

# Adenosine-Mediated Inhibition of the Cytotoxic Activity and Cytokine Production by Activated Natural Killer Cells

Anna Lokshin,<sup>1</sup> Tatiana Raskovalova,<sup>1</sup> Xiaojun Huang,<sup>1</sup> Lefteris C. Zacharia,<sup>2</sup> Edwin K. Jackson,<sup>2</sup> and Elieser Gorelik<sup>1</sup>

<sup>1</sup>Department of Pathology and University of Pittsburgh Cancer Institute, and <sup>2</sup>Departments of Pharmacology and Medicine and Center for Clinical Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania

## Abstract

**Adenosine is an important signaling molecule that regulates multiple physiologic processes and exerts major anti-inflammatory actions. Tumors have high concentrations of adenosine, which could inhibit the function of tumor-infiltrating lymphoid cells. We investigated the ability of adenosine and its stable analogue 2-chloroadenosine (CADO) to inhibit cytokine production and cytotoxic activity of lymphokine-activated killer (LAK) cells and determined whether both these effects are initiated via a common pathway. CADO strongly inhibited cytotoxic activity of LAK cells and attenuated the production of IFN- $\gamma$ , granulocyte macrophage colony-stimulating factor, tumor necrosis factor  $\alpha$ , and macrophage inflammatory protein-1 $\alpha$  by LAK cells stimulated by cross-linking of the Ly49D receptor. These inhibitory effects were associated with the ability of CADO to stimulate cyclic AMP (cAMP) production and activate protein kinase A (PKA). Using cAMP analogues with different affinities for the A and B sites of the regulatory subunits of PKA types I and II, we found that activation of PKA I, but not PKA II, mimicked the inhibitory effects of CADO on LAK cell cytotoxic activity and cytokine production. Inhibitors of the PKA catalytic subunits (H89 and PKI<sub>14-22</sub> peptide) failed to abrogate the inhibitory effects of CADO whereas Rp-8-Br-cAMPS, an antagonist of the RI subunit, blocked the inhibitory effects of CADO. We conclude that the inhibitory effects of adenosine are probably mediated via cAMP-dependent activation of the RI subunits of PKA I but are independent of the catalytic activity of PKA. Tumor-produced adenosine could be a potent tumor microenvironmental factor inhibiting the functional activity of tumor-infiltrating immune cells. (Cancer Res 2006; 66(15): 7758-65)**

## Introduction

Adenosine is an endogenous purine nucleoside (i.e., generated from ATP in the extracellular and intracellular compartments and regulates a wide array of physiologic systems by binding to adenosine receptors that are expressed by most cell types; ref. 1). Four different adenosine receptors have been identified ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ ) that belong to the G protein-coupled seven transmembrane superfamily of cell-surface receptors and are known as purinergic P1 receptors.  $A_1$  and  $A_3$  receptors are negatively coupled to adenylyl cyclase through the  $G_{i/o}$  protein  $\alpha$ -subunits whereas  $A_{2A}$  and  $A_{2B}$  receptors are positively coupled to

adenylyl cyclase through  $G_s$  proteins. Therefore,  $A_{2A}$  and  $A_{2B}$  receptor signaling elevates cyclic AMP (cAMP); in contrast,  $A_1$  and  $A_3$  receptor activation inhibits cAMP production (2).

Adenosine production substantially increases in response to tissue damage, hypoxia, and inflammation (3–5). Adenosine markedly reduces damage induced by ischemia in heart, kidneys, lung, brain, spinal cord, and skin and ameliorates inflammation in various diseases such as pleural inflammation, ischemia-reperfusion injury, rheumatoid arthritis, and endotoxin-mediated shock (1, 3, 5). Adenosine inhibits inflammatory activity of neutrophils, macrophages, and lymphocytes and suppresses production of inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-1, IL-6, IL-8, and IL-12 (4, 6–11).

Inasmuch as adenosine protects normal tissues from excessive damage due to hypoxia and inflammation (4), it is possible that adenosine plays a significant role in protecting malignant tissue as well. Tumors grow under hypoxic conditions as a result of insufficient vascularization, and this may trigger the production of adenosine. Indeed, analysis of the extracellular fluid of solid carcinomas shows that intratumor levels of adenosine can reach as high as 13  $\mu\text{mol/L}$  (12) and we previously showed increased concentrations of adenosine in ascites of Meth A sarcoma (13).

Although intratumor concentrations of adenosine can achieve immunosuppressive levels, little is known about the effects of adenosine on the functional activity of antitumor immune cells. Studies using allospecific CTLs show that adenosine inhibits the cytotoxic activity of T lymphocytes. Similarly, the cytotoxic activity of nonspecific anti-CD3-activated T cells is substantially inhibited by adenosine and its analogues (9, 10, 14, 15). Our recent studies show that adenosine and its analogues are able to inhibit the cytotoxic activity of lymphokine-activated killer (LAK) cells and this inhibitory effect is mediated via the  $A_{2A}$  adenosine receptor (13). Adenosine  $A_{2A}$  receptor signaling also may inhibit production of some cytokines by inflammatory cells (4, 6–11). However, downstream mechanisms responsible for these inhibitory effects of adenosine remain unclear, and it is unknown whether the effects of adenosine on cytotoxic activity and cytokine production are initiated by similar signaling mechanisms.

$A_{2A}$  adenosine receptor signaling activates adenylyl cyclase, and cAMP acts as a major second messenger in various cellular processes. Classically, cAMP signaling leads to the immediate activation of protein kinase A (PKA), resulting in release of two catalytic subunits, which are then able to phosphorylate serine and threonine residues on specific substrate proteins, including various transcription factors such as cAMP-responsive element binding protein (CREB), cAMP-responsive element modulator protein (CREM), and nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ), which are involved in regulation of various genes (16, 17). PKA exists as two different isozymes, types I and II, which can function differently (16–20), and different levels of expression and functional activity of PKA I and

**Requests for reprints:** Elieser Gorelik, University of Pittsburgh Cancer Institute, Hillman Cancer Center, Room 1.46, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-6233217; Fax: 412-624-7736; E-mail: gorelik@pitt.edu.

©2006 American Association for Cancer Research.  
doi:10.1158/0008-5472.CAN-06-0478

PKA II have been found in T cells, B cells, and natural killer (NK) cells (17, 20).

In the present study, we investigated the ability of adenosine and 2-chloroadenosine (CADO), a stable analogue of adenosine, to inhibit the cytolytic activity of LAK cells and to attenuate cytokine production by LAK cells stimulated by cross-linking of the Ly49D receptor. We used a novel Luminex LabMAP technology and multiplex antibody-conjugated beads for simultaneous measurement of concentrations of 20 cytokines, chemokines, and growth factors produced by Ly49D-cross-linked LAK cells in the presence and absence of CADO. To identify which type of PKA is responsible for the inhibitory effects, various cAMP analogues with different affinities for the A and B sites of PKA I and II were used. To obtain more direct evidence of the involvement of cAMP-dependent PKA in the regulation of the effects of adenosine, we tested the ability of various PKA inhibitors for their ability to abrogate the inhibitory effects of adenosine on LAK cell functional activity.

## Materials and Methods

**Mice.** Female C57BL/6 (6–8 weeks old) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Experiments were done in accordance with the approved institutional protocol and guidelines of the Institutional Animal Care and Use Committee.

