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Adenosine A$_{2A}$ Receptor Inactivation Increases Survival in Polymicrobial Sepsis$^1$

Zoltán H. Németh,* Balázs Csóka,* Jeanette Wilmsk,* DaZhong Xu,* Qi Lu,* Catherine Ledent,† Edwin A. Deitch,* Pál Pacher,‡ Zoltán Spolarics,* and György Haskó$^{2*}$

The mechanisms governing the impairment of bacterial clearance and immune function in sepsis are not known. Adenosine levels are elevated during tissue hypoxia and damage associated with sepsis. Adenosine has strong immunosuppressive effects, many of which are mediated by A$_{2A}$ receptors ($A_{2A}R$) expressed on immune cells. We examined whether $A_{2A}R$ are involved in the regulation of immune function in cecal ligation and puncture-induced murine polymicrobial sepsis by genetically or pharmacologically inactivating $A_{2A}R$. $A_{2A}R$ knockout (KO) mice were protected from the lethal effect of sepsis and had improved bacterial clearance compared with wild-type animals. cDNA microarray analysis and flow cytometry revealed increased MHC II expression in $A_{2A}$-inactivated mice, suggesting improved Ag presentation as a mechanism of protection. Apoptosis was attenuated in the spleen of $A_{2A}$ KO mice indicating preserved lymphocyte function. Levels of the immunosuppressive cytokines IL-10 and IL-6 were markedly lower following $A_{2A}R$ blockade. Similar to observations with $A_{2A}$ KO mice, an $A_{2A}R$ antagonist increased survival even when administered in a delayed fashion. These studies demonstrate that $A_{2A}R$ blockade may be useful in the treatment of infection and sepsis. The Journal of Immunology, 2006, 176: 5616–5626.

Sepsis remains the leading cause of morbidity and mortality in critically ill patients with an annual incidence of $\sim$750,000 patients in the United States. Although the treatment of primary infections per se is well-established, $\sim$210,000 deaths per year occur as a result of residual sepsis and multiple organ dysfunction (1, 2). Current treatment options for residual sepsis are mainly supportive, largely because of a failure to fully understand the pathophysiology of this complex and heterogeneous response.

Previous evidence suggested that residual sepsis after control of the primary infection represents the massive uncontrolled inflammatory response of the host to the invading microorganisms (systemic inflammatory response syndrome) (3, 4). In light of the recent failure of clinical trials using anti-inflammatory strategies and with more data accumulating on the immune status of patients with sepsis together with new evidence from more sophisticated animal models of sepsis, it has become clear that the pathophysiology of sepsis is often more complex than can be explained by the systemic inflammatory response syndrome hypothesis (5, 6). Current concepts suggest that even where sepsis causes an initial intense proinflammatory response, this initial hyperimmune or proinflammatory state evolves into a hypoimmune or immune paralytic state in most patients. In fact, the subsequent inability to kill secondary invading pathogens effectively due to immunosuppression is a major cause of late organ dysfunction syndrome (7, 8). Potential mechanisms of immune suppression after a septic insult include decreased phagocytosis of microorganisms by macrophages and neutrophils, immune cell apoptosis, decreased Ag presentation, as well as imbalances in cytokine production (5, 6, 9–11). The mechanisms underlying these immune functional abnormalities are largely unknown.

Adenosine, an endogenous purine nucleoside, is a biologically active extracellular signaling molecule that is formed at sites of metabolic stress associated with hypoxia, ischemia, trauma, or inflammation. Because sepsis can be associated with any of these metabolically stressful conditions, it is not surprising that systemic adenosine levels reach high concentrations in patients with sepsis and septic shock (12–14). Adenosine interacts with one or more of four G-protein-coupled receptors ($A_1$, $A_{2A}$, $A_{2B}$, and $A_3$) through which it can exert substantial anti-inflammatory and immunosuppressive effects (15–22). The most potent anti-inflammatory and immunosuppressive effects of adenosine are attributed to occupancy of $A_{2A}$ receptors ($A_{2A}R$) expressed on APCs (23–25) as well as lymphocytes (26, 27). Activation of $A_{2A}R$ reproduces many phenotypic changes in immune cells that are characteristic of the late “immunoparalytic” phase of sepsis. $A_{2A}R$ stimulation diminishes phagocytosis (28, 29), augments secretion of anti-inflammatory cytokines (30–32), and induces lymphocyte apoptosis (33–35). Thus, we hypothesized that adenosine might contribute to the sepsis-induced onset of immune paralysis via occupancy of $A_{2A}R$ expressed on immune cells. To address this hypothesis, we evaluated whether targeted genetic deletion or pharmacological inactivation of $A_{2A}R$ would reverse the immune-compromised phenotype of septic mice using the clinically relevant cecal ligation and puncture (CLP)$^3$ model of sepsis.

