Anti-Inflammatory Preconditioning by Agonists of Adenosine A1 Receptor

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Abstract

Background: Adenosine levels rise during inflammation and modulate inflammatory responses by engaging with four different G protein-coupled receptors. It is suggested that adenosine exhibits pro-inflammatory effects through its A1 receptor (A1R), and anti-inflammatory effects through A2A receptor (A2AR). Therefore, understanding of the mechanisms that govern adenosine receptor regulation may advance treatment of various inflammatory disorders. We previously reported that peak A1R expression during leukocyte recruitment, is followed by a peak in A2AR during inflammation resolution.

Principal Findings: Here, we examined whether A1R activation sequentially induces A2AR expression and by this reverses inflammation. The effect of adenosine on A1R mediated A2AR expression was examined in peritoneal macrophages (PMMc) and primary peritoneal mesothelial cells (PMC) in vitro. Induction of A2AR was inhibited by pertussis toxin (PTX) and partly dependent on A3AR stimulation. Administration of A1R agonists to healthy mice reduced A1R expression and induced A2AR production in PMC. Mice that were preconditioned with A1R agonists 24 hours before E. coli inoculation exhibited decreased TNFα and IL-6 sera levels and reduced leukocytes recruitment. Preconditioning was blocked by pretreatment with A1R antagonist, as well as, or by late treatment with A2AR antagonist, and was absent in A2AR−/− mice.

Conclusions: Our data suggest that preconditioning by an A1R-agonist promotes the resolution of inflammation by inducing the production of A2AR. Future implications may include early treatment during inflammatory disorders or pretreatment before anticipated high risk inflammatory events, such as invasive surgery and organ transplantation.

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Introduction

Over the past few years, a vast number of investigations have reported the involvement of adenosine in the anti-inflammatory process [1,2]. Adenosine is an endogenous purine nucleoside that is constitutively present in the extracellular spaces at low concentrations. However, in metabolically-stressful conditions such as tissue damage, ischemia and inflammation, adenosine dramatically increases its extracellular levels. Extracellular adenosine levels have been observed to increase by dephosphorylation of ATP in non-immune and immune cells [1] and then to be released through the action of specialized nucleoside transporters [3]. Extracellular adenosine interacts with at least four different G protein-coupled receptors [4–6]. The A2A receptor (A2AR) interacts with the G protein Gαq and the A3 receptor (A3R) interacts with the G protein Gαi to induce adenyl cyclase activity and elevate cAMP levels. In contrast, ligation of adenosine to the A1 receptor (A1R) or to the A2B receptor (A2BR), through interaction with members of the Gs/Gαi family, inhibits adenyl cyclase activity and decreases cAMP levels [7]. A1R exerts a pro-inflammatory response by enhancing phagocytosis [8], promoting chemotaxis [9,10] and enhancing neutrophils adherence to endothelium during inflammatory process [11]. In contrast, engagement of A2AR inhibits neutrophils adherence to endothelium during inflammation [12] and inhibits the activation of neutrophils, monocytes platelets and T-cells [13–15]. In animal models, A2AR-agonists can prevent lethal response to bacterial LPS and sepsis [16,17].

Since each of these receptor subtypes has a unique physiological profile and a particular affinity to its ligand, the inflammatory state is determined by both extracellular adenosine concentrations and by the distribution and expression levels of its receptor subtypes. It has been shown that the expression of adenosine receptors is regulated by factors that are involved in the inflammatory response, such as LPS [18], pro-inflammatory cytokines [19–21], growth factors [22,23] and glucocorticoids [24]. Recently, we have shown in a model of peritonitis that shortly following inoculation, A1R mRNA and protein levels are upregulated on peritoneal mesothelial cells (PMC), reaching a peak in the initial phase of the inflammatory process [19]. Interestingly, concomitant with the resolution phase of peritonitis, we observed a decrease in A1R...
expression levels and an elevation of adenosine and A2AR levels.

The coordinated kinetics of adenosine and its receptors led to the hypothesis that adenosine differentially regulates its own receptors. Since the two receptors, A1R and A2AR, have opposing biological effects, and A1R domination precedes the elevation of A2AR, we sought to examine whether A1R activation would be one of the factors that trigger the anti-inflammatory phase, and whether this action is mediated by upregulation of the A2AR.

