nunclon Surface tissue culture dishes (Nunc®). After 2h incubation at 37°C (5% CO2), non-adherent cells were discarded and 10ml of fresh RPMI supplemented with 10% FBS added (Sigma-Aldrich®) (with antibiotics as above). After culture for 5 days at 37°C (5% CO2), with addition of a further 10ml of fresh 10% FBS/RPMI after 24h (total 20mls), adherent cells were scraped on day 5 and replated in 96-well culture plates (Nunc®) at equal densities (10^5/well) in X-Vivo-15 serum free 0.2% HAS medium (Lonza). Resulting primary MDM, validated by morphological inspection, were incubated overnight at 37°C (5% CO_2) to adhere prior to stimulation with 200ng/ml LPS

(*Salmonella abortus equi*, Enzo Biochem, Inc). To examine the effect of plasma/serum on macrophages, these were added to cell culture 30min prior to LPS to give a final volume of 25% plasma/serum. Similarly eicosanoids of interest were also added to cell culture 30min prior to LPS. AH6809 (50-300μM) was dissolved in X-vivo and added to the macrophages 45min prior to plasma. Macrophage supernatants were collected after 24h stimulation with LPS and stored at -80 °C for cytokine analysis by ELISA as described. Cell viability was ascertained with calcein AM cell viability assay kit (Biotium, Inc). Human serum albumin was obtained from Sigma-Aldrich >99% (agarose gel electrophoresis), essentially fatty acid (0.005%) and globulin free. For bacterial killing, *Escherichia Coli* (clinical isolate, provided by Dr Vanya Gant, Clinical Director for Infection, Consultant Microbiologist, UCLH) was added to healthy volunteer macrophages (ratio 100 *E.coli*: 1 macrophage) in the presence/absence of plasma from patient/healthy volunteer plasma ± AH6809 (50-300μM, Sigma Aldrich Dorset, UK) or albumin (15g/l) as above for 1h in media without antibiotics. Supernatants were plated on blood agar overnight and CFUs counted.

RNA isolation and gene expression studies

Peripheral blood mononuclear cells (PBMC) were isolated from total blood with the Ficoll method (Ficoll-Paque^{TM} PLUS, GE-Healthcare, Cat. No:17-1440-03) from four patients (3 males/1female) and six healthy controls (3 males/3 females). The PBMC cell pellet was immediately frozen at -80^{o}C until cell lysis and total RNA extraction with the RNeasy Mini Kit (Qiagen, Cat. No: 74104). RNA concentration was measured spectrophotometrically at 260 nm (Nanodrop8000, Thermo Fishcer), and RNA purity was determined by the *A*260/*A*280, *A*230/260 ranges and spectral pattern. 500ng of total RNA were reverse transcribed to cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase, RNase H Minus, Point Mutant (M-MLV RT (H–) (Promega, Cat No: M368A) and Promega oligodTs, random primers and

M-MLV X5 RT buffer (Promega). cDNA was then subjected to quantitative real-time PCR using an 7900HT ABI thermal cycler (Applied Biosystems), SYBGR (Applied Biosystems, Cat. No: 4367659) and in-house designed and optimised primers for the genes of interest and internal controls. Primer sequences were: *PTGS1*, forward primer: *TACCAGGTGCTGGATGGAGA*, reverse primer: *CCTTCAGCAGGTCACACAC*; *PTGS2*, forward primer: *GTTTTGACATGGGTGGGAAC*, reverse primer: *CCCTCAGACAGCAAAGCCTA*, *CYPH*: forward primer: *GCCGAGGAAAACCGTGTACT*, reverse primer: *ACCTTGTCTGCAAACAGCTCA; RPL27* (ribosomal protein 27), forward primer: *TCCAAGGGGATATCCACAGA*, reverse primer: *CATGGGCAAGAAGAAGATCG*, *RSP20 (ribosomal protein 20)*, forward primer: *GGAAACGATCCCACGTCTTA*, reverse primer: *AGAGGCGCAAAAGAAAAGAA*. Dissociation curve analysis was performed at 40 cycles to verify the identity of PCR product. For data analysis, the comparative threshold cycle values for constitutively expressed *CYPH* were used to normalize loading variations and data are expressed as arbitrary units (AU). Gene expression data analysis was also undertaken with the use of *RPL27* and *RPS20* as endogenous controls.

Animal models

Mice were maintained in a 12h/12h light/dark cycle at 22 ± 1°C and given food and tap water ad libitum in accordance with UK Home Office regulations. Studies were performed in male C57Bl6/J mice (20-25g) from Charles River UK, Margate, UK. Two models of liver injury were used: Bile duct ligation (BDL, 2 weeks) was performed[37] or Carbon Tetrachloride (CCL_4, Merck, Darmstadt, Germany, 8 weeks) given s.c. (1 ml/kg) twice weekly and 300mg/L phenobarbital in water[38]. We used these to investigate whether PGE_2 inhibitors affected bacterial killing and survival in infection models in vivo.

