



Published in final edited form as:

J Immunol. 2010 July 1; 185(1): 542–550. doi:10.4049/jimmunol.0901295.

A2B Adenosine Receptors Protect against Sepsis-Induced Mortality by Dampening Excessive Inflammation

Balázs Csóka^{*}, Zoltán H. Németh^{*,†}, Peter Rosenberger[‡], Holger K. Eltzschig[§], Zoltán Spolarics^{*}, Pál Pacher[¶], Zsolt Selmeczy^{*}, Balázs Koscsó^{*}, Leonóra Himer^{*,||}, E. Sylvester Vizil^{||}, Michael R. Blackburn[#], Edwin A. Deitch^{*}, and György Haskó^{*,**}

^{*} Department of Surgery, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, NJ 07103

[†] Department of Surgery, Morristown Memorial Hospital, Morristown, NJ 07960

[‡] Department of Anesthesiology and Intensive Care Medicine, University Hospital Frankfurt am Main, Johann Wolfgang Goethe University, Frankfurt, Germany

[§] Mucosal Inflammation Program, Department of Anesthesiology, University of Colorado Denver, Denver, CO 80045

[¶] National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD 20892

^{||} Department of Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest

^{**} Department of Medical Chemistry, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

[#] Department of Biochemistry and Molecular Biology, University of Texas-Houston Medical School, Houston, TX 77030

Abstract

Despite intensive research, efforts to reduce the mortality of septic patients have failed. Adenosine is a potent extracellular signaling molecule, and its levels are elevated in sepsis. Adenosine signals through G-protein-coupled receptors and can regulate the host's response to sepsis. In this study, we studied the role of A_{2B} adenosine receptors in regulating the mortality and inflammatory response of mice following polymicrobial sepsis. Genetic deficiency of A_{2B} receptors increased the mortality of mice suffering from cecal ligation and puncture-induced sepsis. The increased mortality of A_{2B} knockout mice was associated with increased levels of inflammatory cytokines and chemokines and augmented NF-κB and p38 activation in the spleen, heart, and plasma in comparison with wild-type animals. In addition, A_{2B} receptor knockout mice showed increased splenic apoptosis and phosphatase and tensin homolog activation and decreased Akt activation. Experiments using bone-marrow chimeras revealed that it is the lack of A_{2B} receptors on nonhematopoietic cells that is primarily responsible for the increased inflammation of septic A_{2B} receptor-deficient mice. These results indicate that A_{2B} receptor activation may offer a new therapeutic approach for the management of sepsis.

Address correspondence and reprint requests to Dr. György Haskó, Department of Surgery, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, 185 South Orange Avenue, University Heights, Newark, NJ 07103. haskoge@umdnj.edu.

Disclosures

The authors have no financial conflicts of interest.

Systemic illness triggered by microbial invasion of normally sterile parts of the body is referred to as sepsis. Sepsis is a major health care problem; current estimates suggest that the incidence of sepsis is ~750,000 annually, and sepsis is responsible for >200,000 deaths each year in the United States (1). The innate immune system recognizes the presence of invading pathogens and initiates an inflammatory response to control the invading pathogen. This systemic inflammatory response often leads to organ injury and dysregulated apoptosis (2), which are thought to be major contributors to septic mortality (3).

The purine nucleoside adenosine is an endogenous signaling molecule, and its extracellular levels rapidly increase in septic patients owing to the increased breakdown of adenine nucleotides (ATP and ADP) in ischemic, injured, and inflamed tissues (4). Extracellular adenosine elicits its cellular effects by binding to four subtypes of G-protein-coupled adenosine receptors, termed A₁, A_{2A}, A_{2B}, and A₃ (5). The different adenosine receptors activate divergent intracellular pathways in a cell- and tissue-specific manner (6–8). Targeting adenosine receptors for pharmacological intervention in various illnesses is appealing, but the multifaceted regulation of tissue function by adenosine receptors makes their role in regulating complex pathophysiological responses, such as those occurring during sepsis, difficult to predict (9). The role of A₁, A_{2A}, and A₃ receptors in regulating the pathophysiological sequelae of sepsis has been addressed recently using the clinically relevant cecal ligation and puncture (CLP) mouse model of sepsis (10–12). Inactivation of both A₁ and A₃ adenosine receptors was found to result in increased mortality, inflammation, renal dysfunction, and hepatic injury in this model (10,12). In contrast, Nemeth and coworkers (11) showed that A_{2A} receptor inactivation protected from the lethal effect of sepsis, and this protection was associated with improved bacterial clearance, attenuated splenic apoptosis, and diminished inflammatory cytokine levels.

Because the role of A_{2B} receptors in regulating mortality, bacterial growth, and inflammation in sepsis has not been investigated, the potential for targeting these receptors for pharmacological intervention remains unknown. Recent evidence obtained using A_{2B} knockout (KO) mice and selective pharmacological agents has increased our understanding of the influence of A_{2B} receptors in various pathophysiological conditions. The first report using A_{2B} receptor KO mice demonstrated that they exhibit an inflamed phenotype with elevated cytokine levels and adhesion molecule expression in various organs when compared with their wild-type (WT) counterparts (13). Additionally, Yang and coworkers (14) found that genetic inactivation of A_{2B} receptors resulted in increased vascular injury in a femoral artery injury model. Our previous *in vivo* studies have shown that vascular permeability (15) and pulmonary inflammation (16) are significantly increased in A_{2B} KO mice subjected to hypoxia or mechanical ventilation. In contrast, A_{2B} receptor inactivation has been shown to ameliorate the course of inflammatory colitis (17), emphasizing the complex manner in which A_{2B} receptors regulate pathophysiological events during distinct disease processes.

In the current study, using genetic and pharmacological approaches, we examined the role of A_{2B} receptors in regulating the host's response to polymicrobial sepsis. We report that A_{2B} receptors prevent sepsis-induced mortality and inflammation, which indicates that A_{2B} receptors may be therapeutically targeted for the benefit of patients suffering from sepsis.

Materials and Methods

Experimental animals

A_{2B} receptor KO mice were acquired from Deltagen (San Mateo, CA). Mice were backcrossed 10 generations onto a C57Blk/6J background. A_{2B} KO mice were bred as described previously (18). At weaning, a 0.5-cm tail sample was removed for the purpose of DNA collection for genotyping. Genotyping was performed by RT-PCR using primers described previously (18).

