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ADENOSINE NEGATIVE FEEDBACK ON A2A ADENOSINE RECEPTORS MEDIATES HYPORESPONSIVENESS IN CHRONICALLY SEPTIC MICE

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Abstract

Strategies are needed to reverse the immune cell hyporesponsiveness and prevent bacterial overgrowth associated with high mortality rates in septic patients. Adenosine signaling may be mediating immunosuppressive signals within the inflammatory microenvironment that are safeguarding bacteria by rendering immune cells hyporesponsive. We examined A2A adenosine receptor (A2AR)–mediated immune responses in a chronic model of cecal ligation and puncture (CLP)–induced sepsis using both wild-type (WT) and A2AR knockout (KO) mice. In this model, chronic bacterial peritonitis was established that results in the first death on day 4. A2A adenosine receptors promoted bacterial overgrowth that was associated with a high 28-day sepsis mortality (WT 87% vs. A2AR KO 13%; $P < 0.0001$). Chronic bacteremia persisted in both WT and A2AR KO mice over the 28-day study period. Bacteremia was significantly decreased in A2AR KO mice 2 days after antibiotic therapy cessation (day 6 after CLP; $P < 0.005$). Local and disseminated bacteria levels were compared at the end of the 28-day study period or from moribund mice. A2A adenosine receptor deficiency dramatically decreased peritoneal ($P < 0.05$), splenic ($P < 0.05$), and blood ($P < 0.01$) bacterial levels. A2A adenosine receptor deficiency caused an early reduction in inflammatory mediators IL-6, macrophage inflammatory protein 2, TNF- α , and TNF- β ($P < 0.05$), but not in TNF- α , IL-1 β , IL-10, or monocyte chemoattractant protein 1 within 24 h after CLP. In response to an intravenous lipopolysaccharide (day 5 after CLP) challenge, A2AR KO mice showed enhanced secretion of TNF- α (2 h), IFN- γ , IL-6, monocyte chemoattractant protein 1, IL-10, and macrophage inflammatory protein 2 (9 h) ($P < 0.05$), suggesting that A2ARs attenuate inflammatory responses to repeat infectious insults. These data demonstrate that A2AR blockade may be an effective immunotherapy treatment to prevent bacterial overgrowth and reduce mortality secondary to immunosuppression in septic patients.

Keywords

Immune suppression; inflammation; bacterial overgrowth; compensatory anti-inflammatory response syndrome; systemic inflammatory response syndrome; cytokines

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INTRODUCTION

Sepsis is a clinical syndrome associated with mortality rates as high as 47% and has a high financial burden on the health care system (1). Understanding the pathological mechanisms of sepsis is critical for us to better treat septic patients with the goal of decreasing sepsis morbidity and mortality. The complex and dynamic nature of the septic response demands a thorough understanding of the pathological mechanisms associated with the different stages of sepsis to appropriately target therapeutic interventions.

Typically, the systemic inflammatory response syndrome (SIRS) is found in the acute phase of sepsis that has been defined as the first 5 days in mice (2-4). Systemic inflammatory response syndrome is a robust inflammatory response to infection with the release of potent inflammatory mediators that may ultimately lead to severe hypotension, shock, and death. Septic patients undergo a transition, although not always linear, from SIRS to the compensatory anti-inflammatory response syndrome (CARS) to limit damage. Compensatory anti-inflammatory response syndrome suppression of the immune system is thought to result in bacterial overgrowth that is associated with an increase in sepsis mortality (5, 6). Septic monocytes have a decreased capacity to respond to bacterial antigens, which has been used to define CARS in humans (7).

Mice demonstrate a variable prelethal immune response to either secrete or not secrete cytokines in chronic sepsis. Furthermore, a reduced capacity of murine macrophages from moribund mice during chronic sepsis to secrete IL-6 in response to TLR-2 and TLR-4 agonist was found when compared with matched healthy mice (8). Whether a CARS state actually exists in sepsis is still debatable. However, hyporesponsiveness is part of the clinical picture of CARS in septic patients and mice. We postulate that endogenous immune regulators, such as adenosine, play a role in the SIRS to CARS transition.