To test our hypothesis, we examined the effect of adenosine receptor agonists and antagonists in vivo in a model of peritonitis induced by *E. coli* inoculation. This model has particular clinical significance because peritonitis is commonly caused by pathological processes of the gastrointestinal tract or as a complication of abdominal surgery. In vivo, we examined the regulation of the receptors on the cell surface of PMΦ, which are the first line of cellular defense against bacterial invasion in the peritoneum [25], and on PMC, the cells that line the peritoneal membrane and therefore play an important role in transferring inflammatory signals from the peritoneal cavity to the blood vessels [26–30]. We demonstrate that A1R activation triggers the switching of adenosine receptor subtype from A1R to A2AR. By the anti-inflammatory effects of the ligation of adenosine to the A2AR, the described receptor subtype switch alters the progression of inflammation toward resolution.

**Materials and Methods**

**Mice, bacterial strains and drugs**

CD1 female mice aged 10 to 12 weeks (Harlan, Jerusalem, Israel) were maintained in the animal laboratory of the Soroka Medical Center. Experiments were conducted with the permission of the Israel Committee for Animal Experiments. A2AR−/− mice whose phenotype is well established in the literature were graciously kindly donated by Catherine Ledent (Université Libre de Bruxelles) [31].

*Escherichia coli* (*E. coli*) were grown in Luria-Bertani broth (Conda Laboratories, Madrid, Spain) and harvested during the log phase. Bacteria aliquots in Luria-Bertani broth containing 30% glycerol were stored frozen at −70°C. Adenosine (Adenocor) was purchased from Sanofi Winthrop (Auckland, NZ). A2AR antagonist 4-[[7-Amino-2-(2-furyl)[1,2,4]triazin-5-ylamino]ethyl phenol (ZM241385) was purchased from Tocris Cookson (Ellisville, MO). Pertussis toxin (PTX) and other Adenosine receptor agonists and antagonists were purchased from Sigma (St. Louis, MO) [32].

Bacteria aliquots in Luria-Bertani broth containing 30% glycerol were grown in Luria-Bertani broth at 37°C for 24 h. The bacterial strain used was *E. coli* Nissle 1917, which is the first line of defense against *E. coli* and *Salmonella* infections [33].

**Preparation of cultured PMC and PMΦ**

To prepare PMC, the peritoneum was removed from eight newborn (two-week-old) mice and isolated, as previously described [32]. To assess the purity of mesothelial cells, samples of each PMC preparation were morphologically inspected, as previously described [33]. Cells were grown in M199 and supplemented with 10% heat-inactivated FCS, 2 mmol/l L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin (Biological Industries, Bet Haemek, Israel). Experiments were performed on cells from the second to fourth passages. To prepare PMΦ, mice were injected intraperitoneally with 3 ml of 3% thioglycollate (Difco, Sparks, MD). After 3 days, peritoneal cells were collected by lavage and seeded onto 12-well plates in RPMI supplemented with 10% heat-inactivated FCS, 2 mmol/l L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin. Non-adherent cells were subsequently removed by washing with PBS. In experiments, to simulate the gradual increase in adenosine levels found in vivo, cells were treated with increasing doses of adenosine or CHA with or without DPCPX (9 hours with 0.1 μM or 3 hours with 0.1 μM and then 6 hours with 1 μM or 3 hours with 0.1 μM, then 3 hours with 1 μM and then 3 hours with 10 μM).

**Induction of peritonitis and treatment protocol**

Peritonitis was induced in mice by intraperitoneal (i.p.) inoculation of a sub-lethal dose of *E. coli* (3.6×10⁹ CFU). Adenosine agonists and antagonists were injected i.p. before *E. coli* inoculation.

**Sera and peritoneal lavage fluids collection, leukocyte counting and cytokine detection**

At different time points after *E. coli* inoculation, animals were anesthetized. 1 ml syringe flushed with heparin was used to draw intracardial blood sample. The samples were stored on ice before centrifugation at 1,000 g at 4°C for 10 minutes. The cell-free supernatants were collected and frozen at −20°C until assayed by ELISA. Peritoneal lavage was performed with 5 ml phosphate buffer saline (PBS) containing 2% BSA and 5 mM EDTA. After centrifugation at 400 g for 10 minutes, the cell-free supernatants were removed and frozen at −20°C until analysis. TNFα and IL-6 levels were determined by commercial ELISA kits (Bioregen, San Diego, CA and R&D Systems, Minneapolis, MN, respectively). Cells were washed once, and total leukocytes were counted after trypsin blue staining using an improved Neubauer hemocytometer. Cell counts and ELISA were performed blindly on coded samples.