For pharmacological studies with the selective A_{2B} receptor antagonist MRS 1754 8-[4-[[[(4-cyanophenyl)carbamoylmethyl]oxy]phenyl]-1,3-di(*N*-propyl)xanthine (Sigma-Aldrich, St. Louis, MO) and for WT controls, male C57Blk/6J mice were used that were purchased from The Jackson Laboratory (Bar Harbor, ME). 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 (Newark, NJ).

Generation of A_{2B} receptor bone marrow-chimeric mice

A_{2B} receptor bone marrow-chimeric mice were generated as previously described (15,16). Bone marrow was ablated by radiation in WT mice followed by reconstitution with bone marrow derived from previously characterized mice gene-targeted for the A_{2B} receptor and vice versa (A_{2B} KO→A_{2B} WT and A_{2B} WT→A_{2B} KO).

Cecal ligation and puncture

Polymicrobial sepsis was induced by subjecting 8–12-week-old A_{2B} KO or WT mice to CLP as we have described previously (11). In brief, mice were anesthetized with pentobarbital (50 mg/kg), given i.p. Under aseptic conditions, a 2-cm midline laparotomy was performed to allow exposure of the cecum with adjoining intestine. Approximately two-thirds of the cecum was tightly ligated with a 3.0 silk suture, and the ligated part of the cecum perforated twice (through and through) with a 20-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces (~4 mm diameter) from the perforation sites. The cecum was then returned to the peritoneal cavity, and the laparotomy was closed in two layers with 4.0 silk sutures. The mice were resuscitated with 1 ml physiological saline injected s.c. and returned to their cages with free access to food and water. The effect of pharmacological inactivation of A_{2B} receptor in mice subjected to CLP was evaluated using C57BL/6J mice in a similar fashion to that described for the A_{2B} receptor KO or WT mice. The 16-h point was used because in previous studies we have established that cytokine levels and bacterial counts subside sharply after this point (11). In this set of experiments, the mice were injected immediately before the operation with MRS1754 (0.5 mg/kg, s.c.) or its vehicle.

Collection of blood, peritoneal lavage fluid, and organs

Blood samples were obtained aseptically by cardiac puncture using heparinized syringes after opening the chest and were placed on ice into heparinized Eppendorf tubes until further processing for bacteriological analysis. 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. Two milliliters of sterile physiological saline were then installed 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 procedure peritoneal fluid was recovered through the needle. Recovered peritoneal lavage fluid was placed on ice until it was processed for bacteriological examination. After serially diluting 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, heart, and liver were excised and immediately frozen in liquid nitrogen.

Quantification of bacterial CFUs from peritoneal lavage fluid and blood

A total of 100 µl blood or peritoneal lavage fluid was diluted serially in sterile physiological saline, and 50 µl each dilution was aseptically plated and cultured on trypticase blood agar plates (BD Biosciences, San Jose, CA) 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.

Flow cytometric analysis of leukocyte subsets

The percent distribution of leukocyte subsets in blood and spleen cell suspensions was analyzed by specifically staining CD3⁺CD4⁺, CD3⁺CD8⁺ T cells, CD19⁺ B cells, and CD11b⁺ myeloid cells using Abs against CD markers conjugated with FITC, allophycocyanin, PerCP, or PE (BD Biosciences) in three- or four-color incubations. Aliquots of 0.1 ml whole blood or splenocyte suspension were incubated with the respective markers for 15 min followed by incubation with BD FACS lysing solution (BD Biosciences) for 7 min at 37°C. Cells were washed twice with BD FACS wash buffer and then fixed with 1% methanol-free formaldehyde. FACS acquisitions were performed in a centralized flow cytometry facility. At least 30,000 events were collected for each analysis.

Protein extraction and Western blot analysis

Frozen organs were homogenized in a Dounce homogenizer 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, and 1 mM Na₃VO₄). The lysates were centrifuged at 15,000 × g for 15 min, and the supernatant was recovered. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). A total of 40 µg sample was separated on 8–12% Trisglycine gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. The membranes were probed with polyclonal rabbit anti-cleaved caspase-3, polyclonal rabbit anti-cleaved poly (ADP-ribose) polymerase (PARP), polyclonal rabbit anti-IκBα, polyclonal rabbit anti-phospho-Akt, polyclonal rabbit anti-phospho-phosphatase and tensin homolog deleted on chromosome 10 (PTEN), polyclonal rabbit anti-phospho-p38, polyclonal rabbit anti-phospho-ERK1/2, and polyclonal rabbit anti-phospho-JNK Abs (all from Cell Signaling Technology, Beverly, MA). Thereafter, the membranes were incubated with a secondary HRP-conjugated anti-rabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated polyclonal goat anti-β-actin Ab to assess equal loading was used from Santa Cruz Biotechnology. Bands were detected using ECL Western blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ).

Isolation of nuclear protein extract from spleen and heart samples and detection of NF-κB activation

Cytosolic and nuclear protein fractions were isolated from spleen and heart samples using NE-PER Nuclear and Cytosolic Extraction reagent (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. Quantification of NF-κB activation was made using Trans AM NF-κB p65 Chemilumi Transcription Factor Assay kit (Active Motif, Carlsbad, CA) using 2 µg splenic or heart nuclear cell extract according to the manufacturer's suggestions.

Determination of cytokine and chemokine levels

Concentrations of TNF-α, the β form of pro-IL-1 (IL-1β), IL-6, IL-12 p40, IL-10, MIP-2, and MCP-1 were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions (19). The lower detection limit for all of these cytokines was 10 pg/ml.

Statistical analysis

Survival statistics were compared with a Kaplan-Meier curve and log-rank test. Two-tailed *t* testing was used to compare cytokine concentrations, CFUs, and other laboratory parameters. Statistical significance was assigned to *p* values <0.05.

Results

A_{2B} receptor deficiency decreases survival without affecting bacterial load in CLP-induced sepsis

We first studied the effect of A_{2B} deficiency on mortality in CLP-induced septic peritonitis by observing A_{2B} WT and KO mice. As demonstrated in Fig. 1A, A_{2B} receptor KO mice had significantly higher mortality rates compared with A_{2B} WT mice, which became apparent on the second day of observation. On the seventh day following CLP, the mortality rate of A_{2B} KO mice was markedly (by >40%) higher than that of A_{2B} WT mice. No additional changes in mortality were detected when the mice were monitored for up to 10 d (data not shown). Bacterial translocation from the intestines into the peritoneal cavity and bloodstream invasion are important contributors to the mortality of mice in CLP-induced sepsis. As shown in Fig. 1B, we found no difference in the numbers of bacteria in the blood or peritoneal lavage fluid of A_{2B}R KO mice when compared with WT animals at 16 h following the CLP procedure. Taken together, these studies document that despite the fact that A_{2B} receptors do not affect bacterial invasion, A_{2B} receptors prevent mortality in polymicrobial sepsis.

