A1 adenosine receptor knockout mice exhibit increased mortality, renal dysfunction, and hepatic injury in murine septic peritonitis

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Gallos, George, Thomas D. Ruyle, Charles W. Emala, and H. Thomas Lee. A1 adenosine receptor knockout mice exhibit increased mortality, renal dysfunction, and hepatic injury in murine septic peritonitis. Am J Physiol Renal Physiol 289: F369–F376, 2005. First published March 22, 2005; doi:10.1152/ajprenal.00470.2004.—Sepsis is a leading cause of multiorgan dysfunction and death in hospitalized patients. Dysregulated inflammatory processes and apoptosis contribute to the pathogenesis of sepsis-induced organ dysfunction and death. A1 adenosine receptor (A1AR) activation reduces inflammation and apoptosis after ischemia-reperfusion injury. Therefore, we questioned whether A1AR-mediated reduction of inflammation and apoptosis could improve mortality and organ dysfunction in a murine model of sepsis. A1AR knockout mice (A1R knockout) and their wild-type (A1R wild-type) littermate controls were subjected to cecal ligation and double puncture (CLP) with a 20-gauge needle. A1R knockout mice or A1R wild-type mice treated with 1,3-dipropyl-8-cyclopentylxanthine (a selective A1AR antagonist) had a significantly higher mortality rate compared with A1R wild-type mice following CLP. Mice lacking endogenous A1ARs demonstrated significant elevations in plasma creatinine, alanine aminotransferase, aspartate aminotransferase, keratinocyte-derived chemokine, and tumor necrosis factor-α. 24 h after induction of sepsis compared with wild-type mice. The renal cortical-medullary junction from A1R knockout mice also exhibited increased myeloperoxidase activity, intercellular adhesion molecule-1 protein, and mRNA encoding proinflammatory cytokines compared with renal samples from A1R wild-type littermate controls. No difference in renal tubular apoptosis was detected between A1R knockout and A1R wild-type mice. We conclude that endogenous A1AR activation confers a protective effect in mice from septic peritonitis primarily by attenuating the hyperacute inflammatory response in sepsis.

Acute renal failure; multiorgan injury; survival

Sepsis represents a major clinical problem in hospitalized patients. Despite advances in antibiotic, hemodynamic, and ventilatory support, the incidence of sepsis and the numbers of sepsis-related deaths are increasing. On an annual basis, sepsis affects ~750,000 patients in the United States and accounts for nearly 215,000 deaths (2). Development of sepsis-induced organ injury and subsequent progression to acute organ dysfunction are associated with increased morbidity and mortality.

Although the pathogenesis of sepsis-induced acute organ injury and dysfunction is not completely 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 hypoinflammatory phase ensues with enhanced apoptotic cell death occurring in multiple organs including the spleen, kidney, liver, and heart (20, 21). The murine cecal ligation and double puncture model (CLP) resembles the human sepsis syndrome with respect to proinflammatory cytokine generation, apoptosis, and progress to acute sepsis-induced multiorgan injury and failure (11, 13, 33).

We previously demonstrated that endogenous as well as exogenous A1 adenosine receptor (A1AR) activation before renal ischemia and reperfusion significantly protects renal function by reducing inflammation, necrosis, and apoptosis (28). We hypothesized that endogenous A1ARs produce anti-inflammatory and anti-apoptotic effects in renal tubular cells. Therefore, in the present study, we questioned whether mice lacking A1ARs would show increased proinflammatory markers and apoptosis resulting in increased mortality and increased hepatic injury and renal dysfunction in an in vivo murine model of septic peritonitis.

Materials and methods

A1R knockout 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 A1R knockout and A1R wild-type mice have been described previously (38). A1R heterozygous breeding pairs (kind gift of Dr. J. Schermann, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD) were bred and genotyped as described previously (29). The A1R knockout mice used in our studies are descendants of A1AR heterozygous (+/−) breeding pairs. Because these mice are a mixture of the 129/SVJ and C57/BL6 strains and are not congenic, we used littermate progeny from heterozygote parents for our studies. In a blinded fashion, cohorts of littermates were subjected to CLP, and results were obtained after which genotype was revealed. In an effort to maintain consistency, all members (both males and females) of a litter underwent CLP.

