Adenosine is upregulated during peritonitis and is involved in downregulation of inflammation

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Loss of function of the peritoneal membrane is associated with peritonitis. Adenosine levels in sites of inflammation were shown to increase and exhibit immunoregulatory effects. Our aim was to elucidate the regulatory role of adenosine during peritonitis and to test the involvement of peritoneal mesothelial cells (PMC) in adenosine regulation. In a mice model of *Escherichia coli* peritonitis, the adenosine A_{2A} receptor ($A_{2A}R$) agonist (CGS21680) prevented leukocyte recruitment and reduced tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) levels. Peritonitis induced the elevation of adenosine with a peak at 24 h. Analysis of adenosine receptor levels on peritoneum showed that A_1 receptor (A_1R) protein levels peak at 12 h after inoculation and then return to baseline at 24 h, whereas high affinity $A_{2A}R$ protein levels peak at 24 h concomitantly with the peak of adenosine concentration. Low affinity A_{2B} receptor ($A_{2B}R$) levels elevated slowly, remaining elevated up to 48 h. In human PMC (HPMC), the early cytokines, IL-1-α, and TNF-α upregulated the A_{2B} and A_{2A} receptors. However, interferon-γ (IFN-γ) upregulated the $A_{2B}R$ and decreased $A_{2A}R$ levels. Treatment with the $A_{2A}R$ agonist reduced IL-1-dependent IL-6 secretion from HPMC. In conclusion, the kinetics of adenosine receptors suggest that at early stage of peritonitis, the A_1R dominates, and later its dominance is replaced by the G stimulatory (Gs) protein-coupled $A_{2A}R$ that suppresses inflammation. Early proinflammatory cytokines are an inducer of the $A_{2A}R$ and this receptor reduces their production and leukocyte recruitment. Future treatment with adenosine agonists should be considered for attenuating the damage to mesothelium during the course of acute peritonitis.

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The long-term success of peritoneal dialysis is often endangered by structural injury to the peritoneal membrane. This injury is characterized by replacement of the normal peritoneal structure with fibrotic tissue, profusion of capillary vessels, and loss of the mesothelial layer. These morphological alterations are sometimes associated with loss of peritoneal membrane function, leading to the necessity for discontinuation of peritoneal dialysis.[1,2] Apparently, the inflammatory process (which accompanies bacterial peritonitis) is one of the factors which damage the peritoneal membrane. During peritonitis, leukocytes that are shifted into the peritoneal cavity release toxic molecules in order to combat the bacterial invasion. However, these molecules may also be harmful to the peritoneal structure. Moreover, polymorphonuclear neutrophils are recruited into the peritoneal cavity via diapedesis. During this process, these cells strongly adhere to the mesothelium and release proinflammatory compounds such as proteolytic enzymes and reactive oxygen species which possibly injure the peritoneal membrane.[3] Consequently, neutrophil accumulation and their cytotoxic function in the peritoneal exudates must be strictly regulated in order to allow the killing of invading pathogens with minimal damage to the peritoneal tissue.

Peritoneal mesothelial cells (PMC) are not merely bystanders in inflammatory processes, but rather play a central role during inflammation process, within peritoneal cavity.[4] First, as we and others have shown, PMC are a potent source for various proinflammatory mediators, including interleukin (IL)-1α and IL-1β, IL-15, IL-8, monocyte chemotactic protein (MCP)-1, RANTES, IL-6, and growth factors such as transforming growth factor (TGF)-β.[5–9] In addition, PMC constitutively express intracellular adhesion molecule-1,[6] vascular cell adhesion molecule-1, and platelet endothelial cell adhesion molecule-1.[10] PMC are also capable of antigen presentation to lymphocytes and interacting with them.[6] These data underline the role of the mesothelial layer as an important immunomodulator during inflammatory process taking place in the peritoneal cavity.

Over the past few years, a vast number of investigations have reported an involvement of adenosine as an anti-inflammatory mediator.[11,12] Adenosine is an endogenous purine nucleoside that following its release from cells or after

being formed extracellularly diffuses into the cell membrane of surrounding cells where it binds specifically to four known subtypes of adenosine receptors, referred to as A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R.[13–15] All are members of the superfamily of G protein-coupled receptors. The $A_{2A}R$ interacts with the G protein Gs and the ($A_{2B}R$) interacts with the G proteins Gs and Gq (Gq is Gα subunit which stimulates phospholipase C) to stimulate adenylyl cyclase activity thereby causing accumulation of intracellular cAMP levels,[11,16] which have potent immunosuppressive effects.[17] In contrast, the A_1R and A_3R, through interaction with members of the Gi/Go family, inhibit adenylyl cyclase and decrease levels of cAMP.[14] Adenosine has a direct effect on immune cells and contributes to the resolution of inflammation mainly through the $A_{2A}R$.[18–21] By downregulating neutrophil activity and macrophage activation, adenosine inhibits generation of reactive oxygen species and proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-1, IL-6, etc.[12,21–23]