A_{2B} receptor deficiency increases cytokine and chemokine production, and augments NF (NF)-κB activation in CLP-induced sepsis

Increased production of inflammatory cytokines and chemokines and activation of the transcription factor NF-κB are important components of a dysregulated immune response and mortality in septic mice. To investigate whether A_{2B} receptor deficiency alters the release of inflammatory cytokines, we next determined concentrations of TNF-α, IL-1β, IL-12p40, IL-6, and IL-10 in both the plasma and peritoneal lavage fluid. We found markedly elevated cytokine levels in both the blood and peritoneal cavity of A_{2B} KO animals (Fig. 1C–G). We then assessed the levels of MIP-2 and MCP-1, two crucial chemokines that mediate inflammatory responses in the plasma and peritoneal lavage fluid of A_{2B} KO and WT mice subjected to CLP. Fig. 1H and 1I show that the release of both of these chemokines was higher in A_{2B} KO animals than WT mice.

Because the spleen and liver are among the most important sources of inflammatory mediators in sepsis, in the next set of experiments, we studied the cytokine/chemokine response of these organs to CLP-induced sepsis. We found comparable concentrations of inflammatory mediators in the liver of A_{2B} KO and WT mice (data not shown). However, A_{2B} KO mice exhibited elevated splenic IL-6, IL-10, and MIP-2 levels when compared with their WT littermates (Fig. 1J–L). Cytokine levels in all sham-operated animals were below the detection limit (data not shown).

Microbial components and endogenous danger signals trigger the activation of signaling cascades, leading to induction of the NF-κB system during sepsis. Persistent activation of NF-κB may cause excessive inflammatory responses culminating in tissue injury, organ dysfunction, and death. Because we found elevated cytokine and chemokine levels in A_{2B} KO mice undergoing sepsis, we studied the activation of NF-κB by measuring the levels of the inhibitory subunit of NF-κB (IκBα) in whole-cell extracts and the nuclear levels of the p65 NF-κB subunit in the spleen of septic animals. As Fig. 1M shows, the levels of IκBα were lower in the spleen of A_{2B} KO mice than WT mice. In addition, we found that the nuclear level of p65 was significantly enhanced in A_{2B} KO as compared with WT mice (Fig. 1N). These results together indicate that NF-κB activation is increased in A_{2B} KO versus WT mice.

Taken together, these results suggest a prominent role for A_{2B} receptors in harnessing inflammation during polymicrobial sepsis.

Pharmacological inactivation of A_{2B} receptors enhances CLP-induced cytokine and chemokine levels

We then further examined the role of A_{2B} receptors in modulating CLP-induced sepsis using a pharmacological approach. C57BL/6J mice were treated with the selective A_{2B} receptor antagonist MRS1754 (0.5 mg/kg) or its vehicle, and cytokine and chemokine levels were measured in the blood and peritoneal lavage fluid of mice 16 h after the CLP procedure. Similar to genetic inactivation of A_{2B} receptors, the concentration of TNF- α , IL-1 β , IL-6, and IL-10 (Fig. 2A–E), as well as MIP-2 (Fig. 2F) and MCP-1 (Fig. 2G), was higher in both the blood and peritoneal lavage fluid of mice with pharmacological inactivation of A_{2B} receptors. However, there was no difference in IL-12p40 levels between vehicle- and MRS1754-treated animals. These results confirm that inactivation of A_{2B} receptors increases inflammation in polymicrobial sepsis.

Genetic ablation of A_{2B} receptors enhances the levels of inflammatory mediators in the lungs of septic mice

Lung inflammation is a complication of sepsis, often leading to acute respiratory distress syndrome and death. We thus investigated the effect of A_{2B} receptor ablation on pulmonary inflammation in sepsis. We found augmented levels of IL-1 β , IL-6, MIP-2, and MCP-1 in lung tissue of A_{2B} KO versus WT mice when measured 16 h after the induction of sepsis (Fig. 3A–D). Cytokine levels in all sham-operated animals were below the detection limit (data not shown).

These results suggest that A_{2B} receptors prevent the increasing effect of sepsis on lung inflammatory cytokine levels.

A_{2B} receptor deletion augments inflammatory mediator levels and NF- κ B activation in the hearts of septic mice

A growing body of evidence indicates that sepsis induces cardiac injury and myocardial dysfunction in both experimental animals and humans. Moreover, the A_{2B} receptor has an important protective role in preventing inflammation and tissue injury during cardiac ischemia (20). Therefore, we examined the levels of inflammatory mediators in heart tissue homogenates. Fig. 4A and 4B show that protein homogenates from A_{2B} KO heart tissue contained higher levels of IL-6 and MCP-1 when compared with homogenates obtained from WT hearts. Cytokine levels in all sham-operated animals were below the detection limit (data not shown). Sepsis-induced cardiac inflammation is known to correlate with the phosphorylation of MAPK cascades, such as p38, ERK 1/2 (ERK1/2), and JNK (JNK1/2). Hence, we studied the phosphorylation of these kinases in hearts of A_{2B} KO and WT animals after CLP. As Fig. 4C shows, p38 phosphorylation in hearts of A_{2B}-deficient animals was enhanced when compared with WT animals. There was no difference in the phosphorylation of cardiac ERK1/2 and JNK1/2 between WT and KO mice following CLP (data not shown). Previous *in vivo* and *in vitro* studies have demonstrated that cardiac inflammation is generally associated with augmented activation of the NF- κ B system (21). Consequently, we assessed the activation state of the NF- κ B system by measuring I κ B α protein levels and the nuclear level of p65 subunit of NF- κ B in the heart. I κ B α protein levels were decreased, and the nuclear level of p65 was elevated in the heart of animals lacking A_{2B} receptors when compared with WT littermates (Fig. 4D, 4E). Thus, we conclude that A_{2B} receptors diminish cardiac inflammatory mediator production and NF- κ B activation during sepsis.

Lack of A_{2B} adenosine receptors decreases the number of CD19⁺ and CD11b⁺ cells during CLP

CLP-induced sepsis decreased the number of total circulating leukocytes in both A_{2B} receptor KO and WT mice to the same degree (data not shown). Analysis of WBC subsets indicated a decrease in the numbers of circulating CD11b⁺ cells and CD19⁺ B cells in KO versus WT animals, whereas the number of CD4⁺ and CD8⁺ T cells was comparable between WT and KO mice (Fig. 5A). Furthermore, the number of resident splenic CD11b⁺ cells but not other cell populations decreased in A_{2B} KO mice as compared with their WT littermates (Fig. 5B).

