Inhibition of Cytokine Production and Cytotoxic Activity of Human Antimelanoma Specific CD8+ and CD4+ T Lymphocytes by Adenosine-Protein Kinase A Type I Signaling

Tatiana Raskovalova, Anna Lokshin, Xiaojun Huang, Yunyun Su, Maja Mandic, Hassane M. Zarour, Edwin K. Jackson, and Elieser Gorelik

Abstract

The goal of this study was to investigate the effects of adenosine and its stable analogue 2-chloroadenosine (CADO) on the cytotoxic activity and cytokine production by human antimelanoma specific CD8+ and CD4+ T-helper type 1 (Th1) clones. The cytotoxic activity of CD8+ T cells was inhibited by adenosine and CADO. Using Lab MAP multiplex technology, we found that adenosine inhibits production of various cytokines and chemokines by CD8+ and CD4+ T cells. Studies with CGS21680, a specific agonist of adenosine A2A receptor (AdoR2A), and ZM241385, an AdoR2-selective antagonist, indicate that the inhibitory effects of adenosine are mediated via cyclic AMP (cAMP)-elevating AdoR2A, leading to protein kinase A (PKA) activation. Using cAMP analogues with different affinities for the A and B sites of the regulatory subunits of PKA and PKAI, we found that activation of PKAI, but not of PKAI, mimicked the inhibitory effects of adenosine on T-cell cytotoxic activity and cytokine production. Inhibitors of the PKA catalytic subunits (H89 and PKA inhibitor peptide 14–22) failed to abrogate the inhibitory effects of CADO. In contrast, Rp-8-Br-cAMPS that antagonizes binding of cAMP to the regulatory I subunit and PKA activation was efficient in blocking the inhibitory effect of adenosine on the functional activity of T cells. Our findings on the ability of adenosine to inhibit the effector function of antimalanoma specific T cells suggest that intratumor-produced adenosine could impair the function of tumor-infiltrating T lymphocytes. Thus, blocking the inhibitory activity of tumor-produced adenosine might represent a new strategy for improvement of cancer immunotherapy. [Cancer Res 2007;67(12):5949–56]

Introduction

Identification of cancer antigens, discovery of antigen-presenting cells, and new insights into the molecular and cellular basis of antitumor immune responses engender hopes that immunotherapy could become an effective modality in cancer treatment. Numerous types of melanoma-associated antigens have been identified. Some of these antigens belong to the differentiation antigens (Melan-A/MART-1, tyrosinase, gp100, and TRP-2); some are oncofetal/cancer-testis antigens (MAGE, BAGE, GAGE families, and NY-ESO-1). Mutated forms of normal cellular proteins are also expressed in human melanomas (1, 2). Clinical trials using peptide-based vaccinations showed some clinical responses but, in general, the results were disappointing (3).

Low efficacy of immunotherapy can be explained, at least in part, by the ability of tumor to use various mechanisms that help it to escape immune-mediated destruction. In this regard, tumor cells could lose MHC and/or tumor antigen expression, making them unrecognizable by immune T lymphocytes and, in turn, stimulating production of T regulatory cells and various immunosuppressive molecules [such as interleukin (IL)-10, transforming growth factor-β, vascular endothelial growth factor (VEGF), prostaglandin E2, indoleamine 2,3-dioxygenase, and soluble Fas ligand] that inhibit the efficacy of antitumor immunotherapy (4).

Numerous experimental data showed that adenosine could inhibit the functional activity of various immune cells and manifest potent anti-inflammatory properties (5–8).