The A1R knockout mice have been shown to have equivalent renal blood flow and glomerular filtration rate compared with A1R wild-type mice (16). In addition, A1R knockout mice have equivalent mRNA and protein expression of other AR subtypes compared with A1R wild-type mice. For example, our studies show that A1R knockout mice express 102, 110, and 100% of A2a, A2b, and A3 AR mRNA expression of A1R wild-type mice, respectively (n = 2 experiments), confirming the published data of Hansen et al. (16).

Induction of sepsis by cecal ligation and puncture. Mice were anesthetized with intraperitoneal pentobarbital sodium (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 (14). Briefly, the cecum was isolated through a small midline incision and the distal (0.5 cm) portion of the cecum was subjected to CLP, and results were obtained after which genotype was revealed.
below the ileoceleal valve (to avoid bowel obstruction) was ligated with a 4.0 silk suture. The cecum was then punctured through and through along its antimesenteric border (double puncture) with a 20-gauge needle and a small amount of stool was extruded through the puncture site. Because the peritoneal surface is an excellent conduit for absorption, instillation of 0.5 ml of 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 ml every 8 h for 24 h). Twenty-four hours after CLP, some mice were euthanized with an overdose of intraperitoneal pentobarbital sodium, and plasma and kidneys were collected.

**Survival studies.** To determine 7-day survival, A1 wild-type (n = 58; 26 females, 32 males), A1 heterozygous (n = 48; 22 females, 26 males), and A1 knockout (n = 45; 20 females, 25 males) littermates were subjected to CLP with a 20-gauge needle. 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 protocol. To examine whether pharmacological intervention affects survival, A1 wild-type (n = 23) and A1 heterozygous (n = 30) mice received a subcutaneous injection (1 mg/kg) of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; a selective A1AR antagonist) 20 min before and 6 h after CLP. To control for any confounding nonspecific effects of DPCPX which may have contributed to the observed mortality, A1 knockout mice (n = 23) also received DPCPX in the manner outlined above and were observed for 7 days.

**Assessment of renal function and hepatic injury after sepsis.** Renal function was assessed by measuring plasma creatinine 24 h after CLP by a colorimetric method based on the Jaffee reaction (18). Hepatic injury 24 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 corticomedullary myeloperoxidase activity (marker of leukocyte infiltration) and by immunoblotting for intercellular adhesion molecule-1 (ICAM-1) as described previously (29). In addition, renal corticomedullary expression of mRNAs encoding proinflammatory markers was also determined using semiquantitative RT-PCR as described previously (29). The proinflammatory mRNA markers studied included: KC, macrophage inflammatory protein 2 (MIP-2), ICAM-1, monocyte chemoattractant protein 1 (MCP-1), TNF-α, interferon-induced protein 10 (IP-10), and regulated on activation normal T cell expressed and secreted (RANTES). For each experiment, we also performed semiquantitative RT-PCR under conditions yielding linear results for GAPDH to confirm equal RNA input between groups. Primers were designed based on published GenBank sequences for mice (Table 1).

**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 (5, 31). Renal corticosterone was dissected and immersed in 500 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 5% 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 into solution and centrifuged at 18,000 g for 5 min at 4°C. The supernatant was discarded and the pellet was 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 electrophoretic mobility shift assay for NF-κB.

