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ORIGINAL ARTICLE

Extracellular ectonucleotidases are differentially regulated in murine tissues and human polymorphonuclear leukocytes during sepsis and inflammation

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Abstract

Sepsis is life-threatening organ dysfunction caused by a dysregulated inflammatory and immune response to infection. Sepsis involves the combination of exaggerated inflammation and immune suppression. During systemic infection and sepsis, the liver works as a lymphoid organ with key functions in regulating the immune response. Extracellular nucleotides are considered damage-associated molecular patterns and are involved in the control of inflammation. Their levels are finely tuned by the membrane-associated ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) enzyme family. Although previous studies have addressed the role of NTPDase1 (CD39), the role of the other extracellular NTPDases, NTPDase2, -3, and -8, in sepsis is unclear. In the present studies we identified NTPDase8 as a top downregulated gene in the liver of mice submitted to cecal ligation-induced sepsis. Immunohistochemical analysis confirmed the decrease of NTPDase8 expression at the protein level. In vitro mechanistic studies using HepG2 hepatoma cells demonstrated that IL-6 but not TNF, IL-1β, bacteria, or lipopolysaccharide are able to suppress NTPDase8 gene expression. NTPDase8, as well as NTPDase2 and NTPDase3 mRNA was downregulated, whereas NTPDase1 (CD39) mRNA was upregulated in polymorphonuclear leukocytes from both inflamed and septic patients compared to healthy controls. Although the host's inflammatory response of polymicrobial septic NTPDase8 deficient mice was no different from that of wild-type mice, IL-6 levels in NTPDase8 deficient mice were higher than IL-6 levels in wild-type mice with pneumonia. Altogether, the present data indicate that extracellular NTPDases are differentially regulated during sepsis.

Keywords Sepsis · Liver · Ectonucleotidases · NTPDase8 · PMNs · Neutrophils · Hepatocytes

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated inflammatory response to infection [1]. Sepsis is the main cause of mortality in critically ill patients [2] and the leading cause of death in intensive care units (ICU) [3]. The systemic inflammatory response syndrome (SIRS) and the events that trigger multiorgan failure during sepsis such as release of LPS from bacteria in response to infection or release of damage-associated molecular patterns (DAMPs) from tissue injury are yet to be fully understood. The complexity of the host's response in sepsis which involves both exaggerated inflammation and immune suppression [3] explains the lack of treatment options available making urgent the investigation of new cellular and molecular mechanisms that can serve as new drug targets. Purinergic signaling has been described as an important component of tissue homeostasis as well as of immune response and inflammation [4–8]. In the event of inflammation, cell stress, and death, intracellular purines, such as ATP and its degradation products, can be exported to the extracellular space [9]. These signals serve as endogenous DAMPs and promote tissue adaptation to the danger by altering blood flow, cell death/survival and inflammation [5]. Nucleoside triphosphates, including ATP and UTP, can bind to specific P2 receptors or be metabolized to nucleoside diphosphates/monophosphates (ADP, UDP, AMP, UMP) and then adenosine and uridine. Ultimately adenosine can bind P1 or adenosine receptors,

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and ligation of the adenosine receptors has wide ranging immunomodulatory effects [10], [11], [12], [13], [14], [15], [16], [17], [18].

Extracellular levels of nucleotides are regulated primarily by membrane-associated ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) [19]. NTPDase1, -2, -3, and -8 are enzymes anchored to the cellular membrane and face the extracellular compartment [20]. Alterations in their activities have been demonstrated in immune cells and inflammatory diseases [7].

NTPDase1, also known as CD39, has been shown to suppress inflammation and mortality in septic mice [21, 22]. The role of NTPDase2, -3, and -8 in inflammation and sepsis is much less explored. Similar to NTPDase1, NTPDase2 and -3 are also expressed in immune cells [7]. NTPDase8 [23] is expressed highly in the liver [24], where it was detected in the bile canaliculi. In addition, NTPDase8 is also found in the intestine and kidney [24].

The liver is exposed to high levels of circulating antigens, endotoxins, danger signals, and microorganisms [25]. During systemic infection and sepsis, the liver works as a lymphoid organ and is responsible for clearing bacteria and toxins and producing inflammatory mediators [25]. In the liver, extracellular nucleotides have been demonstrated as important regulators of cell volume and proliferation [26, 27], which may be linked to the fact that the liver has one of the highest ATPase and ADPase activities of all the organs [28], [24]. NTPDase1, -2, -3, and -8 are all expressed in the liver, and their function is just beginning to be understood.

Since the role of NTPDase8 in sepsis is unknown, here we investigated the expression, regulation, and function of NTPDase8 in this disease. We also compared alterations of NTPDase8 with that of the other extracellular NTPDases both in septic mice and humans.

Methods

Mice

All experiments were approved by the Columbia University Institutional Animal Care and Use Committee. Ten- to 12-week-old male C57BL/6 J mice were purchased from Charles River Laboratory (Wilmington, MA, USA). All animals were subjected to a 12:12 h dark/light cycle with ad libitum access to standard rodent chow and water. For the cecal ligation and puncture (CLP) studies, male littermate wild-type (WT) and NTPDase8 knockout (KO) mice were produced by heterozygous breeding. For the pneumonia studies, WT (C57BL/6 J) and NTPDase8 KO mice were age and sex-matched mice bred at Charles River.

CLP model

Sepsis was induced experimentally as previously described [29], [30], [21], [31], [32]. Briefly, after being anesthetized with 2% isofluorane, the cecum was exposed and ligated at 50% length and punctured twice with a 22-gauge needle. A small amount of feces was squeezed through the lower puncture. The cecum was then returned into the abdominal cavity, and the incision closed. Sham operation consisted of an abdominal incision, exposure of cecum which was not ligated or punctured, replacement of cecum into the abdominal cavity, and closing of the abdominal wall with sutures. Resuscitation was performed with subcutaneous injection of 1 mL of physiological saline. Pain was managed using Buprenorphine SR – Ethiqa XR (3.25 mg/kg,Fidelis, NJ, USA) injected on the day before the surgery subcutaneously. Mice were euthanized 20- to 24-h post-surgery for tissue collection. Blood samples were obtained aseptically by cardiac puncture. Peritoneal lavage fluid was collected with 3 mL of sterile phosphate-buffered saline (PBS) that was injected into the peritoneal cavity and recovered through the needle. Both blood and peritoneal lavage were serially diluted for bacteriological analysis. Dilutions were aseptically plated and cultured on trypticase blood agar plates (General Laboratory Products, IL, USA) at 37 °C. After 14–18 h of incubation, the number of bacterial colonies was counted. The number of colonies is expressed as colony-forming unit (CFU)s per milliliter of blood or peritoneal lavage fluid. Blood and lavage samples were also centrifuged at 5000 g for 10 min, and the supernatant was collected for further ELISA analysis.

