CD39 limits P2X7 receptor inflammatory signaling and attenuates sepsis-induced liver injury

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Table of contents

Supplementary material and methods

Antibodies and Reagents.

MyD88 (D80F5) (#4283), Phospho-NF-κB p65 (Ser536) (93H1) (#3033), and Phospho-Stat3 (Tyr705) (D3A7) XP® (#9145) from Cell Signaling Technology (Danvers, MA); HRP-conjugated goat anti-mouse and donkey anti-rabbit IgG and the SuperSignal West Femto Maximum Sensitivity Substrate reagents (#PI-34096) were from Thermo Scientific (Rockford, IL). ATP, ADP, AMP, oxidized-ATP, ATP-γS, 2'(3')-O-(4-Benzoylbenzoyl)adenosine-5'-triphosphate tri(triethylammonium) salt (BzATP), Benzyl Alcohol, Brilliant Blue G, phorbol 12-myristate 13-acetate, and Imipramine were from Sigma-Aldrich, MO, USA.

Sepsis induction by cecal ligation and puncture (CLP)

Sepsis was induced by cecal ligation and puncture (CLP) as previously described [1]. Briefly, mice anesthetized by intraperitoneal injection of 80 mg/kg ketamine and 5 mg/kg xylazine, were subjected to laparotomy followed by extracorporeal cecum mobilization and ligation. After double enterotomy with a 21-G needle, a small amount of stool was gently squeezed out, to induce polymicrobial peritonitis, and the peritoneal cavity and abdominal wall were closed by suture. Control animals were subjected to a 'sham' operation, consisting of laparotomy and intraperitoneal replacement, without ligation and puncture. After surgery, mice were injected subcutaneously with 1 mL of 0.9% isotonic NaCl solution to compensate for third-spacing that occurred during the

procedure. Peritoneal fluid and blood samples were collected 3 h and 24 h after surgery.

Animals were periodically assessed and submitted to euthanasia at the first sign of pain or distress such as, lethargy, inability to move, dehydration, shivering and ruffled fur.

For pharmacological inhibition of P2X7 receptor *in vivo*, mice were injected intraperitoneally with 45.5 mg/kg of the P2X7 receptor antagonist Brilliant Blue G or with vehicle control (PBS), 24 h before sepsis induction, as previously described [2]. Some mice were injected with Imipramine 20 mg/kg 1 hour before surgery. For adenosine A_{2A} receptor activation mice were treated with ATL146e (a specific A2A agonist; kind gift of Dr. Joel Linden, La Jolla Institute for Allergy and Immunology, La Jolla, CA) at 1 µg/kg or with vehicle control (PBS) immediately after the surgery as described by Lappas et al. [3].

Peritoneal lavage fluid and blood collection

Peritoneal fluid and blood samples were collected 3 h or 24 h after surgery. Mice were anesthetized and blood samples were obtained from the inferior vena cava using tubes for blood collection with sodium citrate (5 %). The blood samples were centrifuged at 2000 x g for 10 min at 4 °C, and the recovered plasma was stored at -20°C until further use. To collect peritoneal cells, the peritoneal cavity was washed with 5 ml of cold sterile PBS. The peritoneal lavage fluid was collected and centrifuged at 350 x g for 5 min, at 4 °C. The supernatant was stored at -20°C until further analysis and the pellet was

resuspended in 1 mL of PBS or in 1 mL of reaction medium (described below) for the enzymatic assays.

Peritoneal macrophages

Murine macrophages were harvested from the peritoneal cavity of adult WT, $P2X7^{-/-}$ or $CD39^{-/-}$ mice. Peritoneal cavity cell suspensions were plated in 24- or 96-well tissue culture plates (TPP AG, Switzerland) at a density of 2 x 10^5 cells per well, and incubated in non-supplemented Gibco® DMEM for 1 h, at 37 °C, and in a 5% CO_2 atmosphere. Non-adherent cells were removed by washing with PBS, and adherent cells were cultured overnight in Gibco® DMEM complete medium before being used in subsequent experiments.

THP-1 derived human macrophages

THP-1 cells (ATCC® TIB-202™) were cultured in RPMI-1640 Medium (#R0883, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), antibiotics (100 IU penicillin/ ml and 100 mg streptomycin/mL - Gibco®). Cells were tested for mycoplasma contamination using the MycoSEQ™ Mycoplasma Detection Kit, with Discriminatory Positive Control (#4460623, Sigma-Aldrich, MO, USA) every 10 passages. THP-1 cells were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA; #P8139) for 48 h to differentiate into human macrophages.