**EMS A** was performed using the Gel Shift Assay System (Promega, Madison, WI). The oligonucleotides for NF-κB (Promega) consensus sequences were end-labeled with 10 μCi of [γ-32P]ATP (Perkin Elmer Life Technology, Wellesley, MA) and purified using a G-25 spin column (Amersham Biosciences, Piscataway, NJ). Ten micrometers 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 micrometers of Hela cell nuclear extract (Promega) were used for a positive control and 1 μl of NF-κB p65 TransCruz polyclonal antibody (Santa Cruz Biotechnology) was coincubated with the nuclear protein and probe for a

<table>
<thead>
<tr>
<th>Table 1. Primers used to amplify mRNAs encoding proinflammatory cytokines based on published GenBank sequences for mice</th>
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<tr>
<td><strong>Primer</strong></td>
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<tr>
<td>KC</td>
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<tr>
<td>MIP-2</td>
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<td>ICAM-1</td>
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<td>MCP-1</td>
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<td>TNF-α</td>
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<td>IP-10</td>
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<tr>
<td>RANTES</td>
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<td>GAPDH</td>
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Respective primers were designed based on published GenBank sequences for mice. KC, keratinocyte-derived chemokine; MIP-2, macrophage inflammatory protein 2; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein 1; TNF-α, tumor necrosis factor-α; IP-10, interferon-induced protein 10; RANTES, regulated on activation normal T cell expressed and secreted.
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Protein determination. Protein content was determined with the Pierce Chemical (Rockford, IL) bicinchoninic acid protein assay reagent using bovine serum albumin as a standard.

Statistical analysis. One-way ANOVA was used to compare differences in mean values between treatment groups. Survival statistics were compared with a Kaplan-Meier curve and Log Rank Test. In all cases, a probability value <0.05 was taken to indicate significance.

RESULTS

Endogenous A1AR activation protects against CLP-induced mortality. We initially measured the effect of endogenous A1AR activation on mortality from CLP-induced septic peritonitis by comparing 7-day survival statistics for A1 wild-type, A1 heterozygous, and A1 knockout mice. As demonstrated in Fig. 1A, mice lacking endogenous A1AR (A1 knockout) had significantly higher mortality rates compared with A1 wild-type and A1 heterozygous mice. At 24 h following CLP, the mortality rate for A1 knockout mice was 27% compared with 8 and 3% in A1 heterozygous and A1 wild-type mice, respectively. Furthermore, log rank analysis of the 7-day survival statistics revealed significant differences between A1 wild-type (P<0.0001) and A1 heterozygous (P<0.0002) mice compared with A1 knockout mice. Log rank analysis also demonstrated no statistical differences in sepsis-induced mortality between the A1 wild-type and A1 heterozygous groups, suggesting that the presence of even one-half of the genetic complement of the A1AR gene confers survival benefit.

We are aware that evidence in the literature exists to indicate gender differences may affect outcomes in sepsis studies. In fact, we anticipated that we would see a "protective" benefit in morbidity and mortality in our female mice. However, we were unable to demonstrate statistical differences in mortality or morbidity based solely on gender (Table 2). For this reason, both males and females were used. Nevertheless, we were careful to keep the ratio of males to female as equitable as possible between the study groups.

To examine the effect of pharmacological blockade on A1AR activation in the setting of sepsis, A1 wild-type and A1 heterozygous mice were treated with DPCPX. Selective antagonism of A1AR resulted in an adverse shift of both A1 wild-type and A1 heterozygous survival curves (Fig. 1B) mimicking the survival observed with A1 knockout mice. A1 knockout mice treated with DPCPX did not demonstrate a change in sepsis-induced mortality compared with A1 knockout non-treated controls (Fig. 1B).
**A1 knockout mice show significantly increased CLP-induced organ injury and dysfunction.** We examined the degree of renal dysfunction in A1 wild-type, A1 heterozygous, and A1 knockout mice by measuring plasma creatinine (Cr) and the degree of hepatic injury by measuring ALT and AST 24 h after CLP. Twenty-four hours after sham operation plasma creatinine, ALT or AST values in A1 heterozygous and A1 knockout were not different from A1 wild-type values (A1 wild-type Cr: 0.3 ± 0.1 mg/dl, n = 5; A1 wild-type ALT: 20 ± 1.9 SF U/ml, n = 8; A1 wild-type AST: 91 ± 10.6 SF U/ml, n = 8). Creatinine, ALT, and AST significantly increased at 24 h after CLP in all three groups of mice. However, A1 knockout mice showed significantly worse renal dysfunction and hepatic injury compared with A1 wild-type mice at 24 h after CLP (Table 3). A1 heterozygous mice exhibited creatinine, ALT, and AST values that were intermediate between values of A1 wild-type and A1 knockout mice at 24 h after CLP (Table 3).