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$^3$Abbreviations used in this paper: CLP, cecal ligation and puncture; KO, knockout; WT, wild type; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; PARP, poly(ADP-ribose) polymerase.

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Materials and Methods

Experimental animals

The A$_2$AR knockout (KO) mice used in the present study (36) were bred on a CD-1 background in a specific pathogen-free facility, using founder heterozygous male and female mice. All mice were maintained in accordance with the recommendations of the “Guide for the Care and Use of Laboratory Animals”, and the experiments were approved by the New Jersey Medical School Animal Care Committee. Wild-type (WT) and KO littermates of heterozygous parents were used exclusively in all studies. At weaning, a 0.5-cm tail sample was removed for the purpose of DNA collection for genotyping. Genotyping using RT-PCR was performed as described previously (36).

For pharmacological studies with the selective A$_2$AR antagonist 4-[2-(7-amino-2-(2-furyl)-1H-[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino)ethyl](Z)-3-(2H-1,2,4-triazol-2-yl)propyl)benzene (ZM241385; Tocris Cookson), male CD-1 mice were used. [7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385; Tocris Cookson), male CD-1 mice were used

Cecal ligation and puncture

Polymeric salpinx was induced by subjecting mice to CLP, as we have described previously (37), with some modifications. Six- to 8-week-old male A$_2$AR KO or WT mice were anesthetized with Nembutal (80 mg/kg), given i.p. Under aseptic conditions, a 2-cm midline laparotomy was performed to allow exposure of the cecum with adjoining intestine. Approximatively two-thirds of the cecum was tightly ligated with a 3.0 silk suture, and the ligated part of the cecum was perforated twice (through and through) with a 20-gauge needle (BD Biosciences). The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites. The cecum was then returned to the peritoneal cavity and the laparotomy closed in two layers with 4.0 silk sutures. Sham-operated animals underwent the same procedure without ligation or puncture of the cecum. The mice were resuscitated with 1 ml of physiological saline injected s.c. and were returned to their cages with free access to food and water. One group of mice was monitored daily and survival was recorded for 10 days. Another group of mice was reanesthetized with Nembutal (80 mg/kg i.p.) 16 or 48 h after the operation, and blood, peritoneal lavage fluid, and various organs were harvested as described below.

The effect of pharmacological inactivation of A$_2$AR in mice subjected to CLP was evaluated using CD-1 mice in a similar fashion to that described for the A$_2$AR KO or WT mice. In this set of experiments, the mice were injected immediately before or 2 h after the operation and every 12 h thereafter with ZM241385 (15 mg/kg s.c.) or its vehicle (DMSO).

Collection of blood, peritoneal lavage fluid, and organs

Blood samples were obtained aseptically by cardiac puncture using heparinized syringes after opening the chest and placed on ice into heparinized Eppendorf tubes until further processing for hemological and bacteriological analysis. Aliquots of whole blood were analyzed for hemology by flow cytometry (CELL-DYN 3200 System; Abbott Laboratories) in a centralized facility. After serial dilutions for bacteriological analysis were made (see below), the blood was centrifuged at 2000 × g for 10 min and the recovered plasma was stored at −70°C until further use. For peritoneal lavage, the abdominal skin was cleansed with 70% ethanol and the abdominal wall was exposed by opening the skin. Four milliliters of sterile physiological saline were then instilled into the peritoneal cavity via an 18-gauge needle. The abdomen was massaged gently for 1 min while keeping the tip of the needle in the peritoneum, after which peritoneal fluid was recovered through the needle. Recovered peritoneal lavage fluid was placed on ice until processed for bacteriological examination. After serial dilution the peritoneal lavage fluid to determine CFU numbers (see below), the peritoneal lavage fluid was centrifuged at 5000 × g for 10 min and the supernatant was stored at −70°C until further analysis. Samples from spleen, thymus, lung, kidneys, and liver were excised and either immediately frozen in liquid nitrogen or placed in 10% paraformaldehyde for subsequent histological analysis. Snap-frozen tissue samples were transferred to a −70°C refrigerator until analyzed for gene expression and apoptotic markers.

Quantification of bacterial CFUs from peritoneal lavage fluid and blood

One hundred microliters of blood or 10 μl of peritoneal lavage fluid was diluted serially in sterile physiological saline. Ten microliters of each dilution was aseptically plated and cultured on tryptose blood agar plates (BD Biosciences) at 37°C. After 24 h, the number of bacterial colonies was counted. Quantitative cultures are expressed as CFUs per milliliter of blood or peritoneal lavage fluid.

Determination of cytokine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) levels

Concentrations of IL-10, IL-6, IL-12 p70, TNF-α, and MIP-2, in plasma or peritoneal lavage fluid were determined using commercially available ELISA kits (R&D Systems) and according to the manufacturer’s instructions. The lower detection limit for all these cytokines was 10 pg/ml. Plasma concentrations of AST, ALT, and BUN were analyzed using standard laboratory procedures.

