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EVALUATION OF COMPONENTS OF THE EXTRACELLULAR PURINERGIC SIGNALING SYSTEM IN HUMAN SEPSIS

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ABSTRACT—**Objective:** Extracellular purines such as adenosine triphosphate (ATP), uridine triphosphate (UTP), and uridine diphosphate (UDP) and the ATP degradation product adenosine are biologically active signaling molecules, which accumulate at sites of metabolic stress in sepsis. They have potent immunomodulatory effects by binding to and activating P1 or adenosine and P2 receptors on the surface of leukocytes. Here we assessed the levels of extracellular purines, their receptors, metabolic enzymes, and cellular transporters in leukocytes of septic patients. **Methods:** Peripheral blood mononuclear cells (PBMCs), neutrophils, and plasma were isolated from blood obtained from septic patients and healthy control subjects. Ribonucleic acid was isolated from cells, and mRNA levels for purinergic receptors, enzymes, and transporters were measured. Adenosine triphosphate, UTP, UDP, and adenosine levels were evaluated in plasma. **Results:** Adenosine triphosphate levels were lower in septic patients than in healthy individuals, and levels of the other purines were comparable between the two groups. Levels of P1 and P2 receptors did not differ between the two patient groups. mRNA levels of ectonucleoside triphosphate diphosphohydrolase (NTPDase) 1 or CD39 increased, whereas those of NTPDase2, 3, and 8 decreased in PBMCs of septic patients when compared with healthy controls. CD73 mRNA was lower in PBMCs of septic than in healthy individuals. Equilibrative nucleoside transporter (ENT) 1 mRNA concentrations were higher and ENT2, 3, and 4 mRNA concentrations were lower in PBMCs of septic subjects when compared with healthy subjects. Concentrative nucleoside transporter (CNT) 1 mRNA levels were higher in PBMCs of septic versus healthy subjects, whereas the mRNA levels of CNT2, 3, and 4 did not differ. We failed to detect differences in mRNA levels of purinergic receptors, enzymes, and transporters in neutrophils of septic versus healthy subjects. **Conclusion:** Because CD39 degrades ATP to adenosine monophosphate (AMP), the lower ATP levels in septic individuals may be the result of increased CD39 expression. This increased degradation of ATP did not lead to increased adenosine levels, which may be explained by the decreased expression of CD73, which converts AMP to adenosine. Altogether, our results demonstrate differential regulation of components of the purinergic system in PBMCs during human sepsis.

KEYWORDS—Injury; trauma; inflammation; immunosuppression

INTRODUCTION

"The third international consensus definitions for sepsis and septic shock" report defines sepsis as any life-threatening organ dysfunction caused by a dysregulated host response to infection (1). Although leukocytes are crucial in controlling infection, their function is dysregulated during sepsis. An increasing body of evidence demonstrates that the purinergic system, which is an endogenous immunoregulatory system, can regulate leukocyte function in sepsis (2–15).

The purinergic system consists of, among others, the molecules adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine, and their cognate cell surface receptors, as well as a cell surface enzymatic cascade, which degrades extracellular ATP to ADP, AMP, and finally adenosine (16–23). Adenosine triphosphate is a key member of the purinergic system, as it is released from the intracellular into the extracellular space during inflammation, infection, shock, and sepsis. Once released, ATP becomes an extracellular signaling molecule. Detection of released ATP or some pyrimidine molecules such as uridine triphosphate (UTP) and uridine diphosphate (UDP) by P2 purinergic receptors on the cell membrane of immune and inflammatory cells alerts the immune system to danger and launches and choreographs inflammation (24–26). As sustained signaling through P2 receptors, such as P2X4 and P2X7, can be harmful (27), endogenous pathways are in place to terminate P2 receptor activation. Cell surface ectonucleoside triphosphate diphosphohydrolases (NTPDases), enzymes that are members of the evolutionarily conserved apyrase family, are key factors that mitigate P2 receptor signaling by hydrolyzing extracellular ATP/UTP to ADP/UDP and eventually AMP/uridine monophosphate. NTPDase1, 2, 3, and 8 are enzymes anchored to the cellular membrane and face the extracellular compartment. They differ in their preference for ATP/UTP versus ADP/UDP as substrates and tissue expression (20). Adenosine monophosphate is further metabolized mainly by ecto-5′-nucleotidase (CD73) to adenosine, which is broadly anti-inflammatory via P1 purinergic receptors, which are also called adenosine receptors (28,29). Four adenosine receptors have been cloned and characterized: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R (30). Thus, cell surface ectoenzymes serve as molecular switches, which stop pro-inflammatory P2 receptor activation and trigger anti-inflammatory P1 or adenosine receptor

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signaling at the site of injury and inflammation where ATP is released. As leukocytes are endowed with a variety of P1 and P2 receptors and cell surface ectonucleotidases, alterations and activation of these proteins can dictate the nature of the immune response during sepsis (2–15).

Most cell types in the body are endowed with nucleoside transporters, which can transport adenosine across the cell membrane from the intra- to the extracellular space and vice versa. Thus, they can contribute to both the initiation and termination of the adenosine receptor signaling (30,31). However, when extracellular adenosine levels are high, nucleoside transporters remove this extracellular adenosine by transporting it into the cell. Based on their molecular and functional characteristics, nucleoside transporters are classified into the following: (i) equilibrative nucleoside transporters (ENTs; ENT1, ENT2, ENT3, and ENT4), which carry nucleosides across cell membranes along their concentration gradients (32,33) and (ii) concentrative nucleoside transporters (CNTs; CNT1, CNT2, and CNT3), which mediate the cellular uptake of nucleosides against their concentration gradient (33).

One caveat for the results is that the control group was significantly younger (42.8 ± 12.5 compared with 63.1 ± 15.2 years, $P < 0.05$). However, this is common in studies such as ours. It is usually not feasible to recruit age-matched healthy volunteers because most people in their 60s to 70s will have some health conditions that will preclude them from participating as healthy volunteers. For example, 63.1% of the US population over 60 years old will have hypertension (https://www.cdc.gov/nchs/data/databriefs/db289.pdf), and other morbidities that may affect the purinergic system are frequent as well. Although it is not impossible that there is an age-related effect on the extracellular purinergic signaling system, this has not been described in humans.

Several murine studies conducted by our group and others have implicated regulatory roles for the various elements of the purinergic system in sepsis. In addition, although previous studies have begun to study the expression of the various components of the purinergic system on leukocytes of septic patients, no comprehensive analysis has been carried out. In this study, we have interrogated the concentrations of extracellular purines and pyrimidines and expression of purinergic receptors, enzymes, and transporters in peripheral blood mononuclear cells (PBMCs) and neutrophils of septic patients.

PATIENTS AND METHODS

Ethics statement

The studies involving human participants were reviewed and approved by the Columbia University Institutional Review Board (protocol # AAAS0172) and conducted under the Declaration of Helsinki principles. Suitable patients with sepsis admitted to the Surgical (SICU) or Cardiothoracic (CTICU) Intensive Care Units of Columbia University Irving Medical Center were identified by the study team, and informed consent was obtained from the patient or their surrogates. We also obtained information from healthy control subjects before blood draws.

Patient selection criteria

Selection criteria for septic patients were set up in accordance with "The third international consensus definitions for sepsis and septic shock (sepsis-3)" (1). Sepsis was defined as an increase in Sequential Organ Failure Assessment (SOFA) score by 2 or more points (1). The composition of the SOFA score, with adaptations by Vincent et al. (doi:10.1007/BF01709751) is listed in Supplemental Table 1, http://links.lww.com/SHK/B774.

