A₃ adenosine receptor activation decreases mortality and renal and hepatic injury in murine septic peritonitis

H. Thomas Lee,¹ Mihwa Kim,¹ Jin Deok Joo,¹ George Gallos,¹ Jiang-Fan Chen,² and Charles W. Emala¹

¹Department of Anesthesiology, College of Physicians and Surgeons of Columbia University, New York, New York; and ²Department of Neurology, Boston University School of Medicine, Boston, Massachusetts

Submitted 13 January 2006; accepted in final form 22 May 2006

Lee, H. Thomas, Mihwa Kim, Jin Deok Joo, George Gallos, Jiang-Fan Chen, and Charles W. Emala. A₃ adenosine receptor activation decreases mortality and renal and hepatic injury in murine septic peritonitis. Am J Physiol Regul Integr Comp Physiol 291: R959–R969, 2006. First published May 25, 2006; doi:10.1152/ajpregu.00034.2006.—The role of A₃ adenosine receptors (ARs) in sepsis and inflammation is controversial. In this study, we determined the effects of A₃AR modulation on mortality and hepatic and renal dysfunction in a murine model of sepsis. To induce sepsis, congeneric A₃AR knockout mice (A₃AR KO) and wild-type control (A₃AR WT) mice were subjected to cecal ligation and double puncture (CLP). A₃AR KO mice had significantly worse 7-day survival compared with A₃AR WT mice. A₃AR KO mice also demonstrated significantly higher elevations in plasma creatinine, alanine aminotransferase, and aspartate aminotransferase as well as significantly worse kidney and liver injury than A₃AR WT mice. Moreover, A₃AR KO mice also demonstrated significantly higher elevations in inflammatory cytokines and enhanced nuclear translocation of NF-κB compared with A₃AR WT mice.

MATERIALS AND METHODS

AR KO Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University (New York, NY). The generation and initial characterization of the congenic A₃AR KO mice on a C57BL/6 background have been described previously (50) and were provided by Dr. Marlene Jacobson (Department of Neuroscience, Merck Research Laboratories, West Point, PA). The congenic A₂₅AR KO line on a C57BL/6 background was generated as described below.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Previously (7) and provided by Jiang-Fan Chen and Michael Schwarzschild (Department of Neurology, Boston University, Boston, MA). Commercial male C57BL/6 mice (Taconic Farms, Germantown, NY) served as wild-type (WT) controls. Male and female C57 mice are known to have different outcomes following CLP sepsis (2); therefore, only male mice were used in this study. The A3AR KO mice have been shown to have equivalent blood pressure and heart rates compared with WT mice (46). In addition, A1AR KO mice have equivalent mRNA expression of A1, A2a, and A2b ARs compared with WT mice as reported by Salvatore et al. (46) and confirmed by us (data not shown). Breeding pairs of A1AR heterozygous mice were obtained from Dr. Jurgen Schnerrmann (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health) to generate A1AR WT and A1AR KO mice as described previously (31).

### Induction of Sepsis by CLP

Mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg or to effect) and were allowed to spontaneously breathe room air on an electric heating pad under a warming light. CLP was performed as described previously (16). Briefly, the cecum was isolated through a small midline incision and the distal (0.5 cm) portion of the cecum below the ileocecal valve (to avoid bowel obstruction) was ligated with a 4.0 silk suture. The cecum was punctured through the surface and then along its antimesenteric border (double-puncture) with a 22- or 20-gauge needle and a small amount of stool was extruded through the puncture site. We determined in our preliminary studies that the needle size had a significant impact on mortality and hepatic/renal dysfunction after cecal puncture. Since the peritoneal surface is an excellent conduit for absorption, instillation of 0.5 cc normal saline into the peritoneal cavity was performed for fluid resuscitation before closing the abdomen. Fluid resuscitation was continued during the initial 24 h following CLP, by administering subcutaneous saline (1 cc every 8 h for 24 h). Twenty-four or 48 h after CLP, some mice were killed with an overdose of intraperitoneal pentobarbital, and plasma and kidneys were collected.

### Survival Studies

To determine 7-day survival, male A3AR WT mice (n = 32 for 20-gauge and n = 21 for 22-gauge needle) and A3AR KO mice (n = 35 for 20-gauge and n = 23 for 22-gauge needle) were subjected to CLP. All mice had free access to water and food and were observed by dedicated research personnel to determine 7-day survival. All severely moribund animals were euthanized with an overdose injection of anesthetic in adherence with our animal care protocol. This euthanasia was blind, and the moribund animals were counted in the mortality curve. To examine whether pharmacological blockade of endogenous A3AR could exert an impact on survival, A3AR WT mice (n = 12 for 20-gauge and n = 11 for 22-gauge needle) received a subcutaneous injection (1 mg/kg) of 3-ethyl-5-benzyl-2-methyl-6-(3-iodobenzyl)-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191); a selective A3AR agonist), 20 min prior and 6 h following CLP. To determine whether pharmacological stimulation of endogenous A3AR could exert an impact on survival, A3AR WT mice (n = 20 and 16 for 20- and 22-gauge needle, respectively) received a subcutaneous injection (0.5 mg/kg) of N6-(3-iodobenzyl)-AD0-5'-N'-methyleranamide (IB-MECA, a selective A3AR agonist), 20 min before CLP. To determine whether multiple injections were superior to a single injection, some A3AR WT mice (n = 20 and 12 for 20- and 22-gauge needle, respectively) received additional injections of 0.5 mg/kg IB-MECA 4 and 18 h after CLP. We also treated A3AR KO (n = 12), A2aAR KO (n = 10), and A3AR KO mice (n = 10) with either a single dose or multiple doses of IB-MECA and subjected them to 20-gauge CLP sepsis to evaluate the selectivity of IB-MECA for the A3AR.