Isolation, characterization and differentiation of bone marrow derived macrophages

The medullar cells were obtained from the tibia and femur of mice donors. The cell suspension obtained in non-supplemented DMEM Gibco® was passed through a filter for cell culture (Cell Strainer- 40 µm) and centrifuged at 300 x g for 10 min at room temperature. The pellets were resuspended in red blood cells lysis buffer (#A10492-01, Thermo Fisher Scientific, USA). Subsequently, cells were centrifuged at 300 x g for 10 min and washed twice with PBS by centrifugation. Mononuclear bone marrow cells were then plated at a density of 1×10^8 cells in polystyrene flasks and cultured in DMEM Gibco® supplemented with 20% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), antibiotics (100 IU penicillin/ ml and 100 mg streptomycin/mL - Gibco®) and 10 ng/mL macrophage colony-stimulating factor (M-CSF) (#315-02, Perprotech, NJ, USA). The cells were maintained at 37 ° C in an atmosphere of 5% CO_2; after 4 days, the culture medium was replaced and non-adherent cells were removed and discarded. After reaching 80% confluence, cells were removed from the polystyrene plates by the addition of 0.25% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA) and plated again for subsequent experiments. BMDM phenotype was determined by flow cytometry using the following antibodies: APC labeled anti-CD11b APC (# 101226, BioLegend, San Diego, CA, USA) at 1:100 dilution and FITC labeled anti-F4/80 (#MCA497FT, AbDSerotec®, Bio-Rad, Hercules, CA, USA) used at 1:100 dilution. Isotypes were used as negative control.

LPS priming and ATP treatment

For *in vitro* experiments, murine macrophages or THP-1(ATCC® TIB202™) derived macrophages were left untreated or primed with 1 µg/mL LPS for 4 h and then stimulated with P2X7 agonists for 3 h (500 µM ATP, or 100 µM BzATP or 100 µM ATPγs). In some wells, cells were pretreated with P2X7 receptor antagonists (300 µM oATP for 2 h or 100 nM A740003 for 30 min) or with imipramine (a sphingomyelinase inhibitor; 30µM for 30 min) before priming with LPS [4].

Ectonucleotidase activity assays

Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase; CD39) and ecto-5'-nucleotidase (CD73) activities were measured using a reaction medium consisting of 20 mM Hepes buffer (pH 7.5) containing 1 mM $CaCl_2$ (for ATP) or $MgCl_2$ (for AMP), 120 mM NaCl, 5 mM KCl, 60 mM glucose, 1 mM sodium azide and 0.1% mM albumin. Approximately, 10^5 peritoneal cells or 2 x 10^5 peritoneal resident macrophages were resuspended in a final volume of 200 µl, and enzymatic reactions were started by the addition of ATP, ADP or AMP to a final concentration of 2 mM, followed by incubation for 30 minutes at 37 ºC. Reactions were stopped by the addition of 200 µl 10% trichloroacetic acid (TCA) (Sigma-Aldrich, MO, USA). Incubation times, protein concentrations, reaction mixtures, and substrate concentrations were chosen based on a previously published protocol [5]. The amount of inorganic phosphate (Pi) released was measured using the colorimetric method described by Chan et al. [6]. Controls

to correct for non-enzymatic Pi in the samples were performed adding cells after reactions had been stopped with TCA. All reactions were performed in triplicates, and enzyme activities were expressed in nmol Pi released/minute per number of cells.

Flow Cytometry Analysis

Viable peritoneal cavity cells obtained 3 h after surgery were counted using trypan blue, and 3 x 10^5 viable cells were used per sample. Non-specific binding were blocked with 10% normal horse serum and 1% BSA in PBS for 30 min at 4°C, and then stained with 1µg/mL of anti-CD11b PE, anti-Ly-6G Alexa Fluor® 488, and anti-CD39 Alexa Fluor® 647 for a minimum of 30 min at 4°C (eBioscience, CA, USA);10.000 events were acquired per sample. Samples were analyzed by flow cytometry in a FACS Calibur (BD Biosciences, CA, USA). Results were analyzed using the Win-MDI (Multiple Document Interface Flow Cytometry Application, V2.8; Scripps Research Institute, La Jolla, CA., USA) program.