Adenosine regulation through A2A and A2B receptors (A2AR and A2BR) is an evolutionarily conserved mechanism to prevent death as a result of inflammation-induced tissue injury due to trauma or infection. The tissue-protective role of A2ARs has been shown in autoimmune hepatitis (9), cancer (10), and atherogenic (11) models of tissue inflammation/injury. A2AR-mediated immune suppression has been shown to have potent effects on proinflammatory mediator secretion by both arms of the immune system. Activated T cells downregulate the production of IFN- γ in response to A2AR stimulation. Neutrophil chemotaxis and reactive oxygen species generation, as well as macrophage production of TNF- α , IL-6, IL-1 β , and IL-12, are all inhibited by A2AR activation (12). A2AR activation on macrophages limits bacterial-induced inflammation by upregulating the anti-inflammatory cytokine, IL-10, as well (13). A2A adenosine receptors inhibit bacterial clearance within the first 24 h in acute cecal ligation and puncture (CLP)-induced sepsis. However, the role of A2ARs in controlling chronic bacterial infections is unclear, because no significant differences were found in bacterial load between wild-type (WT) and A2AR knockout (KO) mice by 48 h (14). More extensive studies are required to determine the role of A2ARs during chronic sepsis, which is the focus of this article.

Septic patients have increased circulating levels of adenosine compared with age- and sex-matched health volunteers (15). Therefore, it is rational to suspect A2ARs are involved in the downregulation of the SIRS to transition to the CARS due to their role in limiting collateral tissue damage caused by excessive inflammation. Premature attenuation of antibacterial responses to protect tissue from cell-mediated tissue damage may inadvertently protect bacteria leading to bacterial overgrowth.

Based on previous studies showing the effects of A2ARs to reduce inflammatory induced tissue damage, we hypothesized that A2ARs are partially responsible for the functional

hyporesponsiveness of immune cells seen in the CARS as a result of negative feedback inhibitory loops by adenosine on A2ARs. To study the role of A2ARs in CARS, we established a model of chronic bacterial infection by modifying the CLP model of sepsis to sustain bacteria in mice for at least 5 days and to begin sepsis mortality around day 4.

MATERIALS AND METHODS

Mice

Male mice 8 to 12 weeks old were used for all experiments. C57BL/6 were used as age-matched controls for the A2AR KO mice and housed in a pathogen-free facility at Northeastern University, Boston, Mass. A2A adenosine receptor-deficient mice were developed directly on the C57BL/6 background by Ozgene Pty Ltd (Bentley DC, West Australia). All animal experiments were conducted in accordance with IACUC guidelines of Northeastern University and the National Institutes of Health guidelines on the use of laboratory animals.

Cecal ligation and puncture

Polymicrobial sepsis was induced by subjecting mice to CLP as previously described (2, 16) with slight modifications. To achieve the desired final outcomes in the studies outlined below, it was necessary to use a 30-gauge needle to adjust the severity of our CLP model. Antibiotic therapy (25 mg/kg imipenem) was administered by subcutaneous injection every 12 h for 4 days, and saline for fluid resuscitation was administered in all studies to create a more clinically relevant sepsis model as this is standard care for humans. Antibiotic therapy was initiated 1.5 h after CLP.

Blood collection, spleen, and peritoneal content

Small volumes of blood were drawn to prevent hemodynamic alterations that may be misinterpreted as due to the septic response. To test for bacteremia, serial blood draws (25 μ L) were taken on days 6, 13, 20, and 28 and cultured immediately as described below (aerobic bacteria quantification). Additional blood draws were taken to determine circulating cytokine levels after CLP, as described below. Serum was isolated in serum separator tubes (BD Biosciences, San Jose, Calif) for cytokine analysis and stored at -80°C until future analysis.