### Assessment of Renal Function and Hepatic Injury After Sepsis

Renal function was assessed by measuring plasma creatinine 24 and 48 h after CLP by a colorimetric method based on the Jaffee reaction (21). Hepatic injury 24 and 48 h after CLP was assessed by measuring plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using a commercially available colorimetric method (Sigma, St. Louis, MO). Renal and hepatic function at 48 h after CLP were measured for 22-gauge CLP animals only as 20-gauge CLP animals showed severe 24 h mortality: ~50% for A3AR WT mice and >80% for A3AR KO mice.

### Measurement of Blood and Peritoneal Bacterial Load

Bacterial counts were performed on aseptically harvested blood and peritoneal fluid samples. Blood was aseptically obtained by cardiac puncture. Sterile saline (3 ml) was injected into the peritoneal cavity after aseptic preparation of the abdominal wall, and peritoneal fluid was obtained by aspiration. Samples were serially diluted in sterile saline and cultured on tryptic soy agar plates (Fisher Scientific). Plates were incubated (37°C) for 48 h, and colony counts were performed by an operator blinded to the different treatment groups. Results are expressed as colony-forming units per milliliter.

### Measurement of Systemic Cytokines by ELISA

Murine plasma TNF-α (ALPCO, Windham, NH), keratinocyte-derived chemokine (KC; R&D Systems, Minneapolis, MN), and IL-6 and IL-10 (eBioscences, San Diego, CA) concentrations were measured using commercially available ELISA kits according to the manufacturer’s instructions from plasma taken 24 h after CLP.

### Assessment of Renal Inflammation

**MPO activity.** Renal inflammation 24 h after CLP was assessed by measurement of renal MPO activity (marker of leukocyte infiltration) as described previously (31). Renal cortex (~200 mg) was dissected and homogenized for 30 s in 2 ml of 50 mM potassium phosphate buffer, pH 7.4, at 4°C. The samples were centrifuged for 15 min at 16,000 g at 4°C, and the resultant pellet was resuspended in 2 ml of 50 mM potassium phosphate buffer, pH 7.4, with 0.5% hexadecyltrimethyl ammonium bromide at 4°C. The samples were sonicated for 30 s and centrifuged at 16,000 g for 15 min at 4°C. Fifty microliters of supernatant were mixed with 750 μl of 45 mM potassium phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine and 0.3% H2O2. Absorbance (460 nm) was measured over a period of 5 min (unit of enzyme activity = ΔOD min⁻¹ mg protein⁻¹), and the relative MPO activity was expressed as the percentage of the sham-operated group. The remaining supernatant was used to determine protein concentrations.

**Semiquantitative RT-PCR for proinflammatory cytokines.** Twenty-four hours after CLP, renal corticomedullary expression of mRNAs encoding proinflammatory markers were also determined by using semiquantitative RT-PCR as described previously (31). Renal cortices were dissected, total RNA was extracted by using Trizol (Invitrogen, Carlsbad, CA) reagent, and RNA concentrations were determined with spectrophotometric readings at 260 nm. RT-PCR was performed to analyze the expression of proinflammatory [KC, macrophage inflammatory protein 2 (MIP-2), monocyte chemotactic protein -1 (MCP-1), ICAM-1, IL-1β, and TNF-a] genes. Primers were designed based on published GenBank sequences for mice (Table 1). Primer pairs were chosen to yield expected PCR products of 200–600 bp and to amplify a genomic region that spans one or two introns to eliminate the confounding effect of amplifying contaminating genomic DNA. RT-PCR was performed using the Access RT-PCR System (Promega), which is designed for a single tube reaction for first-strand cDNA synthesis (48°C for 45 min) using AMV reverse transcriptase, and subsequent PCR using Taq DNA polymerase. PCR cycles included a denaturation step of 94°C for 30 s, followed by an optimized annealing temperature (Table 1) for 1 min, followed by a 1-min extension period at 68°C. All PCR reactions were completed with
a 7-min incubation at 68°C to allow enzymatic completion of incomplete cDNAs. The PCR cycle number for each primer pair was optimized to yield linear increases in the densitometric measurements of resulting bands with increasing cycles of PCR (15–26 cycles, Table 1). The starting amount of RNA was also optimized to yield linear increases in the densitometric measurements of resulting bands at an established number of PCR cycles. For each experiment, we also performed semi-quantitative RT-PCR under conditions yielding linear results for GAPDH (a housekeeping gene) from the same sample. Semiquantitative analysis of mRNA expression was accomplished by obtaining the ratio of the band density of the mRNAs of interest to that of GAPDH (a housekeeping gene) from the same sample.

![Graph showing band density ratio of mRNAs to GAPDH](image)

**Figure 1.** Band density ratio of mRNAs to GAPDH from the same sample.

### Immunochemistry

Paraffin-embedded mouse kidney sections were deparaffinized in xylene and rehydrated through graded ethanol washes to water. After blocking with 10% normal horse serum/PBS solution, the slides were incubated overnight with primary antibody MAC-2 (Cedarlane Labs, Burlington, NC), neutrophils (cat. no. MCA771G; Serotec, Raleigh, NC), and T-lymphocytes (CD3, pan T cell marker; Serotec) at 4°C in a humidified chamber. Endogenous alkaline phosphatase activity was blocked with 1% levamisole. The primary antibody was localized by using the Vectastain ABC-Alkaline Phosphatase detection system using 5-Bromo-4-chloro-3' indolylphosphate-p-toluidine salt/NitroBlue tetrazolium chloride (BCIP/NBT) as substrate (Vector Laboratories, Burlingame, CA). Control serum IgG was used for negative isotype control experiments.