**Scraping of mice PMC**

Following treatment, animals were anesthetized and PMC were scraped from the peritoneal membrane. The cells were stored on ice before centrifugation at 400g and 4°C for 10 minutes. Cells were harvested with lysis buffer for analyzing mRNA levels or with RIPA (150 mM NaCl, 50 mM Tris HCl pH 7.4, 1% NP-40, 0.25% Na deoxycholate, 1 mM EGTA) including protease inhibitor cocktail (Sigma) for analyzing protein levels.

**Scraper of mice PMC**

At different time points after *E. coli* inoculation, animals were anesthetized and frozen in liquid nitrogen. A2AR mRNA expression levels and an elevation of adenosine and A2AR levels were determined by commercial ELISA kits (Bioregen, San Diego, CA and R&D Systems, Minneapolis, MN, respectively).

**mRNA analysis**

Total RNA was extracted from PMC or PMΦ using the Versagene RNA cell kit (Genra systems, Minneapolis, MN). cDNA was prepared as previously described [29]. Quantitative real time PCR (QPCR) assays were carried out for β-actin, GAPDH, A1R, A2AR, macrophage inflammatory protein-2 (MIP-2) and monocyte chemotactic protein-1 (MCP-1) with the following primers: β-actin sense: 5′-GGG TCA GGA GGA TTC CTA TG-3′, β-actin antisense: 5′-GTT CTT CCC ATA CAT CTT CTG GG-3′, GAPDH sense: 5′-ACA TGC TAT GCT CAC CAA AGC-3′, GAPDH antisense: 5′-ATG AGT TAT GGA AAT GCC AGC AGC-3′, A1R sense: 5′-TAC ATC ATG GCC TAC CAG GTG G-3′, A1R antisense: 5′-AAC AGG TAC CAC AAC ACA-3′, A2AR sense: 5′-ATG GTT GCC AGG CAG GAA GCC C-3′, A2AR antisense: 5′-GCA TCC GGG ACT TTA AAC CAG AGA-3′, MIP-2 sense: 5′-CCT CTC ATG GTT GCA GGT CTG-3′, MIP-2 antisense: 5′-TCC CGG GTG CTG TTT GTT T-3′, MCP-1 sense: 5′-CTC ACC TGC TGC TAC TCA TCC-3′, MCP-1 anti sense: 5′-GGT TCA GGT GGT TGT GAA AAA-3′. cDNAs were diluted x9, mixed with primers (0.2 mM) and Thermo start master mix (ABgene, Surrey, UK).
Adenosine receptors exhibit unique expression kinetics in peritoneal leukocytes following bacterial inoculation

It has been shown that adenosine is upregulated during peritonitis [19]. We therefore examined the regulation of adenosine receptors in peritoneal leukocytes and found that the A1R and A2AR are upregulated during the first 48 hours of peritonitis. However, each of the subtypes exerted unique kinetics. As shown in figure 1, A1R mRNA levels were maximal at 6 hours after inoculation and returned to basal levels at 24 hours, while A2AR mRNA levels gradually increased and reached maximum at 24 hours.

Adenosine induces the expression of A2AR in a dose-dependent manner

Since both adenosine and adenosine receptors are upregulated upon bacterial inoculation [19], we wanted to elucidate whether the regulation of adenosine receptors is adenosine-dependent. In order to simulate the gradual and accumulative increase of adenosine that is observed in vivo, we treated cultured PMCs with multiple and increasing concentrations of adenosine (0.1, 1 and 10 μM at 3 hours intervals). As shown in Figure 2, adenosine induced the expression of A2AR mRNA levels in a dose dependent manner. However, there was no change in A1R mRNA levels upon treatment with the different concentrations of adenosine.