Bacterial dissemination can lead to organ seeding if bacterial growth remains unchecked. To determine if organ bacterial seeding was present, mice were killed at the end of the study period (day 28) or 24 h before death in moribund mice in accordance with IACUC guidelines of Northeastern University. The spleen was excised and homogenized in 2 mL of sterile saline. Spleen homogenates were briefly vortexed before plating on sheep blood agar 5% TSA plates (Remel, Lenexa, Kan).

To determine the peritoneal (local) bacterial load in moribund mice or at the end of the 28-day study period, the peritoneum was lavaged in 1-mL intervals with a total of 5 mL of ice-cold Hanks buffered salt solution (HBSS). Each milliliter collected was strained using a 70- μ m nylon filter (BD Biosciences) to remove peritoneal debris.

Aerobic bacteria quantification

Bacteremia in CLP mice was determined in whole blood taken from the facial vein. To prevent blood contamination with exogenous bacteria, the mandible was shaven and sterilized with an ethanol (70%) swab before blood draw. Twenty-five microliters of undiluted whole blood was immediately plated for bacterial culture unless collected from a severely moribund mouse, which was diluted 2-fold in sterile saline for counting. The local

bacterial load in CLP mice was determined by diluting 100 μ L of peritoneal fluid with sterile HBSS in 10-fold increments to a maximum dilution of 10^7 . All samples were plated on sheep blood agar 5% TSA monolayers (Remel) and incubated overnight at 37°C under aerobic conditions. Colony-forming units (CFUs) were determined by manual counting and multiplication by their dilution factor.

Cytokine production in response to LPS stimulation in chronically infected mice

This study was designed to determine whether A2ARs play a role in the development of the immunosuppressive phenotype typical of sepsis. LPS (10 mg/kg) from *Escherichia coli* 0111:B4 (Sigma, St Louis, Mo) was administered via intravenous injection dissolved in 100 μ L of saline to test the responsiveness of infected mice 5 days after CLP. In either WT or A2AR KO mice, 50 microliters of blood was drawn from the same mouse at 2 and 9 h after LPS injection. Blood was isolated in serum separator tubes (BD Biosciences). Serum for cytokine analysis was separated and stored at -80°C until future analysis. Cytokines were measured by multiplex enzyme-linked immunosorbent assay (ELISA).

Microarray immunoassay

Serum samples collected from septic WT and A2AR KO mice were analyzed using a validated cytokine immunoassay capable of measuring 16 different cytokines using our previous protocol (17).

Statistics

Survival significance was determined by Kaplan-Meier curve and log-rank test. All values are expressed as mean $\pm$ SE. Student *t* test was used to compare data between two groups (GraphPad Prism 4.0; GraphPad Software, La Jolla, Calif).

RESULTS

A2AR KO mice have reduced mortality to chronic sepsis

To study the role of A2ARs during a chronic bacterial infection, a small 30-gauge needle was used to puncture the cecum once. Antibiotic therapy with imipenem, twice per day for 4 days given subcutaneously, was used to prevent rapid dissemination of bacteria from the peritoneal cavity (18) and to mimic the standard patient care for sepsis.

To test the hypothesis that A2AR-mediated immune suppression was associated with an increased risk of mortality, WT and A2AR KO mice were subjected to CLP (as described above) and monitored for mortality over 28 days. The presence of the A2AR in this setting was extremely lethal, resulting in only a 13% survival rate in WT mice (Fig. 1). Conversely, the A2AR KO mice had an 87% survival rate, demonstrating that the presence of A2ARs during chronic bacterial infections has potentially dangerous consequences during sepsis.