**Reagents.** Adenosine, CADO, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), H89, and myristoylated PKI<sub>14-22</sub> peptide were purchased from Sigma-Aldrich (St. Louis, MO). ZM241385 was from Tocris (Ellisville, MO). 8-Hexylaminoadenosine-3',5'-cyclic monophosphate (8-HA-cAMP), *N*<sup>6</sup>-benzoyl-cAMP (6-benz-cAMP), 8-piperidino-cAMP (8-PIP-cAMP), 8-(4-methoxyphenylthio)-2'-*O*-methyl-cAMP, 8-pCPT-2'-*O*-Me-cAMP, and Rp-8-Br-cAMPS were purchased from Axora Platform (San Diego, CA).

**LAK cell generation.** Purified LAK cells were generated from spleens of C57BL/6 mice as described (21). Briefly, a single-cell suspension of spleen cells was prepared and spleen cells ( $50 \times 10^6$ ) were cultured in T-75 flasks for 3 days in the presence of interleukin (IL)-2 (6,000 IU/mL). After removal of nonadherent spleen cells, plastic adherent cells were cultured for additional 3 to 5 days. This approach generates large numbers of purified highly cytotoxic LAK cells (21). Flow cytometry revealed that 80% to 92% of these LAK cells were NK1.1 and asialo GM<sub>1</sub> positive.

**Cytotoxic activity of LAK cells.** The cytotoxic activity of LAK cells was tested against <sup>51</sup>Cr-labeled 3LL Lewis lung carcinoma cells. LAK cells were distributed into V-bottomed 96-well plates, with and without test agents, and, 20 minutes later, radiolabeled 3LL tumor cells ( $5 \times 10^3$  per well) were added. LAK cell cytotoxicity was determined in triplicate at 20:1 effector/target (E/T) ratio. After 4 hours of incubation at 37°C, supernatants (25  $\mu$ L) were transferred into yttrium silicate scintillator-coated white microplates (LumaPlate-96, Perkin-Elmer, Boston, MA) and the level of  $\beta$ -emission by released <sup>51</sup>Cr was measured in a  $\beta$ -counter. The percentage of cytotoxicity was calculated (13).

**Cytokine production.** Rested LAK cells were stimulated with anti-Ly49D monoclonal antibody as described with minor modifications (22, 23). Anti-Ly49D mAb was a gift from Dr. John Ortaldo (National Cancer Institute, NIH, Frederick, MD). Costar 96-well plates were precoated with rabbit anti-rat immunoglobulin G (IgG; 2  $\mu$ g/well) and blocked with RPMI 1640 containing 10% FCS. LAK cells were washed and rested for 2 hours in the absence of IL-2. LAK cells were incubated with test chemicals in tubes for 20 minutes; then anti-Ly49D rat mAb was added (1  $\mu$ g/well) and LAK cells ( $0.5 \times 10^6$  per well) were plated into 96-well plates precoated with rabbit anti-rat IgG. After 6 hours of incubation at 37°C, supernatants (0.1 mL) were collected and concentrations of various cytokines were analyzed using Luminex LabMAP technology.

**Multiplex bead-based cytokine analysis.** We used murine multiplex antibody bead kit for Luminex xMAP (Biosource International, Camarillo, CA) that allows simultaneous testing of 20 murine cytokines, chemokines, and growth factors: IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13,

IL-17, IFN- $\gamma$ , TNF- $\alpha$ , KC, granulocyte macrophage colony-stimulating factor (GM-CSF), MCP-1, macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), IP-10, MIG, fibroblast growth factor- $\beta$  (FGF- $\beta$ ), and vascular endothelial growth factor (VEGF). The multiplexed assay was done at the University of Pittsburgh Cancer Institute Luminex Core Facility as described (24). A filter-bottom 96-well microplate (Millipore, Bedford, MA) was blocked for 10 minutes with PBS/bovine serum albumin. To generate a standard curve, 5-fold dilutions of appropriate standards were prepared in RPMI 1640. Standards and supernatant samples were pipetted at 50  $\mu$ L/well in duplicate and mixed with 50  $\mu$ L of bead mixture. The microplate was incubated for 1 hour at room temperature on microtiter shaker. Wells were then washed thrice with washing buffer using a vacuum manifold. Phycoerythrin-conjugated secondary antibodies were added to the appropriate wells and incubated for 45 minutes in the dark with constant shaking. Wells were washed twice, assay buffer was added, and samples were analyzed using the Bio-Plex suspension array system, which includes a fluorescent reader and Bio-Plex Manager analytic software (Bio-Rad Laboratories, Hercules, CA). Data are expressed as mean  $\pm$  SD (pg/mL).

**cAMP production.** LAK cells were distributed into 4 mL tubes ( $0.5 \times 10^6$  cells/0.5 mL per tube) and incubated with CADO for 10 minutes. Culture medium was removed and 1 mL of ice-cold 1-propanol was added to cells. After shaking, cellular extracts were harvested, frozen, and later used for cAMP analysis by high-performance liquid chromatography using fluorescence detection as previously described (13).

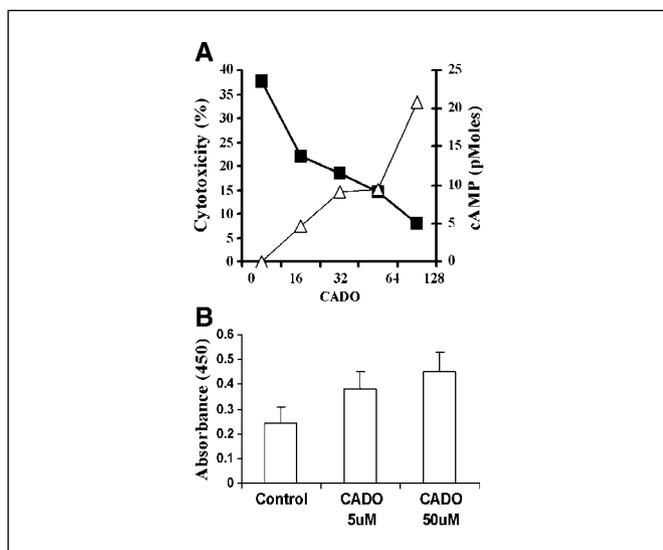
**PKA activity assay.** PKA activity in LAK cell lysates was tested using StressXpress nonradioactive PKA activity kit from Stressgen Bioreagents (Victoria, British Columbia, Canada). This assay uses a specific synthetic peptide as a substrate for PKA and a polyclonal antibody that recognizes the phosphorylated form of the substrate. LAK cells ( $10 \times 10^6$  per group) were treated with CADO (5 or 50  $\mu$ mol/L) for 5 to 120 minutes and were lysed in lysing buffer, centrifuged at 13,000 rpm for 15 minutes, and supernatants were frozen at  $-70^\circ\text{C}$ . A microtiter plate was soaked with 50  $\mu$ L of kinase assay dilution buffer and, after washing, 30  $\mu$ L of each sample were added. The reaction was initiated by adding 10  $\mu$ L of ATP. The plate was incubated for 90 minutes at 30°C and phosphospecific substrate antibody was added. The level of antibody binding was estimated using secondary anti-rabbit IgG-horseradish peroxidase conjugate and corresponding TMB substrate. Absorbance was determined using a microplate reader set at a wavelength of 450 nm. Kinase activity in cell lysates was calculated as follows: [mean absorbance (sample) – mean absorbance (negative control)] / protein in cell lysate. For negative control, cell lysate samples were incubated with substrate without adding ATP or the cell lysates were incubated with ATP in the presence of 5  $\mu$ mol/L of myristoylated peptide PKA inhibitor PKI<sub>14-22</sub> (Sigma).