Isolation of primary neutrophils

Blood was collected from healthy volunteers and critically ill patients with sepsis into BD Vacutainer blood collection tubes coated with K_2ethylene diamine tetraacetic acid (EDTA) (BD Biosciences, San Jose, CA). Samples were kept on ice during handling and processed immediately. MACSxpress Whole Blood Neutrophil Isolation Kit (Miltenyi Biotec, Waltham, MA) was used for the isolation of neutrophil granulocytes according to the manufacturer's protocol. Briefly, blood samples were diluted with phosphate buffered saline (PBS) to the desired volume and incubated with magnetic beads for 5 min at room temperature (RT) using a tube rotator to label nontarget cells. Subsequently, erythrocytes were aggregated and sedimented, whereas nontarget cells were removed by immunomagnetic depletion. Residual erythrocytes were lysed for an additional 2 min at RT using Hybri-Max Red Blood Cell Lysing Buffer (Millipore-Sigma, St. Louis, MO), and neutrophils were washed with PBS and pelleted at 475*g* for 5 min.

For staining of cell surface markers, cells were labeled with various antibodies listed in Supplementary Table 2, http://links.lww.com/SHK/B775. Stainings were carried out in FACS buffer (1% bovine serum albumin-PBS with 2 mM EDTA) at 4°C for 30 min. Subsequently, cells were washed with FACS buffer and labeled with LIVE/DEAD fixable dead cell stain for 10 min at RT. After another washing step, the cells were fixed with 4% paraformaldehyde for 20 min at RT, washed one time, and resuspended in FACS buffer before analysis. All samples were analyzed using BD FACS Canto II flow cytometer (BD Biosciences) equipped with 405-nm, 488-nm, and 633-nm lasers. At least 40,000 events per sample were recorded using BD FACSDiva Software 9.0. Data from the flow cytometer were analyzed using Flowing Software 2 version 2.5.1 (Turku Bioscience Centre, Turku, Finland).

Supplementary Figure 1, http://links.lww.com/SHK/B776, demonstrates that our isolation resulted in a neutrophil purity of 99.3% when assessed based on CD15+/CD16+ double-positive cells.

Isolation of PBMCs

Blood was collected from healthy volunteers and critically ill patients with sepsis into BD Vacutainer blood collection tubes coated with K2EDTA (BD Biosciences). Samples were kept on ice during handling and processed immediately. Blood samples were diluted with equal amounts of PBS + 2% fetal bovine serum and poured into a SepMate tube (STEMCELL Technologies, Cambridge, MA) containing 5 mL of Lymphoprep (STEMCELL Technologies). After a centrifugation step (10 min at RT, 1200*g*, with breaks), plasma was collected from the top section and was placed at −80°C until further use. The PBMC ring was collected into a fresh tube; cells were washed with PBS and pelleted at 475*g* for 5 min. Residual erythrocytes were lysed for an additional 5 min at RT using Hybri-Max Red Blood Cell Lysing Buffer (Millipore). Peripheral blood mononuclear cells were washed for a final time with PBS and pelleted at 475*g* for 5 min.

RNA isolation and real-time polymerase chain reaction

Freshly isolated neutrophils or PBMCs were immediately placed in an ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA) and stored at −80°C until RNA isolation. Total RNA was extracted according to the manufacturer's directions, and then quantified by Nanodrop (Thermo Fisher Scientific, Wilmington, DE) and assessed for quality by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA). Ribonucleic acid was then reverse transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Real-time PCR was performed on an Applied-Biosystem QuantStudio 3 (Thermo Fisher Scientific) using primers specific for targets listed in Table 1. Results were normalized to 18S mRNA content.

Adenosine detection

Plasma adenosine levels of healthy controls and septic patients were determined by Fluorometric Adenosine Assay Kit (Abcam, Waltham, MA) in accordance with the manufacturer's recommendations. Briefly, plasma samples were thawed on ice then diluted with an assay buffer and mixed with reaction mix, containing adenosine deaminase. The product of this reaction interacted with an adenosine probe, creating a fluorescent product. Fluorescence was detected at Ex/Em = 535/587 nm by a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA). Results were calculated based on a standard curve; an assay buffer was used as background control.

ATP detection

Plasma ATP levels of healthy controls and septic patients were determined by ATPlite Luminescence Assay System (Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. Briefly, plasma samples were thawed on ice and pipetted into chilled black 96-well clear bottom microplates. A 50-μL reconstituted substrate was given to each sample, and the mixture was incubated for 5 min in an orbital shaker at 700 rpm. After incubation endpoint, luminescence was measured on a FlexStation 3 microplate reader (Molecular Devices). The substrate alone served as blank; results were calculated based on the ATP standard curve.

TABLE 1. **Human primers used for PCR reactions**

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
18S	CTACCACATCCAAGGAAGCA	TTTTTCGTCACTACCTCCCCG
Adora1	GCCACAGACCTACTTCCACA	CCTTCTCGAACTCGCACTTG
Adora2a	AACCTGCAGAACGTCACCAA	GTCACCAAGCCATTGTACCG
Adora2b	GGTCATTGCTGTCCTCTGG	TCCTCGAGTGGTCCATCAG
Adora3	ACCACTCAAAGAAGAATATG	ACTTAGCTGTCTTGAACTCC
P2X4	GAGATTCCAGATGCGACC	GACTTGAGGTAAGTAGTGG
P2X7	AAGCTGTACCAGCGGAAAGA	GCTCTTGGCCTTCTGTTTTG
ENTPD1	GCCAGCAGAAAAGGAGAATG	TGGGACCTTGGAATCACTTC
ENTPD2	TCAATCCAGCTCCTTGAACC	TCCCCAGTACAGACCCAGAC
ENTPD3	TTGACCTCAGGGCTCAGTTT	TGAGGGGGTTCACTGCTTAC
ENTPD8	ACTGGGCTACATGCTGAACC	GCACCATGAACACCACTTTG
ENT 1	CCTGGCTTTCTCTGTCTGCT	AGTAACGTTCCCAGGTGCTG
ENT 2	CCCTGGATCTTGACCTGGAG	GGTTTTCCTGGCTTCTGGG
ENT 3	AACAGGGTTGCAGTCCACAT	GATCACCATGCAGACAATGG
ENT 4	AACAACGTCCTGGTGGAGAG	CGTCGCAGATGCTGATAAAA
CNT 1	AGGTTCTGCCCATCATTGTC	CAAGTAGGGCCGGATCAGTA
CNT 2	AATGGGTGTTTGCAGGAGTC	GAAGACCTAGGCCCGAAAAC
CNT 3	GACTCACATCCATGGCTCCT	TTCCAGGGAAAGTGGAGTTG
CD64	ATACAGGTGCCAGAGAGGTCTC	CCAGCTTATCCTTCCACGCATG
CD73	ATTGCAAAGTGGTTCAAAGTCA	ACACTTGGCCAGTAAAATAGGG
CD95	GGACCCAGAATACCAAGTGCAG	GTTGCTGGTGAGTGTGCATTCC
CD163	CCAGAAGGAACTTGTAGCCACAG	CAGGCACCAAGCGTTTTGAGCT
CD182	TCCGTCACTGATGTCTACCTGC	TCCTTCAGGAGTGAGACCACCT
CXCR4	CAGCAGGTAGCAAAGTGACG	GTAGATGGTGGGCAGGAAGA
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG

UDP detection

Plasma UDP levels of healthy controls and septic patients were determined by MicroMolar UDP assay kit (Profoldin, Hudson, MA) according to the manufacturer's instructions. Briefly, plasma samples were thawed on ice and then mixed in black 96-well clear bottom microplates with freshly prepared assay buffer. Sample mixtures and standards were incubated for 45 min, and then appropriately diluted fluorescent dye was added. Fluorescence was detected within 5 min with a FlexStation 3 microplate reader (Molecular Devices) with emission at 535 nm and excitation at 485 nm. Results were calculated based on the UDP standard curve.

UTP detection

Plasma UTP levels of healthy controls and septic patients were determined by Uridine Triphosphate ELISA Kit (LSBio, Shirley, MA) in accordance with the manufacturer's recommendations. Briefly, plasma samples, blanks, and standards were added to a 96-well microplate. Detection reagent A was added to each well; the microplate was covered and incubated for 1 h at 37°C. After a wash step, detection reagent B working solution was added to each well, and the microplate was covered with a new plate sealer and incubated for 30 min at 37°C. After a wash step, tetramethylbenzidine substrate solution was added to each well, and the covered microplate was incubated for 10 to 20 min at 37°C in the dark. The color reaction was stopped with stop solution, and optical density was measured by a Spectramax 190 microplate reader (Molecular Devices) set to 450 nm. Results were calculated based on the UTP standard curve.

