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ORIGINAL PAPER

Role of A_{2A} adenosine receptors in regulation of opsonized *E. coli*-induced macrophage function

Balázs Csóka · Zoltán H. Németh · Zsolt Selmeczy · Balázs Koscsó · Pál Pacher · E. Sylvester Vizi · Edwin A. Deitch · György Haskó

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Abstract Adenosine is a biologically active molecule that is formed at sites of metabolic stress associated with trauma and inflammation, and its systemic level reaches high concentrations in sepsis. We have recently shown that inactivation of A_{2A} adenosine receptors decreases bacterial burden as well as IL-10, IL-6, and MIP-2 production in mice that were made septic by cecal ligation and puncture (CLP). Macrophages are important in both elimination of pathogens and cytokine production in sepsis. Therefore, in the present study, we questioned whether macrophages are responsible for the decreased bacterial load and cytokine production in A_{2A} receptor-inactivated septic mice. We showed that A_{2A} KO and WT peritoneal macrophages obtained from septic animals were equally effective in phagocytosing opsonized *E. coli.* IL-10 production induced by opsonized *E. coli* was decreased in macrophages obtained from septic A_{2A} KO mice as compared to WT counterparts. In contrast, the release of IL-6 and MIP-2 induced by opsonized *E. coli* was higher in septic A_{2A} KO macrophages than WT macrophages. These results suggest that peritoneal macrophages are not responsible for the decreased bacterial load and diminished MIP-2 and IL-6 production that are observed in septic A_{2A} KO mice. In contrast, peritoneal macrophages may contribute to the suppressive effect of A_{2A} receptor inactivation on IL-10 production during sepsis.

Keywords Adenosine · Cytokines · Inflammation · Macrophages · Phagocytosis · Sepsis

Abbreviations

IL	Interleukin
MIP-2	Macrophage-inflammatory protein-2
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine, adenosine deaminase inhibitor
ZM241385	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a)[1,3,5]triazin-5-ylamino]ethyl) phenol, selective A2A receptor antagonist

Introduction

Severe sepsis is an extremely costly medical problem in the United States and develops in >750,000 people annually. Although the treatment of primary infections per se is well-established, ~200,000 deaths/year occur as a result of residual sepsis and multiple organ dysfunction [1, 2]. Current theory suggests that the sepsis syndrome is associated with an early overwhelming innate immune response, characterized by unabated activation and release of pro-inflammatory mediators. Subsequently, the exaggerated systemic inflammatory response is counterbalanced by sustained expression of potent anti-inflammatory mediators, which often results in the desensitization of effector cells (such as phagocytes) and development of a hypo-

B. Csóka · Z. H. Németh · E. A. Deitch · G. Haskó (✉)
Department of Surgery, UMDNJ-New Jersey Medical School,
185 South Orange Avenue, University Heights,
Newark, NJ 07103, USA
e-mail: haskoge@umdnj.edu

Z. Selmeczy · B. Koscsó · E. S. Vizi · G. Haskó
Department of Pharmacology, Institute of Experimental Medicine,
Hungarian Academy of Sciences,
Szigony u. 43,
Budapest H-1083, Hungary

P. Pacher
National Institute on Alcohol Abuse and Alcoholism,
12420 Parklawn Dr., MSC-8115,
Bethesda, MD 20892-8115, USA

immune or "immunoparalytic" state. In fact, the inability to kill invading pathogens effectively at later stages during sepsis is due to immunosupression, and it is also a major cause of late organ dysfunction syndrome [3, 4]. Potential mechanisms of immune suppression after a septic insult include decreased antigen presentation, diminished phagocytosis of pathogens, as well as dysregulation in cytokine production [5–9].