Genetic deletion of the A_{2B} receptor increases apoptosis and downregulates PI3K/Akt signaling in the spleen

Sepsis provokes extensive immune cell apoptosis that contributes to immune dysregulation and mortality. Because proteolytic cleavage of caspase-3 and PARP is a good indicator of apoptosis, we tested whether A_{2B} receptor deficiency would affect the cleavage of caspase-3 and PARP in the spleen and thymus of mice subjected to CLP. Fig. 5C shows that 16 h after the onset of sepsis, the cleavage of both caspase-3 and PARP in the spleen was markedly enhanced in A_{2B} receptor KO mice. However, CLP-induced apoptosis in the thymus was comparable between A_{2B} KO and WT mice (data not shown). We next surveyed some of the upstream intracellular signaling mechanisms regulating apoptosis in sepsis. The Ser/Thr kinase Akt is a key regulator of cell survival, and sepsis-induced reduction in its phosphorylation/activation is associated with enhanced apoptosis. Thus, we tested the hypothesis that the elevated apoptosis in A_{2B} receptor KO mice that we observed may be due to impaired Akt phosphorylation/activation. We found decreased levels of phosphorylated/activated Akt in spleens from A_{2B} KO mice subjected to CLP (Fig. 5D). PTEN is an endogenous inhibitor of Akt, and its phosphorylation results in its deactivation (22). We found diminished PTEN phosphorylation in spleens of A_{2B} KO animals when compared with WT mice 16 h following the CLP insult (Fig. 5D), suggesting that A_{2B} KO receptor deletion causes apoptosis via PTEN activation. Taken together, A_{2B} receptors prevent splenic apoptosis in polymicrobial sepsis.

Loss of A_{2B} receptors on nonhematopoietic cells is responsible for the increased cytokine response of A_{2B} receptor-deficient mice

Previous studies employing bone marrow-chimeric mice have helped to clarify the role of the contribution of A_{2B} receptors on hematopoietic versus nonhematopoietic cells to modulating inflammation in various injury models (13,15,16,23). To determine whether the elevated cytokine levels that we detected in A_{2B} KO mice subjected to CLP (Fig. 1) were due to loss of A_{2B} receptors on hematopoietic or nonhematopoietic cells, we generated A_{2B} receptor bone marrow-chimeric mice. A_{2B} WT→A_{2B} KO chimeras resulting in WT bone marrow and KO parenchyma exhibited higher CLP-induced cytokine (TNF- α , IL-1 β , IL-6, and IL-10) (Fig. 6A–D), and chemokine (MIP-2 and MCP-1) (Fig. 6E, 6F) levels than A_{2B} KO→A_{2B} WT mice with WT parenchyma and KO bone marrow. This observation demonstrates that non-bone marrow-associated A_{2B} receptors have a preeminent role in dampening TNF- α , IL-1 β , IL-6, and IL-10 as well as chemokine production in sepsis.

Discussion

Studies employing both KO and pharmacological approaches have provided insights into the role of the various adenosine receptors in regulating the physiological response of the host to sepsis. These studies have revealed that deletion of either A₁ or A₃ adenosine receptors results in impaired survival in response to CLP-induced sepsis (10,12). In contrast to observations with inhibition of A₁ and A₃ receptors, a previous study from our laboratory showed that A_{2A} receptor inactivation protects animals from the lethal effect of sepsis (11). The most important novelty of the current study is that, similar to A₁ and A₃ receptors, blockade of

A_{2B} receptors decreased the survival rate of septic animals, pointing to a protective role of A_{2B} receptors in sepsis.

Studies employing antibiotic therapy have demonstrated that systemic bacterial dissemination is a major factor contributing to the mortality of both experimental animals and humans during sepsis (24,25). Our studies showed that bacterial dissemination was not different between A_{2B} receptor KO and WT mice, suggesting that A_{2B} receptors do not protect against mortality by controlling the systemic infection during sepsis. In addition to direct organ injury caused by multiplying bacteria, excessive inflammation is another factor that precipitates organ injury and eventually leads to death in sepsis. Our studies identify this inflammatory response as a major target of A_{2B} receptors in preventing mortality in septic mice. We found that A_{2B} receptors decreased inflammatory cytokine and chemokine levels both systematically and locally in organs, such as the lung, heart, and the peritoneal cavity. The role of A_{2B} receptors in regulating inflammation is controversial. In some studies, A_{2B} receptor KO mice exhibited elevated plasma levels of inflammatory mediators in the systemic circulation (13), lung (16), vasculature (14), and kidney (23). In contrast, in the dextran sodium sulfate-induced colitis model, A_{2B} receptor KO mice had decreased levels of inflammatory mediators as well as an attenuated clinical course of colitis (17). In addition, A_{2B} receptor inactivation suppressed pulmonary injury in adenosine deaminase-deficient mice (26). These observations underline the concept that the role of adenosine receptors in regulating inflammation is complex and unpredictable (8,9,27–29).

The mechanisms by which A_{2B} receptor activation limits inflammation have not been defined in detail. Increased activation of NF-κB occurs during sepsis, and this activation is associated with higher rates of mortality and worse clinical outcome (30,31). Our results demonstrating downregulation of the NF-κB system in the spleen by A_{2B} receptors indicate that this decreased NF-κB activation may represent a central pathway by which A_{2B} receptors decrease inflammatory cytokine production and injury in sepsis. Earlier studies on A₁ and A₃ receptor KO animals documented increased hepatic and kidney injury in response to CLP (10,12). In the current study, we noted more pronounced cardiac inflammation in septic A_{2B} KO animals, indicating heart protection as an additional potential mechanism of the protective effect of A_{2B} receptors. Although inflammatory cytokine levels were elevated in the lungs of A_{2B} KO animals, this did not translate into increased injury, as assessed using the myeloperoxidase assay and by measuring wet/dry ratio (edema) (data not shown). In contrast, the degree of liver and kidney injury as assessed by histological observation, IκBα degradation, and measuring inflammatory mediator levels was similar in A_{2B} KO and WT mice subjected to CLP (data not shown).