Nitric Oxide production

Nitric oxide (NO) production was evaluated by measuring the concentration of nitrite - a stable NO product - in peritoneal lavage fluid samples using the Griess reaction colorimetric assay. Briefly, 100 µL of the peritoneal fluid was reacted with 100 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrocloride and 2.5% H_3PO_4) for 10 min at room temperature, and the

absorbance was measured at 570 nm in a spectrophotometer. Nitrite concentrations were calculated using a standard curve of sodium nitrite. Results were expressed as the concentration of nitrite in µM. All experiments were performed in triplicates.

Cytokine determination

The concentrations of IL-6, IL-1β, TNF-α, and IL-10 were measured using commercially available ELISA kits, as recommended by the manufacturer (R&D Systems, Minneapolis, MN, USA).

Western Blotting

Samples were lysed in ice-cold modified-RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl) supplemented with Complete Proteinase Inhibitor Cocktails (Roche Diagnostics) and Phosphatase Inhibitor Cocktails (Sigma-Aldrich). Lysates were sonicated briefly on ice and centrifuged at 14,000 rpm for 10 minutes at 4^{o}C. Protein concentrations were determined by Bio-Rad DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as the standard. Proteins (10 µg per lane) were boiled in XT Sample Buffer (Cat#161-0791, Bio-Rad Laboratories), separated on 4-12% Criterion XT Bis-Tris SDS-PAGE gels (Bio-Rad Laboratories) and transferred onto PVDF membrane (Cat#IPVH00010, Millipore) by semi-dry electroblotting. The protein samples were then probed with specific antibodies. Bands were visualized using HRP-conjugated goat

anti-mouse, donkey anti-rabbit, or donkey anti-sheep IgG and the Super Signal West Femto Maximum Sensitivity Substrate reagents (Cat#PI-34096, Thermo Scientific, Rockford, IL) [7].

RT–qPCR

Expression of CD39 in BMDM was determined by RT-qPCR. Total RNA was extracted and purified from cells using an RNeasy Kit (Qiagen) according to the manufacturer's instructions, and the concentration was measured on Nanodrop ND 1000 spectrophotometer (Wilmington, DE, USA). Reverse transcription was conducted on 500 ng of total RNA using iScript cDNA Synthesis Kit (BioRad). The following primers were used: for CD39, 5'-GCCAAGATCATCACTGGACA-3' (forward) and 5'-TTTCTGCCAGAGAGCCTGAT-3' (reverse); and for *Actb*, 5'-TATGCCAACACAGTGCTGTCTGG-3' (forward) and 5'-TACTCCTGCTTGCTGATCCACAT-3' (reverse); *Agr1* 5'-GAAAGTTCCCAGATGTACCAG-3' (forward); 5'-CCAGGGTCTACGTCTCGC-3' (reverse); *Nos2* 5'-ACATCGACCCGTCCACAGTAT-3' (forward); 5'-AGAGGGGTAGGCTTGTCTC-3' (reverse). The RT-qPCR analysis was performed using Quanti Fast SYBR® Green PCR Kit (cat. no 204054; Quiagen) on a Stratagene Fast Real Time Machine (Mx3005P) (Agilent Technologies, Santa Clara, CA). A comparative CT (threshold cycle) was used to determine relative gene expression and analyzed against the endogenous genes of murine β-actin as an internal control.

Liver Histology

Liver biopsies were obtained from WT and CD39$^{-/-}$ septic or sham-operated mice and fixed in 10% buffered formalin, processed and embedded in paraffin. Five-micron sections were cut, stained with hematoxylin-eosin (H&E) and viewed with a Nikon Eclipse E600 microscope [7]. Liver necrosis score was performed as previously described [8] using a semiquantitative grading scale of 0–4: 0, no liver necrosis; 1, single cell necrosis; 2, up to 30% necrosis; 3, up to 31-60% necrosis; and 4, more than 60% necrosis.

Apoptosis

For detection of apoptotic cells in liver tissue 24 hours after sepsis induction, histological sections were deparaffinized and hydrated. The kit ApopTag® Peroxidase In Situ Apoptosis Detection, which detects apoptotic cells by TUNEL method (Terminal deoxynucleotidyl transferase dUTP nick end labeling), was used according to the manufacturer's instructions (# S7100, Millipore, Darmstadt, Germany). Finally, slides were stained with hematoxylin and analyzed in a light microscope and 400 NIKON (Japan).

Alanine aminotransferase (ALT) determination

Plasma ALT levels were measured using a Catalyst Dx Chemistry Analyzer (Idexx Laboratories) according to the manufacturer’s instructions.