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. Systemic adenosine levels are highly elevated in septic patients [10–12]. Adenosine acts by engaging A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors [13], through which it can exert immunosuppressive effects [14–21]. Besides the immunomodulatory effect of A_3 [22, 23] and A_{2B} receptors [24], the most potent immunosuppressive effects of adenosine are attributed to occupancy of A_{2A} receptors on immune cells. Stimulation of A_{2A} receptors induces many phenotypic changes in immune cells that are characteristic of the late "immunoparalytic" phase of sepsis [25–30]. We demonstrated previously, by using genetic and pharmacological inactivation of the A_{2A} receptor in the clinically relevant cecal ligation and puncture (CLP) model of sepsis [31] that stimulation of A_{2A} receptors contributed to the lethal effect of sepsis and led to increased bacterial burden [32]. Furthermore, stimulation of A_{2A} receptors increased levels of the immunoregulatory cytokines IL-10, IL-6, and MIP-2. Because macrophages are important in mediating cytokine production and anti-bacterial defense in septic mice, we questioned whether the increased bacterial burden and cytokine production caused by the activation of A_{2A} receptors in septic animals are mediated by macrophages.

Materials and methods

Experimental animals and cell cultures

A_{2A} receptor KO mice and their WT littermates [32, 33] on the CD-1 background were bred in a specific pathogen-free facility, using founder heterozygous male and female mice. CD-1 male mice were purchased from Charles River Laboratories. 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. 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 was performed by using RT-PCR as described previously [32–34]. CLP-elicited mouse peritoneal macrophages were cultured in Dulbeccos's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, and 1.5 mg/ml sodium bicarbonate in a humified atmosphere of 95% air and 5% CO_2.

Cecal ligation and puncture

Polymicrobial sepsis was induced by subjecting mice to CLP, as we have described previously [32]. Six- to eight-week-old male A_{2A}R KO or WT mice were anesthetized with pentobarbital (50 mg/kg), given intraperitoneally (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 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 was closed in two layers with 4.0 silk sutures. The mice were resuscitated with 1 ml of physiological saline injected subcutaneously (s.c.) and returned to their cages with free access to food and water.

Harvesting of peritoneal macrophages

Sixteen hours after the operation, mice were reanesthetized with pentobarbital (50 mg/kg i.p.), and peritoneal macrophages were harvested in 4 ml of sterile physiological saline. Peritoneal cells were seeded into multiwell tissue culture plates. After 4 h of incubation, nonadherent cells were removed by washing with serum-free DMEM, following which phagocytosis assay was performed.

Opsonization of *E. coli* and phagocytosis assay

Fluorescein isothiocyanate (FITC)-labeled *E. coli* (Molecular Probes) were opsonized by incubation with an equal volume of opsonizing reagent (Molecular Probes) for 1 h at 37°C. The bacteria were then washed twice in phosphate-buffered saline and added to macrophages at a macrophage-to-bacteria ratio of 1:50 for 2.5, 5, or 16 h. At the end of the incubation period, the medium was removed and frozen until use in ELISA experiments (see below). Thereafter, 100 μl of 250 μg/ml Trypan Blue suspension was added to all wells for 1 min. The Trypan Blue solution was then removed by aspiration, and the experimental and control wells (without peritoneal macrophages) were read with a fluorescence plate reader at 480 nm for excitation and 520 nm for emission.

Determination of IL-10, IL-6, and MIP-2 cytokine levels

IL-10, IL-6, and MIP-2 cytokine levels were determined from cell supernatants taken at the indicated points following treatment of macrophages with opsonized bacteria. Cytokine concentrations were measured using DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Values in the figures are expressed as mean ± SEM of *n* observations. Statistical analysis of the data was performed by one-way analysis of variance followed by Dunnett's test, as appropriate.

Results

Effect of A_{2A} adenosine receptor inactivation on macrophage phagocytosis of opsonized *E. coli*

Macrophages play an essential role in the innate immune response against bacterial invasion, and they also eliminate bacteria from the peritoneum and bloodstream during sepsis. Since the inactivation of A_{2A} receptors enhances bacterial clearance, we first examined whether the absence of A_{2A} receptors has any impact on the phagocytic activity of peritoneal macrophages. As illustrated in Fig. 1, no significant differences in macrophage phagocytotic activity were detected between A_{2A} WT (n=5) or A_{2A} KO (n=8) cells.