A2AR deficiency reduced bacteremia in chronic sepsis

To confirm the establishment of a chronic bacterial infection, blood was assayed for the presence of bacteria on the second day after stopping antibiotic therapy, i.e., 6 days after CLP. The presence of bacteria in the blood was tested once every week starting on day 6 (i.e., days 6, 13, 20, and 28). In this manner, CLP-induced bacteremia was detected throughout the 28-day study period despite the administration of antibiotics for 4 days, thereby confirming the presence of a chronic bacterial infection in both WT and A2AR KO mice (Figs. 2 and 3). On day 6, A2AR KO mice had significantly less bacteremia compared with WT mice. The association between better bacterial clearance and sepsis survival in the absence of A2ARs (i.e., A2AR KO mice) suggests that WT mice are dying as a secondary

effect of harboring increased levels of bacteria. Bacterial levels in the peritoneum and spleen of moribund mice and 28-day survivors were compared to establish an association between mortality and poor bacterial containment.

A2AR KO mice clear bacteria more rapidly than do WT mice

Past studies show that mice dying during the chronic phase of sepsis have significant bacterial overgrowth (19). To determine an association between higher mortality in the presence of A2ARs (i.e., WT mice) and bacterial containment, mice were tested for the presence or absence of bacteria in the peritoneum (local bacterial load) and in the spleen. For these studies, mice that survived past day 5 were killed when they appeared moribund (seven WT; one A2AR KO) or on day 28 (two WT; 11 A2AR KO) after CLP. All animals, except for one A2AR KO mouse, had bacteria present in their peritoneum, but the bacterial levels in the peritoneal fluid were significantly reduced in the A2AR KO mice either 24 h before death or in the day-28 survivors (Fig. 3) when killed. In addition, bacterial seeding to the spleen was significantly reduced in A2AR KO mice as seven of 11 A2AR KO mice had detectable levels of bacteria in their spleen, only two of which had greater than 100 (CFUs/mg of tissue) compared with 100% of WT mice with detectable levels of bacteria, and seven of eight had greater than 100 (CFUs/mg of tissue). Significantly higher levels of bacteria present in the spleen and peritoneum of WT mice demonstrate that A2ARs are immunosuppressive and reduce antibacterial responses leading to bacterial overgrowth in chronic sepsis.

A2ARs do not affect early systemic lymphocyte numbers after CLP

Past studies demonstrate that defects in the adaptive immune system are associated with increased sepsis mortality (20-22). Significant lymphopenia is found in mice after CLP (19). Therefore, it is plausible that lymphocytes may play a role in determining the immune cell responsiveness during chronic sepsis. The numbers of lymphocytes were determined 6 and 24 h after CLP. We did not find any significant differences in lymphocyte counts between WT and A2AR KO mice at either time point (Fig. 4).

The effect of A2ARs on inflammatory mediators during infectious challenge by CLP

To determine the effect of A2ARs on cytokine production in our model, serum cytokine levels in WT and A2AR KO mice were determined at 6 and 24 h after CLP. There were significant differences in IL-6, macrophage inflammatory protein 2 (MIP-2), TNF-sr1, and TNF-srII levels between WT and A2AR KO mice at 6 h (Fig. 5A, B, C, and D). TNF-sr1 and TNF-srII levels were also significantly decreased at 24 h in A2AR KO mice compared with WT mice. No differences in TNF- α , IL-1 β , IL-10, or monocyte chemotactic protein 1 (MCP-1) levels were detected at either 6 or 24 h (Fig. 5E, F, G, and H).

A2AR KO mice with chronic bacterial infections increase proinflammatory mediators in response to TLR-4 stimulation

We hypothesized that if the accumulation of extracellular adenosine during persistent bacterial infection was inhibiting antipathogen responses through A2AR-mediated immunosuppression, then inflammatory responses induced by an exogenous stimulus will be enhanced in the absence of A2ARs. Evaluation of inflammatory responses in the presence of adenosine is methodically challenging, because adenosine has multiple sources, including bacteria *in vivo* (23-26). Thus, it was important to test our hypothesis by challenging CLP mice with LPS *in vivo* to maintain adenosine levels at pathophysiological conditions during the septic response. To test this hypothesis, WT mice and A2AR KO mice were subjected to CLP to induce chronic bacterial infections as described in Materials and Methods.