Myocardial injury and cardiac dysfunction are major consequences of sepsis and contribute to the high mortality of this illness (32). It is well appreciated that an NF-κB- and p38-driven inflammatory response leading to overproduction of proinflammatory cytokines, such as IL-6 and TNF-α in the heart, is an important factor in mediating sepsis-induced cardiac injury (33–35). In this regard, our data showing increased cardiac inflammatory mediator production and NF-κB activation in A_{2B} receptor KO mice suggest that A_{2B} receptor may limit septic mortality by attenuating cardiac inflammation and maintaining cardiac contractility. This idea is supported by our recent observations demonstrating that A_{2B} receptor activation is protective in cardiac ischemia (20).

Sepsis provokes extensive immune cell apoptosis, and mortality in this illness is thought to be, at least in part, a consequence of dysregulated immune cell death. The most affected immune cell populations are T and B lymphocytes, as well as dendritic cells (2,36,37). In our model, A_{2B} receptors prevented apoptosis in the spleen. Because we found decreased numbers of CD11b⁺ but not CD4⁺ or CD8⁺ cells in the spleen of A_{2B} KO mice, and dendritic cells express

CD11b, we propose that A_{2B} receptor activation prevents depletion of dendritic cells. Because polymorphonuclear neutrophils also express CD11b, an additional explanation for decreased CD11b expression in the spleen of A_{2B} KO mice is that splenic polymorphonuclear neutrophil infiltration is decreased in KO animals. Because we observed diminished Akt signaling in the spleens of A_{2B} KO animals, it is possible that A_{2B} receptors protect against apoptosis by activating Akt. This proposition is supported by a recent study, which reported that inhibition of Akt signaling augmented apoptosis in the spleen and increased CLP-induced mortality of septic animals (38). In this regard, it is note-worthy that A_{2B} receptors have been reported to activate the Akt system both in vitro (39) and in the ischemic heart (40). Finally, our results that A_{2B} receptors limited activation of PTEN, a negative upstream regulator of Akt highlight a novel mechanism explaining the inhibitory effect of A_{2B} receptor on apoptosis.

Results of a recent study utilizing A_{2B} receptor bone marrow-chimeric mice indicated a primary role for A_{2B} receptors on hematopoietic cells in decreasing the TNF- α and IL-6 response to peritoneal injection of LPS (13). In contrast, our experiments with A_{2B} bone marrow chimeras revealed that regulation of plasma cytokine production in polymicrobial sepsis is associated with A_{2B} signaling on nonhematopoietic cells. These results further emphasize the dissimilarity of our polymicrobial sepsis model and the endotoxemia model, in which LPS injection elicits a cytokine response. Our previous studies using different injury models also underscore the point that protection can be mediated by A_{2B} receptors on nonhematopoietic-derived cells or on both hematopoietic and nonhematopoietic cells (15,16). For example, we showed a critical role of vascular (nonhematopoietic) A_{2B} receptors in limiting vascular leakage caused by hypoxia, and both nonhematopoietic and hematopoietic A_{2B} receptors were responsible for suppressing neutrophil tissue accumulation following a hypoxic episode (15). In summary, the most intriguing result of this study is that inactivation of A_{2B} receptors increases the mortality of mice subjected to polymicrobial sepsis. These results illustrate that A_{2B} adenosine receptors have a beneficial effect on the outcome of sepsis and may represent a new therapeutic target for the management of patient with sepsis.

Acknowledgments

This work was supported by National Institutes of Health Grant R01GM66189 and the Intramural Research Program of the National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism, as well as the Hungarian Research Fund (CK 78275).

Abbreviations in this paper

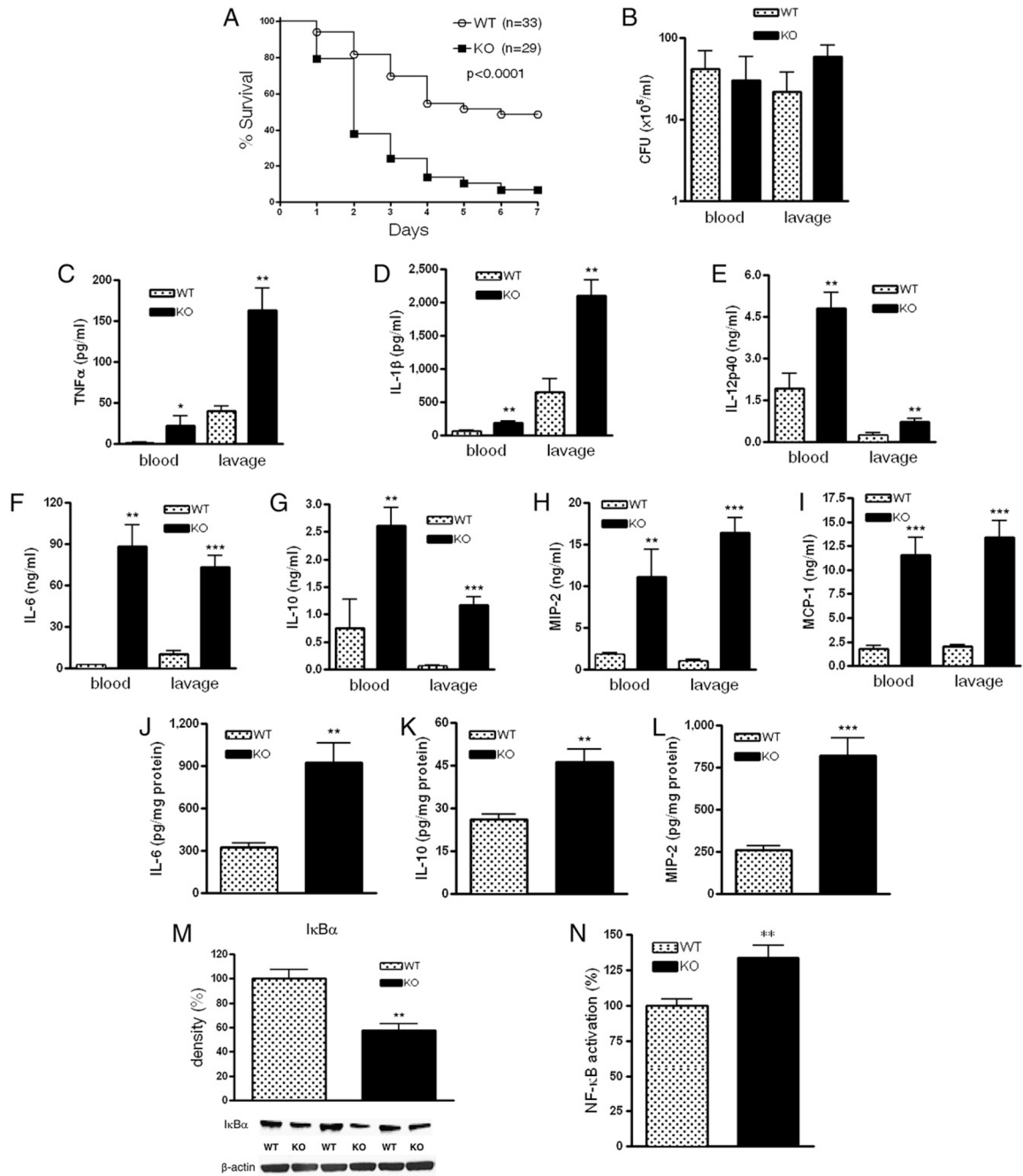
CLP	cecal ligation and puncture
IL-1 β	β form of pro-IL-1
KO	knockout
PARP	poly(ADP-ribose) polymerase
PTEN	phosphatase and tensin homolog deleted on chromosome 10
WT	wild-type