**Statistics.** Statistical analysis of the data was done using Student's *t* test. The significance level was set at  $P < 0.05$ .

## Results

**Analysis of cAMP-PKA pathway in the CADO-mediated inhibition of LAK cell cytotoxicity.** First, we tested the ability of CADO to stimulate cAMP production by LAK cells and to affect their ability to kill 3LL tumor cells. CADO stimulated production of cAMP in LAK cells in a concentration-dependent manner and, in parallel, inhibited LAK cell cytotoxicity (Fig. 1A). These results suggest that cAMP provides the inhibitory signal leading to inhibition of LAK cell cytotoxicity. Because this inhibitory signal is likely mediated via activation of PKA, we tested whether CADO is able to activate PKA activity in LAK cells. The results presented in Fig. 1B indicate that CADO treatment increased PKA activity. Thus, CADO treatment of LAK cells induces cAMP production and increases PKA activity that is associated with inhibition of LAK cell cytotoxicity.

It is unclear which type of PKA mediates the inhibitory effects of adenosine on LAK cell cytotoxic. Both types of PKA form a



**Figure 1.** A, effect of CADO on cAMP production and cytotoxic activity of LAK cells. LAK cells ( $0.5 \times 10^6$ ) were incubated with CADO (16-128  $\mu\text{mol/L}$ ) for 10 minutes and intracellular levels of cAMP analyzed using high-performance liquid chromatography. To test the effect of CADO on the cytotoxic activity, LAK cells were mixed with  $^{51}\text{Cr}$ -labeled 3LL tumor cells at E/T ratio 20:1 in the absence or presence of CADO (16-128  $\mu\text{mol/L}$ ). After 4 hours of incubation, the percentage of  $^{51}\text{Cr}$  release was determined. All tested concentrations of CADO significantly ( $P < 0.05$ ) reduced LAK cytotoxicity and increased cAMP (■ - - ■, % cytotoxicity; △ - - △, cAMP concentration). These experiments were repeated twice. B, CADO-induced activation of PKA in LAK cells. LAK cells were incubated with CADO (5 or 50  $\mu\text{mol/L}$ ) and, 2 hours later, cells were lysed. PKA activity in cell lysates was tested using StressXpress nonradiative PKA activity kit (Stressgen Bioreagents). For negative control, cell lysate samples were incubated with substrate without adding ATP or with ATP in the presence of 5  $\mu\text{mol/L}$  of myristoylated peptide PKA inhibitor PKI<sub>14-22</sub>. Columns, mean; bars, SD. Both concentrations of CADO significantly ( $P < 0.05$ ) increased PKA activity. These experiments were repeated thrice.

tetramer consisting of two regulatory and two catalytic subunits. There are four unique regulatory subunit isoforms (RI $\alpha$ , RI $\beta$ , RII $\alpha$ , and RII $\beta$ ) and three catalytic subunit isoforms (C $\alpha$ , C $\beta$ , and C $\gamma$ ), all encoded by distinct genes. Each RI and RII regulatory subunit has two cooperative cAMP binding sites, A and B. The binding of cAMP

to the A and B sites on the regulatory subunits reduces their affinity for the catalytic units, resulting in dissociation of the holoenzyme complex and releasing two free catalytic subunits, which are then able to phosphorylate serine and threonine residues on specific substrate proteins (16, 17).

Recently, numerous analogues of cAMP have been synthesized that can selectively bind to the A and B sites of the RI or RII subunits (site selectivity). By combining appropriate pairs of cAMP analogues, PKA I and PKA II can be selectively activated (25). Thus, these agents can help to identify the involvement of PKA type I or II in regulation of LAK cell cytotoxicity. Employing this approach, LAK cells were mixed with  $^{51}\text{Cr}$ -labeled 3LL Lewis lung carcinoma cells in the presence or absence of a single or a pair of cAMP analogues. For comparison, the inhibitory activity of CADO (2 and 10  $\mu\text{mol/L}$ ) was tested. The results presented in Table 1 show that CADO substantially inhibited the cytotoxic activity of LAK cells. 6-Benz-cAMP (binds to RIA and RIIA) and 8-PIP-cAMP (binds to RIA and RIIB) used separately did not affect LAK cytotoxicity. A combination of 8-PIP-cAMP and 6-benz-cAMP (binds to A and B sites of RII subunits and therefore selectively activates PKA II) slightly reduced LAK cell cytotoxicity (Table 1). 8-HA-cAMP that activates site B of RI also showed a low level of inhibition, whereas a combination of 8-HA-cAMP and 6-benz-cAMP that activates both A and B sites of RI subunits, and therefore selectively activates PKA I, had a synergistic effect and dramatically inhibited LAK cell cytotoxicity (Table 1). Treatment of 3LL target cells or LAK cells with CADO or PKA activators did not affect their viability. Furthermore, pretreatment of 3LL tumor cells with CADO or PKA I activators did not change their sensitivity to LAK cell-mediated cytotoxicity. In summary, these data indicate that PKA I activation is predominantly responsible for the inhibition of the ability of LAK cells to kill tumor cells.

Recently it was found that cAMP might have an additional target that is independent of PKA (26, 27). This alternative cAMP target is exchange protein directly activated by cAMP (Epac1 and Epac2). Epac1 has an NH<sub>2</sub>-terminal DEP (disheveled, Egl-10, pleckstrin) domain that is involved in membrane docking and cell adhesion, as well as a cAMP binding domain (CNBD), a Ras exchange motif (REM), and a guanine nucleotide exchange factor (GEF) homology

**Table 1.** Effect of PKA I, PKA II, and Epac activators on LAK cell cytotoxicity

Treatment	Concentration	PKA binding site	Cytotoxicity (%)
Control			38.3 ± 4
CADO	10 $\mu\text{mol/L}$		15.0 ± 2*
	2 $\mu\text{mol/L}$		21.0 ± 3*
6-Benz-cAMP	0.25 mmol/L	RI A and RII A	34.3 ± 6
8-PIP-cAMP	0.25 mmol/L	RI A and RII B	35.7 ± 7
8-PIP-cAMP + 6-Benz-cAMP	0.25 + 0.25 mmol/L	PKA II (A + B)	27.1 ± 4*
8-HA-cAMP	0.25 mmol/L	RI B	30.7 ± 3*
8-HA-cAMP + 6-Benz-cAMP	0.25 + 0.25 mmol/L	PKA I (A + B)	8.1 ± 1 <sup>†</sup>
8-pCPT-2'-O-Me-cAMP	0.5 mmol/L	Epac	36.9 ± 3
8-PIP-cAMP + 6-benz-cAMP + 8-pCPT-2'-O-Me-cAMP	(0.25 + 0.25) + 0.5 mmol/L	PKA II + Epac	23.3 ± 4*
8-HA-cAMP + 6-benz-cAMP + 8-pCPT-2'-O-Me-cAMP	(0.25 + 0.25) + 0.5 mmol/L	PKA I + Epac	7.8 ± 1 <sup>†</sup>

NOTE: LAK cells were incubated for 30 minutes with activators of PKA and/or Epac and then incubated for 4 hours with radiolabeled 3LL tumor cells at E/T ratio 20:1.