To test the ability of chronically septic mice to respond to a TLR-4 agonist, mice were challenged intravenously with LPS (10 mg/kg) on day 5 after CLP. Day 5 was selected because previous work showed that circulating cytokine levels are nearly undetectable at this time point (19). Removing the negative immunoregulatory signaling by A2ARs resulted in a more robust response to the bacterial stimulus, LPS (Fig. 6). There was a dramatic increase in TNF- α levels within 2 h after LPS challenge in A2AR KO mice. Conversely, LPS did not have an effect on TNF- α levels in WT mice (Fig. 6A). Similarly, LPS challenge in A2AR KO mice resulted in an increase in IFN- γ , IL-6, IL-10, MCP-1, and MIP-2 levels compared with WT mice 9 h after injection (Fig. 6B, C, D, E, and F). LPS challenge in A2AR KO mice resulted in a time-dependent increase in IFN- γ , IL-6, and MCP-1, but not in WT mice (see Figure, Supplemental Digital Content 1, parts A–C, at <http://links.lww.com/SHK/A67>). More importantly, the decline in IFN- γ , IL-6, and MCP-1 levels from 2 to 9 h in WT mice demonstrates the potent negative feedback immunosuppressive effects of A2ARs on inflammatory responses that may limit bacterial clearance. IL-10 levels stayed elevated in A2AR KO mice, whereas they declined over 9 h in WT mice (Figure S. 1D). Even though MIP-2 levels were significantly increased in A2AR KO mice 9 h after LPS challenge (Fig. 6F), their levels followed a similar decreasing trend from 2 to 9 h as seen in WT mice (Figure S.1E).

DISCUSSION

Compared with our understanding of A2AR regulation during acute inflammatory responses, little is known about the function of A2ARs during chronic bacterial infections. The interaction between adenosine and the A2AR has been shown to trigger downstream signaling to increase intracellular levels of cAMP and decrease inflammation in models of autoimmune hepatitis (9), LPS-induced endotoxemia (9, 14), and cancer (10).

A2A adenosine receptors suppress immune responses to a number of TLR agonists, including CpG DNA and LPS (12), which are critical for the recognition and eradication of bacteria. In addition, A2ARs are shown to negatively regulate immunity, bacterial growth, and mortality in acute sepsis. Conflicting results using LPS-induced endotoxemia versus polymicrobial (CLP) sepsis models to study the role of A2ARs in sepsis have been addressed in past studies (14, 27). Together, these studies show that A2AR inhibition of antibacterial immunity is detrimental in live bacterial models of infection, which may be a consequence of A2ARs' effect to reduce proinflammatory mediators in response to bacterial antigens. In contrast, proinflammatory mediators were decreased in A2AR KO mice compared with WT mice after CLP, suggesting that continued activation of A2ARs promotes inflammatory dysfunction. Taken together, these studies led us to the hypothesis that A2ARs are partially responsible for the functional hyporesponsiveness of immune cells seen in the CARS as a result of negative feedback inhibitory loops by adenosine on A2ARs. We established a model of chronic infection that persisted over a 28-day period to study the effect of A2ARs on bacterial overgrowth. We found that A2ARs attenuated antibacterial responses during chronic bacterial infection and enhanced long-term sepsis survival. Our findings are in agreement with previous studies, suggesting that A2AR blockade is beneficial as a result of more effective bacterial containment (14).

In murine CLP studies, chronic sepsis deaths occur on or after day 5 after CLP. Poor containment of bacteria is a common feature to both acute and chronic sepsis. Previous studies have revealed that A2ARs inhibit antibacterial immunity in acutely septic mice and during chronic infection with *Helicobacter pylori* (28). To our knowledge, the role of A2ARs in a polymicrobial model of chronic bacterial infection has not been examined to date (14). Interestingly, bacteremia persisted for 28 days in both WT and A2AR KO mice, but was associated with decreased mortality only in A2AR KO mice. Furthermore, increased

bacterial dissemination was present at day 6 in WT mice, correlating with a large increase in sepsis mortality by day 7. We found the same effect of A2ARs to limit bacterial clearance and promote organ bacterial seeding in chronically infected mice. Importantly, limiting bacterial growth in the absence of A2ARs, despite ongoing bacterial insults over 28 days, provides a mechanistic clue that A2ARs are partially responsible for the CARS phenotype in sepsis.