Western blot analysis for markers of apoptosis

Samples of spleen and thymus were homogenized in a Dounce homoge- nizer in modified radioimmunoprecipitation assay buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM Na$_3$VO$_4$). The lysates were transferred to Eppendorf tubes and centrifuged at 15,000 × g for 15 min, and the supernatant was recovered. Protein concentrations were determined using the Bio-Rad protein assay kit. A total of 30–40 μg of sample was separated on 8–16% Tris-glycine gel (Invitrogen Life Technologies) and transferred to nitrocellulose membrane. The membranes were probed with polyclonal rabbit anti-cleaved caspase-3 (Cell Signaling Technology; no. 9661S), polyclonal rabbit anti-cleaved poly-(ADP-ribose) polymerase (PARP; Cell Signaling Technology; no. 9544S), or polyclonal goat anti-pi-actin Ab (Santa Cruz Biotechnology; sc-1615), and subsequently incubated with a secondary HRP-conjugated anti-rabbit or anti-goat Ab (Santa Cruz Biotechnology). Bands were detected using ECL Western Blotting Detection Reagent (Amersham Biosciences).

Apoptosis detection by TUNEL

Paraffin blocks containing spleen tissue specimens were cut in 5-μm thick sections and the sections were processed and stained for the detection of apoptosis using the TACS In Situ Apoptosis Detection kit (TACS Klenow; diaminobenzidine) obtained from Trevigen, according to the manufacturer’s instructions. When viewed under a standard light microscope, apoptotic nuclei can be clearly distinguished by brown staining. Quantification of the number of apoptotic cells was performed using an Olympus IX71 microscope, as we have previously described (38). In total, 6,600, 21,440, and 29,655 cells were examined in spleens of control (n = 3), WT-CLP (n = 6), and KO-CLP (n = 6) groups, respectively. The results are expressed as the percent of TUNEL-positive cells, relative to the number of total cells counted in spleen sections.

Flow cytometry determinations for detection of thymocyte apoptosis and MHC II expression on splenic and peritoneal macrophages

To quantitate thymocyte apoptosis, tissue sections from thymi were gently glass-ground to dissociate cells. Tissue debris was then removed from cell suspensions using a 70-μm nylon cell strainer (Falcon; BD Biosciences) and the cells were washed twice and then resuspended in ice-cold PBS. The degree of apoptotic cell death was quantified using a commercially available, fluorescein-labeled annexin V-containing kit (Annexin V/FITC Apo- ptosis Detection Kit I; BD Biosciences). Thymocytes (3 × 10$^6$) were stained with FITC-labeled Annexin V and propidium iodide according to the manufacturer’s instructions. Cells were analyzed in a centralized laboratory using a FACScan Flow Cytometer equipped with a 488-nm laser, 530/30- and 585/42-nm band pass filters, and a 650-nm long-pass filter (BD Biosciences). Instrument calibration was performed daily using Calibrite Beads (BD Biosciences) and also by sphere beads (Spherotech) using target channel values for each of the assays used in the study. Data were analyzed using Cytomation Summit computer software. Electronic compensation of the instrument was conducted to exclude overlapping of the two emission spectra. Cell counts in regions of doublets for annexin V− positive only, propidium iodide-positive only, double-positive, and double-negative were determined and compared.

MHC II expression on splenic and peritoneal macrophages was also determined using flow cytometry (39). Macrophages were identified using PE-labeled anti-mouse F4/80 Abs (eBioscience). MHC II expression was determined using anti-mouse allophycocyanin-labeled MHC II Ab (eBioscience). Cell suspensions from peritoneal lavage and spleen were added to tubes preloaded with the corresponding fluorescent-labeled Abs. After gentle mixing, the tubes were kept at room temperature in the dark for 15 min. RBCs were then lysed with 2.0 ml of FACS Lysing Solution (BD Bio- sciences). After two washes, cells were fixed in 0.3 ml of 3% formaldehyde and kept at 4°C in the dark until acquisition. Analyses were performed.
Affymetrix GeneChip analysis of spleen samples and RT-PCR

RNA isolation, cDNA synthesis, and cRNA transcription were performed, as previously described (40). cRNA was hybridized to Affymetrix murine microarrays which contain probe sets for the whole mouse genome. Hybridization, scanning, and data analysis were performed at the Affymetrix GeneChip Core Facility in the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (technical details are available at [http://info.med.yale.edu/wmkeck/affymetrix/](http://info.med.yale.edu/wmkeck/affymetrix/)). Differentially expressed genes were identified by the Biostatistics Resource Laboratory at the W. M. Keck Foundation by comparing data from spleens taken from CLP-induced A2A WT and KO mice 16 h after the operation (n = 3/group). RT-PCR for IL-10, IL-6, MIP-2, and 18S was conducted as described previously (40) and using the following primers: IL-10, 5'-AAGGAGTTTAACTTCAAGGGCT(antisense) and 5'-AAGGGTTACTTGGGTTGGA-3'; IL-6, 5'-GCTTGGCTAATTTATAACATTTTATACAAACTG(antisense) and 5'-TGGTCTTGGAAGGAGAGCTA(antisense); MIP-2, 5'-ATGGCCCTGTCACCTGTTTC(antisense) and 5'-CGTCTGTGTTTCATGTGTTTGA-3'; and 18S, 5'-GCCACTACGCTTGCTTCGCT-3' (sense) and 5'-CGCAGGCTGTATACCG-3' (antisense).