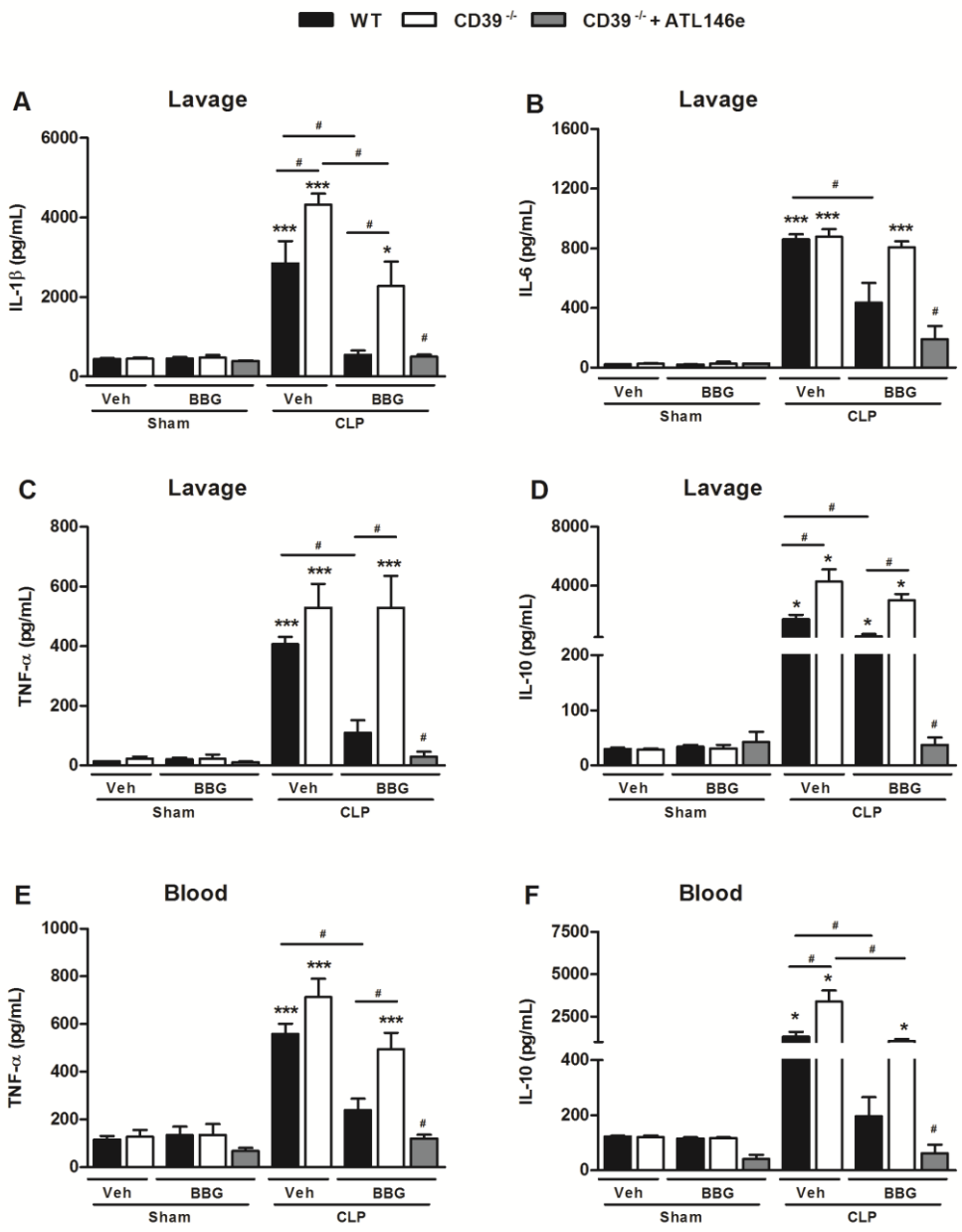

Fig. S1. P2X7 receptor pharmacological inhibition and the adenosine A2A receptor activation inhibit the cytokine production in peritoneal lavage fluid and blood from WT or CD39$^{-/-}$ septic mice. The peritoneal lavage fluid (PLF) and blood were obtained 24 h after sepsis induction and the (A) IL-1β, (B) IL-6, (C) TNF-α and, (D) IL-10 in the PLF and (E) TNF-α and (F) IL-10 in the blood were measured by ELISA. Data are expressed as mean ± S.E.M. of three

independent experiments (n=6). Statistically significant differences between Sham and CLP, and between CLP groups (CLP vehicle vs. CLP BBG, CLP vehicle vs. CLP BBG+ATL146e) are indicated by asterisks (*, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$) and by number sign (#, $p < 0.05$), respectively.

Supplementary references

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