Adenosine is an endogenous adenine nucleoside that is formed from ATP, ADP, and AMP by sequential enzyme-mediated dephosphorylation in both intracellular and extracellular compartments (9). Intracellular adenosine is shunted into the extracellular space through membrane nucleoside transporters (9). Adenosine is quickly metabolized by adenosine deaminase, and adenosine deaminase deficiency leads to accumulation of high concentrations of extracellular and intracellular adenosine with development of severe combined immunodeficiency both in humans and mice (10, 11). Extracellular adenosine binds to adenosine receptors that are expressed by diverse types of cells and mediates various biological effects. Currently, four different adenosine receptors have been identified (A1, A2A, A2B, and A3) that belong to the G protein–coupled seven-transmembrane superfamily of cell receptors. A3 and A2B signaling elevates cyclic AMP (cAMP), whereas A1 and A3 signaling inhibits cAMP production (12). Numerous studies establish the importance of adenosine as an extracellular signal transducer that mediates a variety of physiologic effects in the nervous and cardiovascular systems (9). Adenosine concentrations substantially increased in response to tissue hypoxia or inflammation, and adenosine markedly reduces damage induced by brain or heart ischemia, ischemia-reperfusion injury, tissue inflammation, and sepsis (5–7, 13, 14). It was proposed that adenosine plays an important role in protecting normal tissues from the excessive damage mediated by hypoxia/ischemia and inflammation (5–8). The tissue-protective effects of adenosine are mediated via its ability to inhibit the function of lymphoid cells and production of proinflammatory cytokines (5–8). Although different G protein–coupled receptors (β-adrenergic, prostaglandin, and others) have been equally plausible candidates to play the role of endogenous regulators of immune cells and inflammation, studies done by Ohta...
et al. (6) provided the first direct evidence that it is the endogenous adenosine and adenosine A_{2A} receptor (AdoR_{2A}) that are indeed critical.

It is possible that adenosine is also using the same mechanisms to protect malignant tissue from immune-mediated destruction (7, 8, 15). The oxygen demands of growing tumors may exceed the ability of the existing blood vessels to deliver oxygen to the tumor, leading to development of acute or chronic intratumor hypoxia. Hypoxia is the most powerful stimulator of adenosine production (13). Elevated levels of adenosine are found in various solid and ascitic tumors (15–17).

It is conceivable, therefore, that adenosine might inhibit the efficacy of tumor infiltrating immune cells. This assumption is based on numerous studies showing that adenosine and its analogues are able to inhibit cytotoxic activity of murine T cells by affecting both perforin- and Fas-mediated cytotoxicity of T cells (18, 19). CGS21680, a specific agonist of AdoR_{2A}, significantly inhibited production of IL-2 and tumor necrosis factor (TNF) by murine T-cytotoxic type 1 (Tc1) and type 2 (Tc2) lymphocytes (20). In vivo administration of CGS21680 reduced CD4\(^+\) T-helper type 1 (Th1) and CD8\(^+\) Tc1 cell expansion to alloantigen or pigeon cytochrome c stimulation (20).

Recently, for the first time, it was shown that adenosine could play an important role in the regulation of the antitumor T cell-mediated immunity in vivo (15). This inhibitory effect of adenosine was found to be mediated via AdoR_{2A}. Indeed, in mice with a genetic disruption of AdoR_{2A}, the efficacy of T-cell–mediated immune responses decreased substantially, causing regression or severe inhibition of various experimental tumors. In contrast, these tumors grew progressively in wild-type AdoR_{2A}\(^{–/–}\) mice (15).

In our previous studies, we showed that adenosine is able to inhibit the ability of lymphokine-activated killer (LAK) cells to kill tumor cells and produce various cytokines and chemokines (17, 21, 22). This inhibitory effect is mediated via AdoR_{2A} and LAK cells from AdoR_{2A} knockout mice were resistant to the immunosuppressive effects of adenosine (17, 22). Thus, data obtained in experimental animals indicate that adenosine could reduce the efficacy of antitumor immune responses and thus protect tumor from immune-mediated destruction.