RNAseq

Snap frozen liver samples were homogenized with Qiagen TissueLyser II (Qiagen, Germantown, MD, USA), at 25/s oscillation frequency for 2 min. RNA was extracted using Qiagen miRNeasy (Qiagen) mini kit following the instructions of the manufacturer. RNA samples with RIN ≥8 were submitted for RNAseq analysis. Poly-A pull-down was used to enrich mRNAs from total RNA samples, which was followed by library construction using Illumina TruSeq chemistry. Libraries were sequenced using Illumina NovaSeq 6000. Samples were multiplexed in each lane, yielding a targeted number of paired-end 100-bp reads for each sample. RTA (Illumina) was used for base calling, and bcl2fastq2 (version 2.19) was used for converting BCL to fastq format, coupled with adaptor trimming. Pseudoalignment was performed to a kallisto index created from transcriptomes (mouse: GRCm38) using kallisto (0.44.0). Testing for differentially expressed

genes under various conditions was done using DESeq2 R package designed to test differential expression between two experimental groups from RNAseq counts data. Ingenuity pathway and gene enrichment analysis were performed using IPA software (Qiagen). RNA sequencing was performed one time with three biological replicates per group, and the sample variance plot can be found in the supplemental material (Supplementary Fig. 2). Transcriptome data were deposited in NCBI's Gene Expression Omnibus23 (GEO) and can be accessible through GEO Series accession number: GSE184167.

RT-qPCR

All reagents used for this procedure were purchased from Thermo Fisher – Applied Biosystems, USA. Snap frozen samples were homogenized in TRIzol reagent, and RNA was isolated according to the manufacturer instructions, followed by reverse transcription to obtain cDNA. The RT-qPCR reactions were run (20–100 ng cDNA) on an Applied-Biosystem QuantStudio 3 PCR system using Master Mix Power Up using specific primers (details in supplemental material). Data were analyzed using the $2-\Delta/\Delta CT$ method, as described previously (Livak and Schmittgen, 2011), and data were normalized to the respective housekeeping gene.

Immunohistochemistry (IHC)

After CLP surgery, animals were perfused with PBS, and livers were fixed in 4% paraformaldehyde overnight and embedded in Tissues-Tek optimal cutting temperature (OCT) freezing medium (Fisher, USA). Cryosections ($6-8$ µM) were mounted and fixed in cold acetone and 10% phosphate-buffered formalin (19:1) for 2 min at 4 °C. Sections were incubated for 30 min at room temperature in 5% bovine serum albumin (BSA) diluted in PBS and then incubated overnight at 4 °C with primary antibody (NTPDase8) [33] diluted in 1% BSA. Sections were washed twice with PBS-Tween (0.1%) and incubated with 0.3% hydrogen peroxide in PBS for 10 min. Sections were then incubated with avidin/biotin blocking kit (Vector Laboratories) for 15 min at room temperature followed by 2 washes in PBS-T and then incubated with a biotin-labeled secondary antibody diluted $1000\times$ in PBS for 1 h at room temperature, which was followed by 2 more washes. The complex avidin/biotinylated horseradish peroxidase (Vector Laboratories) was added for 30 min at room temperature to optimize the reaction. After washing twice with PBS-T, peroxidase activity was revealed with the substrate 3,3′-diaminobenzidine (Sigma,MO, USA) for 2 to 3 min. After washing with distilled water, tissues were counterstained with aqueous hematoxylin (Sigma,MO, USA) and mounted in Mowiol (Sigma).

Cell culture and treatment

For in vitro experiments when not mentioned, the reagents were obtained from Sigma. HepG2 cells were obtained from (ATCC, VA, USA) and used at early passages. HepG2 cells were cultivated in DMEM (Gibco, USA) with low glucose supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), penicillin (100 IU/mL), and streptomycin (100 µg/mL). For experimentation, medium containing 0.1% FBS was used and changed 1 h prior to the experiment. Heat inactivated *Escherichia coli* (ATCC; catalogue number: 10798) was used as well as LPS which were incubated for 24 h. IL-6, IL-1β, and TNF-α (RnD, USA) were incubated for different time periods and at different concentrations that are specified in the figures. STAT-3 inhibitor Stattic (Tocris, USA) was added 1 h prior to IL-6. At the end of the experiment, cells were washed twice with PBS and stored in TRIzol reagent. Procedures for RNA isolation and RT-qPCR were followed as described above.

Human studies

The study protocol for blood collection from patients and healthy controls was approved by the Hospital Nossa Senhora da Conceição, Porto Alegre, Rio Grande do Sul, Brazil, Research Ethics Committee (registration numbers 49959315.5.0000.5530 and 49,959,315.5.3001.5345, Brazilian National Ethical Committee-CONEP). Informed consent was obtained from all participants. Inclusion criteria were the presence of acute infection and the concurrent development of acute organic failure. Data of patient characteristics are shown in Supplemetary Table 3. The attending ICU staff defined the acute infection diagnosis. A control group consisted of "inflamed" patients, and this inflamed group included ward patients with chronic diseases but not sepsis admitted to the general internal medicine wards (Supplementary Table 4). A control group consisted of "inflamed" patients, and this inflamed group included ward patients with chronic diseases but not sepsis admitted to the hospital. The non-septic status required the absence of an active infection, as defined by the attending team, and also the lack of signs of clinical deterioration in the last 48 h. Healthy volunteers were also selected as a second control group. We searched for and enrolled septic patients on their first morning after ICU admission. At patient triage, we defined acute organ failure by the presence, in the preceding 48 h before ICU admission, of at least one of the following [34]:

(1) Hypotension: systolic arterial pressure < 90 mmHg or mean arterial pressure < 70 mmHg.
(2) Arterial lactate > 2 mEq/L.
(3) Urine output < 0.5 mL/kg/h for 2 consecutive hours, in the absence of hypovolemia.

(4) PaO_2/FiO_2 ratio < 200, if pneumonia, or < 250 in the absence of pneumonia.
(5) Serum creatinine > 2 mg/dL.
(6) Serum bilirubin > 2 mg/dL.
(7) Platelet count < 100,000/μL.
(8) Coagulopathy (INR > 1.5).

Human polymorphonuclear leukocyte (PMN) isolation

PMNs from peripheral blood were isolated from 5 mL of total blood collected with heparin using Polymorphprep™ solution (density 1.113 g/mL; Accurate Chemical, NY, USA) [35]. Isolated PMNs were immediately harvested in TRIzol reagent (Invitrogen, CA, USA), and RNA isolation was performed as described above.

ELISA

TNF-α, IL-1β, IL-10, IL-6, and MIP-2 in blood and peritoneal and bronchoalveolar lavage were determined using commercially available ELISA kits (Duoset R&D Systems), according to the manufacturer's instructions.