### Assessment of Renal NF-κB Activation

NF-κB activation is associated with transcription pathways of proinflammatory mediators, and suppression of NF-κB activity improves outcome of proinflammatory injuries, such as ischemia reperfusion injury and arthritis (6, 35). Renal cortices were dissected and immersed in 500 μl of buffer A [10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 20% glycerol, 0.2 mM PMSF, 0.5 mM DTT, protease inhibitor cocktail (Mini-complete-EDTA; Roche, Indianapolis, IN)] for 10 min at 4°C. The cells were homogenized using a polytron homogenizer for 5 s to release the nuclei protein and centrifuged at 18,000 g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 50 μl of buffer B (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.5 mM EDTA, 25% glycerol, 0.1% Triton X-100, 0.2 mM PMSF, 0.5 mM DTT, protease inhibitor cocktail), and incubated for 1 h at 4°C with occasional swirling to extract nuclear protein. The nuclei were centrifuged at 16,000 g for 15 min, and the supernatant containing nuclear protein was used for EMSA for NF-κB.

EMSA was performed using the Gel Shift Assay Systems (Promega, Madison, WI). The oligonucleotides for NF-κB (Promega) consensus sequences were end-labeled with 10 μCi of [γ-32P]ATP (PerkinElmer Life Technology, Wellesley, MA) and purified by using a G-25 spin column (Amersham Biosciences, Piscataway, NJ). Ten micrograms of the nuclear extract were incubated with 1 μl of the labeled probe for 20 min at room temperature and electrophoresed on a 4% polyacrylamide gel (200 V at 4°C). Two micrograms of Hela cell nuclear extract (Promega) was used for a positive control and 1 μl of NF-κB p65 TransCruz polyclonal antibody (Santa Cruz Biotechnology, CA) was coincubated with the nuclear protein and probe for a supershift reaction. One hundred-fold concentration of unlabeled probe was coincubated as a competitor in a negative control reaction. In addition to the protein assay, we performed immunoblotting for histones to verify equal loading of nuclear fractions (data not shown). The gel was then transferred to blotting paper and exposed to film or scanned with a PhosphoImager (Molecular Dynamics, Piscataway, NJ).

### Statistical Analysis

A one-way analysis of variance was used to compare mean values across multiple treatment groups with a Dunnett’s post hoc multiple comparison test (e.g., sham vs. CLP). Survival statistics were compared with a Kaplan-Meier curve and log rank test. In all cases, a P < 0.05 was taken to indicate significance.

**Protein determination.** Protein content was determined with the Pierce (Rockford, IL) bicinchoninic acid protein assay reagent using bovine serum albumin as a standard.

### RESULTS

**Endogenous or Exogenous A3AR Activation Protects Against CLP-Induced Mortality**

We initially measured the effect of endogenous A3AR activation on mortality from CLP-induced septic peritonitis by...
comparing 7-day survival for A3AR WT and A3AR KO mice. As demonstrated in Fig. 1, A and B, mice lacking endogenous A3ARs had significantly higher mortality rates compared with A3AR WT mice after CLP sepsis induced with either a 20-gauge (Fig. 1A, \( P < 0.05 \)) or a 22-gauge (Fig. 1B, \( P < 0.01 \)) needle. At 24 h following CLP with a 22- or 20-gauge needle, the mortality rate for the A3AR KO mice was 31.3% and 80% compared with 9.5% and 48.6% in A3AR WT mice, respectively. At 7 days, the 22-gauge needle-induced mortality rate for A3AR KO mice was 95.7% compared with 33.3% in A3AR WT mice. Log rank analysis of the survival statistics revealed significant differences between A3AR WT and A3AR KO mice after CLP sepsis induced with a 20-gauge (\( P < 0.05 \)) or a 22-gauge (\( P < 0.01 \)) needle.

Selective antagonism of A3AR with MRS-1191 resulted in significantly worse mortality in A3AR WT mice after CLP sepsis induced with either the 22- or 20-gauge needle (Fig. 1, A and B) mimicking the decreased survival observed with A3AR KO mice. At 24 h following CLP with a 22- or 20-gauge needle, the mortality rate for A3AR WT mice treated with MRS-1191 was 36.4% and 70% compared with 9.5 and 48.6% in untreated A3AR WT mice, respectively (\( P < 0.05 \)). At 7 days, 22-gauge needle-induced mortality rate for the A3AR WT mice treated with MRS-1191 was 81.8% compared with 33.3% in A3AR WT mice (\( P < 0.05 \)).

A single injection of IB-MECA (a selective agonist for A3ARs, 0.5 mg/kg) resulted in significantly better initial survival in A3AR WT mice after CLP sepsis induced with either a 22- or 20-gauge needle (Fig. 1, A and B). At 24 h following CLP with a 22- or 22-gauge needle, the mortality rate for A3AR WT mice treated with IB-MECA was 0% and 31.3% compared with 9.5% and 48.6% in untreated A3AR WT mice, respectively (\( P < 0.05 \)). However, the survival at day 7 did not differ between mice injected with a single dose of IB-MECA.
and vehicle-treated mice (Fig. 1, A and B). When we gave an additional IB-MECA treatment at 4 and 18 h after CLP, the 7-day survival was significantly better in WT mice. At 7 days following CLP with a 22- or 22-gauge needle, the mortality rate for A1AR WT mice treated with IB-MECA was 10 and 60% compared with 33 and 100% in untreated A1AR WT mice, respectively (P < 0.05).