Based on A2ARs' well-established role as a tissue protector, the absence of A2ARs could increase acute deaths due to overwhelming immune responses to infection. Contrary to this belief, the mechanism of enhanced survival in A2AR KO mice has been postulated to involve the reduction of both proinflammatory and anti-inflammatory mediators during the acute phase of sepsis. Proinflammatory mediators MIP-2 and IL-6 predict 4-day mortality in mice as early as 6 h (29, 30). In our model, CLP induced proinflammatory and anti-inflammatory mediator secretion, albeit at lower levels than those found in acute models of CLP-induced sepsis. These data agree with the absence of death within the first 4.5 days seen in our model. Most WT deaths occurred between days 6 and 13 and were associated with bacterial overgrowth in the peritoneum and spleen, suggesting that the mice died as a result of the infectious insult. Together, our data support that mice are not dying of the pathological consequences of acute sepsis, but rather of bacterial overgrowth that is partially mediated by A2AR pathways. Despite the significant increases in bacteria in WT mice compared with A2AR mice, other mechanisms, such as cardiovascular events, may be responsible for the mortality seen in this model.

A2A adenosine receptor regulation of anti-inflammatory cytokines may explain better bacterial clearance in A2AR KO mice, because they have been shown to impair antibacterial defenses in the CLP model (31). In fact, a previous *in vitro* study showed A2ARs are required for IL-10 secretion in the presence of bacterial infection (13). Additional studies showed that IL-10 declines rapidly in A2AR KO mice subjected to CLP and was consequently proposed as a mechanism of enhanced phagocytosis to decrease bacterial load in A2AR KO mice (14). Similarly, we found that the reduced bacterial load in A2AR-deficient KO mice was clearly associated with a decrease in the anti-inflammatory cytokines, TNF-srI and TNF-srII. Even though we did not observe any differences between IL-10 in WT mice compared with A2AR KO mice, these data support that A2ARs mediate immune suppression by regulating the production of anti-inflammatory cytokines. Importantly, TNF-srI and TNF-srII levels increase in CARS, suggesting that A2ARs might be responsible for the pathogenesis of CARS by increasing the pool of compensatory anti-inflammatory cytokines *in vivo*.

We found that the proinflammatory mediators IFN- γ , IL-6, and MCP-1 were susceptible to negative feedback inhibition via the A2AR in chronically infected mice challenged with LPS. Interpretation of our kinetic studies provides insight into the perceived mechanism of immune hyporesponsiveness during the CARS stage of sepsis. Our data suggest a role for A2ARs to prematurely attenuate proinflammatory responses when rechallenged with bacteria by a negative feedback mechanism (9). This observation is supported by another study that showed that macrophages are not immunoparalyzed during sepsis as they do secrete cytokines (8). If cytokine measurements were compared at a single time point, WT mice may have been interpreted as being hyporesponsive to additional bacterial antigenic challenges. This is not the case, because we observed the initial response to LPS was the same in WT and A2AR KO, with the exception of TNF- α production. The early spike in TNF- α levels may be explained by hypersecretion in response to LPS from nonimmune cell types, such as the endothelium, in the absence of A2ARs. Together with our results demonstrating that the presence of A2ARs increases the susceptibility of mice to bacterial overgrowth, we postulate that A2ARs are partially responsible for the attenuation of

antibacterial responses seen in the CARS stage of sepsis. Our data also challenge the concept of hyporesponsiveness in CARS, because initially mice are equally responsive to a repeat bacterial antigen challenge followed by a rapid decline in cytokines over several hours.