Based on these findings, we hypothesized that adenosine may play an important role in regulating the anti-inflammatory response during acute peritonitis and thus, minimize the peritoneal membrane damage. In order to confirm this hypothesis, we tested the effect of the $A_{2A}R$ agonist on the intraperitoneal influx of leukocytes as well as the proinflammatory cytokine production during peritonitis. We also examined adenosine levels in the peritoneal cavity and the levels and regulation of adenosine receptors on PMC.

RESULTS

$A_{2A}R$ agonist blocks intraperitoneal influx of leukocytes and reduces TNF-α and IL-6 levels

As it has been reported that the $A_{2A}R$ is involved in the anti-inflammatory response,[18–20] we first tested the effect of its specific agonist, CGS-21680 on the intraperitoneal influx of leukocytes during acute peritonitis. As shown in Figure 1, treatment of *Escherichia coli*-inoculated mice with CGS-21680 significantly inhibited the influx of leukocytes into the peritoneal cavity (3.2×10^6 vs 13.2×10^6 cells in control group, $P<0.01$). Similar to this effect, the $A_{2A}R$ agonist strongly reduced TNF-α and IL-6 levels locally and systemically in the infected mice. Levels in the peritoneal fluid (from 48.9 to 22.5 pg/ml, $P>0.05$ and from 101.8 to 20.3, $P<0.05$, respectively) and in the serum (from 471.5 to 182.6 pg/ml, $P<0.001$ and from 150 to 25 pg/ml, $P<0.01$, respectively), as shown in Figure 2.

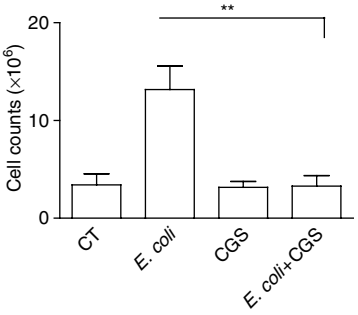

Figure 1 | Effect of adenosine $A_{2A}R$ agonist on leukocyte recruitment in the course of acute peritonitis in mice. CD1 mice were injected with saline or inoculated with a sublethal dose of *E. coli*. At 1 h before inoculation, mice were pretreated with saline or with the $A_{2A}R$ agonist, CGS-21680 (2 mg/kg). Cell exudates were collected at 24 h by peritoneal lavage and counted. The figure is a representative of three similar experiments. Results are presented as mean ± s.e. $N=5$ for each group, $^{**}P<0.01$.

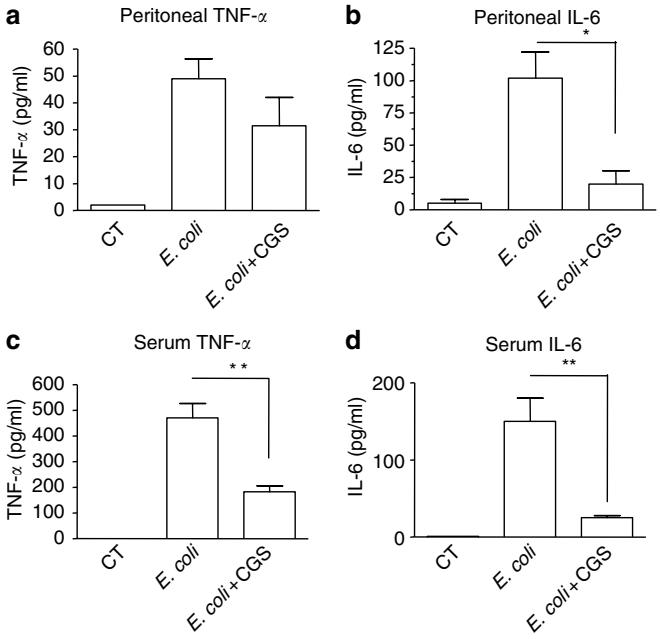

Figure 2 | Effect of $A_{2A}R$ agonist, CGS-21680 on TNF-α and IL-6 levels in the course of *E. coli*-induced peritonitis. CD1 mice were injected with saline or inoculated with a sublethal dose of *E. coli*, and also injected with saline or the $A_{2A}R$ agonist CGS-21680. At 24 h after inoculation, peritoneal lavage was performed, and cell-free supernatants were collected and assayed for (**a**) IL-6, (**b**) TNF-α by enzyme-linked immunosorbent assay kits (R&D Systems). (**c, d**) At 6 or 3 h after inoculation respectively, blood samples from the heart were collected and analyzed for IL-6 and TNF-α, respectively. The figure is a representative of three similar experiments. Results are presented as mean ± s.e. $N=5$ for each group, $^{*}P<0.05$.