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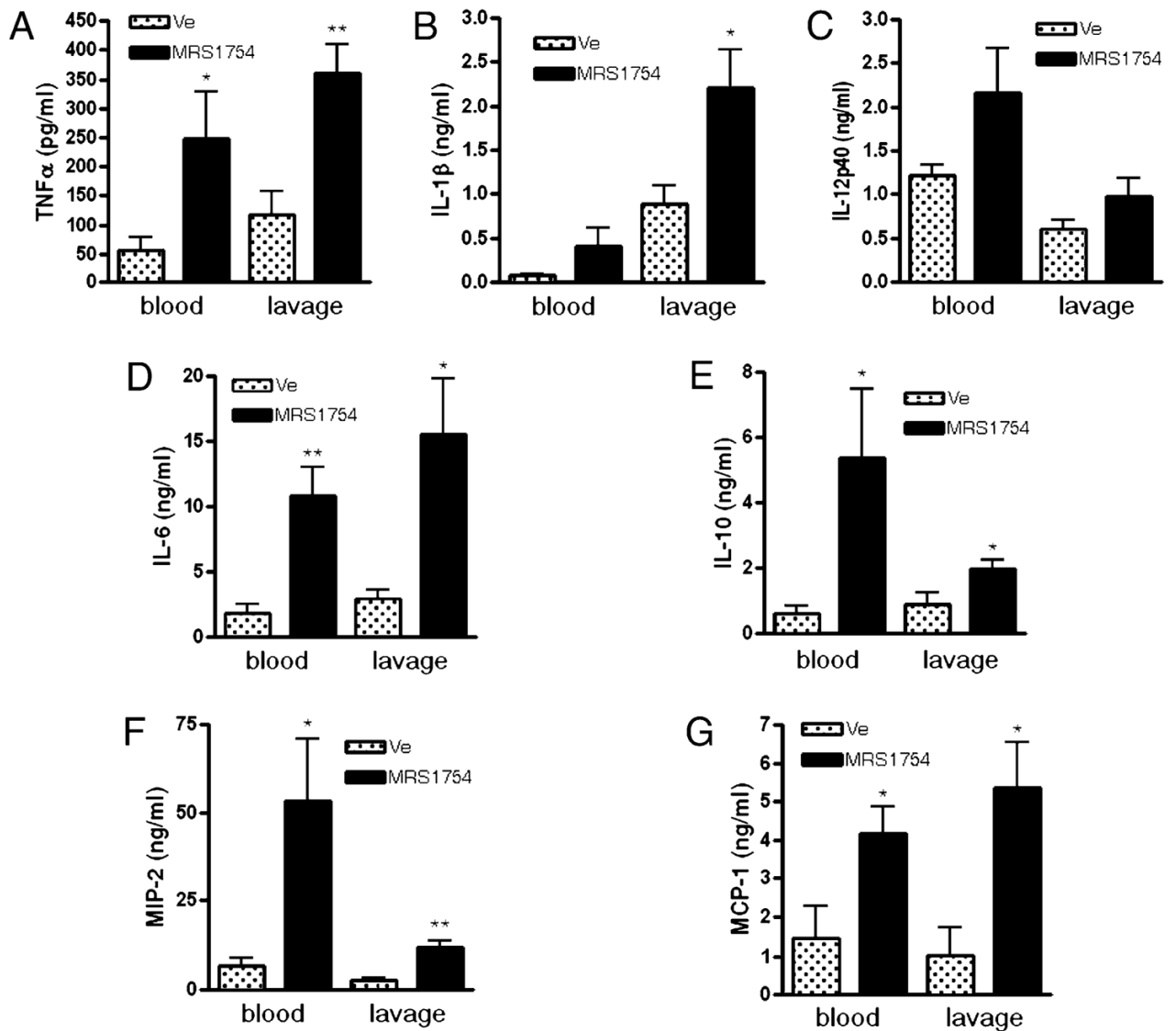
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**FIGURE 1.**

*A*_{2B} deficiency decreases survival and increases inflammation in polymicrobial sepsis. *A*, Surviving *A*_{2B} KO and WT mice were counted every day for 7 d after inducing polymicrobial sepsis by way of CLP. WT, *n* = 33; KO, *n* = 29. *p* < 0.0001 versus WT. *B*, Blood and peritoneal lavage fluid obtained from *A*_{2B} KO or WT mice 16 h post CLP were cultured on soytrypticase agar plates, and the number of bacterial colonies was counted (*n* = 12 mice per group). Serum and peritoneal lavage TNF-α (*C*), IL-1β (*D*), IL-12p40 (*E*), IL-6 (*F*), IL-10 (*G*), MIP-2 (*H*), and MCP-1 (*I*) levels were determined from samples collected 16 h post CLP using ELISA (*n* = 12/group). Splenic levels of IL-6 (*J*), IL-10 (*K*), and MIP-2 (*L*) were measured from protein extracts of spleens of *A*_{2B} KO and WT mice (*n* = 12/group). *M*, IκBα levels were assessed using

Western blotting of spleen protein extracts of A_{2B} WT and KO mice ($n = 6/\text{group}$). Protein extracts were generated from spleen taken 16 h after sepsis induction. Bands were detected by ECL. All results (mean \pm SEM) shown are representative of three experiments. *N*, p65 levels were assessed in nuclear fractions of spleens using NF- κ B (p65) chemiluminescence assay kit. Results (mean \pm SEM) shown are the summary of three independent experiments. WT, $n = 29$; KO, $n = 31$. * $p < 0.05$ versus WT; ** $p < 0.01$ versus WT; *** $p < 0.001$ versus WT.