**Statistical analysis**

Survival curves were analyzed using the two-tailed Fisher’s exact test. Two-tailed t testing was used to compare cytokine concentrations, CFUs, and other laboratory parameters. Statistical significance was assigned to p values smaller than 0.05.

**Results**

Genetic A2AR deficiency protects against CLP-induced mortality

Control (WT) mice had a mortality rate of ~70% when recorded on day 5 after the CLP procedure (Fig. 1). This mortality rate was the result of a gradual process, which was characterized by 10–20% of the mice dying every day. No changes in mortality were detected when the mice were followed for an additional 5 days (data not shown). The mortality rate of A2A KO mice was significantly lower on each day with a 35% mortality rate on day 5 after CLP (Fig. 1). There were no additional deaths in this group until the termination of the experiment (10 days after the surgery, data not shown).

A2AR deficiency improves bacterial clearance

Because persistence of local bacterial infection and bloodstream invasion play important roles in mortality in the CLP model, we next assessed the impact of A2AR inactivation on bacterial levels at the primary peritoneal site of infection and in the bloodstream. We found markedly decreased numbers of bacteria in both the blood and peritoneal lavage fluid of A2AR KO mice when compared with WT animals at 16 h (Fig. 2, A and B). Bacterial numbers fell substantially by 48 h after surgery in both the blood and peritoneal lavage fluid and there were no differences in CFUs between A2A KO and WT mice at this point (Fig. 2, C and D).
and peritoneal lavage fluid remained sterile in sham-operated A2AR KO and WT mice (data not shown).

Effect of genetic A2AR inactivation on cytokine production and markers of organ injury

Because IL-10 appears to be an essential mediator in sepsis-induced impairment in antibacterial host defense (41–44), we compared IL-10 concentrations in the plasma and peritoneal lavage fluid obtained from A2AR KO and WT mice subjected to CLP or sham operation. Sham-operated A2AR WT or KO mice had no detectable levels of IL-10 in their plasma or peritoneal lavage fluid (data not shown). While CLP elevated IL-10 concentrations in both the plasma and peritoneal lavage fluid in both A2AR KO and WT mice, A2A KO mice exhibited markedly lower levels of IL-10.
at 16 h after the CLP procedure (Fig. 3A). IL-10 concentrations subsided to comparable levels in septic A2AR KO and WT mice by 48 h (Fig. 3B).

Because IL-6 blockade with neutralizing Abs has been shown to be protective in CLP-induced sepsis (45), we next explored the role of A2AR in regulating IL-6 production during sepsis. Although IL-6 levels in sham-operated A2AR WT and KO mice were low and comparable between the two groups (38 ± 20 pg/ml in the WT vs 19 ± 0.6 pg/ml in the KOs for the peritoneal lavage fluid and 2.53 ± 0.01 ng/ml in the WT vs 2.53 ± 0.03 ng/ml in the KOs for the plasma), CLP-induced levels of IL-6 were significantly and markedly higher in the peritoneal lavage fluid but not plasma of A2AR WT mice than in the A2A KO animals (Fig. 3C). IL-6 concentrations decreased by 48 h after the CLP procedure and no differences were seen in IL-6 concentrations between the A2A KO and WT mice at this point (Fig. 3D).

To investigate whether A2AR deficiency altered the formation of classical proinflammatory cytokines, we next determined concentrations of TNF-α, IL-12 p70, and MIP-2 in both the plasma and peritoneal lavage fluid. We found that concentrations of IL-12 p70 and TNF-α were below the detection limit for our assays in all groups of mice, including sham- and CLP-operated A2AR WT and KO mice (data not shown). Although MIP-2 was not detectable in sham-operated WT or KO animals (data not shown), CLP-induced concentrations of MIP-2 were diminished in A2A KO mice as compared with their WT counterparts when measured at 16 h (Fig. 3E) but not at 48 h (Fig. 3F).

CLP induced an increase in markers of kidney (BUN) and liver (AST and ALT) injury, when compared with sham-operated animals (Table I). Additionally, white blood cell counts, lymphocyte numbers, and platelet counts dropped significantly in CLP-subjected mice when compared with shams (Table I). However, there were no differences in the levels of these markers or hemological parameters between the WT and KO groups either at 16 (Table I) or 48 h (data not shown) after the CLP procedure.