A1 knockout mice show increased sepsis-induced plasma proinflammatory cytokine levels. With ELISA, TNF-α and KC plasma levels in all three groups of mice undergoing CLP were elevated above normal baseline values (which are typically <18.2 pg/ml, below the sensitivity of the ELISA). However, A1 knockout mice showed significantly elevated TNF-α plasma levels compared with both A1 wild-type and A1 heterozygous mice 24 h after the induction of sepsis (Table 3). KC plasma levels were also elevated above control values (374 ± 65 pg/ml, n = 14). Similarly, A1 knockout mice showed significantly elevated KC plasma levels compared with A1 wild-type mice (Table 3). A1 heterozygous mice showed intermediate KC plasma levels (Table 3).

Renal myeloperoxidase activity 24 h following CLP is increased in A1 knockout mice. Myeloperoxidase (MPO) is an enzyme present in leukocytes and is an index of tissue leukocyte infiltration following injury (42). Because activated leukocyte infiltration is a hallmark of acute inflammation, we sought to determine the effect of endogenous A1AR activation on renal MPO activity 24 h following CLP-induced sepsis. Mice lacking endogenous A1ARs showed significantly higher MPO activity than both A1 wild-type and A1 heterozygous mice 24 h after the induction of sepsis (Table 3).

**Renal mRNA and protein expression of proinflammatory markers following CLP is increased in A1 knockout mice.** We next examined the effects of endogenous A1AR activation on mRNA expression in renal cortices following CLP-induced sepsis. A1 wild-type, A1 heterozygous, and A1 knockout mice had similar levels of KC, RANTES, and IP-10 24 h following sham operation (data not shown). In contrast, A1 knockout mice demonstrated increased mRNA expression of KC, RANTES, and IP-10 compared with A1 wild-type mice 24 h after the induction of sepsis (Fig. 2). We observed no significant differences at the level of mRNA expression for MIP-2, ICAM-1, MCP-1, and TNF-α between groups (data not shown). In a separate set of experiments, immunoblotting performed for ICAM-1 protein from dissected renal cortices taken 24 h following CLP demonstrated ICAM-1 protein expression was significantly higher in the A1 knockout group vs. the A1 wild-type group (P < 0.05; Table 3). A1 heterozygous mice showed intermediate ICAM-1 protein expression.

A1 knockout mice demonstrate increased CLP-induced NF-κB nuclear translocation after sepsis. Renal cortices isolated from A1 wild-type, A1 heterozygous, and A1 knockout mice had similar levels of NF-κB nuclear translocation 24 h following sham operation (data not shown). In contrast, renal cortices from A1 knockout mice exhibit increased NF-κB nuclear translocation 24 h following the induction of sepsis compared with cortices isolated from A1 wild-type mice (Fig. 3). Renal cortices isolated from A1 heterozygous mice 24 h after CLP showed NF-κB nuclear translocation that was intermediate between the levels seen in A1 wild-type and A1 knockout mice (Fig. 3). Preliminary studies demonstrated that the NF-κB-specific band supershifted with a p65 antibody (data not shown).