It is remains unclear, however, whether adenosine affects the functional activity of human antitumor specific T cells. Accordingly, in the present study, we investigated the effects of adenosine and adenosine analogues on the functional activity of antimelanoma specific human CD8\(^+\) and CD4\(^+\) T-cell clones. Particularly, we examined the effects of adenosine and its stable analogue 2-chloroadenosine (CADO), as well as agonists and antagonists of adenosine receptors, on the cytotoxic activity of antimelanoma specific CD8\(^+\) T cells. Using the MAPx Luminex technology, we analyzed the ability of antimelanoma specific CD8\(^+\) and CD4\(^+\) Th1 clones to produce 27 different cytokines, chemokines, and growth factors. The effects of adenosine on the cytokine production by antimelanoma specific T-cell clones were investigated. Finally, we examined the mechanism of adenosine-mediated inhibition of human T-cell activity. The ability of adenosine to induce cAMP and activate protein kinase A (PKA) is well documented (9). By using cAMP analogues with different affinities for the A and B sites of the regulatory subunits of PKA type I and II (PKAI and PKAll, respectively), we examined whether activation of PKA type I or type II is responsible for the inhibition of cytokine production and cytotoxic activity of human antimelanoma specific CD8\(^+\) and CD4\(^+\) T cells. Using various inhibitors of PKA activity, we investigated whether blocking of catalytic or regulatory subunits of PKA can block the inhibitory effects of adenosine on the functional activity of human antimelanoma specific CD8\(^+\) and CD4\(^+\) T cells.

### Materials and Methods

#### Reagents

Adenosine, CADO, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; adenosine deaminase inhibitor), and PKA inhibitors H89 and myristoylated PKA inhibitor peptide 14–22 (PKI_{14–22}) were purchased from Sigma-Aldrich. CGS21680 (AdoR_{2A}-selective agonist) and ZM241385 (AdoR_{2B}-selective antagonist) were from Tocris. Activators of PKAI and PKAll [8-hexylaminoadenosine-3\(^′\),5\(^′\)-cyclic monophosphate (8-HA-cAMP), N\(^′\) benzoyl-cAMP (6-Benz-cAMP), and 8-piperidino-cAMP (8-PIP-cAMP)], Epac activators [8-(4-methoxyphenylthio)-2’-O-methyl-cAMP and 8-(4-chloro-phenylthio)-2’-O-methyladenosine-3\(^′\),5\(^′\)- cyclic monophosphate (8-pCPT-2’-O-Me-cAMP)], and an inhibitor of PKAI (Rp-8-Br-cAMPS) were purchased from Axoxa Platform.

#### Antimelanoma Specific T-Cell Clones

**CTL clone 4/43.** Autologous peripheral blood lymphocytes (PBL) isolated from UPCI-MEL-136 melanoma patient were stimulated in vitro with UPCI-Mel 136.1 melanoma cells in mixed lymphocyte-tumor cell culture as previously described (23). The bulk CTL population was cloned by limiting dilution and then stimulated weekly in the presence of irradiated autologous tumor cells and EBV immortalized B cells in Iscove’s medium supplemented with 10% human serum, IL-2 (100 units/mL), and phytohemagglutinin (PHA-P, Difco). CD8\(^+\) T-cell clone 4/43 produced IFN-γ in ELISPOT assays following melanoma cell stimulation. This clone was found to be highly specific in killing the autologous melanoma cell line UPCI-MEL 136.1 as well as T cells pulsed with Melan-A/MART-1_{27–35} peptide.

**CD4\(^+\) Th1 cell clone 32/28.** CD4\(^+\) T cells isolated from PBLs of patient UPCI-MEL 285 were stimulated with autologous dendritic cells pulsed with LAGE-1 ORF_{25–102} peptide. These cells were cloned by limiting dilution and stimulated biweekly by alternating irradiated allogenic peripheral blood mononuclear cells and EBV cells or autologous peptide-pulsed dendritic cells in Iscove’s medium supplemented with 10% human serum, IL-2 (200 units/mL), and PHA-P (Difco) as previously described (24). CD4\(^+\) T-cell clone 32/28 recognized the HLA-DR epitope LAGE-1 ORF_{25–102} peptide in IFN-γ ELISPOT and cytokine release assays as previously reported (24).