### Apoptotic markers in lymphoid organs of A2AR KO and WT mice undergoing CLP

Increasing evidence shows that widespread lymphocyte depletion induced by apoptosis may contribute to the immunosuppression that occurs in sepsis. In addition, A2AR activation has been reported to induce lymphocyte apoptosis (33–35). Previous studies have documented that the cleavage/activation of caspase-3 is an important early indicator of apoptosis in the spleen (46) and thymus (47) of animals subjected to CLP-induced sepsis. PARP is a major downstream target of activated caspase-3 and is cleaved by this enzyme during apoptosis (48). Therefore, we tested the hypothesis that A2AR deficiency would prevent the cleavage of caspase-3 and PARP in the spleen and thymus of mice subjected to CLP. We found that 16 h after the onset of sepsis, WT mice exhibited substantial cleavage of caspase-3 and PARP (Fig. 4). In contrast, the cleavage of both caspase-3 and PARP was markedly suppressed in A2AR KO mice (Fig. 4, A–D). These indicators of apoptosis were absent in both A2AR WT and KO mice at 48 h, as well as in sham-operated mice (data not shown).

Caspase-3 activation leads to the appearance of late apoptotic signs, such as phosphatidylserine exposure on the outer cell membrane (48). We next examined whether the decreased caspase-3 cleavage/activation in thymus of A2AR KO mice translated into decreased phosphatidylserine exposure 16 h after the onset of sepsis. Using FITC-labeled Annexin V staining and flow cytometry of thymocytes, we found that CLP significantly up-regulated phosphatidylserine exposure on thymocytes from both A2AR KO and WT animals (Fig. 4G). Although, thymocytes from KO animals exhibited 34% lower phosphatidylserine exposure than those from WT animals, this difference did not reach statistical significance (p = 0.116; Fig. 4G).

Because phosphatidylserine exposure is only marginally detectable in the spleen of mice that have undergone CLP (9), we used TUNEL immunohistochemistry to quantify late apoptotic events in septic A2AR KO and WT animals. The percentage of TUNEL-positive cells in spleens of nonseptic control mice was very low 0.13 ± 0.13% (n = 3; mean ± SEM) (Fig. 4H). CLP significantly increased the fraction of TUNEL-positive cells in WT mice to 6.59 ± 1.32% (n = 6, p < 0.001). The percentage of TUNEL-positive cells in spleens of KO mice exposed to CLP was significantly lower (4.08 ± 0.72%; n = 6, p < 0.05) (Fig. 4H).

### Splenic gene expression profile in septic A2AR KO vs WT mice

To further assess the potential cellular and molecular mechanisms that are associated with the decreased mortality of A2AR KO vs WT mice during sepsis, we compared splenic gene expression profiles in these animals. We used oligonucleotide microarray analysis using Affymetrix chips representing the entire mouse genome. There were ~330 genes that were significantly up-regulated and nearly 700 genes that were down-regulated in A2AR KO vs WT mice at least 2-fold (supplemental data). Many of these differentially expressed genes were classified into multiple biological process categories as a result of their biological complexity (gene ontology-supplemental data).

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Table I. Laboratory markers in A2A KO and WT mice 0, 16, and 48 h after cecal ligation puncture

<table>
<thead>
<tr>
<th>Laboratory Parameter</th>
<th>WT (Sham)</th>
<th>KO (Sham)</th>
<th>WT (16 h after CLP)</th>
<th>KO (16 h after CLP)</th>
<th>WT (48 h after CLP)</th>
<th>KO (48 h after CLP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells/µl</td>
<td>3010 ± 105</td>
<td>3340 ± 675</td>
<td>1027 ± 269*</td>
<td>890 ± 138*</td>
<td>1223 ± 215*</td>
<td>1790 ± 310*</td>
</tr>
<tr>
<td>Lymphocytes/µl</td>
<td>930 ± 134</td>
<td>1610 ± 585</td>
<td>85 ± 0.25</td>
<td>9.70 ± 0.19</td>
<td>9.3 ± 0.2</td>
<td>9.12 ± 0.22</td>
</tr>
<tr>
<td>RBC (million/µl)</td>
<td>8.5 ± 0.25</td>
<td>8.9 ± 0.25</td>
<td>405 ± 60*</td>
<td>405 ± 60*</td>
<td>405 ± 60*</td>
<td>405 ± 60*</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.65 ± 0.395</td>
<td>13.1 ± 0.395</td>
<td>14.85 ± 0.25</td>
<td>14.05 ± 0.31</td>
<td>13.59 ± 0.28</td>
<td>13.71 ± 0.39</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>40.5 ± 1.3</td>
<td>42.1 ± 1.15</td>
<td>47.25 ± 1.0</td>
<td>44.80 ± 0.9</td>
<td>43.0 ± 0.9</td>
<td>43.7 ± 1.11</td>
</tr>
<tr>
<td>Platelet (thousands/µl)</td>
<td>990 ± 19.45</td>
<td>995 ± 52.6</td>
<td>824.65 ± 85.4</td>
<td>915.85 ± 67.5</td>
<td>549.87 ± 42.76*</td>
<td>637.53 ± 54.53*</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dl)</td>
<td>28.2 ± 3.81</td>
<td>24.2 ± 3.01</td>
<td>69.33 ± 14.15*</td>
<td>51.38 ± 6.92*</td>
<td>33.71 ± 12.15*</td>
<td>38.29 ± 10.2*</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>428 ± 219.97</td>
<td>209.4 ± 26.07</td>
<td>939 ± 120.9*</td>
<td>726.83 ± 102.5*</td>
<td>424.14 ± 67.23</td>
<td>539 ± 160.08*</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>85.8 ± 27.68</td>
<td>42.6 ± 3.8</td>
<td>340 ± 36.62*</td>
<td>338.17 ± 52.12*</td>
<td>126.29 ± 17.78</td>
<td>179.29 ± 78.6*</td>
</tr>
</tbody>
</table>