We wanted to determine whether protection against mortality induced by CLP sepsis with IB-MECA is truly independent of A1ARs and A2aARs. Sullivan et al. (49) proposed that IB-MECA-induced protection against endotoxemia may be mediated by A2aARs because IB-MECA may activate A2aARs at high doses on the basis of their in vitro binding data (19). Therefore, we treated mice lacking A1AR, A2aAR, or A3AR with IB-MECA (0.5 mg/kg) and determined a 7-day survival following 20-gauge CLP sepsis. Figure 2 shows that even a single dose of IB-MECA improves survival in both A1AR KO and A3AR KO mice, demonstrating that the protective effect of IB-MECA against CLP sepsis is indeed mediated by selective A3AR activation. At 24 h following CLP with a 20-gauge needle, the mortality rate for A1AR KO and A3AR KO mice treated with IB-MECA was 0 and 30% compared with 25.5 and 49.6% in untreated A1AR KO and A3AR KO mice, respectively (P < 0.05). Multiple injections of IB-MECA resulted in even better improvements in survival after CLP sepsis. At 7 days following CLP with a 20-gauge needle, the mortality rate for A1AR KO and A3AR KO mice treated with multiple injections of IB-MECA was 33.3 and 25% compared with 82.76 and 90% in untreated A1AR KO and A2aAR KO mice, respectively (P < 0.05). Multiple injections of the A3AR agonist IB-MECA improved the survival of A1AR KO mice to match that of A1AR WT mice. Surprisingly, the 7-day survival of A3AR KO mice (10%) after 20-gauge CLP sepsis was slightly, but significantly (P = 0.0342) better than the survival of A2aAR WT mice (0%). The A3AR KO mice were not protected against CLP sepsis with IB-MECA (Fig. 1).

Modulation of Hepatic and Renal Dysfunction After CLP Sepsis with A3AR Deletion, Activation, or Antagonism

Twenty-four hours after sham operation, plasma creatinine (Cr), ALT, or AST values in A3AR KO mice (Cr: 0.4 ± 0.1 mg/dl, n = 5; ALT: 20 ± 5.2 Sigma-Frankel (SF) U/ml, n = 8; AST: 66 ± 11.4 SF U/ml, n = 8) were not different from A3AR WT mice (Cr: 0.3 ± 0.1 mg/dl, n = 5; ALT: 20 ± 1.9 SF U/ml, n = 8; AST: 91 ± 10.6 SF U/ml, n = 8). Creatinine, ALT, and AST significantly increased at 24 h after CLP in both A3AR WT and A3AR KO of mice (Table 2). However, A3AR KO mice showed significantly worse renal dysfunction and hepatic injury compared with A3AR WT at 24 h after CLP (Table 2). Treatment of A3AR WT mice with an A3AR agonist (IB-MECA) or antagonist (MRS-1191) improved or worsened renal dysfunction and hepatic injury 24 h after CLP sepsis, respectively (Table 2). Forty-eight hours after CLP, plasma creatinine and ALT improved in all groups to normal levels (Table 2). However, plasma AST levels at 48 h after CLP were significantly higher for A3AR KO mice and significantly lower for A3AR WT mice treated with IB-MECA compared with A3AR WT mice (Table 2).

Modulation of Plasma Proinflammatory and Anti-Inflammatory Cytokine Levels After CLP Sepsis with A3AR Deletion

With ELISA, TNF-α, KC, IL-6, and IL-10, plasma levels were determined 24 h following CLP-induced sepsis. TNF-α and KC plasma levels in mice subjected to 22- or 20-gauge CLP sepsis were elevated above normal baseline values (TNF-α: <18.2 pg/ml and KC: 167 ± 23 pg/ml, n = 4). However, A3AR KO mice showed significantly elevated TNF-α and KC plasma levels compared with A3AR WT mice 24 h after the induction of sepsis (Table 2). Plasma IL-6 levels increased in mice subjected to 22- and 20-gauge CLP sepsis with more severe sepsis (20-gauge CLP) leading to higher IL-6 levels (Table 2). Plasma levels of anti-inflammatory IL-10 levels also increased in mice subjected to 22- or 20-gauge CLP sepsis with A3AR KO mice demonstrating significantly higher IL-10 levels compared with A3AR WT mice (Table 2).

Modulation of Renal MPO Activity After CLP Sepsis with A3AR Deletion or Activation

MPO is an enzyme present in leukocytes and is an index of tissue leukocyte infiltration following injury (53). Since activated leukocyte infiltration is a hallmark of acute inflammation, we sought to determine the effect of endogenous or exogenous A3AR activation on renal MPO activity 24 h following CLP-induced sepsis. Mice lacking endogenous A3ARs subjected to 20-gauge CLP showed significantly higher MPO activity than A3AR WT mice subjected to 20-gauge CLP (Table 2). In addition, activation of A3ARs with IB-MECA reduced the MPO activity in A3AR WT mice after CLP sepsis (Table 2, Sham A3AR KO mice MPO activity = 0.06 ± 0.03
Markers Following CLP Sepsis with A3AR Deletion

Modulation of Renal mRNA Expression of Proinflammatory Markers Following CLP Sepsis with A3AR Deletion or Activation

We next examined the effects of endogenous A3AR activation on mRNA expression in renal cortices following CLP-induced sepsis. A3AR KO (n = 4) and WT (n = 4) mice had similar levels of mRNA encoding KC, IL-1β, TNF-α, ICAM-1, MCP-1, and MIP-2 24 h following sham operation (Fig. 3). In contrast, A3AR KO (n = 6) mice demonstrated increased mRNA expression of these proinflammatory mRNA levels compared with A3AR WT (n = 6) mice 24 h after the induction of sepsis (Fig. 3). In addition, activation of A3ARs with IB-MECA reduced proinflammatory mRNA levels in A3AR WT mice after CLP sepsis (Fig. 3).