Adenosine levels during peritonitis

The next stage was to examine adenosine kinetics during peritonitis in relation to the inflammatory process evolution. At different time points, after intraperitoneal *E. coli* inoculation, peritoneal lavage was performed and serum samples were taken for adenosine determination. No significant changes of adenosine levels were observed in blood following inoculation. Peritoneal leukocytes were counted, and adenosine levels in supernatants were examined concomitantly. As shown in Figure 3a, there is a rapid influx of leukocytes into the peritoneal cavity that reached peak at 12 h, which subsequently recedes slowly over the next 60 h. Adenosine levels rose gradually and reached a maximal level of $2.2 \pm 0.2\ \mu M$ (vs 0.3 ± 0.1) at 24 h and afterwards decreased back to basal levels (Figure 3b). The fact that adenosine levels

peaked at 24 h after inoculation simultaneously to when leukocyte infiltration began to diminish suggests that there is causative linkage between these two events.

Adenosine receptors levels in mesothelial cells and leukocytes

As there is no information reported to date, as whether adenosine receptors exist on peritoneal mesothelial cells, it was required to examine it for a complete picture of our model. As shown in Figure 4, mRNA and protein levels of A_1R, $A_{2A}R$, and $A_{2B}R$ receptors subtypes were upregulated shortly after *E. coli* inoculation. A_1R mRNA levels peaked 6 h after inoculation followed by an increase in protein levels that peaked at 12 h and returned to basal levels at 24 h. Similarly, $A_{2A}R$ mRNA levels peaked at 6 h but remained elevated as long as 24 h and then diminished to levels lower than basal. There was also an increase in $A_{2A}R$ protein levels that peaked at 24 h twofold higher than basal levels and then decreased. The basal mRNA and protein levels of the $A_{2B}R$ were very low and slowly increased during the course of peritonitis up to 48 h. However, A_3R mRNA levels decreased threefold lower than basal shortly after inoculation and remained at the same level up to 48 h.

Correspondingly, in peritoneal leukocytes, $A_{2A}R$, $A_{2B}R$ receptor mRNA levels were upregulated after *E. coli* inoculation, $A_{2A}R$ mRNA levels peaked at 24 h and decreased to levels lower than basal, whereas $A_{2B}R$ level slowly increased up to 72 h after inoculation (Figure 5b).

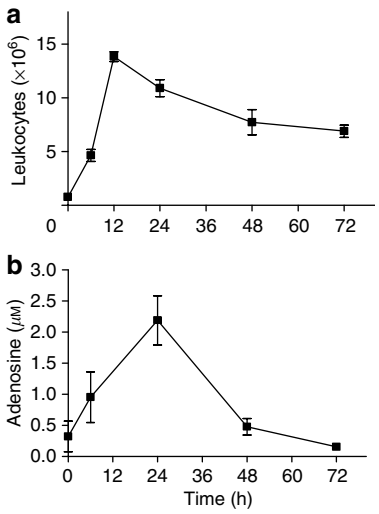

Figure 3 | Kinetics of adenosine generation in course of *E. coli*-induced peritonitis. CD1 mice were inoculated with a sublethal dose of *E. coli*. At the indicated time points after inoculation, peritoneal lavage was performed and (**a**) total leukocyte counts were determined. (**b**) Adenosine levels from peritoneal lavage were determined by HPLC. The figure is a representative of two similar experiments. Results are presented as mean ± s.e. $N = 4$ for each group.

The effect of inflammatory cytokines on adenosine receptors

To elucidate which mechanism regulates the levels of adenosine receptors during peritonitis, primary culture of human PMC (HPMC) were exposed to various proinflammatory cytokines. Different levels of the A_{2A} and A_{2B} receptors on HPMC were demonstrated. In the presence of IL-1, TNF-α, and IFN-γ individually or in combination, there was upregulation in mRNA and protein levels of the $A_{2B}R$ (Figure 6). In a similar manner, mRNA and protein levels of $A_{2A}R$ in the presence of IL-1 and TNF-α were also upregulated. In contrast, IFNγ strongly decreased $A_{2A}R$ mRNA and protein levels both alone and in combination with IL-1 and TNF-α (8.7 ± 0.7 vs 0.7 ± 0.1, $P < 0.01$ for mRNA and 9233.0 ± 200.1 vs 3116.2 ± 514.4, $P < 0.05$ for protein levels).