* Data are the mean ± SEM of n = 5–6 mice. The results are representative of three separate experiments. *, p < 0.05 compared with respective (WT or KO) sham.
Importantly, IL-10, IL-6, and MIP-2 (chemokine (CXC motif) ligand 2) were among the down-regulated genes in A2A KO vs WT mice (supplemental data).<sup>4</sup> RT-PCR confirmed that mRNA levels of IL-10, IL-6, and MIP-2 were decreased in spleens of A2A KO mice when compared with their WT controls (Fig. 5A).<sup>4</sup> Of the up-regulated genes in A2A KO vs WT mice, the most notable differences were observed with members of the MHC II locus. To test whether these changes manifested at the cellular phenotypic level, we compared MHC II expression of septic KO and WT animals using flow cytometry. We found that F4/80<sup>+</sup>/H11001 splenic (Fig. 5B) and peritoneal (Fig. 5C) macrophages from septic KO animals displayed markedly elevated MHC II expression levels as compared with cells from WT mice. These data indicate that there are concordant decreases in the protein and mRNA levels of IL-10, IL-6, and MIP-2, as well as a concordant increase in protein and mRNA of MHC II in septic A2A KO vs WT mice.

Pharmacological inactivation of A2A R decreases CLP-induced mortality

We further examined the role of A2A R in mediating CLP-induced mortality using a pharmacological approach. CD-1 mice treated with the selective A2 R antagonist ZM241385 (15 mg/kg, s.c., twice daily) (49–51) starting at the time of resuscitation exhibited significantly improved survival compared with vehicle-treated mice (Fig. 6A). To explore whether this improved survival of ZM241385-treated vs vehicle-treated mice was associated with a similar cytokine pattern to that one observed in A2A KO vs WT mice, we measured IL-10, IL-6, and MIP-2 concentrations in the plasma and peritoneal lavage fluid at 16 h. Levels of IL-10 and MIP-2 in both the plasma and peritoneal lavage fluid were decreased in ZM241385-treated mice as compared with vehicle-treated animals (Fig. 6C and E). Similar to genetic inactivation of A2A R, levels of IL-6 were lower in the peritoneal fluid of ZM241385-treated mice than in the peritoneal fluid of vehicle-treated mice, however, IL-6 concentrations in the plasma were comparable between the two groups (Fig. 6D).

Finally, we explored the effect of delayed administration of ZM241385 relative to resuscitation. We observed that ZM241385 administration starting 2 h after resuscitation (15 mg/kg, s.c., twice daily) was still protective (Fig. 6B), indicating a potential clinical use of A2A R blockade in acutely developing septic conditions.
levels of the cytokines IL-10, IL-6 and MIP-2. Our results, therefore, are the first to demonstrate a harmful role of A2AR stimulation in invasive bacterial infection.

Several recent studies have addressed the role of A2AR in regulating injury in animal models of systemic overwhelming inflammation. Pharmacological studies using exogenous A2AR agonists show that the activation of A2AR protects both organs and the organism from early overwhelming inflammation triggered by endotoxin (52, 53). The recent demonstration of the tissue protection by endogenous adenosine acting at A2AR in acute endotoxemia (19, 54) further suggests the relevance of A2AR to protection from hyperacute systemic inflammation. The mechanistic link between A2AR stimulation and protection from inflammatory organ injury is suggested by studies showing that A2AR KO mice injected with endotoxin have increased activation of NF-κB and higher plasma levels of proinflammatory TNF-α compared with similarly treated WT controls (19, 54), which we confirmed in our current study (Fig. 7).