Immunohistochemistry

Immunohistochemistry for three leukocyte subtypes (neutrophils, T-lymphocytes and macrophages) in kidneys of WT and A3AR KO mice subjected to CLP showed that very few (1–2 cells per field of ×400) neutrophils and lymphocytes infiltrate the kidney 6–48 h after CLP. Slightly increased macrophage infiltration (including resident macrophages) was observed for both WT and A3AR KO mice; however, there were no qualitative differences between the two groups of mice (data not shown).

A3AR Activation Reduces and A3AR Deletion Increases CLP-Induced NF-κB Nuclear Translocation After Sepsis

Renal cortices isolated from A3AR WT (n = 4) or A3AR KO (n = 5) mice had similar levels of NF-κB nuclear translocation 24 h following sham operation (Fig. 4). In contrast, renal cortices from A3AR KO (n = 6) mice exhibit increased NF-κB nuclear translocation 24 h following the induction of sepsis compared with cortices isolated from A3AR WT mice (n = 6, Fig. 4). In contrast, renal cortices from A3AR WT mice treated with IB-MECA (n = 6) show reduced NF-κB nuclear translocation 24 h following the induction of sepsis compared with cortices isolated from A3AR WT mice subjected to CLP sepsis. The NF-κB-specific band supershifted with a p65 antibody (data not shown).

Bacterial Counts in Blood and Peritoneal Fluid After CLP Sepsis

Aerobic bacteria in blood and peritoneal fluid were counted 24 h after CLP in A3AR WT mice (n = 8 for 22-gauge and n = 7 for 20-gauge), A3AR KO mice (n = 6 for 22-gauge and n = 6 for 20-gauge) and A3AR WT mice treated with 0.5 mg/kg IB-MECA (n = 7 for 22-gauge and n = 6 for 20-gauge). The average bacterial counts in blood did not differ between A3AR WT mice, A3AR KO mice and A3AR WT mice treated with 0.5 mg/kg IB-MECA subjected to either 22- or 20-gauge needle (Fig. 5A). In contrast, the average aerobic bacterial count in the peritoneal fluid was significantly higher for A3AR KO mice compared with A3AR WT mice subjected to 22-gauge needle (Fig. 5B). The average peritoneal bacterial counts for mice subjected to more severe sepsis with 20-gauge needle did not differ between A3AR WT mice, A3AR KO mice, and A3AR WT mice treated with 0.5 mg/kg IB-MECA (Fig. 5B).

DISCUSSION

The major finding of the present study is that exogenous A3AR activation reduced mortality and improved renal and hepatic function after CLP-induced sepsis. Improved survival

Table 2. Markers of organ injury and dysfunction, markers of systemic inflammation, and marker of renal inflammation 24 or 48 h after the induction of sepsis with 20-gauge or 22-gauge cecal ligation and double puncture (CLP)

<table>
<thead>
<tr>
<th>Test</th>
<th>CLP Needle Size (gauge) and Time After CLP</th>
<th>A3AR WT CLP</th>
<th>A3AR KO CLP</th>
<th>A3AR WT + IB-MECA CLP</th>
<th>A3AR WT + MRS-1191 CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Creatinine, mg/dl</td>
<td>22 (24 h)</td>
<td>0.8±0.05 (12)</td>
<td>1.1±0.04 (11)*</td>
<td>0.6±0.04 (8)*</td>
</tr>
<tr>
<td></td>
<td>ALT, SF U/ml</td>
<td>22 (24 h)</td>
<td>86±19 (6)</td>
<td>155±9 (10)*</td>
<td>29±2.4 (8)*</td>
</tr>
<tr>
<td></td>
<td>AST, SF U/ml</td>
<td>22 (24 h)</td>
<td>226±32 (8)</td>
<td>365±16 (10)*</td>
<td>116±7.5 (8)*</td>
</tr>
<tr>
<td></td>
<td>TNF-α ELISA, pg/ml</td>
<td>22 (24 h)</td>
<td>23±10 (N=5)</td>
<td>139±40 (5)*</td>
<td>443±979 (6)*</td>
</tr>
<tr>
<td></td>
<td>KC ELISA, pg/ml</td>
<td>22 (24 h)</td>
<td>712±3814 (4)</td>
<td>55643±18604 (6)*</td>
<td>553 (5) 4434</td>
</tr>
<tr>
<td></td>
<td>IL-6 ELISA, pg/ml</td>
<td>22 (24 h)</td>
<td>2158±406 (6)</td>
<td>2006±382 (6)</td>
<td>2212±469 (6)</td>
</tr>
<tr>
<td></td>
<td>IL-10 ELISA, pg/ml</td>
<td>22 (24 h)</td>
<td>435±1122 (5)</td>
<td>3896±1127 (5)</td>
<td>4997±1161 (5)</td>
</tr>
<tr>
<td></td>
<td>MPO assay, ΔOD·min⁻¹·mg protein⁻¹</td>
<td>20 (24 h)</td>
<td>0.48±0.1 (6)</td>
<td>1.04±0.2 (6)*</td>
<td>0.13±0.05 (5)*</td>
</tr>
</tbody>
</table>

| Markers of Systemic Inflammation |
|----------------------------------|------------------|--------------|------------------|
| TNF-α ELISA, pg/ml | 22 (24 h) | 23±10 (N=5) | 139±40 (5)* |
| KC ELISA, pg/ml | 22 (24 h) | 712±3814 (4) | 55643±18604 (6)* |
| IL-6 ELISA, pg/ml | 22 (24 h) | 2158±406 (6) | 2006±382 (6) |
| IL-10 ELISA, pg/ml | 22 (24 h) | 435±1122 (5) | 3896±1127 (5) |

Results are expressed as means ± SE; number in parentheses is the number of mice. *P < 0.05 vs. A3 adenosine receptor wild-type (A3AR WT) mice subjected to CLP. ALT, alanine aminotransferase; AST, aspartate aminotransferase; KO, knockout.