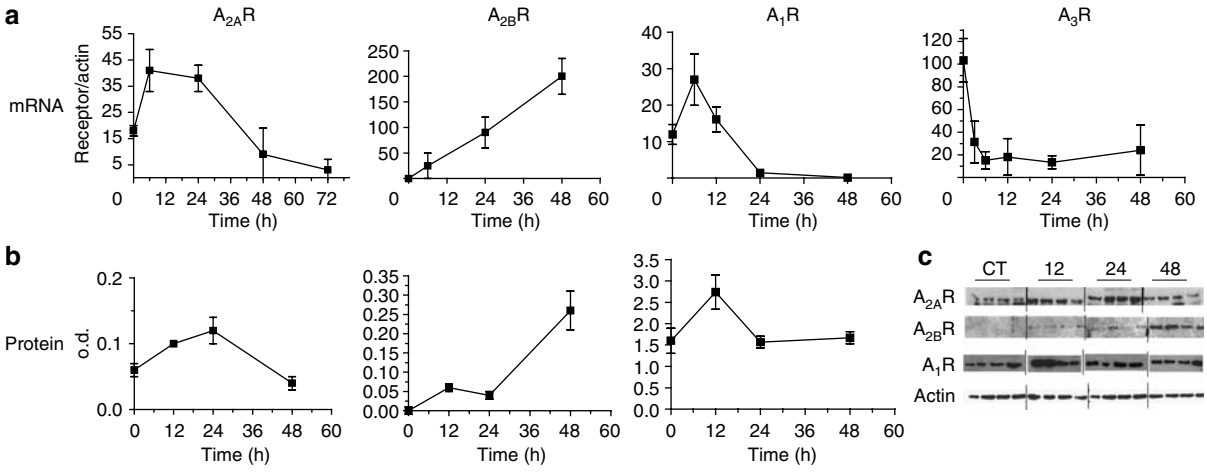

Figure 4 | Adenosine receptors levels in peritoneal mesothelial cells during peritonitis. CD1 mice were inoculated with a sublethal dose of *E. coli*. At the indicated time points after inoculation, mesothelial monolayers covering the liver were analyzed for (**a**) RNA levels of $A_{2A}R$, $A_{2B}R$, A_1R, and A_3R by real-time PCR and (**b, c**) for protein levels by Western blot analysis of $A_{2A}R$, $A_{2B}R$, and A_1R, using specific antibodies. (**b**) Represent the densitometry (mean ± s.e.) of the protein blot depicted in (**c**). The figure is a representative of three similar experiments. Results are presented as mean ± s.e. $N = 4$ for each group.

The effect of adenosine, and adenosine receptor agonists and antagonists on cytokine secretion from HPMC

To better understand the regulatory role of adenosine during peritonitis, one must take into consideration the complexity of receptor interactions. We used specific adenosine receptor agonists and antagonists in order to distinguish the expression of each receptor, on IL-1-dependent IL-6 secretion from HPMC. As shown in Figure 7, adenosine alone (0.1–10 μM) had no significant effect on IL-6 secretion. However, when adenosine was combined with the A_1R antagonist DPCPX (8-cyclopentyl-1, 3-dipropylxanthine), it significantly reduced IL-6 levels in a dose-dependent manner (57% inhibition at 10 μM of adenosine, $P<0.01$). In the presence of the A_2 receptor antagonist, DMPX (1,3-dimethyl-7-propargylxanthine), adenosine significantly increased IL-6 production at 10 μM (722$\pm$72 vs 1033$\pm$59). Conversely, adenosine had no significant effect on IL-6 secretion in the presence of the A_3 receptor antagonist 9-chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,4]triazolo[1,5-c]quinazoline (MRS). To further test the involvement of the A_{2A}R in the inhibition of IL-6, we tested the effect of the A_2R agonist CGS21860 on IL-6 secretion. As shown in Figure 6d, CGS-21860 at doses of 1–10 μM significantly reduced IL-6 secretion in a dose-dependent manner (21% inhibition at 10 μM, $P<0.01$).