Bile duct ligation/sham procedures were carried out under anesthesia (isoflurane 1.5%) as described previously[18]. Carbon tetrachloride (CCL_4, Merck, Darmstadt, Germany) was given subcutaneously (s.c., 1:1 dissolved in olive oil; 1 ml/kg) twice weekly and 300mg/L phenobarbital added to drinking water[19]. Sham mice received s.c. injections of olive oil. After 2 weeks for BDL mice or 8 weeks for CCL_4 mice, interventional models were undertaken or blood and liver were taken and prepared for histology or further experimental use. Experiments were performed on CCL_4 mice on the same day as the final injection.

Interventional models: peritonitis and intra-venous bacterial inoculation

Group B Streptococcus (GBS) (NCTC10/84, serotype V) was grown in Todd Hewitt broth without agitation at 37°C to an OD600 of 0.4, equivalent to 10^8 Colony Forming Units (CFU)/mL, centrifuged/washed with sterile PBS and injected intraperitoneally (i.p.) at $30x10^6$ colony forming units (CFUs) in 300µl sterile PBS or for bacterial killing assays. A clinical isolate of GBS was provided by Dr Vanya Gant, Clinical Director for Infection, Consultant Microbiologist, UCLH and $1x10^7$CFUs in 300µl sterile PBS i.p. was used for mouse survival studies; $6x10^7$ CFUs for i.p saline vs albumin infusion studies and $4x10^7$ in 200µl sterile PBS for intravenous (i.v.) studies. For bacterial killing assays in mice with/without the non-selective COX-inhibitor indomethacin ($3mg.kg^{-1}$ p.o. 1h prior to bacterial challenge, Sigma-Aldrich®. or equivalent volume of vehicle), heparinized blood was collected 3h after GBS injection, centrifuged (10,000g, 4°C, 10min), plated on agar overnight and CFUs counted the following day. For the survival experiments, mice were observed every 6h for up to 80h. In accordance with Home Office guidance, mice were humanely culled if they had a "sickness score" of 4 or 3 for greater than 24h (Supplementary Table 2).

Cytokine analysis

Murine cytokine expression profiles were determined using Th1/Th2 10-plex FlowCytomix Mouse Kits (Bender MedSystems). Fluorescence was measured on a flow cytometer (BD FACSCanto II™, BD bioscience) and data analyzed using manufacturer's software. The expression profile of a cytokine panel in human macrophage supernatants was measured using the MSD® Bio-Plex human cytokine assay (Merck, Sharp & Dohme Ltd.). Our assay was customized to detect/quantify IL-1, IL-6, IL-8, IL-10 and TNFα. Key cytokine changes were confirmed by dedicated ELISA (TNFα and IL10 - mouse eBioscience or human Duoset®, R&D systems). All samples were run in duplicate.

Mouse blood analysis, macrophage isolation/culture

Blood was collected by intracardiac puncture into heparin and centrifuged (10,000g, 4^{O}C, 10min). Plasma was analyzed for liver and renal function tests using the COBAS® INTEGRA 400 plus multianalyser with appropriate diagnostic kits (Roche - Diagnostics) or stored at -80°C. Peritoneal macrophages from healthy animals were isolated as described previously[20] and incubated with/without LPS (*Salmonella Typhosa*, 0.1μg/mL for 24h; Sigma-Aldrich®) in the presence of plasma (50μl) from naïve, sham, BDL or CCL_4 mice in cell culture media (complete DMEM; Life Technologies™) to give a final concentration of 25% by volume. In other experiments plasma was added to cell culture, as above, from BDL mice given indomethacin (3mg.kg^{-1} po 6h prior to sampling); the cytochrome P450 inhibitor SKF525A (Enzo® Life Sciences; 50mg.kg^{-1} s.c. 1h prior to sampling); or the 12/15 lipoxygenase (LOX) inhibitor Bacalein (Sigma-Aldrich®; 100mg.kg^{-1} s.c. 90min prior to sampling). In experiments examining the effect of the DP1/EP1-3 receptor antagonist AH6809 (300μM; Sigma-Aldrich®), this compound was dissolved in DMEM and added to macrophages 45min prior to addition of plasma. Cell viability was ascertained with the calcein AM cell viability assay kit (Biotium, Inc) in accordance with manufacturer's instructions. For heat inactivation, plasma

was placed at 56°C in a water bath for 1h and samples stored overnight at 4°C and used for cell culture the following day.

Immunohistochemistry

For COX 1 and COX 2 expression, immunohistochemistry was carried out on formalin-fixed, wax-embedded mouse tissues using COX 1 polyclonal antibody (Cayman Chemical, catalogue number 160109) and COX-2 polyclonal antibody **(**Cayman Chemical, catalogue number 160106) using rabbit anti-mouse as secondary.