Cytokine assays

chemokine (C-X-C motif) ligand 8, monocyte chemoattractant protein-1, interleukin-1 beta, and tumor necrosis factor-alpha concentrations in plasma from healthy controls and septic patients were determined by ELISA Duosets (R&D systems, Minneapolis, MN). Plasma samples were kept at −80°C until measurement and defrosted on ice. Assays were performed according to the manufacturer's instructions. Plates were read at 450 nm with a reference measurement at 540 nm by a Spectramax 190 microplate reader (Molecular Devices.) Results were calculated based on respective standard curves.

Statistical analysis

Values in the figures and texts are expressed as mean ± standard error of means (SEM) of n observations. Statistical analysis comparing two groups was performed

TABLE 2. **Demographics of critically ill patients, mean ± SD or n (%)**

	All (N = 14)
Female sex	7 (50.0%)
Age, y	63.1 ± 15.2
Height, cm	170.6 ± 10.5
Weight, kg	81.3 ± 21.5
Body mass index, kg/m^2	25.9 ± 6.6
Race	
White	7 (50.0%)
Black/African American	5 (35.7%)
Other	1 (7.1%)
Ethnicity: Hispanic or Latino or Spanish origin	4 (28.6%)
Body mass index, kg/m^2	25.9 ± 6.6
Cardiac surgery	5 (35.7%)
Abdominal surgery	4 (28.6%)
Other surgery	3 (21.4%)
No surgery	2 (14.3%)
SOFA score (total)	10.9 ± 4.2
SOFA subscores	
Renal	2.57 ± 1.89
Respiratory	1.71 ± 1.54
Coagulation	1.62 ± 1.00
Liver	0.79 ± 0.89
Central nervous system	0 ± 0
Serum creatinine, mg/dL	1.25 ± 0.74
On continuous renal replacement therapy	8 (57.1%)
Mechanical ventilation	11 (78.6%)
pO_2 to F_iO^2 (*P*/*F*) ratio, mm Hg	291 ± 161
Platelet count (1,000/μL)	134 ± 174
Total bilirubin, mg/dL	1.64 ± 1.36
90-d mortality (%)	8 (57.1%)
Length of ICU stay—survivors only, d	27.0 ± 7.0

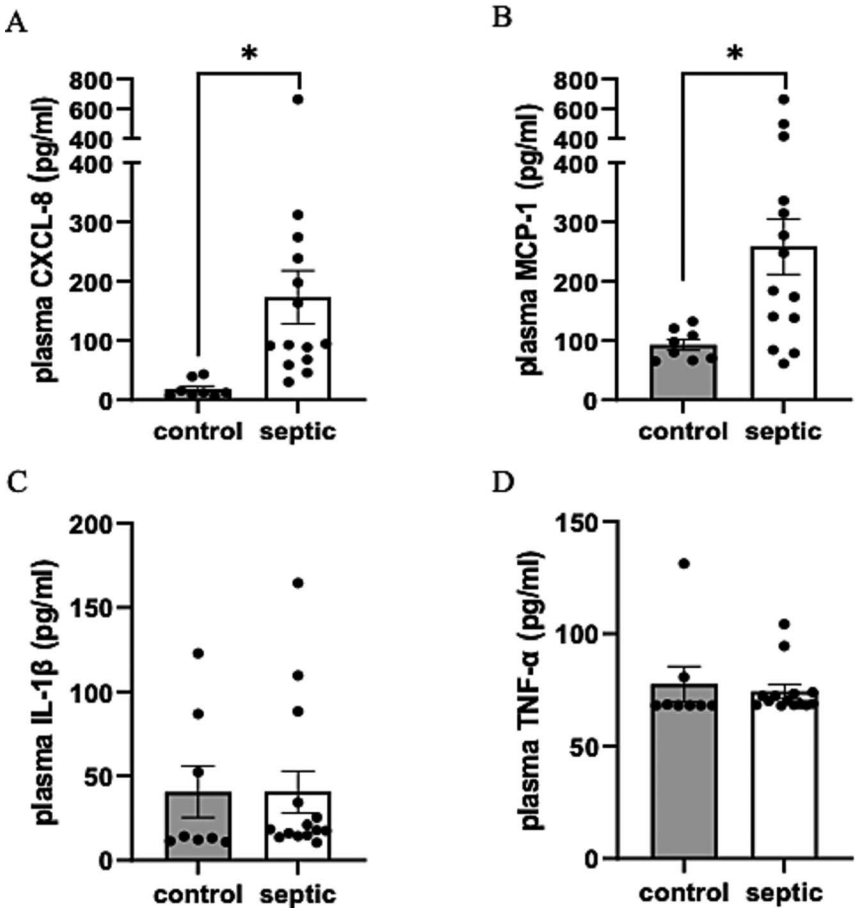

FIG. 1. **Cytokine levels in septic patients compared with healthy controls**. Plasma levels of CXCL-8, MCP-1, IL-1β, and TNF-α were measured using ELISA (A–D). Results are presented as mean ± SEM (n = 8 controls/14 septic patients; $^*P \leq 0.05$). CXCL-8, chemokine (C-X-C motif) ligand 8; MCP-1, Monocyte chemoattractant protein-1; IL-1b, Interleukin-1 beta; TNF-a, Tumor necrosis factor-alpha; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean.

by using two-tailed unpaired Student *t* test. GraphPad Prism 9.1.2 was used for data illustration. Results were considered statistically different when *P* value was ≤0.05.

RESULTS

Patients

Patients were selected from a patient population admitted to the SICU or CTICU of Milstein Hospital. Selection criteria were based on *Sepsis-3* (1), through which 14 patients were included in our studies with a SOFA score of 2 or more. Seven men and seven women with Hispanic or Latino, Black or African American, White, and other racial backgrounds were selected. The average age was 63.1 ± 15.2 years, and the average body mass index was 25.9 ± 6.6 kg/m^2. Twelve patients had undergone surgery before admission to the intensive care unit (five cardiac, four abdominal, and three other surgeries). Eleven of 14 subjects (78.6%) required mechanical ventilation, and 8 of 14 (57.1%) required continuous renal replacement therapy. The median SOFA score at the time of recruitment was 10.9 ± 4.2. The 90-day mortality was 57.1%. Demographics are listed in Table 2. We further recruited eight healthy controls and obtained blood after informed consent through venopuncture. Of the controls, 50% were women and the age was 42.8 ± 12.5 years.

Effect of sepsis on the host's inflammatory and immune response

We first investigated the host's inflammatory and immune response to sepsis. Plasma levels of the chemokines chemokine (C-X-C motif) ligand 8 and monocyte chemoattractant protein-1 were increased in septic patients (Fig. 1, A and B), whereas plasma interleukin-1 beta and TNF levels did not differ (Fig. 1, C and D). Although IL-6 mRNA decreased in septic PBMCs but not in neutrophils, mRNA concentrations of TNF were comparable in both PBMCs and neutrophils (Fig. 2, A–D). Peripheral blood mononuclear cells isolated from septic patients had higher CD64 and CD163 mRNA levels than PBMCs isolated from healthy individuals, whereas CD95, CD182, and CXCR4 mRNA levels were comparable in septic and nonseptic individuals (Fig. 3, A–E). CD182 mRNA expression was significantly lower in neutrophils isolated from septic patients, whereas no differences were seen in the levels of CD64, CD95, CD163, and CXCR4 mRNA from neutrophils between healthy and septic patients (Fig. 3, F–K). These results confirmed that septic patients presented with an exacerbated inflammatory and altered immune response in our study.