**FIGURE 2.**

Pharmacological inactivation of A_{2B} receptors enhances inflammatory mediator production in polymicrobial sepsis. Blood and peritoneal lavage fluid was obtained from mice injected with the A_{2B} receptor-specific antagonist MRS1754 or vehicle (DMSO) 16 h after the CLP procedure, and TNF- α (A), IL-1 β (B), IL-12p40 (C), IL-6 (D), IL-10 (E), MIP-2 (F), and MCP-1 (G) concentrations were determined using ELISA ($n = 10$ /group). All results (mean \pm SEM) shown are representative of three experiments. * $p < 0.05$ versus vehicle-injected mice; ** $p < 0.01$ versus vehicle-injected mice.

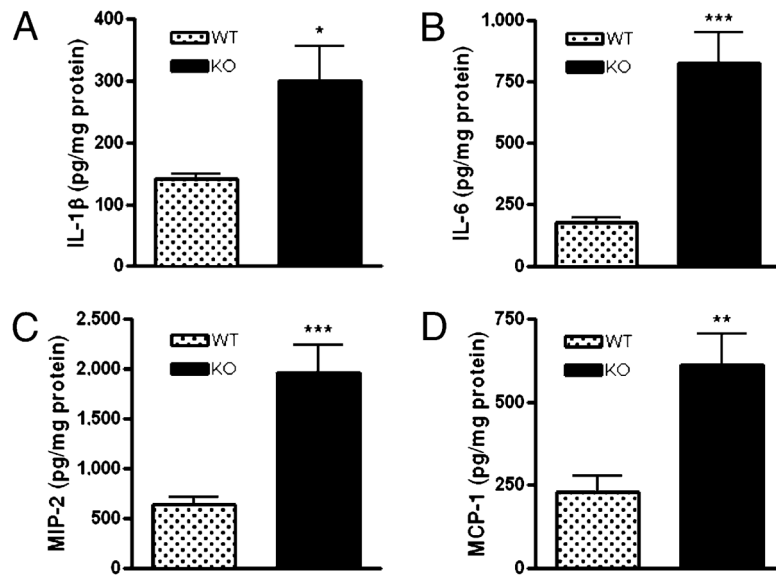
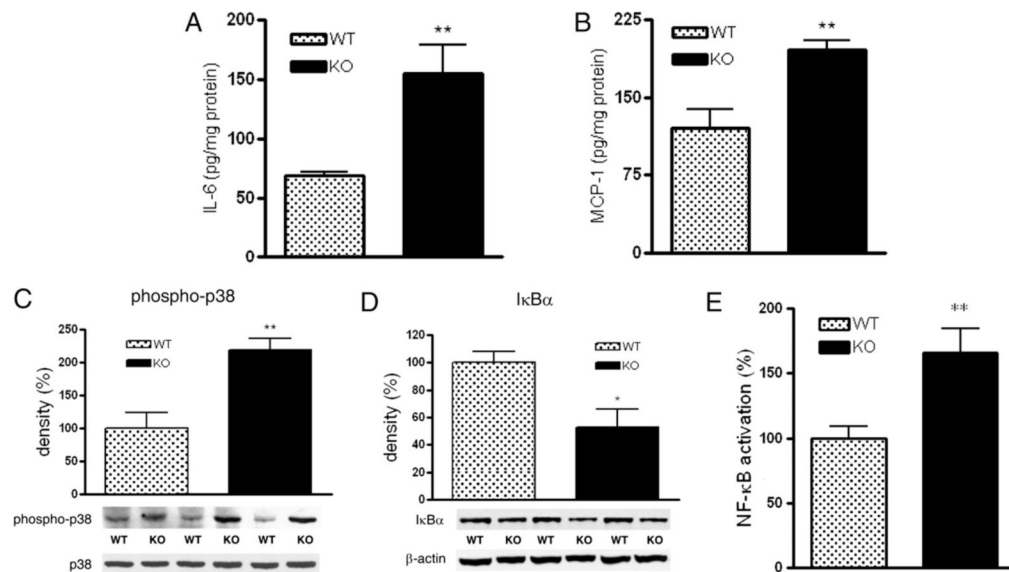
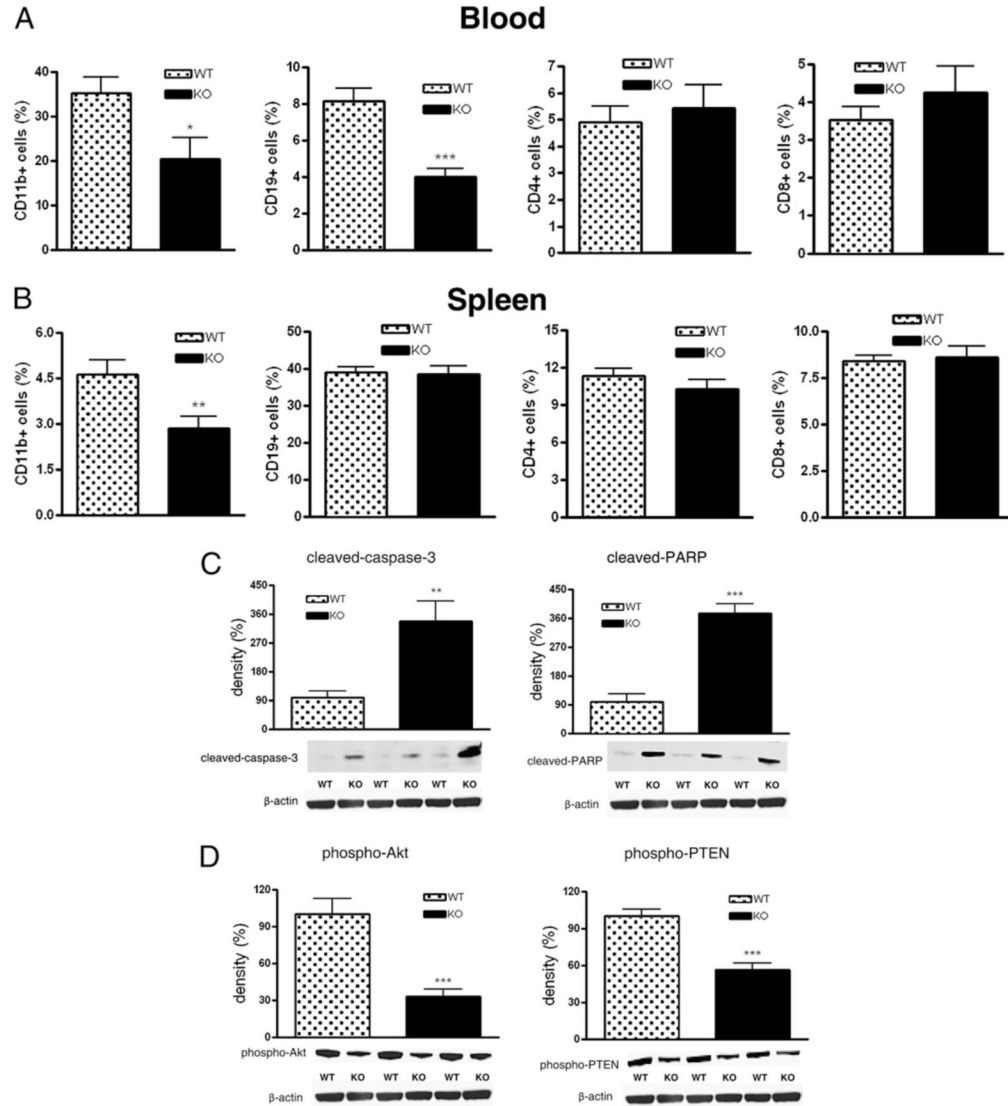