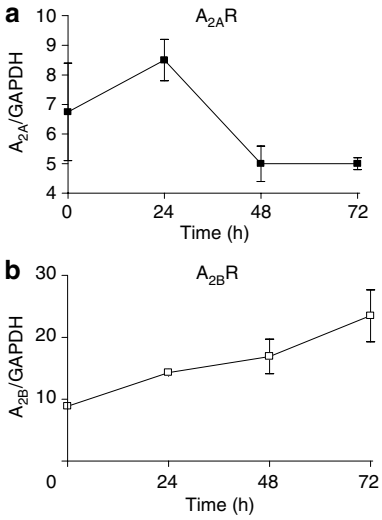

Figure 5 | Expression of adenosine receptors on peritoneal leukocytes during peritonitis. CD1 mice were injected with saline or inoculated with a sublethal dose of *E. coli*. At the indicated time points after inoculation, peritoneal lavage was performed, peritoneal leukocytes were harvested for RNA extraction and (**a**) A_{2A}R mRNA levels, (**b**) A_{2B}R mRNA levels were determined by real-time polymerase chain reaction. The figure is a representative of two similar experiments. Results are presented as mean$\pm$s.e. $N=3$ for each group.

DISCUSSION

The results of the present study show that administration of the A_{2A}R agonist, CGS-21680, causes a significant decline in leukocyte count in peritoneal exudates. Similarly, Zhang *et al.* showed that propentofylline, an atypical xanthine derivative that increases adenosine levels by blocking adenosine uptake, reduced polymorphonuclear leukocyte recruitment during

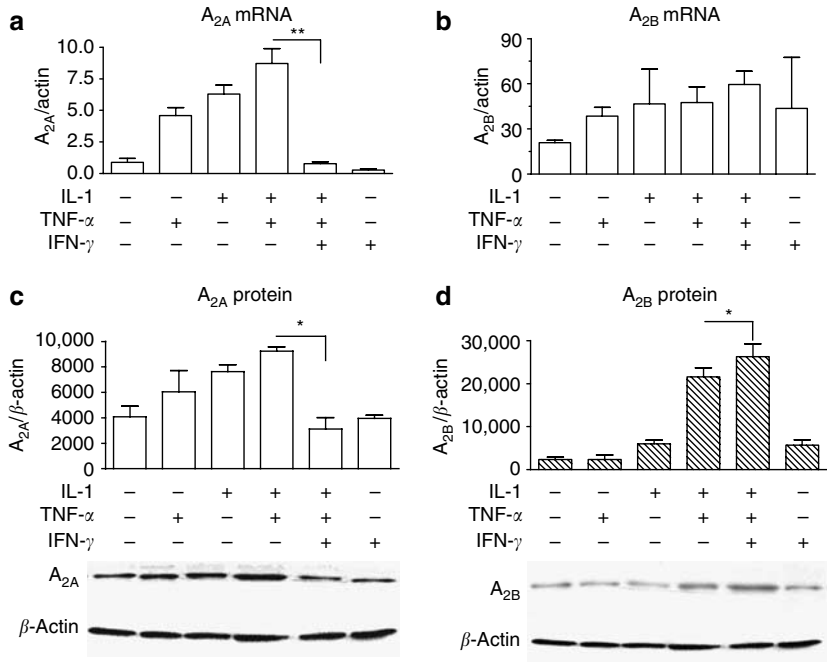

Figure 6 | The effect of pro-inflammatory cytokines on A_{2A} and A_{2B} receptor levels in HPMC. HPMC were stimulated with IL-1 (100 U/ml), TNF-α (10ng/ml), IFN-γ (100 U/ml) alone or in combination. (**a, b**) After 3 h of stimulation, total RNA was extracted from cells and real-time PCR was performed for (**a**) A_{2A}R, (**b**) A_{2B}R. (**c, d**) After 8 h of stimulation, total protein was extracted from cells and analyzed by Western blot for (**c**) A_{2A}R, (**d**) A_{2B}R. The figure is a representative of three similar experiments. Results are presented as mean$\pm$s.e. $N=3$ for each group, $^*P<0.05$, $^{**}P<0.01$.