\*Significantly differs from the Control group ( $P < 0.05$ ).

<sup>†</sup>Significantly differs from all other groups ( $P < 0.001$ ). This experiment was repeated thrice.

**Table 2.** The effect of Ly49D cross-linking on the cytokine production by LAK cells

Groups	Cytokines (pg/mL)					
	IFN- $\gamma$	GM-CSF	TNF- $\alpha$	MIP-1 $\alpha$	IP-10	VEGF
LAK	308 $\pm$ 51	822 $\pm$ 30	224 $\pm$ 5	4,406 $\pm$ 1,239	0	211 $\pm$ 77
LAK + Ly49D	968 $\pm$ 33	2,467 $\pm$ 409	496 $\pm$ 68	11,942 $\pm$ 958	92.5 $\pm$ 10	368 $\pm$ 84

Groups	Cytokines (pg/mL)					
	IL-1 $\beta$	IL-2	IL-4	IL-6	IL-10	IL-12
LAK	362 $\pm$ 23	113 $\pm$ 7	79 $\pm$ 1	39 $\pm$ 1	121 $\pm$ 5	323 $\pm$ 3
LAK + Ly49D	21 $\pm$ 5	92 $\pm$ 3	15 $\pm$ 1	16 $\pm$ 4	2 $\pm$ 0	21 $\pm$ 1

Groups	Cytokines (pg/mL)				
	IL-17	KC	MCP-1	G-CSF	FGF- $\beta$
LAK	93 $\pm$ 1	59 $\pm$ 24	33 $\pm$ 1	34 $\pm$ 1	81 $\pm$ 21
LAK + Ly49	1 $\pm$ 0	35 $\pm$ 9	12 $\pm$ 2	10 $\pm$ 1	29 $\pm$ 11

NOTE: IL-2-activated LAK cells were rested for 2 hours without IL-2. After washing, LAK cells ( $0.5 \times 10^6$  per well) were treated with anti-Ly49D mAb (1  $\mu$ g/well) and were plated onto 96-well plates coated with rabbit anti-rat IgG. The supernatants were harvested after 6 hours of incubation in the absence of IL-2. Cytokine concentrations (pg/mL) in the supernatants of LAK cells were analyzed using Luminex LabMAP technology. All groups of LAK + Ly49D significantly differ from LAK groups ( $P < 0.05$ ). This experiment was repeated thrice.

domain. Epac2 has an additional CNBD (26, 27). Previous studies show that activation of Epac, but not PKA, suppresses macrophage phagocytosis whereas PKA is responsible for inhibition of leukotriene B<sub>4</sub> and TNF- $\alpha$  production (28). Bactericidal activity and H<sub>2</sub>O<sub>2</sub> production are suppressed by either PKA or Epac-1 activation (29). Therefore, it is important to investigate whether Epac activation affects LAK cell cytotoxicity. 8-pCPT-2'-O-methyl-cAMP is a potent activator of Epac-1 and Epac-2 (28). When LAK cells were incubated with 8-pCPT-2'-O-methyl-cAMP (0.5 mmol/L), their ability to kill 3LL tumor cells was not affected (Table 1). Furthermore, this Epac activator did not provide any additional inhibitory signal when combined with activators of PKA I or PKA II. These results indicate that activation of PKA I, but not PKA II or Epac, is responsible for the inhibition of LAK cell cytotoxicity.

**Inhibitory effect of CADO on cytokine production by activated LAK cells.** To assess the effects of adenosine on cytokine production by LAK cells, resting LAK cells were activated by cross-linking of the Ly49D receptor as previously described (22, 23, 30). To do a more comprehensive analysis of cytokines produced by activated LAK cells, we used a novel multiplexed immunobead-based Luminex LabMAP technology that allows simultaneous measurement of numerous cytokines in the same sample of serum, plasma, or cell culture supernatant (24). Purified population of LAK cells was expanded by culturing for 6 days with IL-2. Then LAK cells were rested for 2 hours without IL-2 and then were stimulated by cross-linking the Ly49D receptor for 6 hours as described (22, 23). Supernatants were collected and concentrations of 20 cytokines were analyzed using Luminex LabMAP technology. The results presented in Table 2 show that rested LAK cells produced various cytokines and cross-linking of Ly49D stimulated production of IFN- $\gamma$ , GM-CSF, MIP-1 $\alpha$ , TNF- $\alpha$ , and VEGF. IP-10 was not detectable in unstimulated cells and its levels increased

following cross-linking of Ly49D. Three cytokines (IL-1 $\alpha$ , IL-5, and MIG) had very low or undetectable levels that did not change following cross-linking of Ly49D. Rested LAK cells produced 11 other cytokines [IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, KC, granulocyte colony-stimulating factor (G-CSF), MCP-1, and FGF- $\beta$ ]. However, the levels of these cytokines were substantially reduced following cross-linking of Ly49D (Table 2).

When LAK cells were stimulated with cross-linking of Ly49D in the presence of CADO (5  $\mu$ mol/L), the production of IFN- $\gamma$ , GM-CSF, MIP-1 $\alpha$ , and TNF- $\alpha$  was substantially inhibited (Table 3). The inhibitory effects of CADO were blocked when LAK cells were pretreated with ZM241385, an antagonist of adenosine receptors A<sub>2A</sub> and A<sub>2B</sub>, suggesting involvement of these receptors in the inhibitory effects of CADO (Table 3). Although cross-linking of Ly49D stimulated VEGF production, CADO did not significantly affect VEGF production by LAK cells (data not shown). Failure of CADO to inhibit VEGF production suggests that VEGF, in comparison with IFN- $\gamma$ , GM-CSF, MIP-1 $\alpha$ , and TNF- $\alpha$ , has different mechanisms of regulation. Previous studies showed that adenosine could affect VEGF production but these effects varied in cells of different origins (31–33).

The levels of 11 cytokines (IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, KC, G-CSF, MCP-1, and FGF- $\beta$ ) that were reduced following Ly49D cross-linking became undetectable following treatment with CADO (data not shown). In general, using different preparations of LAK cells on different days of culture, the levels of these 11 cytokines varied and often were undetectable. However, in all experiments, cross-linking of Ly49D consistently stimulated production of IFN- $\gamma$ , GM-CSF, MIP-1 $\alpha$ , and TNF- $\alpha$ , and CADO consistently was able to inhibit production of these cytokines.