Pneumonia model

Ten- to 12-week-old male WT and NTPDase8 KO mice were anesthetized by briefly placing them into an isoflurane chamber (2%, 1 L/min) followed by intraperitoneal ketamine-xylazine injection (100 mg/kg and 10 mg/kg, respectively). Adequate depth of anesthesia was checked by hind toe pinch reflex. *Streptococcus pneumoniae* (ATCC, Manassas, VA, USA; catalogue number: 6303) and *Escherichia coli* (ATCC; catalogue number: 10798) bacteria were administered at 10^{10} CFU at a 1:1 ratio in 40 μL of PBS, 20–20 μL in each nostril. At the 6-h timepoint, bronchoalveolar lavage was collected in 1 mL of PBS. Blood was collected by heart puncture, and samples were spun down for 10 min at 3500 × rpm at 4 °C. Samples were stored at − 20 °C until further analysis.

Statistics

For RNAseq data processing, DESeq2 differentially expressed gene analysis was applied. log2 fold change values were determined, standard error (lfcSE) was calculated, and Wald statistic was applied to determine p-value. Data shown depicts Benjamini–Hochberg adjusted *p*-values (padj). Volcano plot depicts FDR corrected *p*-values. When comparing 2 groups, 2-tailed unpaired Student *t* test was used. When comparing 3 or more groups, one-way ANOVA was used. GraphPad Prism Software version 8 was used. Statistically different results were considered when p value ≤ 0.05.

Results

Sepsis alters gene expression in the murine liver

Considering the important function of NTPDases in the liver and the important immunological function of the liver during sepsis, we first performed liver bulk RNAseq analysis in sham-operated and CLP-induced septic mice 20–24 h after sham operation or the induction of sepsis. We confirmed that septic mice had systemic infection and inflammation (Supplementary Fig. 1). As expected, a very different gene expression profile was observed between mice subjected to CLP and sham-operated animals (Fig. 1A) and the CLP *vs.* sham samples clustered close together in a general sample profile analysis (Supplementary Fig. 2). The top 50 differentially expressed genes are listed within the heatmap presented in the Fig. 1A and a volcano plot representing the top 28 up- and downregulated genes based on their log2 fold change against the *p* value (Fig. 1B). Interestingly, *Entpd8* was identified among the top 28 downregulated genes in the septic mouse liver (Fig. 1B).

Sepsis induces different biological pathways and gene networks in the murine liver

An analysis of pathways altered in the murine liver during sepsis (Fig. 2A) as well as an upstream gene network analysis was performed (Fig. 2B). Pathways such as EIF2 signaling, LPS/IL-1-mediated inhibition of RXR function, tumor microenvironment pathway, mTOR signaling, and eIF4 and p7056K signaling were found upregulated. Other pathways such as RAR activation and hepatic cholestasis were also found as differentially expressed as expected. In the upstream gene network panel (Fig. 2B), the prediction for up- and downregulated molecules and transcription factors is demonstrated. Inflammatory molecules such as cytokines and chemokines are predicted as upregulated as expected. The predictions indicate that CXCL3 upregulates, and IL1A/B downregulates the expression of the transcription factor *PPARG* which inhibits *NR1H* nuclear receptor superfamily. NR1H are key regulators of macrophage function, controlling their transcriptional programs that are involved in lipid homeostasis and inflammation.

Entpd8 gene expression is downregulated in the septic mouse liver, gut, and kidney

We next analyzed extracellular NTPDase gene expression using quantitative real-time PCR in sham mice and mice with CLP. As expected, we found that transcripts of NTPDase8 were suppressed in the septic *vs.* sham liver,

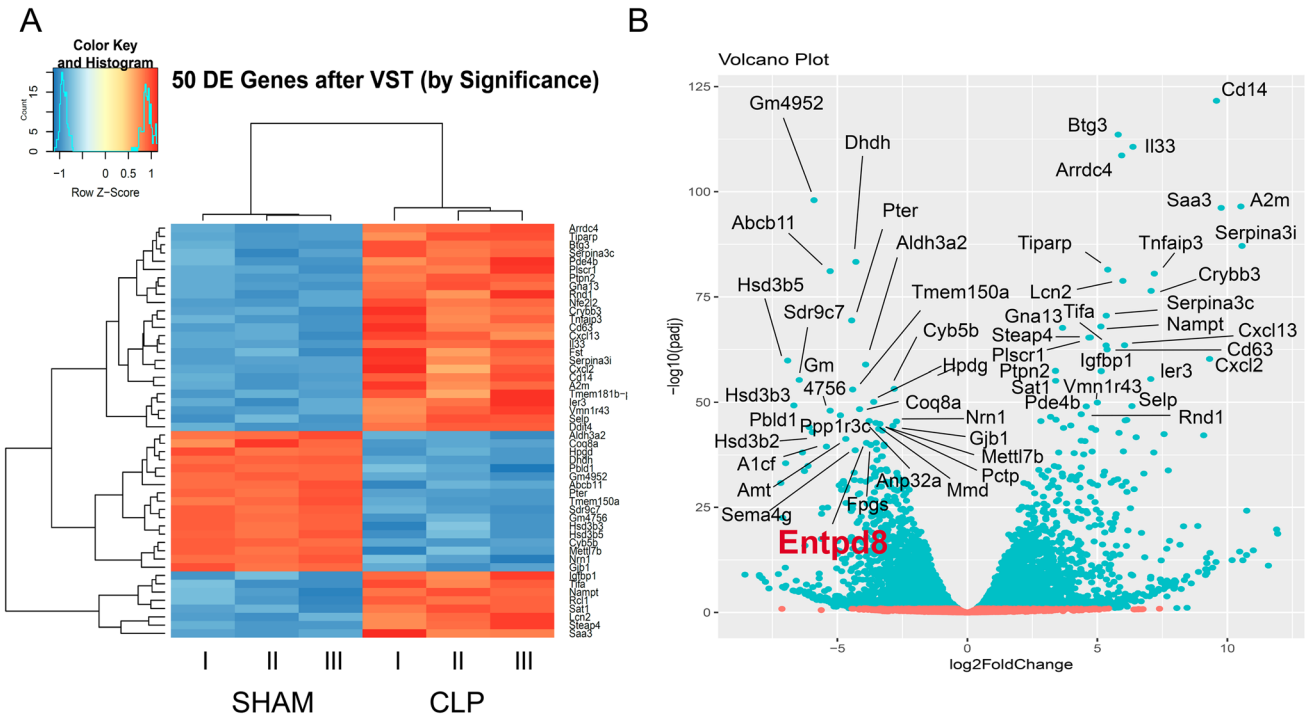

Fig. 1 Sepsis alters gene expression in the murine liver. Bulk liver RNASeq analysis of mice with SHAM and CLP surgery ($n=3$ per group). **A** Heatmap representing variance-stabilizing transformation (VST) analysis showed clear differences in the 50 top differently expressed genes between mice subjected to SHAM vs. CLP surgery. **B** Volcano plot depicts main genes (blue) that were identified to be differentially expressed in CLP when compared to SHAM, determined by adjusted p values (Wald statistic; Benjamini-Hochberg; padj ≤ 0.05) between the two experimental groups. Entpd8 localization in the volcano plot is indicated in red