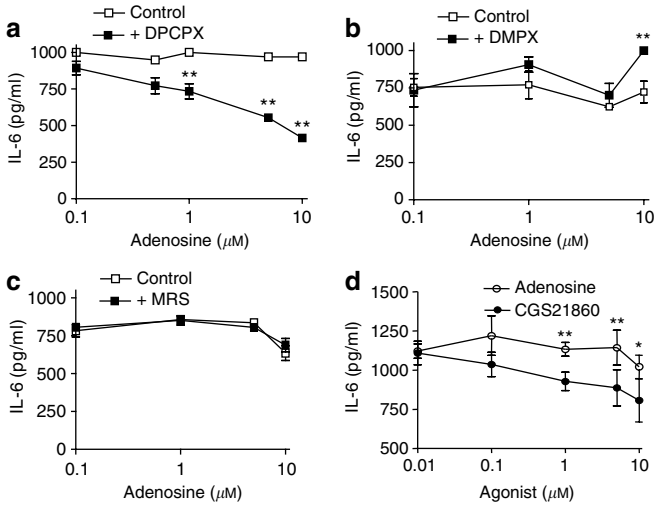

Figure 7 | Production of IL-6 by HPMC after stimulation of IL-1 in the presence of adenosine and adenosine receptor agonists and antagonists. HPMC were stimulated with IL-1 (100 U/ml) and treated with different concentrations of adenosine and with (**a**) A_1R antagonist DCPCX (10 nM), (**b**) A_2R antagonist DMPX (1,3-dimethyl-7-propargylxanthine) (10 μM), (**c**) A_3R antagonist MRS (10 μM), and (**d**) A_2AR agonist CGS-21860 (1–10 μM). At 24 h after incubation, the medium was collected and analyzed for IL-6 levels by enzyme-linked immunosorbent assay. The figure is a representative of four similar experiments. Results are presented as mean $\pm$ s.e. $N = 3$ for each group, $^*P < 0.05$, $^{**}P < 0.01$.

acute peritonitis, an effect that was counteracted by selective adenosine $A_{2A}R$ antagonists.[24] Moreover, administration of $A_{2A}R$ agonist reduced TNF-α and IL-6 levels in the serum and peritoneal fluid of infected mice. This effect of reducing TNF-α was shown also in *in vitro* studies on lipopolysaccharide-induced RAW 264.7 macrophages and U937 cells and *in vivo*, in plasma of LPS-induced mice treated with the $A_{2A}R$ agonist.[18,25] These results support the notion that adenosine moderates the intensity of the inflammatory response to bacterial invasion of the peritoneum, as the anti-inflammatory effect of adenosine is mediated mainly through the $A_{2A}R$.[18–20]

A steep rise in leukocyte count was observed soon after *E. coli* inoculation peaking at 12 h, gradually declining in the succeeding hours. Noteworthy is the rise of adenosine levels in the peritoneal exudate that peaked at 24 h to a level approximately 400% higher than the level before inoculation with bacteria, whereas a sharp drop in leukocyte count occurred suggesting that these two events are cause related.

The next stage was to investigate the expression and kinetics of the four subtypes of adenosine receptors in peritoneal mesothelial cells and leukocytes during bacterial peritonitis. Expression of A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R was found both in mRNA and protein levels on the peritoneal mesothelial cells (see Figure 4). A_1 receptor protein levels peaked at 12 h and then returned to baseline at 24 h, whereas high-affinity $A_{2A}R$ protein levels remained at their summit up to 24 h at which point adenosine concentration achieved its highest level in the peritoneal fluid. In contrast low-affinity $A_{2B}R$ protein and mRNA levels showed a different pattern: a slow and sustained elevation up to 48 h. According to Olah and Stiles,[16] $A_{2A}R$ interaction with Gs and $A_{2B}R$ interaction with Gs and Gq stimulate cAMP production which is endowed with strong immunosuppressive properties. Therefore, high expression of A_1R immediately after inoculation causes a decrease in cAMP levels, which give a rise for local proinflammatory cytokines and leukocyte migration, hence allowed an adequate and effective immune response to the invading microorganisms. In contrast, the increase in $A_{2A}R$ and $A_{2B}R$ at late phases of peritonitis is probably associated with increased cAMP levels, which markedly decreases local proinflammatory cytokines levels and leukocyte recruitment, hence restraining inflammatory flames. Therefore, the sequential increase of A_1R, $A_{2A}R$, and $A_{2B}R$ observed in our study suggests that they are finely regulated to allow an adequate signal from adenosine during the different phases of peritonitis. The early increase of the A_1R that peaks at 12 h allows leukocyte influx into the inflamed area thus enabling an effective attack of the bacteria. The upregulation of the A_{2A} and A_{2B} receptors that appear at a later stage moderates the number of leukocytes in the exudates, thereby reducing the potential damage to normal peritoneal tissue caused by cytotoxic and destructive molecules released by leukocytes. Furthermore, the dominant $A_{2A}R$ in PMC and leukocytes is replaced by the dominance of $A_{2B}R$ at 48 h. This switch between the high-affinity $A_{2A}R$ and the low-affinity $A_{2B}R$ suggests that the anti-inflammatory pathway of adenosine is desensitized at this stage.