We next tested whether adenosine, similar to its analogue CADO, is able to inhibit cytokine production by LAK cells. Because

**Table 3.** Effect of CADO and adenosine on cytokine production by LAK cells stimulated by cross-linking of Ly49D

Groups	IFN- $\gamma$	GM-CSF	MIP-1 $\alpha$	TNF- $\alpha$
Experiment 1				
Control	308 $\pm$ 51	822 $\pm$ 30	4,406 $\pm$ 1,239	224 $\pm$ 5
Ly49D	968 $\pm$ 302	2,467 $\pm$ 409	11,942 $\pm$ 958	497 $\pm$ 68
Ly49D + CADO	94 $\pm$ 17	420 $\pm$ 54	1,195 $\pm$ 71	129 $\pm$ 15
Ly49D + ZM241385 + CADO	537 $\pm$ 34	1,426 $\pm$ 245	9,712 $\pm$ 3,109	296 $\pm$ 21
Experiment 2				
Control	612 $\pm$ 24	407 $\pm$ 13	862 $\pm$ 349	124 $\pm$ 7
Ly49D	836 $\pm$ 141	606 $\pm$ 41	8,195 $\pm$ 77	161 $\pm$ 13
Ly49D + adenosine + EHNA	257 $\pm$ 22	83 $\pm$ 24	738 $\pm$ 300	101 $\pm$ 11
Ly49D + EHNA	640 $\pm$ 9	399 $\pm$ 16	4,135 $\pm$ 459	139 $\pm$ 16

NOTE: Plastic adherent purified LAK cells were washed and rested for 2 hours in the absence of IL-2. LAK cells were incubated with test chemicals in tubes for 20 minutes, then anti-Ly49D rat mAb was added (1  $\mu$ g/well) and LAK cells were plated into 96-well plates precoated with 2  $\mu$ g/well rabbit anti-rat IgG (0.5  $\times$  10<sup>6</sup> per well). After 6 hours of incubation at 37°C, cytokine production was analyzed using a multiplex kit and Luminex LabMAP technology. Anti-Ly49D mAb significantly increased cytokine production ( $P < 0.05$ ) and CADO or ADO + EHNA significantly attenuated this response ( $P < 0.05$  and  $P < 0.01$ , respectively). The inhibitory effect of CADO was significantly reversed by ZM241385 ( $P < 0.05$ ; experiment 1). The differences between groups EHNA alone and ADO + EHNA are significant ( $P < 0.05$ ; experiment 2). The experiments were repeated twice.

adenosine is very quickly metabolized by adenosine deaminase, these experiments were done in the presence of EHNA, an inhibitor of adenosine deaminase. Adenosine plus EHNA substantially inhibited production of IFN- $\gamma$ , GM-CSF, TNF- $\alpha$ , and MIP-1 $\alpha$ . EHNA also show a low level of inhibition that was probably mediated by inhibition of the metabolism of adenosine endogenously produced by LAK cells (Table 3).

**Effect of PKA I and PKA II activation on cytokine production by LAK cells.** The inhibitory effect of adenosine and its analogues is probably due to induction of cAMP and activation of PKA. However, it remains unknown which subtype of PKA is responsible for inhibition of cytokine production. To determine this, we tested the ability of cAMP analogues with different specificities for binding RI or RII subunits of PKA to affect cytokine

production by LAK cells stimulated by cross-linking of Ly49D. LAK cells were activated with anti-Ly49D mAb and cultured for 6 hours in the presence of cAMP analogues.

Treatment of activated LAK cells separately with 6-Benz-cAMP, 8-PIP-cAMP, and 8-HA-cAMP at concentrations of 0.12 and 0.06 mmol/L significantly ( $P < 0.05$ ) inhibited cytokine production (Table 4). Each of these agonists binds separately to A or B site of RI that might lead to partial activation of PKA I and inhibition of cytokine production. However, when both A and B sites of RI were activated by a combination of 6-Benz-cAMP and 8-HA-cAMP, a synergistic inhibitory effect was observed that substantially exceeded the effect of each compound used separately ( $P < 0.001$ ). Production of IFN- $\gamma$ , GM-CSF, and MIP-1 $\alpha$  was reduced by 92% to 99% whereas TNF- $\alpha$  production was completely inhibited

**Table 4.** Comparative effects of PKA I and PKA II activation on cytokine production in LAK cells stimulated by cross-linking Ly49D

Treatment	Concentration (mmol/L)	PKA binding site	IFN- $\gamma$	GM-CSF	MIP-1 $\alpha$	TNF- $\alpha$
LAK			631 $\pm$ 46	576 $\pm$ 33	16,225 $\pm$ 997	222 $\pm$ 11
+CADO	0.005		115 $\pm$ 10	95 $\pm$ 14	1,022 $\pm$ 164	38 $\pm$ 3
+6-Benz-cAMP	0.12	RI A/RII A	381 $\pm$ 18	364 $\pm$ 11	7,249 $\pm$ 578	98 $\pm$ 0.2
	0.06		327 $\pm$ 35	316 $\pm$ 39	6,456 $\pm$ 557	84 $\pm$ 6
+8-PIP-cAMP	0.12	RI A/RII B	491 $\pm$ 26	459 $\pm$ 35	9,863 $\pm$ 1,556	119 $\pm$ 7
	0.06		527 $\pm$ 14	478 $\pm$ 7	13,310 $\pm$ 181	122 $\pm$ 1
8-PIP-cAMP + 6-benz-cAMP	0.12 + 0.12	PKA II (A + B)	365 $\pm$ 17	353 $\pm$ 30	5,599 $\pm$ 903	76 $\pm$ 6
	0.06 + 0.06		360 $\pm$ 11	346 $\pm$ 6	5,360 $\pm$ 163	71 $\pm$ 0.8
8-HA-cAMP	0.12	RI B	238 $\pm$ 16	219 $\pm$ 9	3,502 $\pm$ 126	60 $\pm$ 1
	0.06		322 $\pm$ 21	293 $\pm$ 20	5,606 $\pm$ 1,108	79 $\pm$ 7
8-HA-cAMP + 6-benz-cAMP	0.12 + 0.12	PKA I (A + B)	52 $\pm$ 6*	19 $\pm$ 0.5*	191 $\pm$ 47*	0*
	0.06 + 0.06		58 $\pm$ 7*	35 $\pm$ 10*	220 $\pm$ 69*	0*

NOTE: IL-2-activated LAK cells were rested for 2 hours without IL-2. After washing, LAK cells were activated with anti-Ly49D mAb and incubated for 6 hours with CADO or cAMP analogues that specifically bind to A and B sites of RI or RII. The levels of cytokine in supernatants were determined using Luminex LabMAP technology.

\*Significantly differs from other groups ( $P < 0.01$ ). The experiment was repeated twice.

(Table 4). In contrast, a combination of 6-Benz-cAMP and 8-PIP-cAMP that stimulates PKA II activity did not show any further increase in the inhibitory effects in comparison with each compound used separately (Table 4). These results indicate that activation of PKA I is mostly responsible for adenosine-mediated inhibition of the cytokine production.

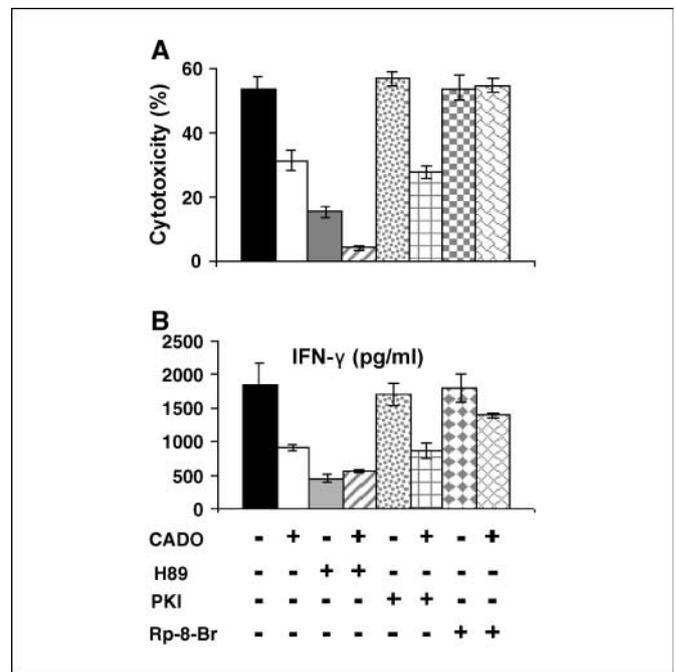
Activation of Epac with two different activators, 8-(4-methoxyphenylthio)-2'-O-methyl-cAMP and 8-pCPT-2'-O-Me-cAMP, even at high concentrations (1 and 0.5 mmol/L), showed no effect on cytokine production in activated LAK cells (data not shown). Thus, our data indicate that activation of PKA I, but not PKA II or Epac, inhibits cytokine production by LAK cells.