Sepsis differentially affects the concentrations of extracellular purines in blood

Concentrations of the purine nucleotide ATP were decreased in the plasma of septic patients (Fig. 4A), and although levels of the purine nucleoside adenosine trended upward in septic versus healthy patients (Fig. 4B), there were no statistically significant differences between the two cohorts. The plasma levels of UDP and UTP were comparable between septic and healthy individuals (Fig. 4, C and D).

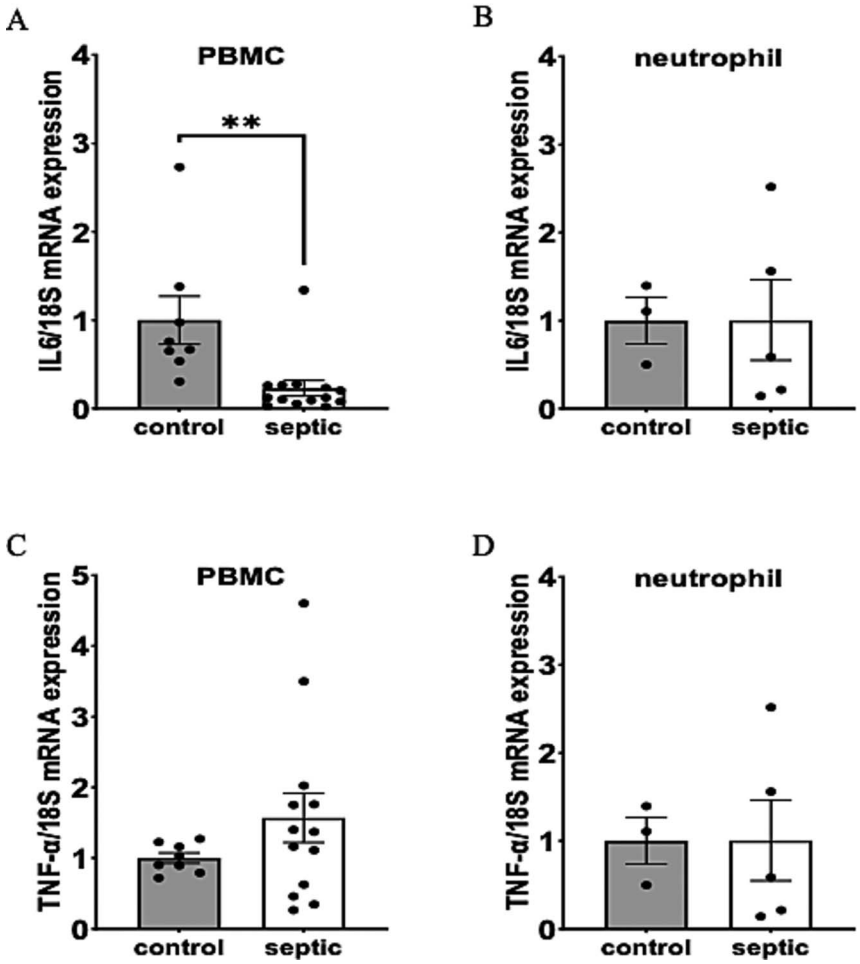

FIG. 2. **IL-6 and TNF-α mRNA levels in PBMCs and neutrophils of septic patients compared with healthy controls**. mRNA expression of IL-6 and TNF-α was measured in PBMCs and neutrophils from septic versus healthy donors (A–D). Results are presented as mean ± SEM (n = 8 controls/14 septic patients; $*P \leq 0.05$). Results were normalized for the expression of the internal control 18S and are presented as mean ± SEM ($**P \leq 0.01$). IL-6, Interleukin-6; TNF-a, Tumor necrosis factor-alpha; mRNA, messenger ribonucleic acid; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

Effect of sepsis on adenosine and P2X4 and P2X7 receptor expression

Given that several murine studies by our group and others have evaluated the role of adenosine receptors in regulating the host's response to sepsis (2,3,6,8,34), we first investigated the expression of adenosine receptors. Although we noted a trend toward increased $A_{2A}R$ expression in both PBMCs and neutrophils from septic versus healthy patients, no differences were observed in the expression of A_1Rs, $A_{2B}Rs$, and A_3Rs (Fig. 5). Given that our murine studies have indicated that both P2X4 (12) and P2X7 (13) receptors but not P2Y receptors (data not shown) have important regulatory roles in sepsis, we determined P2X4 and P2X7 receptor expression in human leukocytes. There were no differences in P2X4 and P2X7 receptor mRNA expression between healthy and septic human leukocytes (Fig. 6.)

Sepsis differentially affects ectonucleotidase and nucleoside transporter expression in PBMCs and neutrophils

Murine studies have also implicated ectonucleotidases in the orchestration of the host's inflammatory/immune response to sepsis (7,35). In human PBMCs, NTPDase1 expression was increased and NTPDase2, NTPDase3, NTPDase8, and CD73 expression was decreased in the septic when compared with the nonseptic group (Fig. 7, A–E). There were no differences in the expression of ectonucleotidases in neutrophils isolated from septic versus healthy patients (Fig. 7, F–K).

ENT1 expression was increased while the expression of ENT2, 3, and 4 was decreased in septic versus healthy PBMCs (Fig. 8, A–D), whereas the expression of all ENTs was comparable between healthy and septic neutrophils (Fig. 8, E–H). The expression of CNT1 and 2 in both neutrophils and PBMCs was similar between healthy and septic patients (Fig. 9, A, B, D, and E). CNT3 expression was significantly increased in septic versus healthy PBMCs, and although not significant, the same trend was observed in neutrophils (Fig. 9, C–F).

DISCUSSION

Here we undertook a comprehensive but not exhaustive evaluation of the purinergic signaling system in human sepsis. We initially determined the levels of some of the major extracellular purines and found that ATP levels were decreased in septic patients when compared with healthy individuals, whereas no differences were noted for UTP, UDP, and adenosine. This finding of decreased ATP levels was surprising, because in a previous study

