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

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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.—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, congenic 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, aspartate aminotransferase, keratinocyte-derived chemokine, and TNF-α 24 h after induction of sepsis compared with A3AR WT mice. Renal cortices from septic A3AR KO mice exhibited increased mRNA encoding proinflammatory cytokines and enhanced nuclear translocation of NF-κB compared with samples from A3AR WT mice. A3ARs displayed improved renal function after ischemia reperfusion injury (30). A3AR KO Mice

Seppsis represents a major clinical problem without effective therapy. Despite advances in antibiotic, hemodynamic, and ventilatory support, the incidence of sepsis and the number of sepsis-related deaths is very high. Approximately 750,000 annual cases of severe sepsis occur in the United States leading to 215,000 deaths (9.3% of deaths from all causes) (3, 11, 34). Although the pathogenesis of sepsis-induced multiorgan injury leading to death is incompletely understood, the initial hyperinflammatory process and subsequent hypoinflammatory phase contribute to mortality and morbidity in sepsis. The initial hyperinflammatory response seen in sepsis is associated with uncontrolled, hyperexuberant cytokine production that can be deleterious to various tissues and leads to organ injury and dysfunction. After this hyperinflammatory phase, a hypoinnune phase ensues with enhanced apoptotic cell death occurring in multiple organs including the spleen, kidney, liver, and heart (23, 24).

Adenosine receptors (ARs) modulate inflammation and cell death in many organs including the heart, kidney, lung, and liver, and these organs are subject to multiorgan injury in sepsis (17, 26, 29, 42). We have demonstrated that ARs play important roles in modulating outcome after renal ischemia and reperfusion injury, as well as after cecal ligation and puncture (CLP)-induced sepsis, in part by modulating inflammation (17, 28–31). A3ARs in particular appear to have a complex role in inflammation as both proinflammatory and anti-inflammatory effects have been demonstrated (15, 46, 51). We have previously shown that A3AR activation before renal ischemia results in the worsening of renal function and that mice lacking A3ARs displayed improved renal function after ischemia reperfusion injury (30). In contrast, a selective A2aAR agonist N6-(3-iodobenzyl)ADO-5’N-methyluronamide (IB-MECA) reduced inflammation in mouse models of colitis and reduced LPS-induced mortality in mice (19, 32). However, Sullivan et al. (49) speculated that the protective effects of IB-MECA against endotoxemia may actually be mediated by A2aARs because high doses of IB-MECA may activate both A2aARs and A1ARs. Therefore, the role of A2aARs in protecting against sepsis and inflammation is not clear. In addition, the role of A3ARs in CLP-induced sepsis has never been studied. In the present study, we tested the effects of a selective A3AR agonist (IB-MECA) against CLP-induced sepsis, a better model of sepsis than the endotoxin model of sepsis. We determined the effects of genetic deletion of A3ARs on sepsis-induced mortality and hepatic and renal dysfunction by subjecting A3AR knockout (KO) mice to CLP sepsis. We also tested the effects of IB-MECA in mice genetically lacking the A1AR or A2aAR to determine the specificity of IB-MECA activating A3ARs in mediating protection from sepsis.

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 A3AR KO line on a C57BL/6 background was generated as described...
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 A2AR 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, A2aR, and A2bR 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 Schnermann (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 (n = 35 for 20-gauge and n = 23 for 22-gauge needle) mice 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 blinded, and the moribund animals were counted in the mortality curve. To examine whether pharmacological blockade of endogenous A1AR could exert an impact on survival, A3AR WT mice (n = 12 for 20-gauge and n = 11 for 22-gauge needle, respectively) received a subcutaneous injection (1 mg/kg) of 3-ethyl-5-benzyl-2-methyl-phenethyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191); a selective A3AR antagonist), 20 min prior and 6 h following CLP. To determine whether pharmacological stimulation of endogenous A1AR could exert an impact on survival, A3AR WT (n = 20 and 16 for 20- and 22-gauge needle, respectively) mice received a subcutaneous injection (0.5 mg/kg) of N9-(3-isodobenzyl)-ADO-5'-N'-methyluronamide (IB-MECA, a selective A1AR 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 A1AR KO (n = 12), A2aR KO (n = 10), and A2bR 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 A1AR.
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 (15 cycles) to confirm equal RNA input. Five microliters of the RT-PCR product was analyzed on a 6% acrylamide gel stained with SYBR Green (Invitrogen, Carlsbad, CA) for analysis with a UVP Bio-imaging System (Upland, CA). 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. Comparisons of the control group (sham group) between one gel and another can vary (e.g., due to staining differences). Therefore, the data presented are actually the normalized values of cytokine mRNA/GAPDH ratio to the sham A3AR WT group in each gel. These ratios are presented in the figure with the sham animals normalized to one.

**Immunohistochemistry**

Paraffin-embedded mouse kidney sections were deparaffinized in xylene and rehydrated through graded ethanolos 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 Phospho Imager (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 A2aAR 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 A2aAR KO mice treated with IB-MECA was 0 and 30% compared with 25.5 and 49.6% in untreated A1AR KO and A2aAR 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 A2aAR 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 A2aAR 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 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 quantitative 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 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) showed 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).

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...
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.
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 cultures (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...
IB-MECA may be nonselective at high doses and a selective antagonist of A2aARs (4-[(2-[7-aminopyrazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)-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, A3AR 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 A3ARs, we treated A1AR KO, A2aAR KO and A3AR 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 A1AR KO mice and A2aAR KO mice but not in A3AR KO mice.

In fact, our study demonstrated that the A3AR 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 A3AR 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 A2aAR (from a CD-1 background) as well as antagonism of A2aAR showed improved outcome after CLP sepsis.

The effect of endogenous or exogenous A3AR 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 A3AR in sepsis because A3AR KO mice had worse survival and renal/hepatic dysfunction after sepsis. Moreover, even a single injection of an A3AR 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 A3AR antagonist MRS-1191 equalized A3AR WT survival with A1AR KO survival. The protective role of A3ARs 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). A2aAR KO and the A3AR 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 A1AR KO mice are not congenic and therefore, WT littermates were used as controls of the A1AR KO mice. We noted significant differences in the survival rates between A1AR WT mice (noncongenic) and C57BL/6 mice (congenic WT mice for A2aAR KO and A3AR 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 rephosphorylation 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 A3AR antagonist to illustrate that the results we observed in our A3AR KO mice can be demonstrated in A3AR WT mice treated with an A3AR antagonist. By using both models, we provide conclusive data that endogenous A3AR 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, A3AR activation improved and A3AR 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 A3AR KO mice subjected to 22-gauge CLP sepsis showed higher peritoneal bacterial counts compared with the A3AR WT mice. We believe that the lack of anti-inflammatory A3ARs led to higher bacterial counts in the peritoneum of A3AR 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, A3AR 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 A3AR 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 A3WT 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-dependent 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 A3KO KO mice compared with A3WT 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

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