**Effects of PKA inhibitors on CADO-induced inhibition of LAK cell activity.** The data presented above show that activation of PKA I mimics the inhibitory effects of adenosine on LAK cell cytotoxic activity and cytokine production. This suggests that the immunoinhibitory effects of adenosine are mediated via the PKA pathway. If this hypothesis is correct, one would expect that inhibition of PKA activity would block the inhibitory effects of CADO. To test this prediction, we first examined the ability of the PKA inhibitor H89 to abrogate the inhibitory effect of CADO on LAK cell cytotoxic activity. H89 inhibits the activity of the catalytic subunits of PKA by blocking the ATP binding site and preventing phosphorylation of the substrate. However, H89 at 10  $\mu\text{mol/L}$  almost completely inhibited the cytotoxic activity of LAK cells (data not shown). At 5  $\mu\text{mol/L}$ , H89 still substantially inhibited LAK cell cytotoxicity, which was further inhibited in the presence of CADO (Fig. 2A). Similar results were obtained when H89 at 2.5  $\mu\text{mol/L}$  was tested (data not shown). Although H89 is a potent PKA inhibitor, it also inhibits at least eight other protein kinases (34), and this might explain the strong inhibitory effect of H89 on LAK cell functional activity.

We next used a more specific PKA inhibitor, myristoylated peptide PKI<sub>14-22</sub>, which specifically blocks the catalytic activity of PKA. PKI peptide at 10  $\mu\text{mol/L}$  did not affect the cytotoxic activity of LAK cells. When PKI was used in combination with CADO, no changes in the inhibitory effect of CADO were observed (Fig. 2A). Similarly, PKI peptide at 5 or 20  $\mu\text{mol/L}$  failed to abrogate the inhibitory effect of CADO (data not shown).

These findings indicate that the inhibitory effect of CADO on LAK cell cytotoxicity is not mediated via the catalytic subunits of PKA; however, these results do not exclude the involvement of the regulatory subunits of PKA. The regulatory subunits are the primary receptors for cAMP. Recently, several Rp-cAMPS analogues were identified that specifically antagonize the regulatory RI subunits of PKA I. In this regard, Rp-8-Br-cAMPS was found to be the most potent antagonist of RI. By binding to RI, Rp-8-Br-cAMPS prevents the PKA I holoenzyme from dissociating and thereby prevents the release of RI (35). If RI subunits have signaling functions per se and are involved in mediating adenosine-induced inhibition of LAK cell cytotoxicity, Rp-8-Br-cAMPS should block this effect of adenosine. To test this hypothesis, LAK cells were mixed with Rp-8-Br-cAMPS (1 mmol/L) for 20 minutes and then CADO (5  $\mu\text{mol/L}$ ) and  $^{51}\text{Cr}$ -labeled 3LL tumor cells were added. Rp-8-Br-cAMPS completely blocked the inhibitory effect of CADO (Fig. 2A). Rp-8-Br-cAMPS at lower concentrations (500  $\mu\text{mol/L}$ ) only partially reversed the inhibitory effects of CADO and, at 250  $\mu\text{mol/L}$ , had no blocking effect (data not shown).

We next examined the ability of PKA inhibitors to block the inhibitory effects of CADO on cytokine production. The IFN- $\gamma$  production was inhibited either in the presence of CADO or H89.



**Figure 2.** A, effects of H89 and PKI peptide, inhibitors of the catalytic activity of PKA, and Rp-8-Br-cAMPS, antagonist of RI, on the inhibitory effects of CADO on LAK cell cytotoxicity. LAK cells were mixed with  $^{51}\text{Cr}$ -labeled 3LL tumor cells at E/T ratio 20:1 in the absence or presence of CADO (5  $\mu\text{mol/L}$ ). In some groups, LAK cells were preincubated for 20 minutes with H89 (5  $\mu\text{mol/L}$ ), PKI peptide (10  $\mu\text{mol/L}$ ), or Rp-8-Br-cAMPS (1 mmol/L) before CADO and target cells were added. After 4 hours of incubation, the percentage of  $^{51}\text{Cr}$  release was determined. The H89 + CADO and PKI + CADO groups differ significantly ( $P < 0.01$ ) from the Control group. The Rp-8-Br-cAMPS + CADO group differs significantly ( $P < 0.05$ ) from the CADO alone group but not from the Control or Rp-8-Br-cAMPS alone group. Experiments were repeated thrice. B, analysis of the ability of H89, PKI and Rp-8-Br-cAMPS to block the inhibitory effects of CADO on cytokine production by LAK cells. Ly49D cross-linked LAK cells were incubated for 6 hours with CADO (5  $\mu\text{mol/L}$ ). In some groups, the PKA inhibitors, H89 (5  $\mu\text{mol/L}$ ), PKI (10  $\mu\text{mol/L}$ ), or Rp-8-Br-cAMPS (1 mmol/L), were added 20 minutes before CADO. Supernatants were collected and the concentration of IFN- $\gamma$  was analyzed using multiplex immunobeads kit. The H89 + CADO and PKI + CADO groups significantly ( $P < 0.01$ ) differ from the Control group. The Rp-8-Br-cAMPS + CADO group significantly ( $P < 0.05$ ) differs from the CADO alone group but not from the Control or Rp-8-Br-cAMPS alone group. Experiments were repeated twice.

When H89 was used in combination with CADO, H89 did not block the inhibitory effects of CADO (Fig. 2B). Similarly, PKI peptide did not block the inhibitory effects of CADO on IFN- $\gamma$  production. In contrast, Rp-8-Br-cAMPS significantly ( $P < 0.05$ ) blocked the inhibitory effect CADO (Fig. 2B). Similarly, Rp-8-Br-cAMPS, but not H89 and PKI peptide, blocked the inhibitory effects of CADO on production of TNF- $\alpha$ , GM-CSF, and MIP-1 $\alpha$  (data not shown).

Thus, these results indicate that adenosine-mediated inhibition of LAK cell cytotoxic activity and cytokine production is not abrogated by blocking the catalytic subunits of PKA I. However, inhibiting dissociation of RI subunits from PKA I blocks the inhibitory effects of CADO. Thus, release of RI from the holoenzyme is required for inhibition of LAK cell cytotoxic activity and cytokine production.

## Discussion

Our data show that adenosine receptor signaling affects two major functions of LAK cells: their ability to kill tumor cells and produce cytokines. It was shown that cross-linking of the Ly49D

receptor leads to the association of this receptor with DAP12, which contains an immunoreceptor tyrosine-based activation motif. Studies with various pharmacologic inhibitors show that cross-linking of Ly49D stimulates IFN- $\gamma$  production via DAP12 tyrosine phosphorylation mediated by Src family kinases whereas *MIP-1 $\alpha$*  and *MIP-1 $\beta$*  gene activation is regulated by different pathways (23). The finding that CADO is able to inhibit production of IFN- $\gamma$ , MIP-1 $\alpha$ , TNF- $\alpha$ , and GM-CSF suggests that adenosine-induced inhibition of the production of these cytokines is mediated by upstream signaling events.