ΔOD/mg protein, n = 5, and Sham A3AR WT mice MPO activity = 0.05 ± 0.03 ΔOD/mg protein, n = 5.)
and organ function with A3AR activation was associated with reduced inflammation. Moreover, mice lacking endogenous A3ARs and mice treated with a selective A3AR antagonist demonstrated increased mortality, increased acute renal and hepatic injury, and increased inflammation associated with murine septic peritonitis. Importantly, the A3AR agonist IB-MECA protected against CLP-sepsis in A1KO and A2aKO mice without protecting the A3KO mice, ruling out the in-

Fig. 3. A: representative gel images of semiquantitative RT-PCR of proinflammatory markers TNF-α, IL-1β, keratinocyte-derived chemokine (KC), macrophage inflammatory protein 2 (MIP-2), and ICAM-1 from renal cortices of sham-operated A3AR WT (n = 4) mice, sham-operated A3AR KO (n = 4) mice, sham-operated A3AR WT mice treated with IB-MECA (n = 4), A3AR WT mice subjected to CLP with 20-gauge needle (n = 6), A3AR KO mice subjected to CLP with 20-gauge needle (n = 6), and A3AR WT mice treated with IB-MECA and subjected to CLP with 20-gauge needle (n = 6). B: densitometric quantifications of relative band intensities normalized to GAPDH from RT-PCR reactions for each indicated mRNA. *P < 0.05 vs. A3WT sham, #P < 0.05 vs. A3WT CLP. Error bars represent the mean ± 1 SE.

AJP-Regul Integr Comp Physiol • VOL 291 • OCTOBER 2006 • www.ajpregu.org
volvement of A1AR or A2aAR in IB-MECA mediated protection.

Our previous studies (16, 17), as well as studies by others (10, 18, 23, 35, 47), demonstrate that modulation of the hyperactive inflammatory response after CLP sepsis can improve outcome. The role of A3ARs in the modulation of inflammation is complicated by seemingly conflicting reports. A3AR activation can produce proinflammatory or anti-inflammatory states depending on the cell type and the organs studied. For example, A3ARs in murine mast cells contribute to the inflammatory changes in the lung (52). In addition, antigen-dependent degranulation of bone marrow-derived mast cells are mediated by A3ARs (43, 46). We have previously demonstrated that A3AR activation exacerbates renal dysfunction and mice lacking A3ARs had better renal function following renal ischemia reperfusion injury (30). On the other hand, A3AR agonists inhibit LPS-mediated release of TNF-α in vivo and in vitro in macrophages (37, 46). More importantly, the A3AR agonist IB-MECA reduces mortality after endotoxin treatment in mice (19). Other investigators demonstrated reduced inflammation and increased survival with activation of A3ARs in two murine models of colitis (32).

However, Sullivan et al. (49) suggested that the protective effects of IB-MECA may be due to the activation of A2aARs as

Fig. 4. A: representative gel image of NF-κB EMSA of nuclear extracts from renal cortices of sham-operated A3AR WT (n = 4) mice, sham-operated A3AR KO (n = 5) mice, sham-operated A3AR WT mice treated with IB-MECA (n = 5), A3AR WT mice subjected to CLP with 20-gauge needle (n = 6), A3AR KO mice subjected to CLP with 20-gauge needle (n = 6), A3AR WT mice treated with IB-MECA and subjected to CLP with 20-gauge needle (n = 6). B: densitometric quantifications of relative band intensities from NF-κB EMSA. *P < 0.05 vs. A3WT sham, #P < 0.05 vs. A3WT CLP. Error bars represent the mean ± 1 SE.

Fig. 5. Comparison of bacterial load expressed in colony-forming units per milliliter (CFU/ml) in blood (A) and peritoneal lavage fluid (B) of A3WT mice (n = 8 for 22-gauge and n = 7 for 20-gauge), A3KO mice (n = 6 for 22-gauge and n = 6 for 20-gauge), and A3WT mice pretreated with IB-MECA (n = 7 for 22-gauge and n = 6 for 20-gauge) and subjected to 22- or 20-gauge CLP sepsis. *P ≤ 0.05 compared with A3WT mice subjected to 22-gauge CLP sepsis.
IB-MECA may be nonselective at high doses and a selective antagonist of A<sub>2a</sub>ARs (4-(2-[7-aminopiperazin-2-yl]methyl)phenol, [ZM-241385]) blocked IB-MECA’s protective effects against LPS-mediated mortality. However, equating the in vitro binding affinity of a drug with systemic effects in vivo can be problematic. Moreover, in Sullivan et al.’s study, A<sub>3</sub>AR KO mice were not utilized to test the effects of IB-MECA. In addition, ZM-241385 may have adversely affected the mortality after LPS injection. To further confirm that IB-MECA protects via activation of A<sub>3</sub>ARs, we treated A<sub>1</sub>AR KO, A<sub>2a</sub>AR KO and A<sub>3</sub>AR KO mice with IB-MECA and subjected them to CLP sepsis. Our data do not support Sullivan et al.’s hypothesis because IB-MECA produced protection in both A<sub>1</sub>AR KO mice and A<sub>2a</sub>AR KO mice but not in A<sub>3</sub>AR KO mice.