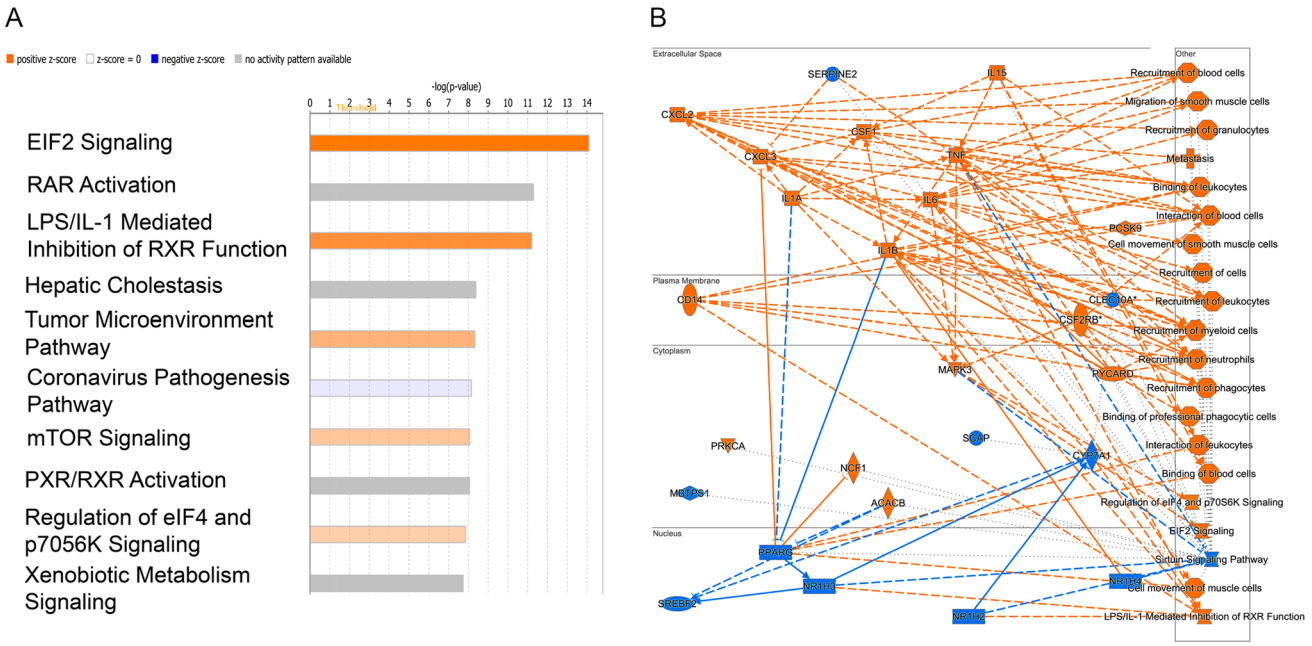

Fig. 2 Sepsis induces different biological pathways and gene networks in the murine liver. **A** Stacked bar chart displays the percentage of genes that were upregulated (orange), and genes differentially regulated (white) in IPA biological function analysis. The 10 top biological pathways were considered significant based on Fisher's exact test with a $-\log 10$ p-value > 1.3 (corresponds to a p-value < 0.05). The X axis on the bottom shows the significance ($-\log$ (p-value). **B** Upstream gene network panel of the prediction for up and downregulated molecules and transcription factors analyzed by IPA software

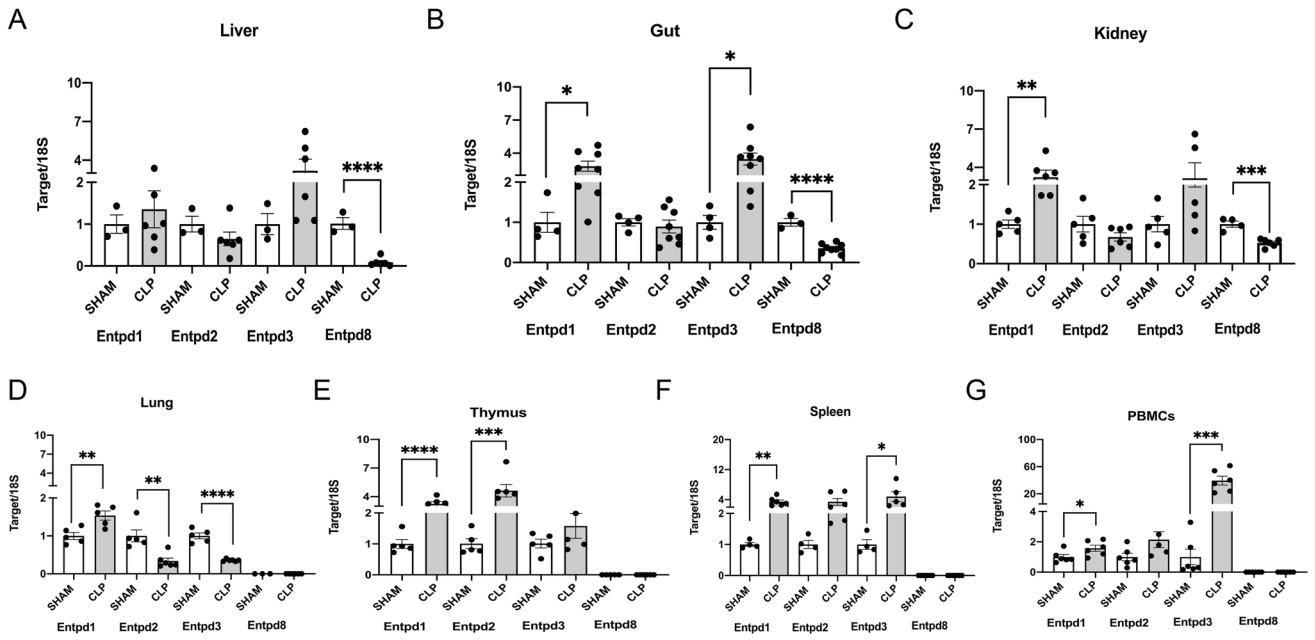

Fig. 3 *Entpd8* gene expression is downregulated in the septic mouse liver, gut, and kidney. RT-qPCR analysis of NTPDase1, -2, -3, and -8 in several organs isolated from mice after CLP and sham surgery. (**A**) Gene expression of NTPDase1, -2, -3, and -8 in liver, (**B**) gut, (**C**) kidney, (**D**) lung, (**E**) thymus, (**F**), spleen, and (**G**) peripheral blood mononuclear cell (PBMC)s. Graphs corresponding to one representative experiment from 2–4 experimental replicates. Results are expressed as average ± SEM with biological replicates of the experiment represented by dots. $*p \leq 0.05$; $**p \leq 0.01$, $***p \leq 0.005$, $****p \leq 0.0001$, ns: $p > 0.05$. 2-tailed unpaired Student *t* test

but transcripts of NTPDase1, -2, and -3 were not altered (Fig. 3A). As NTPDase8 is expressed mainly in the liver, gut, and kidney, we also analyzed the gene expression levels of extracellular NTPDases in these tissues and also found that NTPDase8 mRNA is downregulated in both the gut (Fig. 3B) and kidney (Fig. 3C). NTPDase8 was undetectable in the lung (Fig. 3D), thymus (Fig. 3E), or PBMCs (Fig. 3G). In addition, in both the gut and kidney, NTPDase1 mRNA was upregulated (Fig. 3B and C), as was NTPDase3 mRNA in the gut (Fig. 3B).