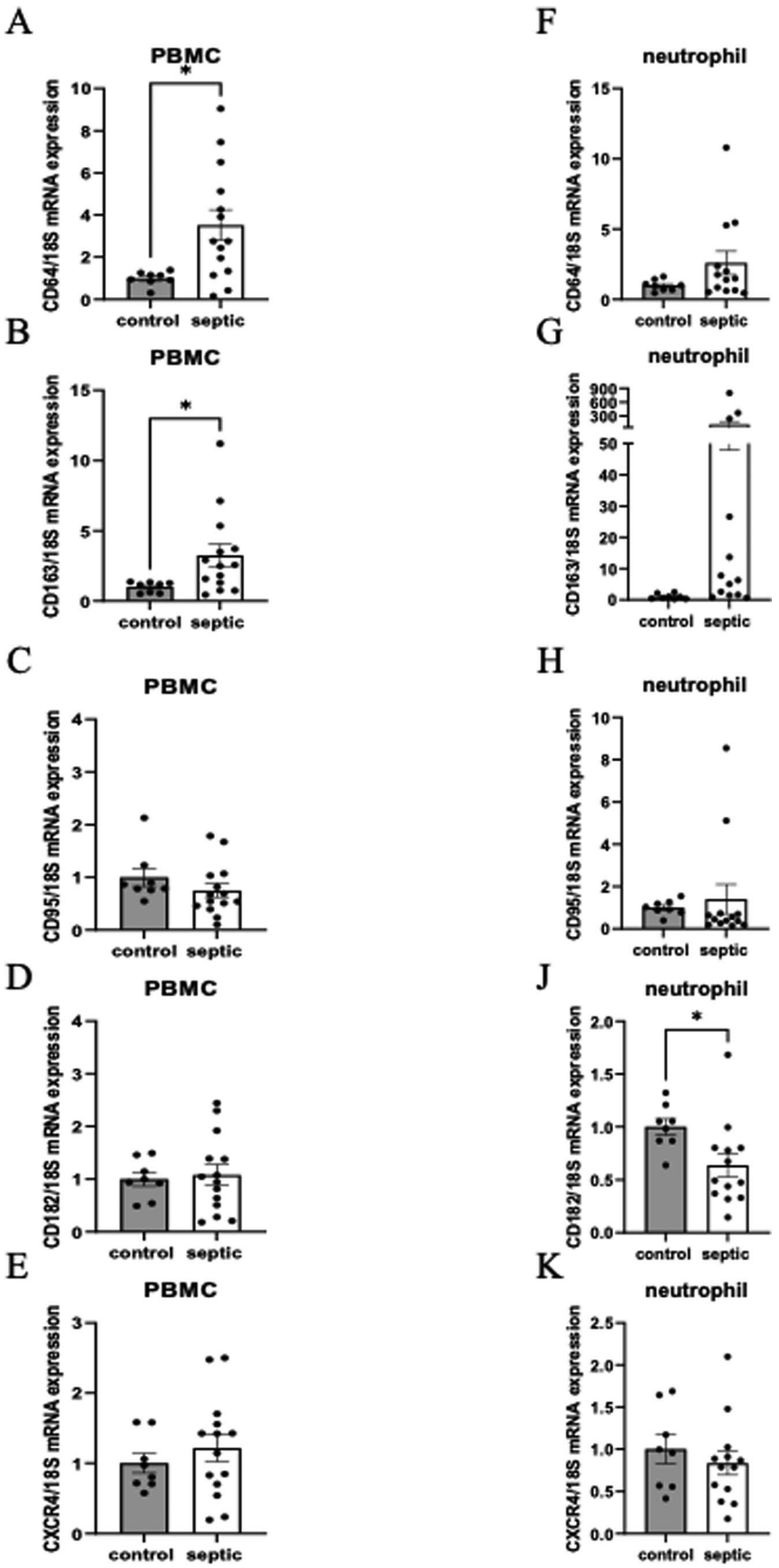

FIG. 3. **Cluster of differentiation mRNA expression in PBMCs and neutrophils of septic versus healthy donors.** CD64, CD163, CD95, CD182, and CXCR4 mRNA expressions were measured in PBMCs (A–E) and neutrophils (F-K) from septic versus healthy donors. Results were normalized for the expression of the internal control 18S and are presented as mean ± SEM (*$P \leq 0.05$). CD, cluster of differentiation; mRNA, messenger ribonucleic acid; CXCR4, C-X-C chemokine receptor type 4; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

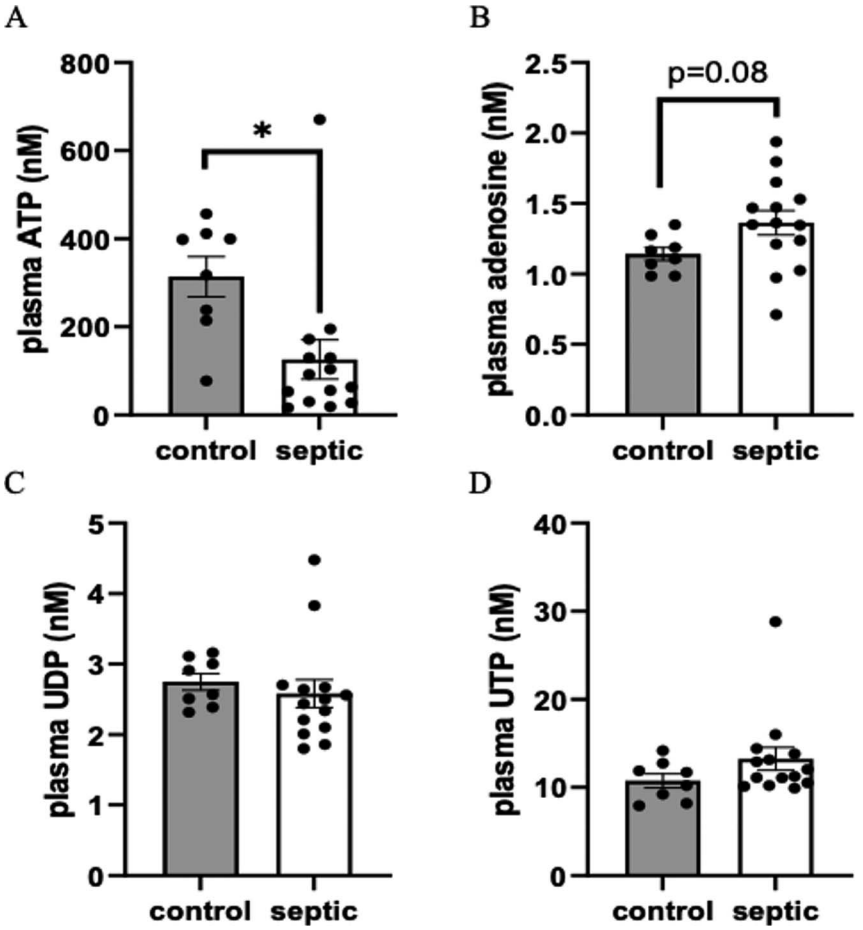

FIG. 4. **Comparison of plasma nucleotides in septic patients versus healthy donors.** Plasma ATP, adenosine, UDP, and UTP levels were measured by specific detection kits in samples from septic versus healthy donors (A–D). Results are presented as mean ± SEM (*$P \leq 0.05$). ATP, adenosine triphosphate; UDP, uridin diphosphate; UTP, uridin triphosphate; SEM: standard error of the mean.

conducted in a different medical center, we observed increased circulating ATP concentrations in septic patients (36). The current results also differed from our previous ones in that ATP levels in the current study were in the mid-nanomolar range, whereas in the previous one, they averaged 5 to 10 μM. Of note, the patients included in this study were very sick, with high SOFA scores representing severe multiorgan failure and an overall 90-day mortality of 50%. We focused our current studies on the P2X4 and P2X7 ATP receptors, because murine studies previously implicated both of these receptors in promoting macrophage bacterial killing during sepsis (10,12–14). However, P2X4 and P2X7 receptor expression was unchanged in both neutrophils and PBMCs. These results are in agreement with the previous study we conducted, where P2X7 receptors on neutrophils were comparable between septic patients and controls (36). However, in a study by another group, septic monocytes had increased P2X7 receptor expression (14). This discrepancy can be explained by the fact that the study by Martinez-Garcia et al. (14) evaluated cell surface P2X7 receptor expression in monocytes, whereas our studies analyzed P2X7 mRNA in neutrophils and PBMCs.

In contrast to disparate results with regard to ATP levels between our two studies (current and reference [36]), both of our studies find comparable adenosine levels in septic and control patients. This, however, is inconsistent with results from other studies, in which adenosine levels were higher in septic patients than in controls (37,38). Potential explanations for this discrepancy include differential patient cohorts, differential diagnostic criteria, and disparate management of patients. We also determined levels of the pyrimidine nucleotides UTP and UDP, which were no different in healthy and septic individuals. Although we did not investigate the expression of UTP/UDP receptors on leukocytes here, in a series of murine studies examining the role of P2Y1, P2Y4, P2Y6, and P2Y14 receptors, which detect extracellular UTP and UDP, we failed to find a regulatory role for these receptors in governing immunity during sepsis (data not shown). Nevertheless, further studies on the role of pyrimidinergic signaling in human sepsis may reveal unexpected immunoregulatory roles.

Our observation of increased NTPDase1 in PBMCs of septic patients can potentially explain the decreased ATP levels, because NTPDase1 or CD39 is the major ATP-degrading enzyme in immune cells. This observation of increased NTPDase1 is also confirmatory of our earlier data demonstrating increased serum ATPase and ADPase activities in septic compared with healthy patients (36), where the source of circulating ATPase and ADPase activities is likely NTPDase1 (39). In a recent study, NTPDase1 expression and ecto-ATPase activity were shown to be upregulated on B lymphocytes from septic patients, which was associated with increased adenosine levels in septic shock but not sepsis patients (7).