In fact, our study demonstrated that the A<sub>3</sub>AR KO mice had slightly, but significantly, improved 7-day survival after 20-gauge CLP. This was a surprising finding as it is well known that the A<sub>2a</sub>AR activation reduces inflammation and improves outcome in many disease models including ischemia and reperfusion injury of the kidney (39, 40) and liver (9), as well as LPS-induced endotoxemia (49). Our finding is consistent with the recently published work of Nemeth et al. (38). In their study, mice lacking the A<sub>2a</sub>AR (from a CD-1 background) as well as antagonism of A<sub>2a</sub>AR showed improved outcome after CLP sepsis.

The effect of endogenous or exogenous A<sub>3</sub>AR activation in sepsis is measured in this study by tracking animal survival and by assessing kidney and liver dysfunction. In this study, we demonstrate a cytoprotective role of A<sub>3</sub>AR in sepsis because A<sub>3</sub>AR KO mice had worse survival and renal/hepatic dysfunction after sepsis. Moreover, even a single injection of an A<sub>3</sub>AR agonist at the onset of 22- or 20-gauge needle puncture CLP sepsis protected against sepsis-induced early mortality and organ dysfunction. However, a single IB-MECA injection resulted in equivalent 7-day mortality. Multiple injections of IB-MECA resulted in drastic improvement of 7-day survival. In addition, the A<sub>3</sub>AR antagonist MRS-1191 equalized A<sub>3</sub>AR WT survival with A<sub>3</sub>AR KO survival. The protective role of A<sub>3</sub>ARs in CLP-induced sepsis is in stark contrast to their detrimental role in other forms of acute renal failure including ischemia and reperfusion injury and glycerol-induced myoglobinuria, indicating fundamental differences in the mechanisms of pathogenesis of ARF due to sepsis and isolated renal ischemia and reperfusion injury (30).

The genetic background of mice has been shown to be an important factor in outcome after several types of injury including sepsis, inflammation, and ischemia and reperfusion (4, 10, 36). A<sub>2a</sub>AR KO and the A<sub>3</sub>AR KO mice used in this study are congenic on a C57BL/6 background with more than 12 backcrossings. We therefore used commercially available C57BL/6 mice as their WT controls. The A<sub>1</sub>AR KO mice are not congenic and therefore, WT littermates were used as controls of the A<sub>1</sub>AR KO mice. We note significant differences in the survival rates between A<sub>1</sub>AR WT mice (noncongenic) and C57BL/6 mice (congenic WT mice for A<sub>2a</sub>AR KO and A<sub>3</sub>AR KO mice) after equivalent degrees of CLP sepsis. Our data further provide evidence that the genetic background of mice is an important determinant of survival after CLP sepsis.

In animal models, potentiating adenosine’s effects (by either administration of adenosine receptor agonists or by inhibition of adenosine enzyme pathways to limit repurification or prevent degradation) has been shown to improve outcomes in sepsis (1, 8, 14, 41, 48). Use of AR KO mice further complements these prior studies. However, since compensatory physiological changes are inherent concerns with studies using KO mice, we also used a selective A<sub>3</sub>AR antagonist to illustrate that the results we observed in our A<sub>3</sub>AR KO mice can be demonstrated in A<sub>3</sub>AR WT mice treated with an A<sub>3</sub>AR antagonist. By using both models, we provide conclusive data that endogenous A<sub>3</sub>AR activation serves protective functions in CLP-induced sepsis.

We adopted the CLP model to more accurately recapitulate the complex immunology seen in human sepsis. Unlike models employing endotoxin or bacteria, this model induces septic peritonitis that more closely resembles human sepsis with regard to proinflammatory cytokine generation, progression to multiorgan injury and failure, and response to certain therapeutic interventions (44). Another advantage of this septic model is the ability to manipulate the magnitude of the inflammatory response by modulating the needle size used for puncture. We employed a 22- or 20-gauge needle to induce in our mice a level of sepsis with moderate predilection for mortality and organ injury in the absence of any interventions. We demonstrate that the 22-gauge needle produces a milder form of sepsis with less mortality, organ dysfunction, and inflammation. However, in both needle size-induced CLP sepsis, A<sub>3</sub>AR activation improved and A<sub>3</sub>AR antagonist worsened mortality, organ dysfunction, and inflammation, respectively.

With CLP, cecal ischemia produces deterioration of mucosal integrity leading to bacterial translocation into the peritoneum as well as into the systemic circulation. Several studies demonstrated that bacterial translocation is an important initiating mechanism for the induction of systemic inflammation and organ injury after CLP as removal of the cecal contents or treatment with powerful antibiotics reduce mortality and organ dysfunction (12, 13). We showed in the present study that high bacterial counts were observed in blood and peritoneal cavity (Fig. 5) 24 h after CLP sepsis. In particular, the A<sub>3</sub>AR KO mice subjected to 22-gauge CLP sepsis showed higher peritoneal bacterial counts compared with the A<sub>3</sub>AR WT mice. We believe that the lack of anti-inflammatory A<sub>3</sub>ARs led to higher bacterial counts in the peritoneum of A<sub>3</sub>AR KO mice, reflecting a state of more severe sepsis (higher mortality and renal and hepatic injury). Subjecting mice to more severe sepsis (20-gauge CLP) led to significantly increased bacterial counts in the peritoneum in all groups of mice. Interestingly, A<sub>3</sub>AR modulation had no impact on blood bacterial counts and a similar degree of bacteremia was observed between mice subjected to 22- and 20-gauge CLP. Therefore, the mechanism(s) of bacterial translocation may be different between the peritoneum and the blood.