**Renal tubular necrosis or apoptosis following CLP is not increased in A1 knockout mice.** Virtually no necrosis was detected in the kidney 24 h after CLP in A1 knockout, A1

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### Table 3. Markers of organ injury and dysfunction, markers of systemic inflammation, and markers of renal inflammation 24 h following induction of sepsis

<table>
<thead>
<tr>
<th>Test</th>
<th>Markers of Organ Injury</th>
<th>Markers of Systemic Inflammation</th>
<th>Markers of Renal Inflammation</th>
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<tr>
<td></td>
<td>A1 wild-type</td>
<td>A1 heterozygous</td>
<td>A1 knockout</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.63±0.04 (23)*</td>
<td>0.76±0.10 (20)*</td>
<td>1.24±0.11 (21)</td>
</tr>
<tr>
<td>AST, SF U/ml</td>
<td>174±13 (23)*</td>
<td>253±35 (22)</td>
<td>308±38 (14)</td>
</tr>
<tr>
<td>ALT, SF U/ml</td>
<td>59±5 (23)*</td>
<td>82±15 (22)</td>
<td>94±13 (13)</td>
</tr>
<tr>
<td>TNF-α ELISA, pg/ml</td>
<td>1,309±272 (7)*</td>
<td>1,438±242 (6)*</td>
<td>4,424±771 (6)</td>
</tr>
<tr>
<td>KC ELISA, pg/ml</td>
<td>38,225±10,262 (9)*</td>
<td>48,139±18,668 (7)</td>
<td>91,803±5,725 (5)</td>
</tr>
<tr>
<td>MPO assay, ΔOD-min⁻¹mg protein⁻¹</td>
<td>0.03±0.023 (7)*</td>
<td>0.13±0.08 (4)</td>
<td>0.57±0.18 (4)</td>
</tr>
<tr>
<td>ICAM-1 protein expression, % of A1 WT</td>
<td>100±8 (9)*</td>
<td>172±36 (8)</td>
<td>224±61 (8)</td>
</tr>
</tbody>
</table>

Results are expressed as average ± SE (n). AST, aspartate aminotransferase; ALT, alanine aminotransferase; SF, Sigma-Frankel; WT, wild-type. *P < 0.05 vs. A1 knockout.
heterozygous, or A1 wild-type mice. Only occasional single-cell necrosis was observed in high-power fields with H&E staining. The Jablonski grading scale for necrosis in A1 wild-type mice subjected to CLP was 0\$/H_{1001}/0, 1\$/H_{1005}/0 and in A1 knockout mice was 0\$/H_{1001}/0.2, 1\$/H_{1005}/0.4. We failed to detect TUNEL-positive cells in kidney sections from sham-operated A1 wild-type or A1 knockout mice (Fig. 4, A and B). Twenty-four hours after CLP A1 wild-type (Fig. 4C) and A1 knockout (Fig. 4D) mice showed near equivalent numbers of TUNEL-positive cells in the corticomedullary junction. Although all mice subjected to CLP demonstrated DNA laddering in DNA isolated from renal cortices, there were no differences between A1 wild-type or A1 knockout mice (data not shown).

**DISCUSSION**

The major findings of the present study are that mice lacking endogenous A1AR demonstrate increased mortality, increased acute renal and hepatic injury, and increased inflammation associated with murine septic peritonitis induced by cecal ligation and puncture. Moreover, blockade of endogenous A1ARs in A1 wild-type mice converted the A1 wild-type response to an A1 knockout response. A1 wild-type and A1 knockout mice had similar degrees of renal tubular apoptosis with sepsis.

We previously demonstrated that modulation of renal adenosine receptors can result in powerful protection against acute renal cell injury in vivo (rats and mice) and in vitro (human proximal tubule cells) (23–27). We showed that A1AR activation protects against ischemia- and reperfusion-induced renal failure via mechanisms involving Gi, PKC, and ERK MAPK signaling pathways. Moreover, we demonstrated that A1 knockout mice had increased renal failure and inflammation following ischemia-reperfusion injury. This suggests that the A1AR imparts a protective benefit by reducing inflammation after ischemia-reperfusion injury. Because a hyperactive inflammatory response contributes to the development of multiorgan injury and dysfunction in sepsis, we hypothesized that mice lacking A1ARs would have worse outcomes compared with A1AR wild-type mice.