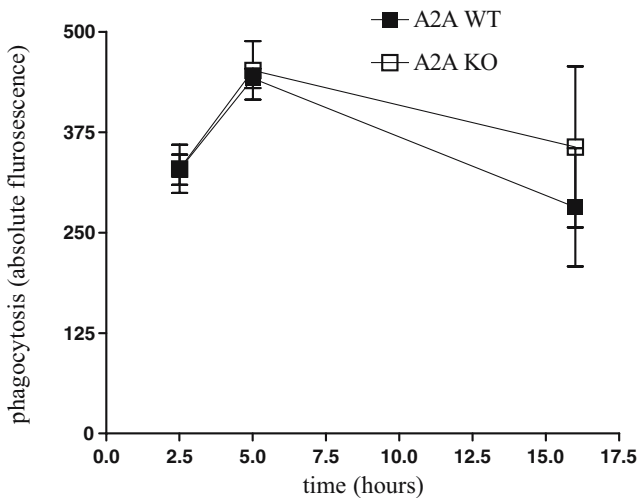

Fig. 1 Phagocytic activity of A_{2A} WT and KO CLP-elicited peritoneal macrophages. Peritoneal macrophages were harvested from A_{2A} WT and KO mice after 16 h of polymicrobial sepsis induced by cecal ligation and puncture. Cells were seeded at 10^6/ml density in 96-well plates. Peritoneal macrophages were then stimulated with IgG-coated, FITC-labeled *E. coli* for 2.5, 5 or 16 h, and phagocytosis was quantitated by measuring fluorescence. Results (mean ± SEM, n=5–8) shown are one representative experiment from three separate studies

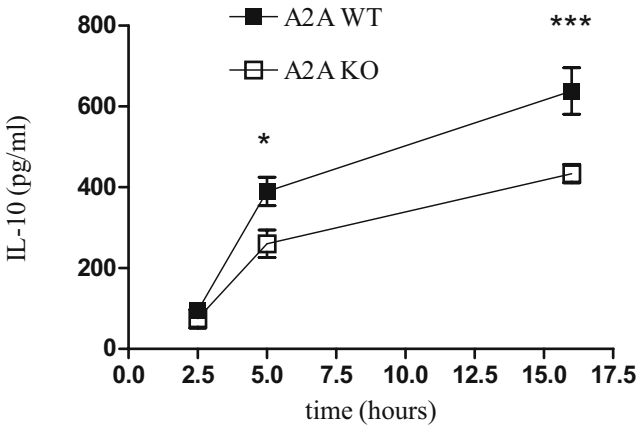

Fig. 2 IL-10 release is decreased by peritoneal macrophages obtained from A_{2A} KO vs. WT mice. Peritoneal macrophages were obtained from A_{2A} WT and KO mice 16 h after cecal ligation and puncture. Cells were plated at a density of 10^6/ml in 96-well plates. Cells were stimulated with IgG-coated FITC-labeled *E. coli* for the indicated time periods, and IL-10 release was measured using ELISA. Results (mean ± SEM) depicted are from n=30–48 wells from six separate experiments. *P<0.05, ***P<0.005 vs. A_{2A} WT group

Effect of A_{2A} adenosine receptor inactivation on macrophage cytokine release

IL-10 is a potent anti-inflammatory and immunosuppressive cytokine that has been shown to be elevated in patients with sepsis. We have previously shown that its level was markedly lower in A_{2A} KO mice than its WT littermates under septic conditions. Therefore, we measured the IL-10 level after stimulation with opsonized *E. coli* in CLP-elicited peritoneal macrophages. We observed that opsonized *E. coli* enhanced IL-10 levels (n=30 in case of A_{2A} WT), the induction of which was inhibited by genetic inactivation of A_{2A} receptors (n=48 in case of A_{2A} KO) (Fig. 2).