Adenosine stimulates cAMP production and activates PKA, and therefore it is conceivable that the cAMP/PKA pathway transmits the signal leading to inhibition of LAK cell cytotoxicity and cytokine production. Using isozyme-selective cAMP analogue pairs that preferentially activate PKA I or PKA II, we found that activation of PKA I, but not PKA II, mimics the suppressive effects of CADO on the cytotoxic activity and cytokine production of murine LAK cells.

PKA I and PKA II have differential effects on the functional activity of other lymphoid cells, such as B and T cells. The very early events of T-cell activation are associated with activation of PKA I, but not PKA II (36). In addition, activation of PKA I, but not PKA II, is responsible for inhibition of receptor-induced B and T cell proliferation (20). PKA I hyperactivation is found in HIV-infected T cells and is associated with their functional impairment (20). Abnormally high levels of RII $\beta$  expression are found in T cells of patients with systemic lupus erythematosus, and RII $\beta$  directly interacts and suppresses CREB transcriptional activity in activated T cells (20). Activation of PKA I by specific cAMP-elevated agents (*S<sub>p</sub>*-8-Br) inhibits the cytotoxic activity of rat NK cells (37).

It is unclear why activation of PKA I and PKA II results in different effects on the LAK cell activity. Adenosine-induced cAMP can bind to the regulatory units of both PKA I and PKA II and this would release the catalytic units from both types of PKA. The catalytic subunits are similar in both PKA isozymes and, on activation, they would phosphorylate various targets including transcription factors, such as CREB, CREM, and NF- $\kappa$ B, and affect expression of cytokine genes (17). The fact that activation of only PKA I, but not PKA II, is responsible for the observed adenosine-mediated effects might have several explanations. NK cells as well as other lymphoid cells have ~70% PKA I and only 30% PKA II (37). In addition, PKA I is mostly soluble and is predominantly cytoplasmic, whereas the vast majority of PKA II is insoluble and associated with PKA anchoring proteins (AKAP) that target PKA II to different subcellular sites (centrosomes, actin cytoskeleton, endoplasmic reticulum, mitochondria, the nuclear matrix, etc.; ref. 38). Furthermore, regulatory RI and RII subunits may have distinctive functions (16, 18, 20).

Although adenosine induces cAMP, activates PKA, and inhibits the cytotoxic activity of LAK cells and cytokine production by LAK cells similar to the PKA I agonists, this does not necessarily imply that these effects of adenosine are mediated via the PKA pathway. Some studies show various agents, such as forskolin, dibutyryl cAMP define, *S<sub>p</sub>*-cAMPS, cholera toxin, rolipram, prostaglandin *E<sub>2</sub>*, albuterol, and isoproterenol, induce cAMP and inhibit T-cell proliferation and IL-2 and IL-5 production, but these effects are PKA independent (39, 40). This conclusion is based on the failure of PKA inhibitors (H89 and PKI peptide) to block the inhibitory effects of cAMP-elevating agents. Studies show that although H89 inhibits PKA activity and CREB phosphorylation, it does not

abrogate the inhibitory effects of cAMP-inducing agents on human T-cell proliferation (40).

PKA activity can be inhibited either by blocking directly the activity of the catalytic units or by blocking the dissociation of the regulatory subunits. Indeed, we found that H89 (5  $\mu$ mol/L), PKI (10  $\mu$ mol/L), and Rp-8-Br-cAMPS (500  $\mu$ mol/L) completely block CADO-induced PKA activity of LAK cells (data not shown). In our experiments, direct blocking of the catalytic subunits with H89 and PKI peptide does not attenuate the inhibitory effects of CADO, arguing against the involvement of catalytic subunits in these effects. On the other hand, blocking the release of RI subunits by Rp-8-Br-cAMPS is associated with attenuation of the inhibitory effects of CADO. These findings indicate that the inhibitory effects of CADO on LAK cell cytotoxic activity as well as cytokine production are mediated via cAMP-mediated release of regulatory RI subunits from PKA I. Similarly, previous studies show that the inhibitory effects of cAMP agonist (*S<sub>p</sub>*-8-Br) on cytotoxic activity of rat NK cells can be blocked Rp-8-Br-cAMPS (37). The precise mechanisms by which RI mediates these inhibitory effects need to be elucidated.

Increasing evidence suggests that the regulatory subunits of PKA per se have functional activity. Mice with a selective knockout of genes encoding regulatory subunits (RI $\alpha$ , RI $\beta$  or RII $\alpha$ , RII $\beta$ ) reveal distinctive phenotypic changes (16). Knockout of *RI $\alpha$*  gene causes early embryonic lethality whereas knockout of the *RI $\beta$*  gene results in hippocampal alterations, reduced inflammatory responses, and nociceptive pain without changes in a total PKA activity. Knockout of *RII $\alpha$*  shows no detectable abnormality in spite of a reduction of PKA activity. Mice with knockout of *RII $\beta$*  gene manifest metabolic changes (lean phenotype, increased lipolysis, reduced plasma insulin and VLDL and LDL cholesterol), diminished motor learning, increased alcohol consumption, and decreased alcohol-induced sedation (16).

Experiments with transfection of tumor cells showed that RI $\alpha$  promotes cell growth whereas RII $\beta$  inhibits growth and induces cell differentiation (16, 18, 19). The importance of the regulatory unit RI $\alpha$  in carcinogenesis was recently shown. Mutation in RI $\alpha$  causes the Carney complex tumor syndrome with development of multiple tumors (schwannomas and endocrine tumors) and cardiac and extracardiac myxomatosis (41). These data clearly indicate that regulatory subunits of PKA have functional activity and PKA has biological significance beyond its catalytic activity.

In summary, adenosine inhibits LAK cell cytotoxic activity and cytokine production. These inhibitory effects involve a common mechanism that includes cAMP induction and activation of PKA type I. Moreover, these inhibitory effects depend on the involvement of RI and are independent of the activity of catalytic subunits of PKA. Findings that adenosine levels are elevated within growing tumors suggest that adenosine could inhibit the effector functions of tumor-infiltrating immune cells and thus protect malignant tissues from immune-mediated destruction.

## Acknowledgments

Received 2/6/2006; revised 4/10/2006; accepted 5/17/2006.

**Grant support:** Department of Defense Breast and Prostate Cancer Research Program grants DAMD17-03-1-0599 and DAMD-02-1-0127 (E. Gorelik), NIH grants RO1 CA098642-01A1 and R21CA102061-01A1 (A. Lokshin), and NIH grant DK68575 (E. K. Jackson).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. John Ortaldo for providing anti-Ly49D mAb.