NTPDase8 protein expression is decreased in the septic murine liver

We next performed IHC to detect NTPDase8 protein in the liver (Fig. 4). We detected decreased expression of NTPDase8 in septic compared to sham mice. In both groups, bile canaliculi expressed high levels of NTPDase8. NTPDase8 KO mice submitted to CLP or sham operation failed to stain for NTPDase8 confirming the specificity of our staining (Fig. 4C).

IL-6 downregulates NTPDase8 in human hepatocytes via *STAT-3*

We investigated the mechanisms behind NTPDase8 downregulation in sepsis in a reductionist model using the human hepatocyte cell line HepG2. First, the cells were exposed to both heat-inactivated bacteria and LPS to investigate whether bacteria or bacterial LPS mediate the septic downregulation of NTPDase8 in hepatocytes. Both bacteria and bacterial LPS failed to affect NTPDase8 mRNA levels (Fig. 5A). Given that the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β are major mediators of organ injury in sepsis [36], we treated cells with IL-6, TNF-α, and IL-1β. We observed that IL-6 downregulated NTPDase8, but the other cytokines failed to do so (Fig. 5B). The maximum decrease of NTPDase8 mRNA levels was found at 6 h after IL-6 treatment (Fig. 5C). To begin to study the cellular mechanisms by which IL-6 downregulates NTPDase8 mRNA, we exposed the cells to the STAT-3 inhibitor Stattic before IL-6, as STAT-3 is a major signaling molecule downstream of the IL-6 receptor. We found that Stattic reversed the IL-6 suppression of NTPDase8 mRNA (Fig. 5D). In addition, Stattic alone was able to increase the expression of NTPDase8 mRNA (Fig. 5D), indicating a role for endogenous STAT-3 signaling. At the concentrations used, neither IL-6 or Stattic was toxic to the cells (Fig. 5E).

NTPDase8 gene expression is decreased in PMNs of inflamed and septic patients

In an attempt to translate our findings to humans and to better understand the function of extracellular

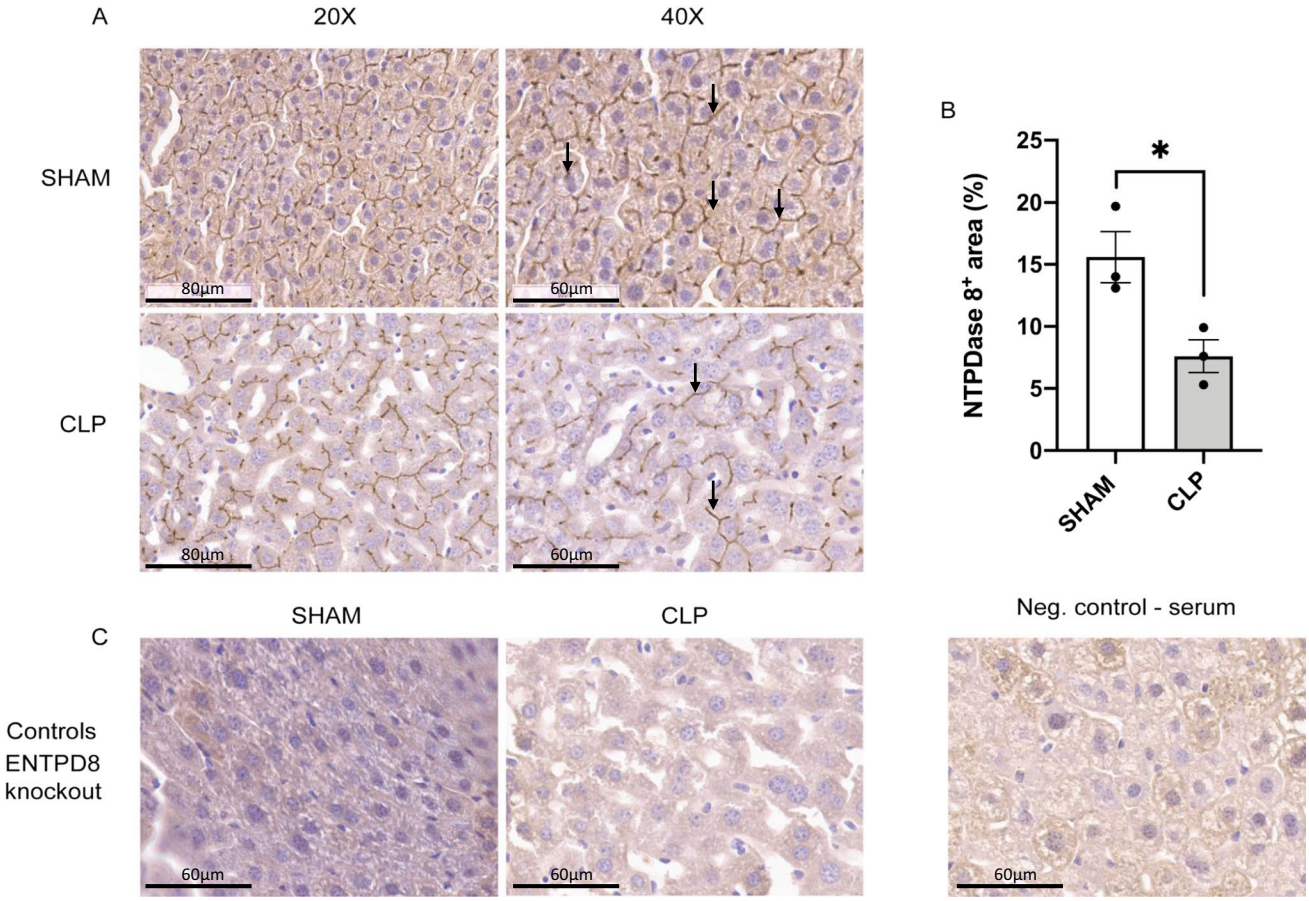

Fig. 4 NTPDase8 protein expression is decreased in the septic murine liver. IHC detection of NTPDase8 protein in the mouse liver submitted to CLP and or SHAM operation. **A** Representative images of microscopy in 20× and 40×. Brown color represents NTPDase8 immunodetection. **B** Quantification of the NTPDase8-stained area related to the total tissue area. **C** Representative images of the respective controls: SHAM *Entpd8*$^{-/-}$ mouse, CLP Entpd8$^{-/-}$ mouse, and negative control with respective anti-serum in 40× magnification. Arrows indicate NTPDase8 positivity. Results are expressed as average ± SEM with biological replicates of the experiment represented by dots. *$p \leq 0.05$. 2-tailed unpaired Student *t* test