Our previous work has shown that the level of IL-6 was markedly lower in the bloodstream of A_{2A} KO receptor mice compared with their WT counterparts in CLP-induced polymicrobial sepsis [32]. To assess the effect of A_{2A} receptor inactivation on IL-6 release by macrophages, CLP-elicited peritoneal macrophages were stimulated with opsonized *E. coli*. We found that the IL-6 level was markedly higher in A_{2A} KO macrophages stimulated with opsonized *E. coli* (n=48) than in WT (n=30) macrophages at 2.5 h, and this difference was even more robust at 5 and 16 h (Fig. 3).

We previously observed that MIP-2 production was decreased both in the bloodstream and in the peritoneum of A_{2A} KO vs. WT mice under septic conditions [32]. Consequently, we studied the effect of opsonized *E. coli* on the release of the MIP-2 in CLP-elicited macrophages.

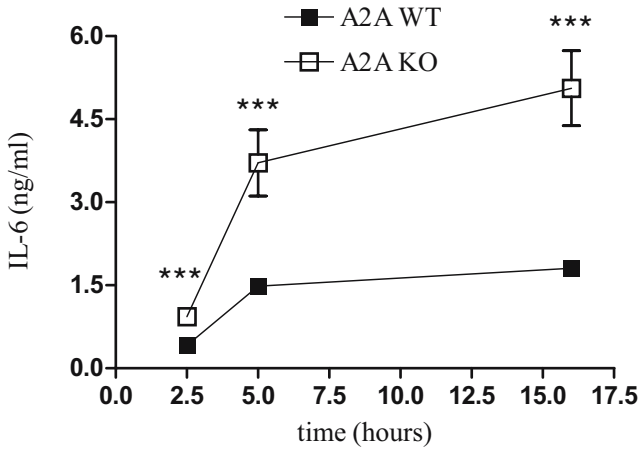

Fig. 3 Effect of genetic inactivation of the A_{2A} receptor on IL-6 production by CLP-elicited peritoneal macrophages. Peritoneal macrophages were obtained from A_{2A} WT and KO mice 16 h after cecal ligation and puncture. Cells were plated at a density of 10^6/ml in 96-well plates. Cells were stimulated with IgG-coated FITC-labeled *E. coli* for the indicated time periods, and IL-6 release was measured using ELISA. Results (mean ± SEM) depicted are from n=30–48 wells from six separate experiments. ***P<0.005 vs. A_{2A} WT group

As illustrated in Fig. 4, the MIP-2 release induced by opsonized *E. coli* was higher in A_{2A} KO (n=48) than WT (n=30) macrophages.

Discussion

The most important result of this study is that the increased bacterial clearance we found in septic A_{2A} KO mice [32] is not due to the enhanced phagocytic activity of peritoneal macrophages. Bacterial clearance is an elaborate process that includes bacterial killing as well as phagocytosis by macrophages and neutrophils. We reported in a previous study that A_{2A} receptor inactivation decreased bacterial burden in septic mice [32]. Thus, in the present study we questioned whether this decreased bacterial load in A_{2A} KO mice was due to enhanced phagocytosis by macrophages. Our results indicated that this was not the case, because A_{2A} KO macrophages did not ingest greater numbers of opsonized *E. coli* than WT macrophages.