Adenosine regulates A2AR expression through A1R

Since A1R is elevated shortly after bacterial inoculation (Figure 1) and is followed by elevation of A2AR expression, we wanted to examine whether the induction of A2AR by adenosine may be mediated by the A1R. Therefore, we treated PMC and PMΦ with 0.1, 1 and 10 μM at 3 hour intervals with A1R agonist (CHA) or adenosine in the presence or absence of the A1R antagonist (DPCPX, 50 nM). As shown in Figure 3A and B, CHA upregulated mRNA levels of the A2AR while treatment with adenosine in the presence of the DPCPX blocked A2AR upregulation both in PMΦ and PMC respectively. In contrast, stimulation with CGS, an A2AR agonist failed to induce A2AR (Figure 3D).

Ligation of adenosine to the A1R is mediated through the interaction with members of the G\textsubscript{i}/G\textsubscript{o} family and inhibits adenylyl cyclase activity. To elucidate the mechanism by which A1R induces A2AR elevation, we pretreated PMC with PTX, a G\textsubscript{i} inhibitor (Figure 3C). Pretreatment with PTX blocked the effect of CHA on A2AR mRNA levels.

For effective induction of A2AR a sequential induction with increasing doses of adenosine or CHA (0.1, 1, 10 μM) were
necessary suggesting the involvement of an additional adenosine receptor. CCPA, a specific A1R agonist, was less effective than CHA, an A1R agonist with lower specificity (Figure 3D). ZM241385, an A2AR antagonist, partially blocked the induction of A2AR mRNA that was induced by adenosine (Figure 3D) or CHA (data not shown), which suggests that in addition to the requirement of A1R stimulation, A2AR ligation supports its own induction. Treatment with adenosine in the presence of A3R (MRS1220, 100 nM) or A2BR antagonist (MRS1754, 50 nM) did not alter A2AR mRNA levels (data not shown).

**Effect of A1R agonist on the expression of A2AR and A1R in vivo**

We examine whether the A1R agonist also regulates the levels of the A2AR in mice that were administered an A1R agonist (CHA, 0.1 mg/kg). We found that A2AR mRNA levels increase ~3 fold and that A2AR protein levels increase ~2.5 fold, compared to vehicle. In contrast, as shown in Figure 4, both A1R mRNA and protein levels decreased in the presence of A1R agonist by ~6 and ~2 fold, respectively.

**Pretreatment with the A1R agonist reduces serum cytokine levels and peritoneal leukocyte recruitment during inflammation**

Since we showed that A2AR levels are upregulated through the activation of A1R both in vitro and in vivo, we wanted to elucidate whether pretreatment of A1R agonist before inoculation would upregulate the expression of A2AR and lead to advancement of the anti-inflammatory response via A2AR. For this, mice were treated

![Figure 3. A1R trigger the induction of A2AR in vitro.](image-url)
with an A1R agonist (CHA, 0.1 mg/kg) 24 hours before inoculation of *E. coli*, after which sera were analyzed for IL-6 and TNFα levels. As shown in Figure 5A, we found a significant reduction both in serum IL-6 and TNFα levels 12 hours after inoculation (to 25% and 38% from vehicle, respectively).

Since PMC express an array of chemokines which cause accumulation and activation of leukocytes in tissues, we wanted to examine changes in the levels of CXC chemokines, MCP-1 and MIP-2, following pretreatment with A1R agonist. As a result of pretreatment with the A1R agonist (CHA 0.1mg/kg), MCP-1 and MIP-2 mRNA levels decreased in comparison to vehicle, as determined 12 hours after inoculation (Figure 5B). In accordance with reduced chemokine levels, leukocyte recruitment significantly decreased 24 hours after inoculation to 66% from vehicle, as determined in lavage fluid (Figure 5C).

**A1R-agonist preconditioning is blocked by a selective A1R antagonist**

To ensure that the anti-inflammatory state was mediated by selective activation of the A1R, we examined the anti-inflammatory effect of low-dose CHA and an additional specific A1R-agonist CCPA, in the presence of a specific A1R antagonist (DPCPX). As shown in Figure 6, treatment with either CCPA (A) or CHA (B) significantly reduced serum and lavage IL-6 and TNFα levels. However, pretreatment with an A1R antagonist (DPCPX, 1 mg/kg) 2 hours before administration of A1R agonist blocked the effect of 0.02 mg/kg CHA, 0.1 mg/kg CHA (data not shown) and 0.1 mg/kg CCPA.