In animal models, potentiating adenosine effects (by either administration of adenosine receptor agonists or inhibiting reuptake mechanisms) have been shown to improve outcomes
in sepsis (1, 12, 34, 37). The A2aAR is known to have anti-inflammatory effects and therefore has been an adenosine receptor subtype evaluated for its potential role in sepsis. Sullivan et al. (37) in an in vivo endotoxemia model of sepsis demonstrated increased survival mediated by ATL146e through the A2aAR. However, not all of adenosine’s protective effects can be attributable to activation of the A2aAR. For example, Hasko et al. (17) found that adenosine is capable of inhibiting TNF-α and IL-12 production in A2a knockout mice, suggesting that other adenosine receptor subtypes may also mediate anti-inflammatory effects in sepsis. To date, no study has addressed the role that endogenous A1AR activation may play in 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 (35). Another advantage of this septic model is the ability to manipulate the magnitude of the inflammatory response by modulating the needle gauge used for puncture. We employed a 20-gauge needle to induce in our A1 wild-type mice a level of sepsis with moderate predilection for mortality and organ injury in the absence of any interventions. Given that our central hypothesis was to demonstrate the effect of endogenous A1AR activation on outcomes in murine sepsis, we chose not to possibly confound our results by introducing antibiotics into our studies. In fact, we were able to demonstrate differences in survival and morbidity without

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Fig. 4. Representative fluorescent photomicrographs of kidney sections from identical fields illustrate apoptotic nuclei (left, TUNEL fluorescent stain) or total nuclei (right, Hoescht 33342 fluorescent stain). Sham-operated A1 wild-type mice (A) and sham-operated A1 knockout mice (B) showed no apoptotic nuclei. C. A1 wild-type mice subjected to CLP showed TUNEL-positive cells at 24 h. D: A1 knockout mice subjected to CLP showed similar degrees of TUNEL-positive cells at 24 h. Corticomedullary areas are shown (magnification of ×200) and arrows indicate TUNEL-positive cells. Photomicrographs are representative of 4 independent experiments for each group of animals.
having to manipulate survival outcomes with antibiotic administration.

The sepsis model of CLP has two distinct phases. An early hyperdynamic stage is characterized by increased cardiac output, tissue perfusion, and decreased vascular resistance, whereas the later hypodynamic stage is characterized by decreased cardiac output and tissue perfusion (39–41). At 2–10 h following CLP, the hyperdynamic phase predominates, which then transitions into the later hypodynamic phase (38). By 20 h after CLP, decreased tissue perfusion as well as direct inhibition of mitochondrial respiration by bacterial toxins contribute to tissue hypoxia. In this milieu, evidence exists that increased ATP breakdown and hypoxia-dependent inhibition of adenosine kinase can drive the accumulation of adenosine (10). In fact, elevated plasma and cerebrospinal fluid levels of adenosine have been reported in patients with severe sepsis (30). Adenosine has been coined as a “retaliatory molecule” due, in part, to its abundant overexpression during cellular stress and its ability to provide protection in the setting of tissue and cellular injury. Under normal conditions, extracellular adenosine levels are tightly held at 30–300 nM, but during episodes of hypoxia these levels can rise to ~10 μM (15, 43). In one study, severe septic patients’ adenosine levels were ~4 μM, and as such, are well within the binding affinity range for A1ARs. Elevated adenosine levels during sepsis may therefore serve to protect the host via activation of A1ARs.

Given the high mortality associated with sepsis (which ranges between 30 and 60%), therapeutic effects are primarily judged by their impact on survival (3, 8). Therefore, we first sought to determine whether survival differences would exist between mice lacking endogenous A1AR and their A1AR wild-type littermates in response to a septic insult. By 24 h following CLP, we observed a greater level of mortality and morbidity in the A1 knockout mice. Furthermore, log rank analysis of these 7-day survival studies demonstrated a significant survival benefit for both the A1 wild-type and A1 heterozygous vs. the A1 knockout mice.