NTPDase2, 3, and 8 were downregulated in PBMCs of septic patients in our study, the significance of which is unclear at this

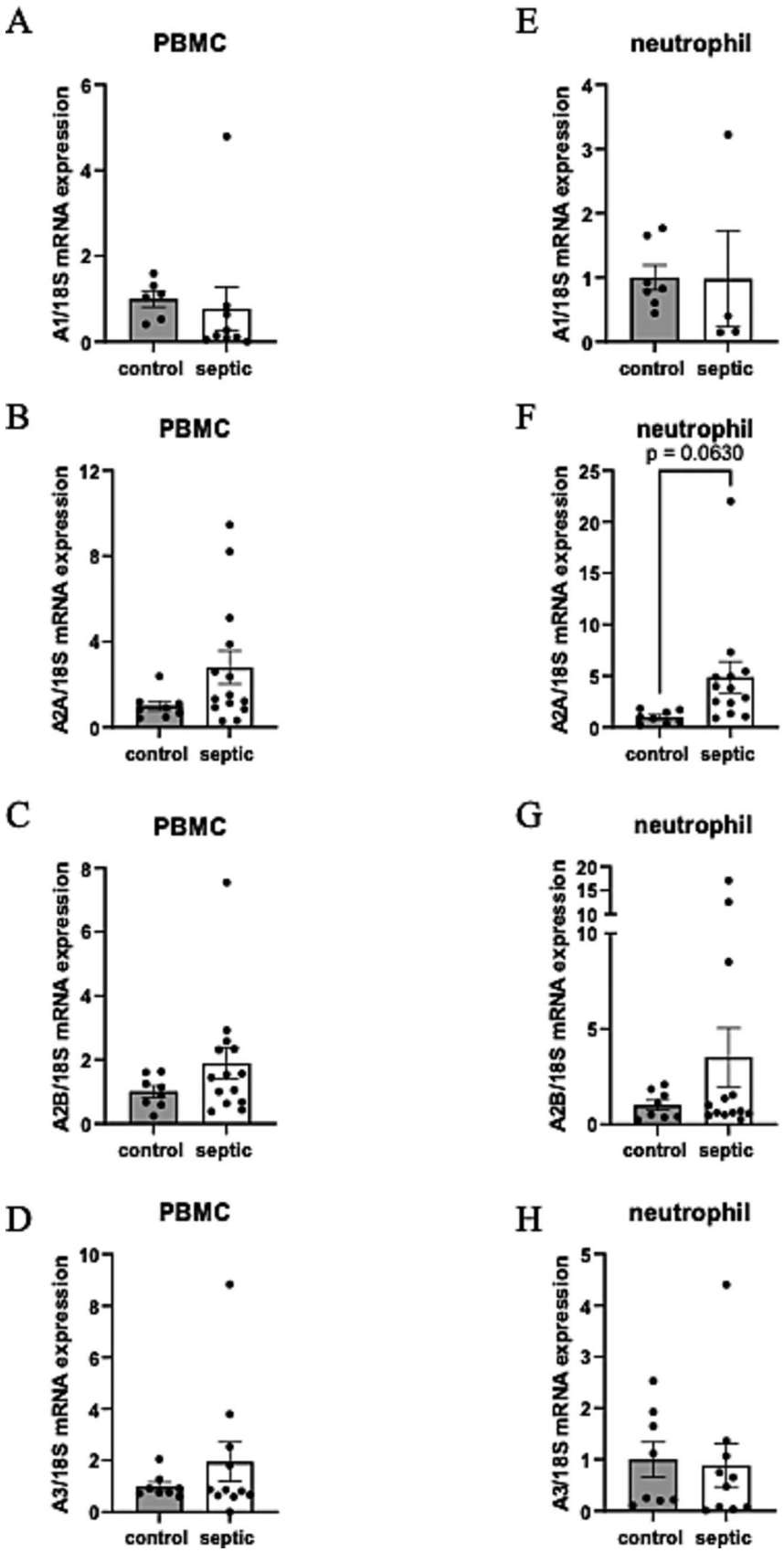

FIG. 5. **mRNA expression of adenosine receptors in leukocytes of septic patients versus healthy donors.** mRNA expression of A_1, A_{2A}, A_{2B}, and A_3 receptors was evaluated in PBMCs (A–D) and neutrophils (E–H) from septic versus healthy donors. Results were normalized for the expression of the internal control 18S and are presented as mean ± SEM. mRNA, messenger ribonucleic acid; A, adenosine; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

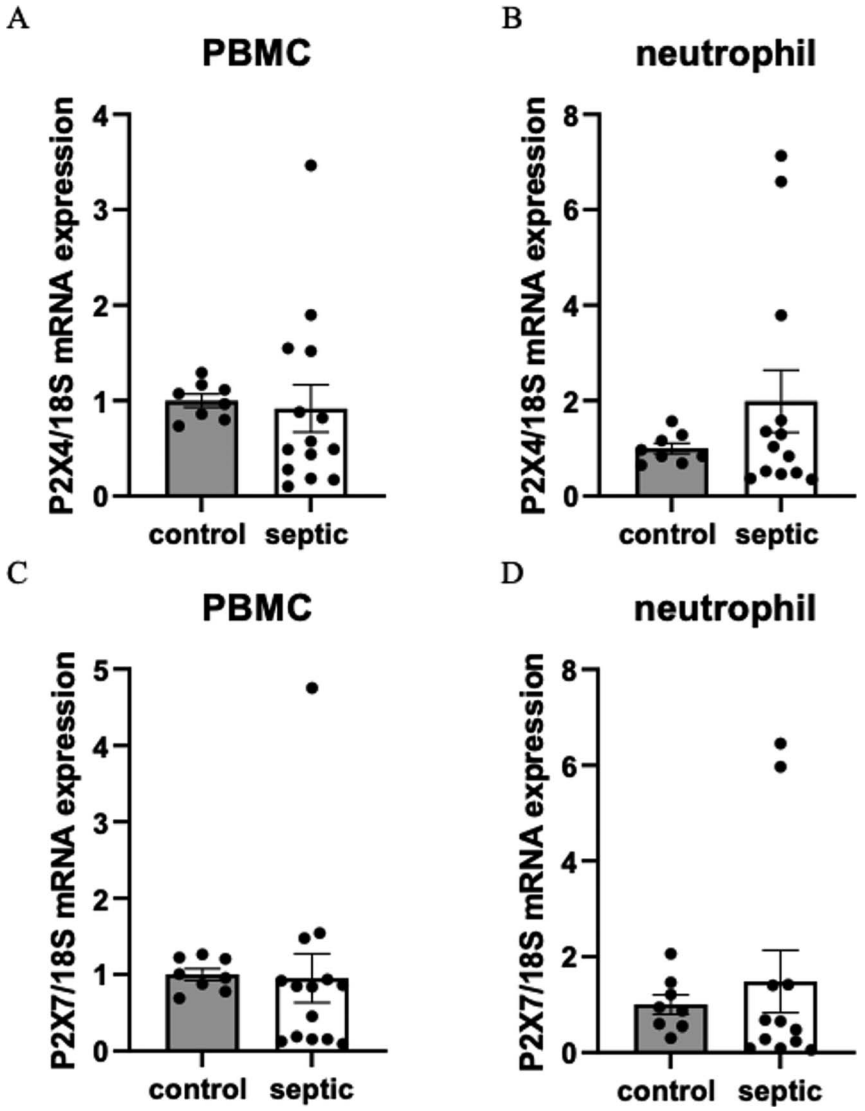

FIG. 6. **mRNA expression of P2X receptors in leukocytes of septic patients versus healthy donors.** mRNA expression of P2X4 and P2X7 was measured in PBMCs (A, C) and neutrophils (B, D) from septic and healthy donors. Results were normalized for the expression of the internal control 18S and are presented as mean ± SEM. mRNA, messenger ribonucleic acid; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

point, given that our understanding of the function of these enzymes is rudimentary. Interestingly, although in the current study we found no alterations in neutrophil NTPDase expression, in another of our previous studies, we demonstrated that NTPDase1 was upregulated and NTPDase2, 3, and 8 were downregulated in neutrophils from septic individuals (40). Thus, the patterns of changes in NTPDase expression in septic neutrophils in one study and PBMCs in the other were similar. Unlike NTPDase1 and 3 where ADP is not released from the enzymes during ATP or UTP catabolism to AMP or uridine monophosphate, respectively, NTPDase2 and NTPDase8 can release ADP and UDP transiently, which can produce, at least transient, proinflammatory and prothrombotic effects through P2 receptors on immune cells and platelets (41,42). Although NTPDase8 does not appear to have an immunoregulatory role based on our recent murine sepsis study (40), the role of NTPDase2 and 3 is unknown. Downstream from NTPDases CD73 catabolizes AMP to adenosine. CD73 expression decreased in PBMCs but not in neutrophils from septic patients. Because CD73 is protective in sepsis (43), this decrease in PBMC CD73 levels appears to be maladaptive.