**Figure 4. Effect of A1R agonist on A1R and A2AR levels in vivo.** Mice were administered i.p. with the A1R agonist (CHA, 0.1 mg/kg) or with vehicle. PMC were scraped from the peritoneal surface and analyzed for (A) A2AR and A1R mRNA levels at 4 hours or (B+C) A2AR and A1R protein levels at 24 hours. (B) Densitometry of protein blot depicted in (C). A1R and A2AR mRNA levels were normalized to GAPDH and protein levels were normalized to β-actin. Results are presented as fold change from vehicle-treated animals. Data represent three experiments and are expressed as mean±SEM. * p<0.05 between conditions per receptor, n = 4 for each experiment. doi:10.1371/journal.pone.0002107.g004

**Figure 5. The anti-inflammatory effect of pretreatment with the A1R agonist.** Mice were treated with the A1R agonist (CHA, i.p., 0.1 mg/kg) or vehicle 24 hours prior to bacterial inoculation. (A) Sera levels of IL-6 and TNFα at 12 hours. (B) Chemokine mRNA levels. 12 hours after inoculation PMC were scraped from the peritoneal membrane and total RNA was extracted, analyzed for MCP-1 and MIP-2 mRNA levels and normalized to β-actin. (C) Total cell count at 24 hours after inoculation. Cell exudates were collected from peritoneal lavage fluid. Data represent five experiments and are expressed as mean±SEM for serum cytokine levels and as mean±SEM fold of control for chemokine mRNA levels.* p<0.05, ** p<0.01, n = 5 for each experiment. doi:10.1371/journal.pone.0002107.g005
Modulation of the inflammatory response due to pretreatment with the A1R agonist is A2AR-dependent

To prove that the modulation in the inflammatory response (Figure 5) is mediated by A2AR, we treated animals with an A2AR antagonist (30 min before inoculation, ZM241385, 1 mg/kg). As shown in figure 7, blockade of the A2AR caused an increase in serum and lavage IL-6 and TNFα levels to similar levels found in infected mice administrated with vehicle alone. As expected, administration of A2AR agonist (30 min before inoculation, CGS21680, 1 mg/kg) reduced IL-6 and TNFα levels in serum and lavage fluids to levels comparable to those found in CHA-treated animals. In concordance, pretreatment of A2AR2/2 mice with A1R agonist resulted in unchanged serum IL-6 and TNFα levels (Figure 7C), as well as chemokine mRNA levels in PMC (data not shown). However, in WT mice there was a significant reduction both in cytokine levels and mRNA chemokine levels (data not shown). These data suggest that the modulation of the inflammatory response caused by pretreatment with A1R agonist is, indeed, mediated by A2AR.

Discussion

The study presented here demonstrates a novel mechanism of adenosine receptor subtype autoregulation. Since adenosine action is mediated through at least four different receptors, each of which exhibits a unique affinity and opposing signaling pathways, the regulation of subtypes expression is critical for determining the outcome of adenosine activity [5]. Others and we have shown that adenosine receptors are regulated by various inflammatory mediators and multiple endogenous factors [24]. For example, we found that A2AR mRNA and protein levels are upregulated in human PMC following treatment with IL-1β and TNFα, while treatment with IFNγ strongly decrease A2AR expression both alone and in combination with IL-1β and TNFα [19]. In the same study, we show that following inoculation, adenosine receptor levels on PMCs are sequentially upregulated and that adenosine is induced following inoculation and reaches peak levels at 24 hours [19]. The A1R is induced during the first phase of leukocyte recruitment and the A2AR is induced later, at the resolution phase of peritonitis [19]. In the present study, we obtained the same pattern of adenosine receptor expression on peritoneal leukocytes.
These results suggest that both mesothelial cells and the recruited leukocytes are highly synchronized in their response to adenosine. Furthermore, this sequential elevation of the A1R and the A2AR on PMC and leukocytes suggests that adenosine may regulate its receptors. Both our in vitro and in vivo data in the current study support this suggestion; we found that adenosine significantly upregulates A2AR expression levels in isolated PMC in a dose dependent manner.