Because compensatory physiological changes are inherent concerns with studies using knockout mice, we included a selective A1AR blocking agent to illustrate that the results we observed in our A1 knockout mice can be demonstrated in A1 wild-type mice treated with an A1AR antagonist. By using both models, we provide conclusive data that endogenous A1AR activation serves protective functions in CLP-induced sepsis. Selective blockade of A1AR activation on sepsis-induced mortality was assessed following administration of DPCPX. The effect of DPCPX in the setting of sepsis was a profound elimination of the survival advantage observed in both A1 wild-type and A1 heterozygous groups. However, A1 knockout mice administered DPCPX failed to demonstrate a change in survival, suggesting that the effect was indeed specific to A1AR blockade. These results support the hypothesis that endogenous A1AR activation has a protective role in sepsis and can lead to increased survival.

It is well known that mortality in sepsis is strongly influenced by the development of organ injury and dysfunction (6, 7, 9, 36). In concordance, our study showed that the lack of endogenous A1AR not only increased CLP-induced mortality, but it also significantly increased the magnitude of CLP-induced renal and hepatic injury.

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., IL-8). In the present study, the inflammatory processes elicited during CLP-induced sepsis contributed to greater organ injury and dysfunction observed in the A1 knockout group as they do not possess the counterbalancing anti-inflammatory benefits afforded by endogenous A1AR activation. This is supported by the findings that TNF-α levels increase in the plasma by 24 h following CLP; however, TNF-α levels in A1 knockout mice were significantly elevated compared with A1 wild-type mice. This observation correlates well with studies performed by Meldrum et al. (32), which demonstrated that adenosine was capable of attenuating TNF-α levels in a cardiac ischemia-reperfusion model. Given the diminution of TNF-α levels in A1 wild-type mice compared with A1 knockout mice, our results draw into question whether this phenomenon is actually mediated via A1AR-related mechanisms. Murine KC is a proinflammatory chemokine that putatively represents the functional homolog 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 (19). We also demonstrated an increase in plasma KC levels in the A1 knockout mice compared with A1 wild-type mice, providing further evidence that endogenous A1AR activation serves an anti-inflammatory and protective role during sepsis.

Further evidence that the A1AR contributes to decreased inflammation is the finding that increased mRNA encoding KC, RANTES, and IP-10 is found in the kidneys of A1 knockout mice 24 h following CLP compared with A1 wild-type mice. Similarly, protein levels of ICAM-1 and MPO were elevated in kidneys of A1 knockout mice 24 h following CLP compared with A1 wild-type mice.

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 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 increased NF-κB expression is predictive of poor prognosis in sepsis (4). In other models of sepsis, suppression of NF-κB activation decreased acute inflammatory processes and organ dysfunction (31). Further evidence for the anti-inflammatory role of endogenous A1AR activation in sepsis is demonstrated in the present study by the significant increase in activation of NF-κB shown in A1 knockout mice compared with A1 wild-type mice in response to sepsis, which underscores the potential signaling pathways by which A1AR may be exerting its protective effects in sepsis.

In conclusion, we demonstrate that endogenous A1AR activation provides protection from CLP-induced mortality and acute organ dysfunction. Because the pathogenesis of organ dysfunction in sepsis is largely mediated by an imbalanced inflammatory response, A1AR activation improves organ function after septic insult by attenuating this hyperinflammatory process. This attenuation is global in nature and culminates in a reduction in organ injury and thereby protects from progression to multiple organ dysfunction. We speculate that endogenous A1AR activation exerts cytoprotective mechanisms that counteract inflammation and injury in CLP-induced sepsis.
Given the protective benefit of the A1AR on survival and organ dysfunction, our findings may have important future therapeutic implications for patients in sepsis.

GRANTS

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