The regulation of FcγR-mediated phagocytosis by adenosine was one of the first effects described for this purine nucleoside in modifying macrophage functionality [35]. It is well known that adenosine augments FcγR-mediated phagocytosis by human monocytes [30]. In contrast to its stimulatory effect on phagocytosis by human monocytes, adenosine reduced FcγR-mediated erythrocyte phagocytosis by mouse peritoneal macrophages [36, 37], suggesting that depending on the cellular stimulus and source, adenosine may have differential effects on phagocytosis. In this study, we found that there was no difference in the FcγR-mediated phagocytotic activity of A_{2A} KO and WT peritoneal macrophages. These results suggest that the decreased bacterial burden in A_{2A} KO mice under septic conditions is not due to enhanced phagocytosis by macrophages. Since actual bacterial burden is determined by both bacterial dissemination and clearance, and clearance is a complex process that includes not only phagocytosis but also killing of pathogens by macrophages and other cell types, it will be important in the future to study how A_{2A} receptors govern all these bactericidal functions.

In a previous study [32], we showed that A_{2A} receptor inactivation decreased IL-10, IL-6, and MIP-2 production in the bloodstream and in the peritoneum in CLP-induced sepsis. In this study we observed that opsonized-*E. coli*-induced IL-10 release was diminished in A_{2A} KO peritoneal macrophages in vitro. This finding is in accord with our previous in vivo data obtained in the CLP model and suggests that under septic conditions the decreased IL-10 level of A_{2A} KO mice is due to a decreased production of IL-10 by macrophages. In contrast to the in vivo results showing decreased IL-6 and MIP-2 production in A_{2A} receptor-inactivated mice [32], we found that peritoneal macrophages isolated from A_{2A} KO mice showed enhanced IL-6 and MIP-2 production as compared with WT macrophages. These results suggest that other cell types or other macrophage populations may be responsible for IL-6 and MIP-2 production under septic conditions. In this regard, it is noteworthy that Riedemann et al. [38] demonstrated that serum IL-6 concentrations during sepsis in rats are reduced when neutrophils are depleted. Furthermore, it has been reported that $GdCl_3$ (a Kupffer cell-depleting agent)

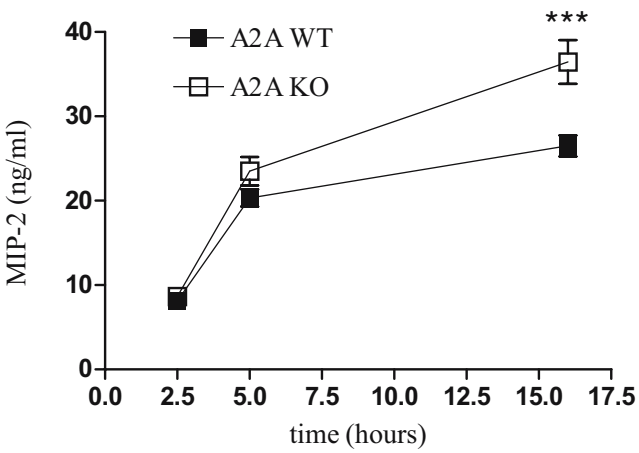

Fig. 4 Effect of genetic inactivation of the A_{2A} receptor on MIP-2 production by CLP-elicited peritoneal macrophages. Peritoneal macrophages were obtained from A_{2A} WT and KO mice 16 h after CLP. Cells were plated at a density of 10^6/ml in 96-well plates. Cells were stimulated with IgG-coated FITC-labeled *E. coli* for the indicated time periods, and MIP-2 release was measured using ELISA. Results (mean ± SEM) depicted are from n=30–48 wells from six separate experiments. ***P<0.05 vs. A_{2A} WT group

prevents the up-regulation of IL-6 in a mouse septic peritonitis model [39]. In addition, it has been shown that MIP-2 production by liver mononuclear cells in mice with peritonitis is significantly increased compared with sham-operated mice, confirming the important role of Kupffer cells in cytokine production during sepsis [40]. Therefore, it is possible that either neutrophils or Kupffer cells, both of which express A_{2A} receptors [14, 25], are responsible for the decreased IL-6 and MIP-2 levels that we detected in A_{2A} KO vs. WT mice [32].

In summary, A_{2A} receptors differentially regulate immune function in vitro and in vivo. In the future it will be interesting to determine the mechanisms that are responsible for these differences.

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