NTPDases in sepsis, the gene expression of NTPDases 1, 2, 3, and 8 was measured in freshly isolated PMNs from septic patients compared to control groups. We enrolled 9 patients in the sepsis group and in both control groups. All septic patients fulfilled sepsis-3 definitions. Septic patients' mean age was 61.8 years (range 31 to 72), and the mean SOFA score was 7.2 (range 3 to 10). Five were female. Among septic patients, we found two patients having no concurrent disease, two patients had diabetes, 5 had arterial hypertension, two had atherosclerotic disease, one had cancer, and two had chronic pulmonary disease (Supplementary Table 3). The first control group, comprising "inflamed" but not septic patients, had a mean age of 68.5 years (range 55 to 91). Four were female. We found diabetes in four, arterial hypertension in 7, atherosclerotic disease in three, cancer in one patient, and chronic pulmonary disease in three patients (Supplementary Table 4). The second control group comprised healthy individuals.

All patients provided written consent, and the study was approved by Institutional Research Ethics Committee and was adherent to all applicable regulations. As expected based on our previous murine study [21], we observed higher NTPDase1 transcript levels in septic patients compared to both healthy and inflamed controls (Fig. 6A). In addition, inflamed controls presented with higher NTPDase1 than healthy controls (Fig. 6A). We found that NTPDase8 was downregulated in inflamed patients as well as in septic patients when compared to healthy controls (Fig. 6D). Both NTPDases 2 and 3 mRNA were also decreased in systemic inflammation and sepsis when compared to healthy controls (Fig. 6B and C).

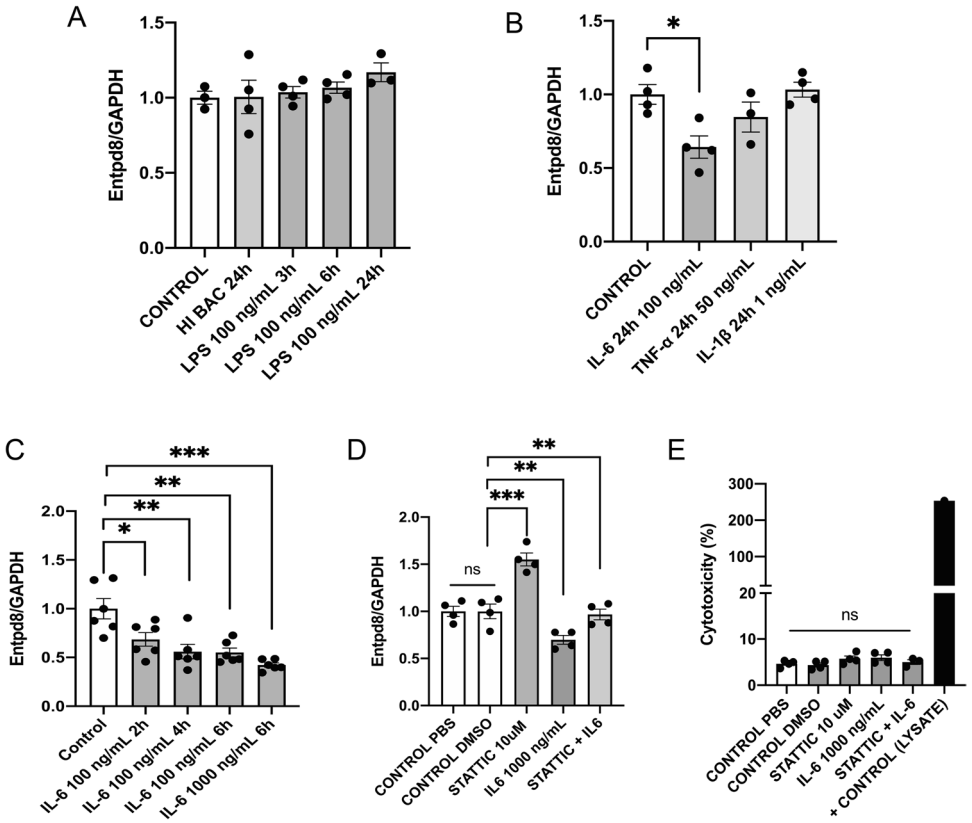

Fig. 5 IL-6 downregulates NTPDase8 mRNA in human hepatocytes via STAT-3. RT-qPCR analysis of *ENTPD*1, -2, -3, and -8 gene expression in human hepatocytes cell line HepG2. **A** Cells were exposed to heat-inactivated *E. coli* (HI-BAC) for 24 h or LPS (100 ng/mL) for different time periods as indicated in the graph. **B** Cells were exposed to three different cytokines for 24 h. **C** *ENTPD*8 gene expression in HepG2 cells exposed to IL-6 for different time periods. **D** Cells were treated with Stattic (STAT-3 inhibitor) for 1 h prior to incubation with IL-6 (100 ng/mL) for 6 h. (**E**) Cell viability analysis of the respective treatments in (**D**) evaluated by LDH activity measured in the cells supernatant. Graphs corresponding to one representative experiment from 3 experimental replicates. Results are expressed as average ± SEM with experimental replicates of the experiment represented by dots. One way ANOVA followed by multiple comparisons test. *$p \leq 0.05$; **$p \leq 0.01$, ***$p \leq 0.005$, ****$p \leq 0.0001$, ns: $p > 0.05$

NTPDase8 loss of function fails to affect the host's response to abdominal sepsis but modulates inflammation in pneumonia

In an attempt to unravel the role of NTPDase8 in sepsis, we studied the host's response to CLP of WT and NTPDase8 KO mice. We detected no difference in bacterial load both in blood and peritoneal lavage when comparing WT and NTPDase8 KO mice (Fig. 7A and B). In addition, inflammatory cytokine levels were no different in both peritoneal lavage (Fig. 7C–G) and blood (Fig. 7H–L) in WT compared to NTPDase8 KO mice after CLP. However, both blood and bronchoalveolar lavage IL-6 levels were higher in NTPDase8 KO compared to WT mice (Supplementary Fig. 3C) 6 h after pneumonia. There was no difference in the levels of other cytokines (Supplementary Fig. D–G) and bacterial load (Supplementary Fig. 3A and B) after pneumonia.

Discussion

In the present studies, we investigated the expression and function of extracellular NTPDases in the context of inflammation and sepsis. In an RNAseq of the mouse liver 20–24 h after inducing sepsis, NTPDase8 was identified as an important downregulated gene. This finding was confirmed using RT-qPCR and IHC indicating that NTPDase8 is specifically modulated in sepsis at least in mice. In vitro mechanistic studies demonstrated that IL-6 but not IL-1β or TNF-α decreased the gene expression of NTPDase8 in human hepatocytes. It was also shown that PMNs from both inflamed and septic patients admitted to the ICU have decreased expression of NTPDase8 when compared to healthy controls. NTPDase8 failed to influence the host's response to abdominal sepsis but suppressed IL-6 production in pneumonia.