Thus, at first glance, our observations that A2AR activation is harmful during CLP-induced sepsis might seem contradictory to observations that A2AR activation is beneficial in acute inflammation due to endotoxosis. But we believe the data seen as a whole suggests that differences in outcome in the two models are mainly due to immunosuppression being beneficial in acute endotoxemia but detrimental in more clinically relevant models of infection-induced sepsis where mortality depends more upon the loss of control of bacterial growth (6, 55, 56). Consistent with the distinct pathophysiology of endotoxicosis models and the CLP model, LPS is not a major contributor to the mortality of mice subjected to CLP, because genetic ablation of TLR4, the most important LPS receptor, fails to influence the survival of mice undergoing CLP (57). Circulating LPS levels in the CLP model were found to be very low, an observation that is also consistent with minute amounts of LPS found in human sepsis (6). Our observations that A2AR KO mice have increased circulating levels of IL-6 following i.p. LPS injection, but decreased IL-6 levels after CLP, indicate that A2AR activation distinctly affects the production of TLR4-mediated IL-6 production and IL-6 production induced by other stimuli present during CLP. The exact nature of these stimuli are at present unknown, but they may include Fcγ-mediated processes or stimuli that activate other members of the TLR family of receptors, such as TLR2, which is sensitive to Gram-positive bacterial cell wall products present during CLP.

A gradual long-lasting increase in IL-10 production is a pivotal factor contributing to the potentially lethal impairment in immune function and antibacterial defense observed in the CLP model (43, 44). One potential explanation for the decreased bacterial load in mice lacking A2AR is a decrease in the levels of the immunosuppressive IL-10 leading to a better preserved phagocytic response (43, 44). IL-6 levels are up-regulated in septic humans and are correlated with poor outcome (58, 59). Although controversial because IL-6 KO mice do not show improved survival in CLP-induced sepsis (60), high IL-6 levels may be disadvantageous in CLP-induced sepsis. Riedemann et al. (45) demonstrated a dose-dependent beneficial effect of IL-6 blockade in this sepsis model, using anti-IL-6 in mice. These authors suggested that although IL-6 blockade is beneficial for the outcome in CLP-induced sepsis in mice, some low levels of IL-6 may be necessary for an appropriate acute-phase response. Because the levels of IL-6 were substantially, but not completely, inhibited in the A2AR KO mice at the 16-h time point, it can be proposed that A2AR inactivation improves survival, at least in part, by decreasing IL-6 production. Interestingly, IL-6 concentrations were decreased in the peritoneum, but not in the systemic circulation of A2AR KO mice. It is

Bolus high dose endotoxin increases levels of TNF-α and IL-6 in A2AR KO mice when compared with WT animals

Endotoxin (LPS) treatment of mice induces an overwhelming inflammatory response with no infectious component. To investigate the role of A2AR in regulating this inflammatory response, we compared cytokine levels of A2AR KO and WT mice injected i.p. with LPS (5 mg/kg). The plasma level of both TNF-α and IL-6 was increased in A2AR KO mice when compared with WT mice, whereas IL-10 and MIP-2 levels were comparable (Fig. 7). Thus, A2AR differentially modulate cytokine responses in sepsis and in overwhelming endotoxemia.

Discussion

The current study shows that A2AR blockade either genetically or pharmacologically protects mice from the lethal effect of CLP-induced sepsis. This protection following A2AR blockade is paralleled by a decrease in bacterial burden as well as an increase in macrophage MHC II expression. Concurrently, A2AR blockade leads to decreased apoptosis in the spleen as well as decreased

FIGURE 5. A, RT-PCR analysis demonstrates that levels of IL-10, IL-6, and MIP-2 mRNA are decreased in spleens of A2AR KO mice when compared with WT mice. F4/80+ macrophages from spleens (B) or peritoneal cavity (C) of A2AR KO mice (n = 5) exhibit increased MHC II expression (mean fluorescence intensity) when compared with WT (n = 8) animals. Spleens or peritoneal cells were taken 16 h after CLP. *p < 0.05.
possible that the cellular sources of IL-6 are different in the peritoneal cavity and blood. We also found that levels of the CXC chemokine MIP-2 were highly elevated in A2AR WT but not KO mice at 16 h after CLP. Because the neutralization of MIP-2 was protective in a similar CLP model to ours (61), it is plausible that the salutary effect of A2AR deficiency on survival following CLP was partially due to the decreased MIP-2 levels.

CLP-induced sepsis triggers extensive apoptosis in the thymus and spleen (46, 47, 62). Guo et al. (47) found that caspase-3 activity elevated to a greater extent than other caspases in the thymus and these authors suggested that caspase-3 plays an important role in sepsis-induced thymocyte apoptosis. Similarly, a high degree of caspase-3 activation occurs in CLP-induced sepsis in the spleen (46). The mechanisms triggering the apoptotic machinery in lymphoid organs during sepsis are not well understood. Our data showing decreased levels of caspase-3 cleavage as well as PARP cleavage indicate that A2AR are essential contributors to early apoptotic processes in both the spleen and thymus. Interestingly, although late apoptotic events in the spleen were also suppressed in A2A KO mice, this was not as evident in the thymus. A similar divergence between early and late apoptotic events in the septic thymus has been previously reported in mice overexpressing Bcl-2 (63). As both caspase-dependent and -independent pathways may be involved in thymocyte apoptosis during sepsis, our results suggest that caspase-independent pathways may dominate caspase-dependent pathways in the thymus following sepsis.