The aim in our experiments was to explore the regulatory effect of the early pro-inflammatory cytokines, TNF-α, and IL-1, on the expression of these two A_2R. We found that TNF-α and IL-1 stimulated the expression of the A_{2A} and A_{2B} receptors. This phenomenon might explain the increase of these receptors at the early phase of peritonitis. In contrast, IFN-γ significantly reduced the levels of the $A_{2A}R$ but increased the $A_{2B}R$. These data suggest that IFN-γ, which is secreted at the late phase of peritonitis from T cells and natural killer (NK) cells, is responsible for the *in vivo* late desensitization of the A_2 system by converting the dominance to the low-affinity $A_{2B}R$ (Figure 6). These data are consistent with other studies which have shown that proinflammatory cytokines modulate $A_{2A}R$ and $A_{2B}R$ expression and function on human dermal microvascular endothelial cells and in human monocytic THP-1 cells.[26,27]

The complex relationship between the different adenosine receptors can be demonstrated by their mutual effect on IL-6 modulation. Only unopposed stimulation of $A_{2A}R$ by its specific agonist CGS-21680 or by adenosine itself but in the presence of concomitant blockade of A_1R (DPCPX (8-cyclopentyl-1, 3-dipropylxanthine)) inhibited IL-1-induced IL-6 production by HPMC. The simultaneous stimulation of all receptor types by adenosine had no effect on IL-6 production, which demonstrates the balance of opposite stimuli. The combination of four types of receptor with their

diverse expression, affinity, and link to G-protein different subunits is an essential condition for the realization of precise and variable regulatory functions by a single molecule.

In conclusion, we have shown that the $A_{2A}R$ agonist reduces cytokine production and leukocyte recruitment in infected animals. Furthermore, we provided evidence that adenosine is upregulated at the initial phase of peritonitis and have traced the expression and regulation of its receptors by various cytokines during this process. These results are consistent with our hypothesis that adenosine when released during a bacterial attack of the peritoneum plays an important regulatory role in the intensity of the inflammatory response. This findings may have important implications for patients who forced to discontinue peritoneal dialysis treatment as a result of bacterial peritonitis.[1,2]

MATERIALS AND METHODS

Mice, bacterial strains, and drugs

CD1 female mice aged 10–12 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.

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 $-70^{\circ}C$.

Adenosine (Adenocor) was purchased from Sanofi Winthrop (Auckland, NZ). Adenosine receptor agonists and antagonists were purchased from Sigma (Rehovot, Israel). These included CGS-21680 which is an agonist of A_{2A} receptor; A_2 receptor antagonist DMPX (1,3-dimethyl-7-propargylxanthine); A_1 receptor antagonist DPCPX (8-cyclopentyl-1, 3-dipropylxanthine); and A_3 receptor antagonist MRS-1220.

Induction of peritonitis and drug injection

Peritonitis was induced in CD1 mice by intraperitoneal (i.p.) inoculation of the sublethal dose of *E. coli* (1.5×10^7 colony-forming unit). Adenosine agonists and antagonists were injected i.p. before *E. coli* inoculation.

Lavage, leukocyte counting, and cytokine detection

At different time points after *E. coli* inoculation, animals were killed and peritoneal lavage was performed with phosphate-buffered saline containing 2% bovine serum albumin and 5 mM ethylenediaminetetraacetic acid. After centrifugation at 400 *g* for 10 min, the cell-free supernatants were removed and kept frozen at $-20^{\circ}C$ until analysis. TNF-α and IL-6 concentrations were measured by duoset enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA). Leukocytes from lavage after trypan blue staining were counted using an improved Neubaur hemocytometer. Cell count was carried out blindly on coded tubes by the same person for all the experiments.

Serum cytokine detection

Peritonitis was induced by inoculation of *E. coli*. At different time points, animals were killed, and a 1 ml syringe flushed with heparin was used to draw a blood sample from their hearts. In preliminary experiments, we found that following bacterial inoculation, serum TNF-α levels peak at 1.5–3 h and IL-6 levels peak at 6–10 h. Therefore, animals were bled at 3 h for TNF-α determination and 6 h for IL-6 determination. The samples were stored on ice before centrifugation at 1000 *g* and $4^{\circ}C$ for 10 min. The cell-free supernatants were collected and frozen at $-20^{\circ}C$ for future analysis. TNF-α and IL-6 serum concentrations were quantified by duoset enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA).