Randomisation

Cages of animals (n=5/cage) were randomly allocation to surgical or chemical-induced liver injury. Thereafter, groups of mice were allocated to receive drug intervention, infectious stimuli or both. In addition, groups of liver disease mice and sham controls were chosen for plasma extraction for *ex vivo* bioassays, where appropriate. For human studies, randomisation was not possible as human monocyte-derive macrophages received plasma from either healthy volunteers or from liver disease patients.

Blinding

For all mouse and human cell culture and bacteria experiments and mouse survival experiments, the investigator was blinded to the sample source during both the experiment and analysis of the data.

Statistical analysis

For calculation of group sizes, from experiments with murine peritonitis, cellular profiles, inflammatory protein expression and lipid mediator production is extremely reproducible. We found with random allocation of animals to each group that intra-animal replicate variability is much less than inter-animal biological variability. An effect size of ~40% of parameter mean is considered biologically relevant. Using this and population statistics, to enable statistical determination at a $P<0.05$ in a primary ANOVA screen followed by *post-hoc* Bonferroni corrected T-test at 90% power, a group size of 5 animals is necessary with a maximum of 5 groups per experiment. Applying this approach to humans using human cirrhotic plasma on humans cells with bacterial killing and TNFα as a readout, a minimum of n=10/group was required in order to discern significant changes in immune function. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software). For comparisons between multiple groups, 1-way ANOVA with repeated measures was performed followed by Bonferroni post-test. Comparisons between 2 groups were made by 2-tailed (un)paired t test. Differences between time-response curves were assessed by 2-way ANOVA. Correlations between variables were calculated using linear regression with Pearson statistic. For data not normally distributed (clinical data presented in Table 1) the Mann-Whitney test was used. $P < 0.05$ was considered statistically significant. No data, either rodent or human, were excluded from analysis.

Figure S1. PGF2α, 5- and 15-HETE are elevated in plasma of patients with Acute Decompensation (AD) when compared with healthy volunteers. (**a-g**) Lipidomic LC/ESI-MS/MS analysis of plasma from AD patients and healthy volunteers (n=8 healthy volunteers and 14 AD patients). Data are represented as mean ± SEM. * $P<0.05$ t-test.

Figure S2. Aside from PGE_2 other plasma eicosanoids are not elevated in bile duct ligation (BDL) liver injury mice. (**a-p**) Lipidomic LC/ESI-MS/MS analysis of plasma lipids from naïve, sham and BDL mice (n=6-8 mice/group). Data are represented as mean ± SEM.

Figure S3. The immunosuppressive properties of BDL liver injury mice plasma are mediated by PGE_2. (**a**) IL-6 and (**b**) IL-10 release from LPS stimulated peritoneal macrophages from naive mice in the presence of healthy (naïve) or BDL mice plasma with or without prior administration of indomethacin, SKF525A or baicalein (n=6-8 mice/group). (**c**) TNFα and (**d**) IL-10 release from LPS stimulated peritoneal macrophages from naive mice in the presence or absence of PGE_2 at concentrations found in plasma of BDL or CCL_4 mice (n=8-10 mice per group). (**e**) TNFα and (**f**) IL-10 release from from LPS stimulated peritoneal macrophages from naive mice in the presence of healthy (naïve) or BDL mice plasma with or without AH6809 (300μM) (n=6 mice/group). (**g**)TNFα, (**h**) IL6 and (**i**) IL-10 release from LPS stimulated peritoneal macrophages from BDL mice in the presence of healthy (naïve) or BDL mice plasma (n=4 mice/group). Data are represented as mean ± SEM. * $P<0.05$ ** $P<0.01$ ***$P<0.001$, ANOVA or t-test where appropriate.

Figure S4. Aside from PGD_2 and PGE_2, no other eicosanoid was elevated in plasma from CCL_4 mice when compared to shams. (**a-p**) Lipidomic LC/ESI-MS/MS analysis of

plasma from sham and CCL4 mice (n=6-8 mice/group). Data are represented as mean ± SEM. * $P<0.05$, t-test.

Figure S5. PGE_2 antagonism with AH6809 has a greater immune restorative effect on AD^{MIS} (most immunosuppressive) compared with AD^{LIS} (least immunosuppressed) **plasma samples.** (**a-d**) TNFα and IL-10 release from human monocyte-derived macrophages stimulated with LPS in the presence of plasma from AD patients, patients awaiting liver transplant sampled 24 weeks apart and healthy volunteers with and without AH6809; with cirrhosis patients grouped into into AD^{MIS} and AD^{LIS}. (**e**) LPS-stimulated human monocyte-derived macrophages TNFα synthesis in the presence of healthy volunteer or AD plasma samples in patients administered 0.9% saline on days 1(400-600ml) & 3 (200-400ml) of admission with samples obtained during admission and at 10-20 or 30-60 days post discharge (n=10). Data are represented as mean ± SEM. * $P<0.05$ ** $P<0.01$ *** $P<0.001$, ANOVA.