In conclusion, adenosine signaling via A2AR/A2BR-mediated pathways may be the immunological switch governing the transition from SIRS to an immunosuppressed phenotype by prematurely attenuating inflammatory responses to bacteria. Thus, the adenosine-rich inflammatory environment allows immune cells to coexist with bacteria without significant restriction on their ability to proliferate that leads to bacterial overgrowth and increased sepsis mortality. From a therapeutic standpoint, it was shown in the past that patients may benefit from the early administration of an A2AR antagonist, whereas our genetic studies suggest that patients with longer disease progression may benefit from blocking A2ARs as well (14). Therefore, the most effective treatment strategy to prevent bacterial overgrowth may be the pharmacological blockade of both A2AR and A2BR(s).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Increased lethality to CLP-induced chronic bacteremia is associated with A2ARs
 Twenty-eight-day survival curve for WT (solid line) and A2AR KO (broken line) mice subjected to CLP. Cecal ligation and puncture were performed with a 30-gauge needle, producing 13% survival over 28 days in WT mice. A2A adenosine receptor KO mice had increased resistance to CLP mortality, with an 87% in survival. Antibiotic therapy was stopped 4 days after CLP. Survival experiments were performed in two independent experiments. $P < 0.001$ comparing WT to A2AR KO mice by log-rank test.

Fig. 2. Effect of CLP on bacteria levels in blood after stopping antibiotic therapy

A small aliquot of blood (25 μL) was collected on day 6 of the survival study period to check for bacterial growth. All WT and A2AR KO mice had detectable levels of bacteremia on day 6 (WT, $n = 10$; A2AR KO, $n = 14$) after antibiotic therapy cessation on day 4. Of the surviving mice on day 6, A2AR KO mice had significantly less bacteremia than WT mice.

** $P < 0.005$; data are expressed as the mean of two independent experiments comparing WT to A2AR KO mice.

Fig. 3. Effect of A2ARs on bacteria levels during infection

Peritoneal fluid (PF), spleen, and blood were collected from moribund mice or mice on day 28 to determine bacterial levels. The presence of A2ARs was associated with bacterial overgrowth and poor bacterial containment. Results are expressed as the mean $\pm$ SEM from two independent experiments. * $P < 0.05$; ** $P < 0.01$ comparing WT to A2AR KO mice.

Fig. 4. Peripheral lymphocyte counts are similar between WT and A2AR KO mice
Whole blood was collected at 6 and 24 h after CLP. There were no significant differences in the number of circulating lymphocytes between WT (n = 13) and A2AR KO (n = 13) mice.

Fig. 5. Effect of A2ARs on inflammatory mediators

Serum IL-6 (A), MIP-2 (B), TNF-α srI (C), and TNF-α srII (D) levels were decreased by 6 h after CLP. No significant differences in TNF-α (E), IL-1β (F), IL-10 (G), or MCP-1 (H) levels were found between WT and A2AR KO mice at 6 or 24 h after CLP by multiplex ELISA. Results are expressed as the mean ± SEM from two independent experiments. * $P < 0.05$, ** $P < 0.01$ comparing WT to A2AR KO mice.

Fig. 6. *In vivo* TLR-4 activation stimulates increased cytokine secretion in chronically infected A2AR KO mice

Mice were subjected to CLP and given antibiotics twice daily for 4 days. Mice were injected intravenously with LPS (10 mg/kg) to stimulate TLR-4 receptors in WT ($n = 5$) and A2AR KO ($n = 5$) mice with chronic bacterial infections. Sera collected after the LPS challenge were analyzed for TNF- α (A), IFN- γ (B), IL-6 (C), IL-10 (D), MCP-1 (E), and MIP-2 (F) by multiplex ELISA. Cytokine/chemokine levels are represented 9 h after the LPS challenge, except for TNF- α levels (2 h). Results are expressed as the mean $\pm$ SEM from two independent experiments. * $P < 0.05$, ** $P < 0.01$ comparing WT to A2AR KO mice.