FIGURE 3.

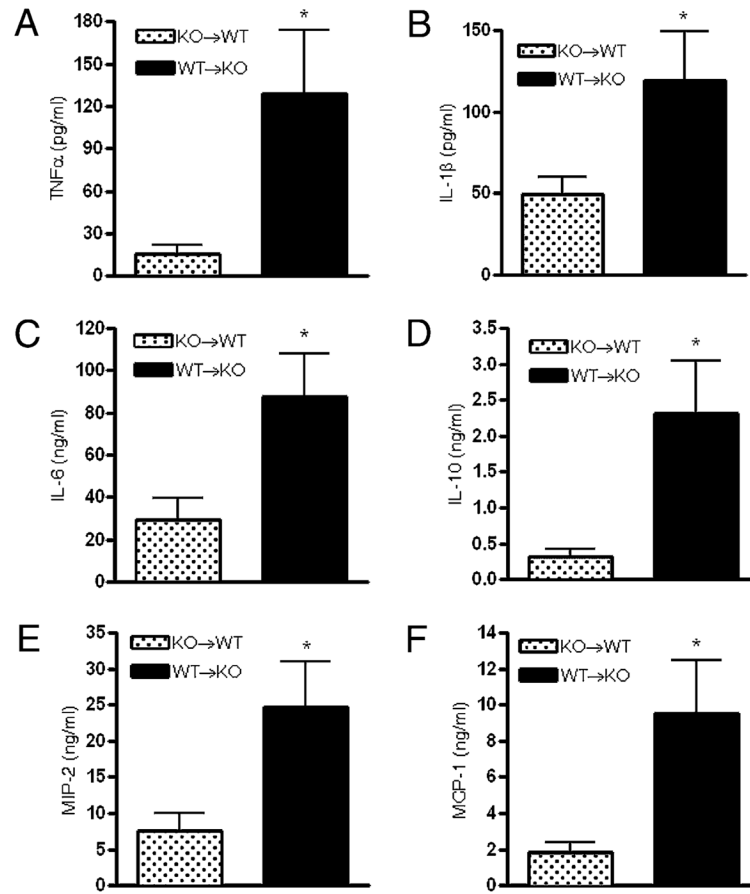
Genetic ablation of A_{2B} receptor enhances sepsis-induced pulmonary inflammatory cytokine and chemokine levels. Pulmonary concentrations of IL-1 β (A), IL-6 (B), MIP-2 (C), and MCP-1 (D) were measured from pulmonary extracts of A_{2B} KO and WT mice taken after 16 h of sepsis ($n = 12$ /group). All results (mean \pm SEM) shown are representative of three experiments. * $p < 0.05$ versus WT; ** $p < 0.01$ versus WT; *** $p < 0.001$ versus WT.

**FIGURE 4.**

A_{2B} receptor deletion augments sepsis-induced inflammatory mediator levels and NF- κ B activation in the heart. Cardiac levels of IL-6 (A) and MCP-1 (B) were assessed using ELISA. Protein extracts were prepared from hearts of A_{2B} KO and WT mice isolated after 16 h of sepsis ($n = 12$ /group). p38 phosphorylation (C) and I κ B α (D) levels in heart protein extracts of A_{2B} KO and WT mice were examined using Western blotting. Bands were detected by ECL. All results (mean \pm SEM) shown are representative of three experiments ($n = 6$ /group in each experiment). E, p65 levels were assessed in nuclear fraction of hearts using NF- κ B (p65) chemiluminescence assay kit. Results (mean \pm SEM) shown are the summary of three independent experiments. WT, $n = 29$; KO, $n = 31$. * $p < 0.05$ versus WT; ** $p < 0.01$ versus WT.

**FIGURE 5.**

A_{2B} receptor deficiency decreases the number of CD19⁺ and CD11b⁺ lymphocyte subsets and increases apoptosis. Numbers of various lymphocyte subsets were monitored in blood (A) and spleen (B) by flow cytometric analysis after 16 h of sepsis induced by CLP ($n = 8$ /group). Proteolytic cleavage of caspase-3 and PARP (C) as well as phosphorylation of Akt and PTEN (D) in spleen protein extracts from A_{2B} WT and KO mice was examined using Western blotting. Bands were detected by ECL. All results (mean \pm SEM) shown are representative of three experiments ($n = 6$ /group in each experiment). * $p < 0.05$ versus WT; ** $p < 0.01$ versus WT; *** $p < 0.001$ versus WT.

**FIGURE 6.**

Lack of signaling through A_{2B} receptors on nonhematopoietic cells is responsible for the increased cytokine response of A_{2B} receptor-deficient mice. A–F, Blood was obtained from (A_{2B} KO→A_{2B} WT; A_{2B} WT→A_{2B} KO) A_{2B} receptor bone marrow-chimeric mice 16 h post CLP. Plasma TNF-α (A), IL-1β (B), IL-6 (C), IL-10 (D), MIP-2 (E), and MCP-1 (F) concentrations were assessed using ELISA. Results are depicted as mean ± SEM of *n* = 10 A_{2B} KO→A_{2B} WT and *n* = 8 A_{2B} WT→A_{2B} KO mice. **p* < 0.05 versus A_{2B} WT→A_{2B} KO bone marrow-chimeric mice.