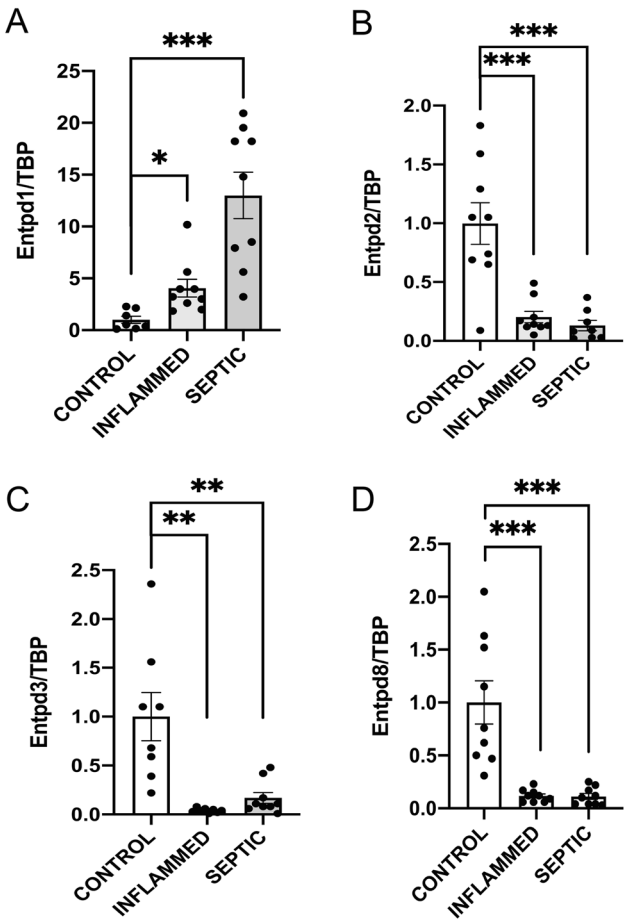

Fig. 6 *ENTPD8* gene expression is decreased in PMNs of inflamed and septic patients. RT-qPCR analysis of *ENTPD*1, -2, -3, and -8 gene expression in human PMNs freshly isolated from septic patients, inflamed patients and healthy controls. (**A**) *ENTPD*1 gene expression, (**B**) *ENTPD*2 gene expression, (**C**) *ENTPD*3 gene expression, and (**D**) *ENTPD*8 gene expression. Results are expressed as average ± SEM of *n* patients, where *n* in the various groups is indicated in the text. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.005$. 2-tailed unpaired Student *t* test

NTPDases are important regulators of nucleotide levels in the extracellular compartment. As ATP is a DAMP or danger signal, high concentrations of extracellular ATP are related to inflammation. In this sense, NTPDase expression has been linked to infection and inflammation [7]. NTPDase1 (CD39) is the most studied NTPDase, and its expression is altered by inflammation. NTPDase1 increases survival in an experimental model of polymicrobial sepsis by suppressing inflammation [21]. NTPDase1 was also described as protective in sepsis-induced liver injury [22]. NTPDase8 has a higher capacity for ATP/UTP over ADP/UDP hydrolysis when compared to NTPDase1/CD39 and is more like NTPDase2 functionally [37]. NTPDase8 has absolute requirement for divalent cations for the catalytic activity (Ca(2 +) > Mg(2 +)) with an optimal pH between 5.5 and 8.0 for ATP and 6.4 for ADP hydrolysis. Hydrolysis of triphosphonucleosides by NTPDase8 results in a transient accumulation of the corresponding diphosphonucleoside, e.g., ADP, as expected from the apparent K(m) values and as also noted for NTPDase2. Thus, the various NTPDases can function differently in terms of elevating ADP *vs*. AMP, and their function also depends on the cellular microenvironment. Thus, it is important to study all ectonucleotidases simultaneously. Prior to this study, the role of other extracellular NTPDase2, -3, and -8 during sepsis has not been evaluated.

The most striking finding of this study is the strong downregulation of NTPDase8 in septic liver, gut, and kidney. This is especially striking in light of the observations that NTPDase1 and 3 are upregulated following sepsis (this study and [21] in the lung. We find that liver NTPDase1 was not upregulated in the currect study as opposed to what we observed inour previous study [21], which may be explained by differences in experimental conditions (e.g., animal facility. The reason for the decrease in NTPDase8 is unclear. NTPDase8 KO mice failed to fare worse, at least in the short term, than their WT counterparts, indicating that the sepsis-induced suppression of NTPDase8 is not a protective mechanism. It is however possible that NTPDase8 downregulation in the liver and kidney has effects beyond dictating acute organ injury (liver or kidney injury was not altered in NTPDase8 KO mice,Supplementary Fig. 4. For example, it is possible that NTPDase8 in liver canaliculi alters bile flow or composition and long-term liver function after sepsis, which should be studied in the future. It is also noteworthy that NTPDase8 is downregulated in PMNs from septic patients. This indicates that NTPDase8 suppression in inflammation is not tissue or species specific. This contrasts with NTPDase1, which is upregulated in sepsis both in humans (this study and mice [21]. The cellular and molecular mechanisms and the consequences of this differential regulation of NTPdase8 and NTPDase1 in inflammation are unclear and need further study.

Colitis induced in NTPDase8 KO mice triggered higher gene expression of pro-inflammatory cytokines such as MIP-2, IL-1β, and IL-6 than in WT controls [38]. In accordance, we also observed increased levels of IL-6 in the serum of NTPDase8 KO mice with pneumonia when compared to WT mice (Supplementary Figure 3). Different from the observation that NTPDase8 general KO presented with worse colitis disease score when compared to WT [38], we did not observe increased exacerbated disease course in general NTPDase8 KO mice when submitted to sepsis or pneumonia. Since both sepsis and pneumonia are acute inflammation models (hours) and colitis is more chronic (days), these differences could explain why NTPDase8 differentially regulates our acute disease models *vs.* the colitis model.