Adenosine exerts its immunosuppressive effects mainly through A_{2A}Rs. In our previous study on septic patients, A_{2A}R expression was augmented on neutrophils. Similarly, Thiel et al. have reported increased A_{2A}R expression on neutrophils of septic patients (4,44), but despite this, A_{2A}R signaling is dysregulated in septic patients (4,38,44). Our current data demonstrate trends toward increased A_{2A}R expression in both neutrophils and PBMCs. Several rodent models have indicated A_{2A}R-mediated immunosuppression contributes to septic mortality (6,8,34). Thus, increased A_{2A}R expression also seems to be maladaptive, and blocking A_{2A}Rs is a viable strategy to treat septic patients with immunosuppression.

Intestinal epithelial cells express both ENT1 and ENT2, which are both downregulated during tissue inflammation and hypoxia via the actions of hypoxia-inducible factor-1α (45). This downregulation of ENTs increases adenosine levels in the gut, and

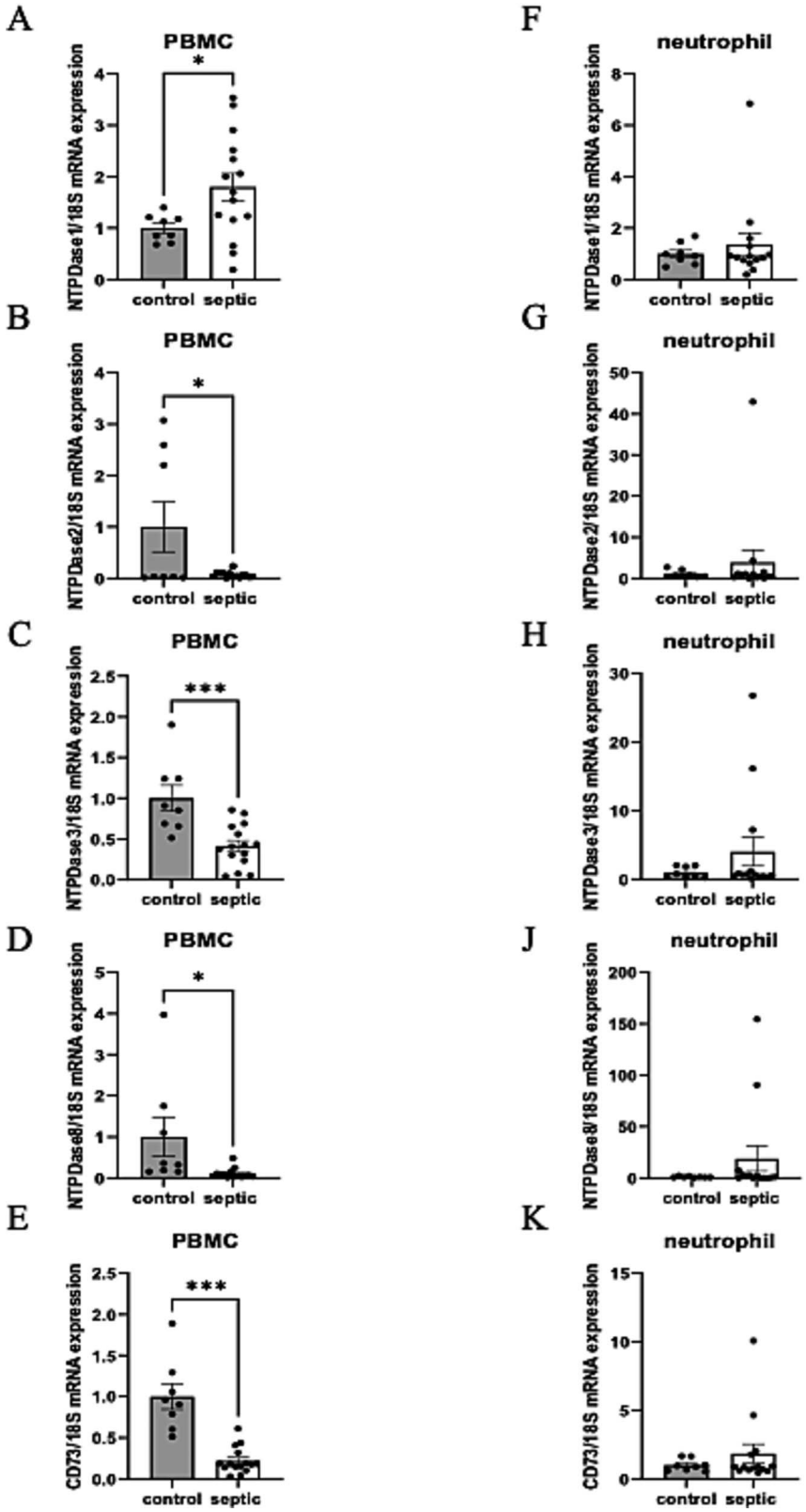

FIG. 7. **mRNA expression of NTPDases in leukocytes of septic patients versus healthy donors.** mRNA expression of NTPDase1, 2, 3, 8, and CD73 was measured in PBMCs (A–E) and neutrophils (F–K) from septic versus healthy donors. Results were normalized for the expression of the internal control 18S and are presented as mean ± SEM (*$P \leq 0.05$, ***$P \leq 0.001$). mRNA, messenger ribonucleic acid; NTPDase, nucleoside triphosphate diphosphohydrolase; CD, cluster of differentiation; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

the increased adenosine protects against colitis through A_{2B}Rs (46,47). The regulation and function of ENT and CNT expression in immune cells are less well understood (48,49). Our observation of downregulation of ENT2, 3, and 4 in septic PBMCs indicates that similar to intestinal epithelial cells, inflammation also downregulates expression of some ENTs in leukocytes. However, because