Given that our central goal was to evaluate the effect of endogenous A<sub>3</sub>AR activation on outcomes in murine sepsis, we chose not to possibly confound our results by introducing antibiotics into our studies. We recognize that antibiotics themselves have therapeutic value. In fact, we were able to demonstrate differences in survival and morbidity without having to manipulate survival outcomes with antibiotic administration. However, we did give fluid resuscitation to produce and mimic hemodynamic changes seen during early (hyperdynamic) sepsis. We show that the bacterial load of blood and...
peritoneum did not differ between A3AR WT mice, A3AR WT mice treated with IB-MECA, and A3AR KO mice subjected to 20-gauge CLP sepsis, indicating that the modulation of survival, and renal and hepatic function is not impacted by bacterial contamination after CLP sepsis. Rather, potent anti-inflammatory effects of A3AR activation may directly produce survival and organ function preservation in mice treated with an A3AR agonist (IB-MECA).

Sepsis represents a systemic inflammatory response that initially manifests as an overproduction of stimulatory mediators including proinflammatory cytokines (i.e., TNF-α), and chemokines (i.e., KC). In the present study, the inflammatory processes elicited during CLP-induced sepsis contributed to a greater organ injury and dysfunction observed in A3AR KO mice because they do not possess the counterbalancing anti-inflammatory benefits afforded by endogenous A3AR activation. This is supported by the findings that TNF-α and KC levels increased in the plasma by 24 h following CLP; however, these cytokine levels in A3AR KO mice were significantly elevated compared with A3AR WT mice. Murine KC is a proinflammatory chemokine that putatively represents the functional homologue of human IL-8. As such, KC not only serves as a potent neutrophil attractant and activator, but its overexpression has also been associated with various inflammatory conditions and is a marker for increased mortality in the CLP sepsis model (22).

Previous studies suggested that IL-6 serves as both a marker and a mediator for the severity of sepsis (45). Our study also supports these findings in that more severe sepsis induced with a 20-gauge needle resulted in higher plasma levels of IL-6 compared with plasma levels after CLP with a 22-gauge needle (Table 2). However, unlike plasma TNF-α and KC levels, modulation of A3ARs failed to have an impact on plasma IL-6 levels because we found no significant differences in IL-6 levels between A3AR WT mice, A3AR KO mice, and A3AR WT mice treated with IB-MECA. Recent studies also propose an important role for the production of anti-inflammatory mediators, such as IL-10 for the modulation of the septic response (27, 33). We demonstrate in this study that A3AR KO mice with a more severe septic injury showed higher plasma levels of IL-10 mice, perhaps indicating an increased endogenous anti-inflammatory response against the more severe septic response (Table 2). The A3AR activation failed to modulate plasma IL-10 levels. In addition, as the mortality rate for the A3AR KO mice was higher compared with the A3AR WT mice, we conclude that plasma IL-10 levels did not modulate survival after CLP sepsis in these mice. Taken together, IL-6 or IL-10 modulation is not a major mechanism of A3AR-mediated protection against CLP sepsis.

Generation of cytokines and LPS during sepsis leads to toll receptor activation and propagation of the pathogenesis of sepsis. A central downstream element of toll receptor-dependant signaling is activation of the pleiotropic transcription factor NF-κB. NF-κB has been implicated in the regulation of multiple biological phenomena and disease states, including apoptosis, cell growth, stress response, innate immunity, and septic shock. Studies have demonstrated that increased NF-κB expression is predictive of poor prognosis in sepsis (5). In other models of sepsis, suppression of NF-κB activation decreased acute inflammatory processes and organ dysfunction (35). Further evidence for the anti-inflammatory role of endogenous A3AR activation in sepsis is demonstrated in the present study by the significant increase in activation of NF-κB shown in A3AR KO mice compared with A3AR WT mice in response to sepsis that underscores the potential signaling pathways by which A3AR may be exerting its protective effects in sepsis.

The two major limitations of this study are that 1) the cell type(s) involved in protection against CLP sepsis and 2) the signal transduction mechanism(s) after activation of A3AR leading to anti-inflammatory and protective effects are not elucidated. Multiple immune, as well as nonimmune, cells play an intricate role in defending against septic pathogenesis including macrophages, lymphocytes, as well as, neutrophils in several organs including spleen, liver, and thymus. Several of these cell types may be involved in the in vivo protective effects of IB-MECA because the presence of A3ARs in these cell types has been demonstrated (25). In particular, A3AR activation produces anti-inflammatory effects in monocyte/macrophage cell lines (20, 25).

In conclusion, we demonstrate that endogenous or exogenous A3AR activation provides protection from CLP-induced mortality and acute organ dysfunction. Since the pathogenesis of organ dysfunction in sepsis is largely mediated by an imbalanced inflammatory response, A3AR activation improves organ function after septic insult by attenuating this hyperinflammatory process. Given the protective benefit of the A3AR on survival and organ dysfunction, our findings may have important future therapeutic implications for patients in sepsis.

GRANTS

This work was supported by the Intramural Research Fund of the Department of Anesthesiology at Columbia University and by National Institute of Health Grant R01-DK-58547

REFERENCES

9. Day YJ, Marshall MA, Huang L, McDuffie MJ, Okusa MD, and Linden J. Protection from ischemic liver injury by activation of A2A adenosine receptors during reperfusion: inhibition of chemokine induc-