Imprints of mice PMC and adenosine receptor determination

Peritonitis was induced by inoculation of *E. coli* as described above. At the indicated time points, microscope slides (Superfrost plus, Menzel-Glaser, Germany) were applied to the anterior liver surface for 15–20 s by peeling off the mesothelial monolayer. The slides were washed with phosphate-buffered saline to remove blood and non-adherent cells. Two imprints were taken from each animal.

mRNA analysis

Total RNA was extracted from mesothelial monolayers imprinted on the microscopic slide, from HPMC, or peritoneal leukocyte using RNAeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was prepared as previously described.[8] Quantitative real-time polymerasr chain reaction (QPCR) assays were carried out for β-actin, glyceraldehyde 3-phosphate dehydrogenase, A_1, A_{2A}, A_{2B}, and A_3 receptors with the following primers: β-actin sense, GGG TCA GGA GGA TTC CTA TG, β-actin antisense, GGT CTC AAA CAT GAT CTG GG; glyceraldehyde 3-phosphate dehydrogenase sense, CAA TGC ATC CTG CAC CAC CAA, glyceraldehyde 3-phosphate dehydrogenase antisense, GTC ATT GAG AGC AAT GCC AGC; A_1 sense, TAC ATC TCG GCC TTC CAG GTC G, A_1, anti-sense, AAG GAT GGC CAG TGG GAT GAC CAG; $A_{2A}R$ sense, ATT TGT GCC AGC CAG GAA GCC, $A_{2A}R$ antisense, GCA TCC GGG ACT TTA AAC CAC AGA; $A_{2B}R$ sense, ATT TGT GCC AGC CAG GAA GCC, $A_{2B}R$ antisense, GCA TCC GGG ACT TTA AAC CAC AGA; A_3R sense, ACC ACT CAA AGA AGA ATA TG, A_3R anti-sense, ACT TAG CTG TCT TGA ACT CC. cDNAs were diluted ninefold, mixed with primers (0.2 mM) and Thermo start master mix (ABgene, Surrey, UK). Reaction was carried out in Rotor-Gene real-time PCR machine (Corbett-Research, Northlake, Australia).

Western blot analysis

For determination of protein levels, mesothelial cells were harvested with RadioImmunoProtection Assay (RIPA) (150 mM NaCl, 50 mM Tris HCl pH-7.4, 1% NP-40, 0.25% Na deoxycholate, 1 mM ethylene glycol tetra acetate) after which protease inhibitor cocktail (Sigma, Rehovot, Israel) was added. After centrifugation at 13000 *g* for 30 min, the supernatants were taken for total protein determination by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). In total, 20 μg of total protein from each sample was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions and after heating. The gels were blotted onto polyvinylidine difluoride membrane (Bio-Rad, Hercules, CA, USA) and probed with specific antibodies: rabbit anti-adenosine $A_{2A}R$ or $A_{2B}R$ affinity-purified polyclonal antibody (Chemicon, Temecula, CA, USA), rabbit anti-rat A_1R (Alpha Diagnostic International, San Antonio, TX, USA), and Actin Ab-1 monocloonal antibody (Oncogene, San Diego, CA, USA). The membrane was then probed with goat anti-rabbit immunoglobulins conjugated to peroxidase agent (Dako, Copenhagen, Denamark). Antigen–antibody complexes were subsequently visualized by Western Lightning™ Chemiluminescence Reagent *Plus* (PerkinElmer, Boston, MA, USA).

Preparation of HPMC

HPMC were isolated from pieces of omentum of approximately 6 cm^2 obtained during elective abdominal surgery (with informed consent of the patients) and were isolated, as previously described.[28] As the harvested cells had to be identified as mesothelial cells, samples of each HPMC preparation were morphologically inspected as previously described.[29] Cells were grown and experiments were carried out in M199 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and Biogro 2™ (2%, Biological Industries, Beit Haemek, Israel). Experiments were performed on cells from the second to fourth passages in 12-well plates after cells reached confluence.

Adenosine determination and HPLC analysis

For adenosine determination in mice peritoneal lavage, 900 μl lavage fluid was placed in a polystyrene tube containing 100 μl stop solution (2.3 μM erytro-9(2-hydroxynon-3-yl)-adenine and 20 μM dipyridamole) designed to inhibit the metabolism and cellular uptake of adenosine.

Adenosine levels were measured in deproteinated fluid by HPLC as previously described.[30,31]

Statistical analysis

Data are presented as mean $\pm$ s.e. Statistical analysis was performed by analysis of variance. *P*-values below 0.05 were considered significant.

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