Of all adenosine receptor subtypes, A1R exhibits the highest affinity for adenosine \( K_i = 10 \text{ nM} \) [34], implying that A1R is activated at the low levels of adenosine produced during the initiation of inflammation. This early activation of A1R receptor may enable the induction of A2AR. The A1R agonist, CHA, significantly induced the expression of A2AR, while treatment with the A2AR antagonist in the presence of adenosine partially blocked A2AR induction. Therefore, one can conclude that A1R ligation by elevated levels of adenosine is sufficient to support the initial signal of A1R.

According to our in vitro data, mice treated with CHA exhibited a significant 2-3 fold increase in A2AR mRNA and protein levels as determined, in PMCs compared to untreated animals. Interestingly, mRNA and protein A1R levels were significantly down-regulated by some treatments in PMCs (6- and 2-fold decrease, respectively), suggesting that A1R receptor may be responsible for the “switching” between the two receptor subtypes during inflammation. In Support of our findings, Schnurr et al. showed that in immature plasmacytoid dendritic cells (PDCs) adenosine activates A1R, which induces chemotaxis; however, in mature PDCs, A1R is replaced by the A2AR, which inhibits cytokine production [9].

In order to understand the physiological role of the exchange between the two receptors, we examined whether ligation of the A1R will trigger the induction of the A2AR and lead to an advancement of the resolution phase of the inflammatory process. We found that preconditioning with an A1R agonist significantly reduces the inflammatory response to bacterial challenge. CHA or CCPA administration at 24 hours before inoculation significantly reduced sera and peritoneal levels of the pro-inflammatory cytokines TNF\(\alpha\) and IL-6, and reduced mRNA levels of chemokines on PMC as well as leukocyte recruitment to the peritoneum. The anti-inflammatory effect induced by pre-treatment (24 hours) with A1R agonist was also achieved by a specific A2AR agonist (CGS21680) administered to animals 30 minutes before bacterial inoculation. Pre-treatment with CHA or CCPA had no anti-inflammatory effect in animals that were administered with the A1R antagonist, 2 hours before agonists or A2AR antagonist, ZM241385 30 minutes before inoculation or when A2AR antagonist animals were examined. The marked blocking effect of ZM241385 and the lack of effect of CHA in A2AR knockout animals clearly indicate that the anti-inflammatory effects of the A1R agonist are mediated by the A2AR.

Elevation of cAMP usually down-regulates the inflammatory response [5]. Since A1R is a \( G_i \) coupled receptor that suppresses the induction cAMP, it is not surprising that this receptor had no direct anti-inflammatory effect. High expression of A1R implies that immediately after inoculation, decreased cAMP levels give rise to local pro-inflammatory cytokines and leukocyte migration, hence allowing an adequate and effective immune response to the invading microorganisms. In contrast, the increase in A2AR at late phases of peritonitis is probably associated with elevated cAMP levels, which markedly decrease local pro-inflammatory cytokine levels and leukocyte recruitment, hence restraining inflammatory flames (Figure 8).

In summary, our study sheds light on the sequential autoregulation of adenosine receptor subtypes. The mechanism we have describes may directly participate in the propagation of the compensatory anti-inflammatory response syndrome (CARS), which follows systemic inflammation in trauma patients. Whether patients with CARS exhibit elevated adenosine levels pursuing traumatic insult should be explored. These findings may also have future implications for clinical treatments by combining pre-treatment with an A1R agonist and subsequent A2AR agonist to enhance the anti-inflammatory effect, or to promote anti-inflammation by endogenous adenosine at the site of inflammation. As such, preconditioning with an A1R-agonist could be used in preparation of tissue for transplantation or to induce an anti-inflammatory and immunosuppressive state in patients before invasive surgery and organ transplantation.

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Author Contributions
Conceived and designed the experiments: EL AD SN CC YS. Performed the experiments: SN YS. Analyzed the data: AD SN. Contributed reagents/materials/analysis tools: AD GS DC MZ. Wrote the paper: EL AD SN.

Figure 8. Effect of adenosine receptor subtype autoregulation on the inflammatory process. (A) Early expression of A1R after bacterial inoculation decreases cAMP levels, enhances production of local pro-inflammatory cytokines and promotes leukocyte migration. (B) In a later phase of peritonitis A2AR expression increase by A1R which leads to increase in cAMP levels. High cAMP markedly decreases local pro-inflammatory cytokines and leukocyte recruitment, hence restraining inflammatory flames.

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