## References

1. Burnstock G. The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* 1997;36:1127-39.
2. Klinger M, Freissmuth M, Nanoff C. Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell Signal* 2002;14:99-108.
3. Sitkovsky MV, Lukashov D, Apasov S, et al. Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annu Rev Immunol* 2004;22:657-82.
4. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in down-regulation of inflammation and protection from tissue damage. *Nature* 2001;414:916-20.
5. Cronstein BN. Adenosine, an endogenous anti-inflammatory agent. *J Appl Physiol* 1994;76:5-13.
6. Panther E, Corinti S, Idzko M, et al. Adenosine affects expression of membrane molecules, cytokine and chemokine release, and the T-cell stimulatory capacity of human dendritic cells. *Blood* 2003;101:3985-90.
7. Szabo C, Scott GS, Virag L, et al. Suppression of macrophage inflammatory protein (MIP)-1 $\alpha$  production and collagen-induced arthritis by adenosine receptor agonists. *Br J Pharmacol* 1998;125:379-87.
8. Lukashov D, Ohta A, Apasov S, Chen JF, Sitkovsky M. Cutting edge: Physiologic attenuation of proinflammatory transcription by the Gs protein-coupled A2A adenosine receptor *in vivo*. *J Immunol* 2004;173:21-4.
9. Koshiba M, Kojima H, Huang S, Apasov S, Sitkovsky MV. Memory of extracellular adenosine A2A purinergic receptor-mediated signaling in murine T cells. *J Biol Chem* 1997;272:25881-9.
10. Huang S, Apasov S, Koshiba M, Sitkovsky M. Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood* 1997;90:1600-10.
11. Hasko G, Kuhel DG, Chen JF, et al. Adenosine inhibits IL-12 and TNF- $\alpha$  production via adenosine A2a receptor-dependent and independent mechanisms. *FASEB J* 2000;14:2065-74.
12. Blay J, White TD, Hoskin DW. The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. *Cancer Res* 1997;57:2602-5.
13. Raskovalova T, Huang X, Sitkovsky M, Zacharia LC, Jackson EK, Gorelik E. Gs protein-coupled adenosine receptor signaling and lytic function of activated NK cells. *J Immunol* 2005;175:4383-91.
14. Hoskin DW, Reynolds T, Blay J. 2-Chloroadenosine inhibits the MHC-unrestricted cytolytic activity of anti-CD3-activated killer cells: evidence for the involvement of a non-A1/A2 cell-surface adenosine receptor. *Cell Immunol* 1994;159:85-93.
15. Hoskin DW, Butler JJ, Drapeau D, Haeryfar SM, Blay J. Adenosine acts through an A3 receptor to prevent the induction of murine anti-CD3-activated killer T cells. *Int J Cancer* 2002;99:386-95.
16. Chin KV, Yang WL, Ravatn R, et al. Reinventing the wheel of cyclic AMP: novel mechanisms of cAMP signaling. *Ann N Y Acad Sci* 2002;968:49-64.
17. Skalhegg BS, Tasken K. Specificity in the cAMP/PKA signaling pathway. Differential expression regulation, and subcellular localization of subunits of PKA. *Front Biosci* 2000;5:D678-93.
18. Cho-Chung YS, Nesterova M, Becker KG, et al. Dissecting the circuitry of protein kinase A and cAMP signaling in cancer genesis: antisense, microarray, gene overexpression, and transcription factor decoy. *Ann N Y Acad Sci* 2002;968:22-36.
19. Neary CL, Nesterova M, Cho YS, Cheadle C, Becker KG, Cho-Chung YS. Protein kinase A isozyme switching: eliciting differential cAMP signaling and tumor reversion. *Oncogene* 2004;23:8847-56.
20. Torgersen KM, Vang T, Abrahamsen H, Yaqub S, Tasken K. Molecular mechanisms for protein kinase A-mediated modulation of immune function. *Cell Signal* 2002;14:1-9.
21. Gunji Y, Vujanovic NL, Hiserodt JC, Herberman RB, Gorelik E. Generation and characterization of purified adherent lymphokine-activated killer cells in mice. *J Immunol* 1989;142:1748-54.
22. Ortaldo JR, Bere EW, Hodge D, Young HA. Activating Ly-49 NK receptors: central role in cytokine and chemokine production. *J Immunol* 2001;166:4994-9.
23. Ortaldo JR, Winkler-Pickett R, Wiegand G. Activating Ly-49D NK receptors: expression and function in relation to ontogeny and Ly-49 inhibitor receptors. *J Leukoc Biol* 2000;68:748-56.
24. Gorelik E, Landsittel DP, Marrangoni AM, et al. Multiplexed immunobead-based cytokine profiling for early detection of ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2005;14:981-7.
25. Beebe SJ, Blackmore PF, Chrisman TD, Corbin JD. Use of synergistic pairs of site-selective cAMP analogs in intact cells. *Methods Enzymol* 1988;159:118-39.
26. Kawasaki H, Springett GM, Mochizuki N, et al. A family of cAMP-binding proteins that directly activate Rap1. *Science* 1998;282:2275-9.
27. de Rooij J, Zwartkruis FJ, Verheijen MH, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 1998;396:474-7.
28. Aronoff DM, Canetti C, Serezani CH, Luo M, Peters-Golden M. Cutting edge: macrophage inhibition by cyclic AMP (cAMP): differential roles of protein kinase A and exchange protein directly activated by cAMP-1. *J Immunol* 2005;174:595-9.
29. Enserink JM, Christensen AE, de Rooij J, et al. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol* 2002;4:901-6.
30. Mason LH, Anderson SK, Yokoyama WM, Smith HR, Winkler-Pickett R, Ortaldo JR. The Ly-49D receptor activates murine natural killer cells. *J Exp Med* 1996;184:2119-28.
31. Fischer S, Knoll R, Renz D, Karliczek GF, Schaper W. Role of adenosine in the hypoxic induction of vascular endothelial growth factor in porcine brain derived microvascular endothelial cells. *Endothelium* 1997;5:155-65.
32. Olah ME, Roudabush FL. Down-regulation of vascular endothelial growth factor expression after A(2A) adenosine receptor activation in PC12 pheochromocytoma cells. *J Pharmacol Exp Ther* 2000;293:779-87.
33. Leibovich SJ, Chen JF, Pinhal-Enfield G, et al. Synergistic up-regulation of vascular endothelial growth factor expression in murine macrophages by adenosine A(2A) receptor agonists and endotoxin. *Am J Pathol* 2002;160:2231-44.
34. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000;351:95-105.
35. Gjertsen BT, Mellgren G, Otten A, et al. Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell culture. Basal cAMP-kinase activity modulates interleukin-1 $\beta$  action. *J Biol Chem* 1995;270:20599-607.
36. Laxminarayana D, Berrada A, Kammer GM. Early events of human T lymphocyte activation are associated with type I protein kinase A activity. *J Clin Invest* 1993;92:2207-14.
37. Torgersen KM, Vaage JT, Levy FO, Hansson V, Rolstad B, Tasken K. Selective activation of cAMP-dependent protein kinase type I inhibits rat natural killer cell cytotoxicity. *J Biol Chem* 1997;272:5495-500.
38. Dell'Acqua ML, Scott JD. Protein kinase A anchoring. *J Biol Chem* 1997;272:12881-4.
39. Bryce PJ, Dascombe MJ, Hutchinson IV. Immunomodulatory effects of pharmacological elevation of cyclic AMP in T lymphocytes proceed via a protein kinase A independent mechanism. *Immunopharmacology* 1999;41:139-46.
40. Staples KJ, Bergmann M, Tomita K, et al. Adenosine 3',5'-cyclic monophosphate (cAMP)-dependent inhibition of IL-5 from human T lymphocytes is not mediated by the cAMP-dependent protein kinase A. *J Immunol* 2001;167:2074-80.
41. Kirschner LS, Kusewitt DF, Matyakhina L, et al. A mouse model for the Carney complex tumor syndrome develops neoplasia in cyclic AMP-responsive tissues. *Cancer Res* 2005;65:4506-14.