Although inflammatory cytokines are primarily produced by immune cells and secondarily stimulate hepatocytes, hepatocytes are recognized as important players in the septic host's response [39, 40]. We found that IL-6-STAT-3

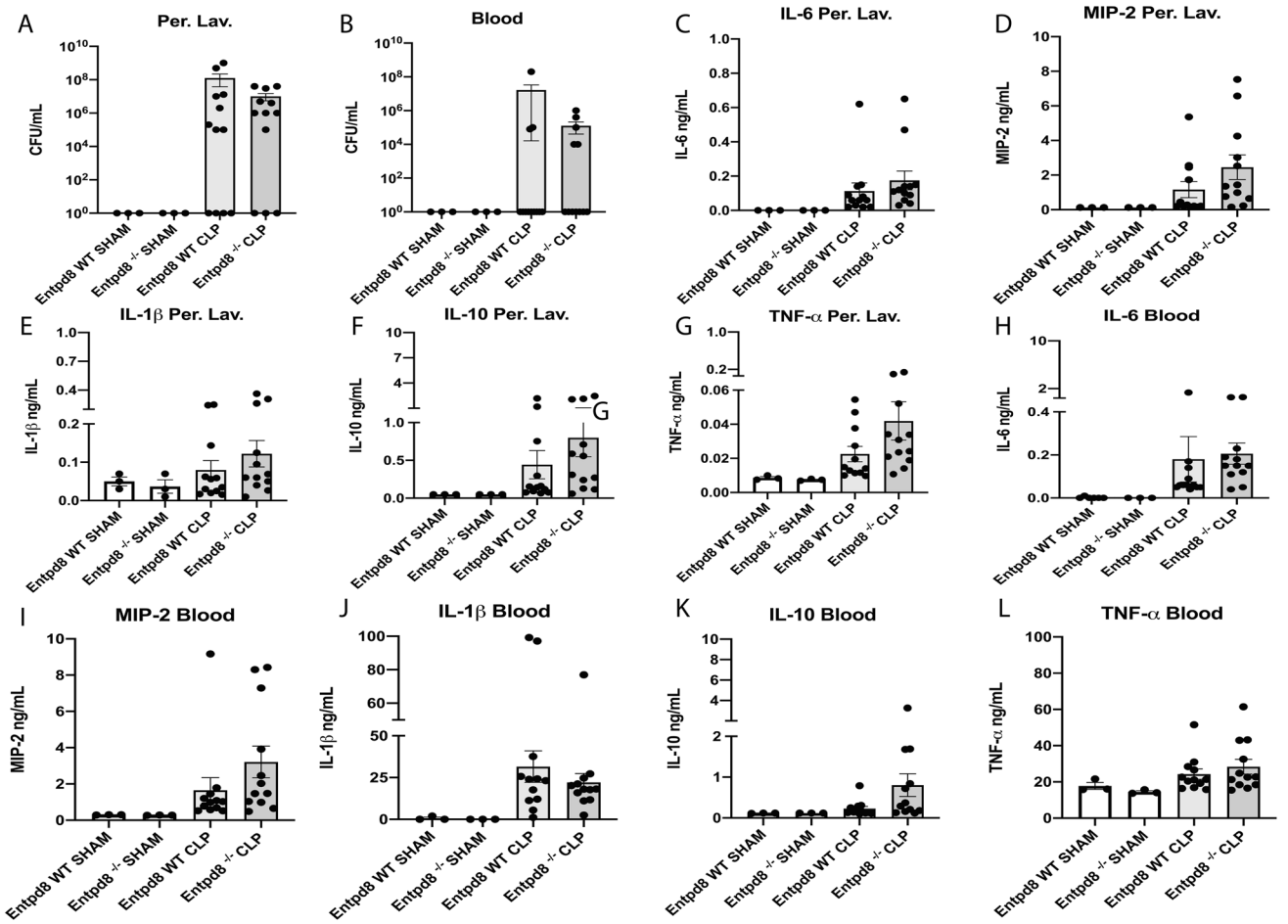

Fig. 7 NTPDase8 loss of function fails to affect the host's response to CLP. CFU counts were measured in peritoneal lavage (**A**) and blood (**B**) in both SHAM and CLP groups of both *Entpd8*$^{-/-}$ and WT animals. Cytokines in the peritoneal (Per. Lav.) lavage (**C**–**G**) and blood (**H**–**L**) were detected using ELISA. Results are expressed as average ± SEM with biological replicates of the experiment represented by dots. 2-tailed unpaired Student *t* test

axis is linked to NTPDase8 regulation in human hepatocytes in vitro. IL-6 decreased NTPDase8 expression, and the inhibition of STAT-3 reversed the IL-6 suppression of NTPDase8. As STAT-3 can actually promote NTPDase1 expression in Th17 cells [41] and in macrophages [42], we conclude that the STAT-3 regulation of NTPDase expression is cell type-dependent. Hepatocytes express TLR4, which in the presence of LPS promotes inflammasome activation [43]. However, LPS did not affect NTPDase8 expression in vitro (Fig. 5). Hepatocytes are also central to the acute-phase response. IL-1, TNF-α, and IL-6 can activate this process. IL-6 triggers the release of classic acute-phase proteins, including serum amyloid A, C-reactive protein, haptoglobin, α1-antichymotrypsin, and fibrinogen [39]. Based on our in vitro data, we speculate that NTPDase8 is involved in the acute-phase response of hepatocytes.

PMNs rapidly infiltrate the liver parenchyma during infection. They are engaged in the phagocytosis and killing of bacteria and in the release of antimicrobial granule proteins. They are also key components of the inflammatory response during sepsis [25]. During sepsis, a reprogramming of PMN gene expression takes place with reduced pro-inflammatory and immunomodulatory genes [44]. Our data indicate that NTPDases 2, 3, and 8 are additional downregulated genes in septic PMNs. It is worth mentioning that this study represents the first demonstration of NTPDases -2, -3, and -8 expression in PMNs. While NTPDases -2 and -3 have been found on immune cells (reviewed in [7], prior to this study, NTPDase8 had not been detected on immune cells (reviewed in [7]. One caveat of our results is that NTPDase expression was studied at the mRNA level. Future studies should aim to measure NTPDase protein levels in and surface expression on blood cells.

Given the differential function (ADP *vs.* AMP production) of NTPDases and their differential dependence on the extracellular milieu, we posit that NTPDases are differentially regulated to achieve a certain ratio of ADP *vs.* AMP in the septic environment. This may explain why NTPDase1 is protective in sepsis, whereas NTPDase8 is not. Further

studies will be needed to examine the reason for the differential role of NTPDase subtypes in sepsis.

Altogether, the present data indicate that extracellular NTPDases have a different pattern of expression during sepsis and that the specific suppression of NTPDase8 in the septic murine liver is probably related to the acute-phase response activated by IL-6. The specific functions of NTPDase8 and other extracellular NTPDases in vivo in mice and in patients during systemic inflammation and sepsis will be an important subject for further studies.

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Data availability Transcriptome data will be deposited in NCBI's Gene Expression Omnibus23 (GEO) upon acceptance.

Declarations

Conflict of interest G.H. owns stock in Purine Pharmaceuticals, Inc., and has a patent to develop NTPDases for the treatment of sepsis. The other authors have no financial conflict of interest.

Ethical approval All animal experiments were approved by the Columbia University Institutional Animal Care and Use Committee. The human study protocol for blood collection from patients and healthy controls was approved by the Hospital Nossa Senhora da Conceição, Porto Alegre, Rio Grande do Sul, Brazil Research Ethics Committee (registration numbers 49959315.5.0000.5530 and 49959315.5.3001.5345, Brazilian National Ethical Committee-CONEP).

Informed consent Written informed consent was obtained from all the participants.

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