In a recent study, Jenner and Young (64) have collated and compared published transcriptional-profiling data from 32 prior studies that involved 77 different host-pathogen interactions, and have defined a common host-transcriptional response. Using cluster analysis, a common host-transcriptional program was defined that is shared among different cell types in response to a range of pathogen species (64). In addition, several functional groups of gene products were identified. The group of genes that was most strongly and most consistently up-regulated across the various studies was termed the inflammatory/chemotactic cytokine cluster, which consisted of genes that encoded TNF, IL-1β, IL-6, IL-8, CSF3, CCL3, CCL4, CXCL1, CXCL2, CXCL3, and PTGS2 (COX2). Our microarray study demonstrated that with the exception of TNF, CCL20, and CXCL3, expression of all members of IL-10, MIP-2, and IL-6 are decreased in the plasma and peritoneum of mice treated with the selective A2AR antagonist ZM241385 (15 mg/kg, s.c., twice daily). Concentrations of IL-10, MIP-2, and IL-6 were measured by ELISA in plasma and peritoneal lavage fluid that were obtained 16 h after cecal ligation and puncture. Data are the mean ± SEM of n = 6–9 mice per group. Results are representative of three separate experiments. *, p < 0.05; **, p < 0.01.
this cluster of genes was down-regulated at least 2-fold in spleens of CLP-induced A2A KO mice as compared with their WT counterparts. Another group of genes contained mostly IFN-inducible genes, including chemokines, four metallothionein (M1T) genes, as well as a number of other genes, which included members of the 2’,5’-oligoadenylate cyclase group (64). A2AR KO mice had decreased levels of expression of all of these IFN-inducible genes. Such concerted down-regulation of inflammatory and IFN-regulated genes suggests that A2AR may modulate a pathway(s) that is intrinsic to the induction of inflammatory responses in bacterial sepsis.

It is less clear whether any gene expression patterns can be defined based on the up-regulated genes in A2A KO mice. Our analysis identified two functional clusters using the gene ontology nomenclature, in which most genes were up-regulated: lysosomal and endosomal clusters. The most notable of these genes were at least four members of the MHC II locus. Importantly, expression of the CIITA, which is the master regulator of MHC class II genes (65), was also increased in A2A KO mice. We confirmed increased MHC II expression on macrophages from A2A KO animals using flow cytometry. Because MHC II proteins expressed on cells of the innate immune system are major players in Ag presentation to CD4+ lymphocytes, cells that are crucial for adaptive immune responses against bacteria, increased expression of MHC II molecules in A2A KO mice might constitute an important mechanism resulting in an improved antibacterial defense in these mice. This idea is supported by the observation that patients suffering from bare lymphocyte syndrome, caused by genetic defects in the MHC2TA gene encoding CIITA, often die of severe infectious and septic complications (65).

Our findings that markers of organ damage are similar in A2A KO and WT mice demonstrate that the survival advantage conferred by A2AR deficiency does not result from protection of the liver, kidney, or lung. Two key determinants in sepsis are bacterial clearance and the inflammatory response to the infection. A2AR deficiency potentiated bacterial clearance and decreased the expression of IL-6, which is a major initiator of the acute phase response, suggesting that the mechanisms leading to improved survival in A2A KO mice may be several-fold. These mechanisms may also include various effects on the cardiovascular system or the CNS, as the function of all of these organ systems can be influenced by the ubiquitously expressed A2AR (66, 67).

In addition to our results showing that adenosine via A2AR regulates immunity and mortality in sepsis, it was recently reported that A1R are also important in governing mortality in mice subjected to CLP (68). These results confirm the hypothesis that endogenous adenosine is an important regulator of immune events in mice undergoing sepsis.

From a therapeutic standpoint, it is worth emphasizing that pharmacological blockade of A2AR was as protective as genetic deletion in preventing CLP-induced death. In addition, pharmacological blockade of A2AR using a selective A2A antagonist produced a similar change in the cytokine profile of CLP-induced mice to that achieved by targeted deletion of the A2A gene. Even more importantly, delayed administration of the A2A antagonist was also protective. Recent studies have demonstrated that a variety of targets hold promise as possible therapies of sepsis. These include activated protein C (69), high-mobility group box 1 protein (70), macrophage migration inhibitory factor (71), C5a (72), and the cholinergic system (73). Our results reveal that blockade of A2AR may offer a new strategy for the management of patients with sepsis and septic shock.

Disclosures
The authors have no financial conflict of interest.

References