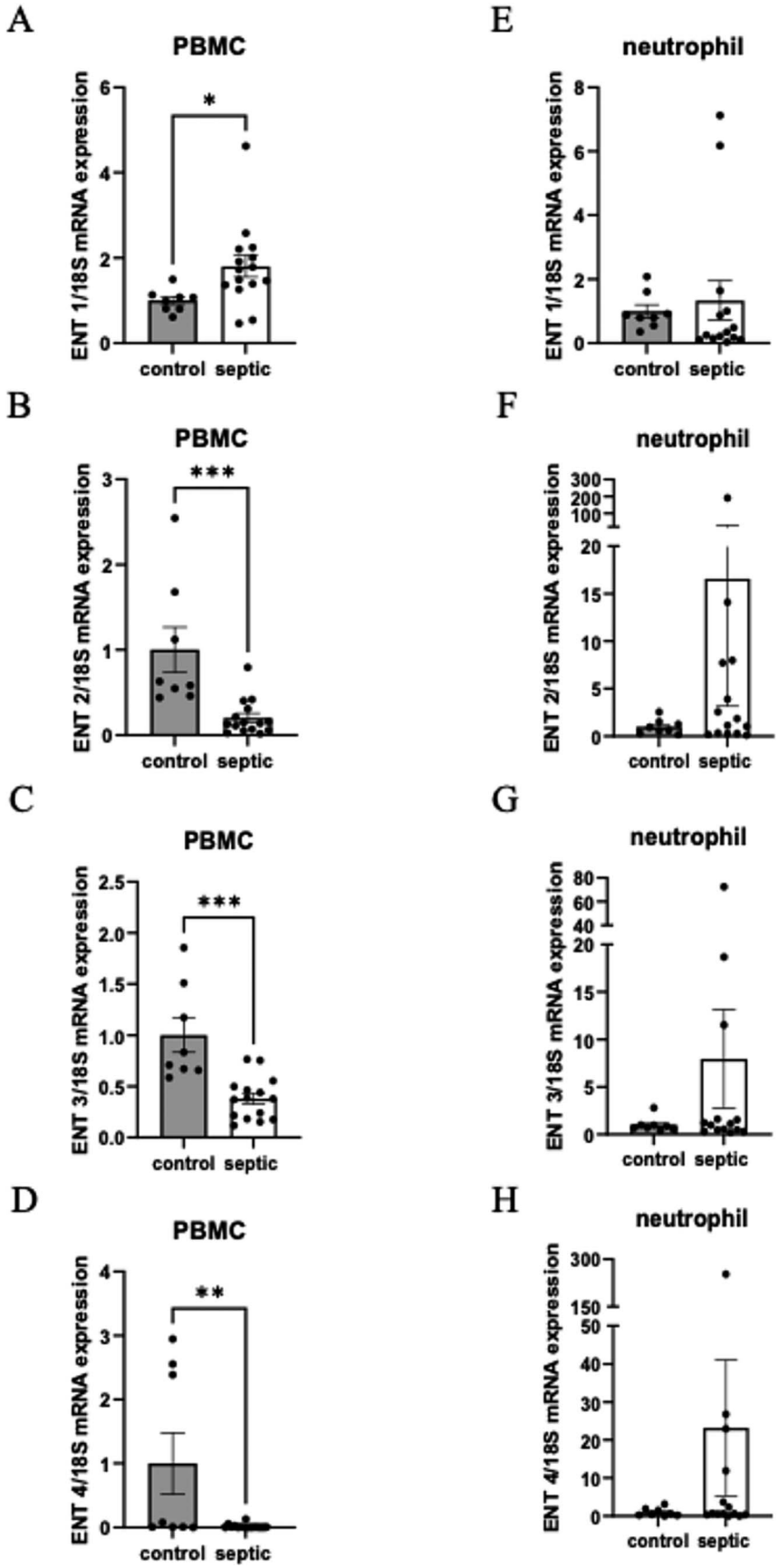

FIG. 8. **mRNA expression of ENTs in leukocytes of septic patients versus healthy donors.** mRNA expression of ENT1, 2, 3, and 4 was measured in PBMCs (A–D) and neutrophils (E–H) from septic versus healthy donors. Results were normalized for the expression of the internal control 18S and are presented as mean ± SEM (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). mRNA, messenger ribonucleic acid; ENT, Equilibrative nucleoside transporter; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

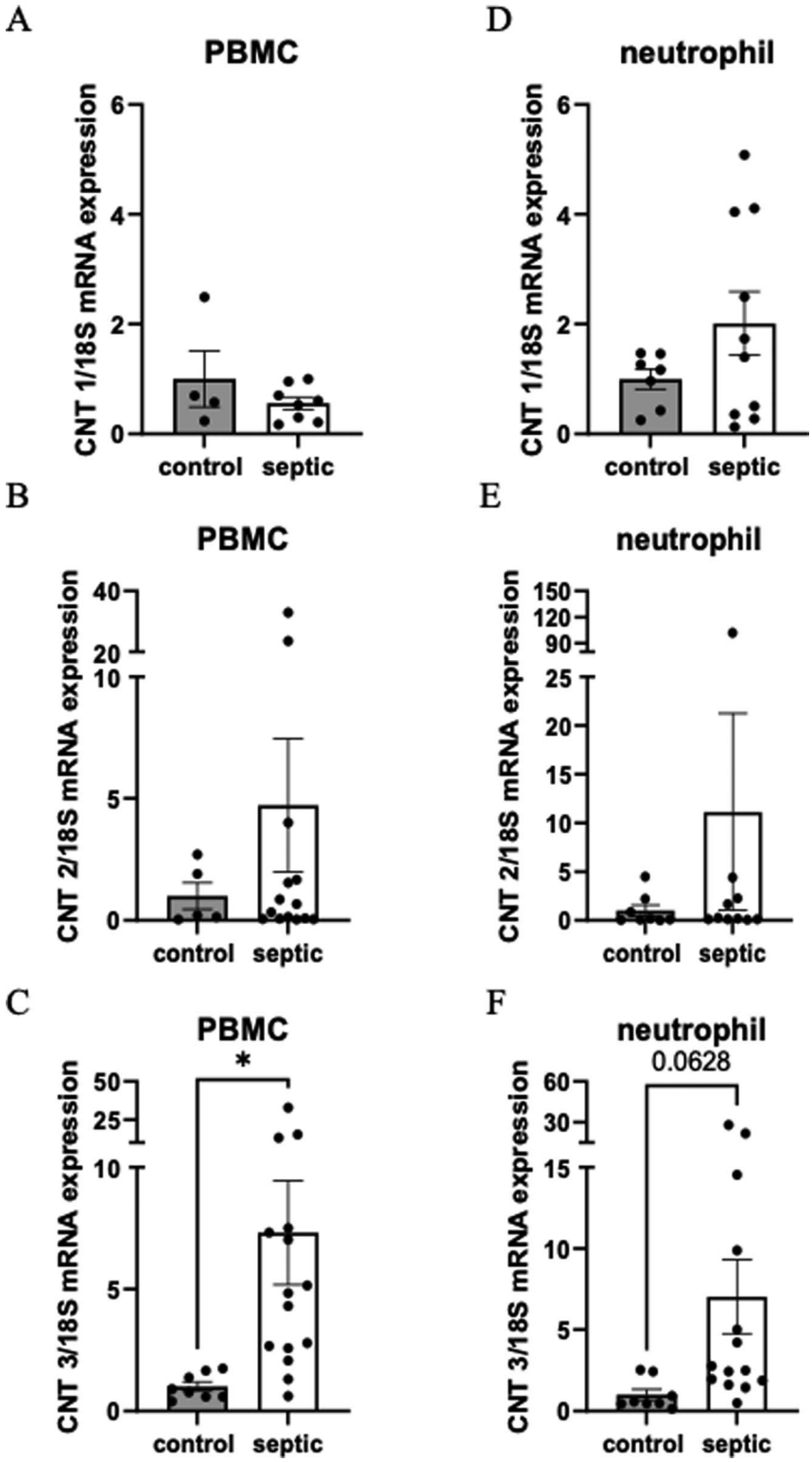

FIG. 9. **mRNA expression CNTs in leukocytes of septic patients versus healthy donors.** mRNA expression of CNT1, 2, and 3 was measured in PBMCs (A–C) and neutrophils (D–F) from septic versus healthy donors. Results were normalized for the expression of the internal control 18S and are presented as mean ± SEM (*$P \leq 0.05$). mRNA, messenger ribonucleic acid; CNT, concentrative nucleoside transporter; PBMC, peripheral blood mononuclear cell; SEM: standard error of the mean.

ENT1 expression was increased, this is different from intestinal epithelial cells. The clinical significance of adenosine transport through ENTs remains to be explored during sepsis.

In summary, our results demonstrate differential regulation of components of the purinergic system in PBMCs during human sepsis. Our observation of decreased ATP levels in blood of septic patients indicates decreased signaling through P2X4 and P2X7 receptors. At the same time, we failed to find alterations in adenosine levels and adenosine receptor expression indicating continued signaling through adenosine receptors.

Our results also raise several questions, such as whether changes in mRNA levels translate into alterations of protein levels and

function and whether these alterations have functional roles in regulating the natural history of immune activation and organ injury in septic patients. Given the central role in the purinergic system in immunity (11,16,27,29,50), answering these questions should be a priority in sepsis research.

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