#### Cytotoxic Activity of CD8\(^+\) T Cells

The cytotoxic activity of CD8\(^+\) T cells was tested against \(^{51}\)Cr-labeled T2 cells that were loaded with MART-1_{27–35} peptide (10 \(^{6}\) cfu/mL). T cells were distributed into V-bottomed 96-well plates, with and without test agents, and, 30 min later, radiolabeled T2 cells loaded with MART-1_{27–35} peptide (5 \(^{10}\) per well) were added. The cytotoxic activity of CD8\(^+\) T cell was tested in triplicates at various effector-to-target (E:T) ratios. After 4 h of incubation at 37\(^\circ\)C, supernatants (25 \(^{\mu}\)L) were transferred into yttrium silicate scintillator–coated white microplates (LumaPlate-96, Perkin-Elmer) and the level of \(^{51}\)Cr emission by released \(^{51}\)Cr was measured in a beta-counter. The percentage of cytotoxicity was calculated. The SD among the triplicates was always <7% of mean.

#### Cytokine Production

Cultured CD8\(^+\) and CD4\(^+\) Th1 cells were washed and rested for 2 h without cytokines. After washing, rested T cells were incubated with test chemicals for 30 min and were plated onto 96-well plates precoated with anti-CD3 monoclonal antibody (mAb; 1 \(^{10}\) per well) in duplicates. After overnight incubation, supernatants (0.1 mL) were collected and concentrations of various cytokines were analyzed using Luminex Lab MAP technology.

#### Multiplex Bead–Based Cytokine Analysis

We used human multiplex antibody bead kit (Biosource International) that allows simultaneous testing of 29 human cytokines, chemokines, and growth factors: IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-18, IFN-γ, TNF-α, GM-CSF, MIP-1a, MIP-1b, MCP-1, IP-10, GRO-α, RANTES, Eotaxin, IL-6, IL-8, IL-10, IL-12p40, IL-18, IL-22, IL-23, IL-29, IL-33, IL-35, IL-36γ, IFN-α, IFN-β, IFN-κ, and IFN-λ.
were calculated using Bio-Rad software. Beads were counted for each analyte per well. Mean values (pg/mL) were calculated using Bio-Plex Manager analytic software (Bio-Rad Laboratories). One hundred standards and supernant samples were pipetted at 50 μL/well in duplicate and mixed with 50 μL of bead mixture. After 1 h incubation, wells were washed twice, 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). One hundred beads were counted for each analyte per well. Mean values (pg/mL) ± SE were calculated using Bio-Rad software.

Statistics

All experiments were repeated two to three times and representative experiments are presented. The statistical analysis was done using t test and two-tailed ANOVA and column statistics by GraphPad PRISM software program. All data were expressed as mean ± SD. The significance level was set at P < 0.05.

Results

Inhibitory effect of adenine on the cytotoxic activity of human melanoma specific CD8+ T cells. Adenosine is quickly metabolized by adenosine deaminase and, thus, the effect of adenosine on the cytotoxic activity of CD8+ T cells was tested in the presence of EHNA, inhibitor of adenosine deaminase. Antimelanoma specific CD8+T cells were highly efficient in killing T2 cells pulsed with MART-127–35 peptide even at the E/T ratio 2:1. Adenosine at 5 μmol/L significantly (P < 0.05) inhibited killing of melanoma cells (Fig. 1A). Similarly, CADO, a stable adenine analogue, and CGS21680, a specific agonist of AdoRA2A, inhibited the cytotoxic activity of CD8+ T cells (Fig. 1A). These results suggest that the inhibitory effect of adenosine and CADO is mediated via AdoRA2A. This conclusion is further supported by experiments in which ZM244385, an AdoRA2A-selective antagonist, was able to block the inhibitory effects of CADO and restore the cytotoxic activity of CD8+ T cells (Fig. 1B).

Adenosine or its analogues, by binding to AdoRA2A, stimulate cAMP production, leading to activation of PKA (17, 22). Two types of PKA have been identified consisting of two regulatory and two catalytic subunits. Each regulatory (RI) and regulatory II (RII) subunit has two cooperative cAMP binding sites, A and B. The binding of cAMP molecules to the A and B sites on the two regulatory subunits reduces their affinity for the catalytic subunits, resulting in dissociation of the holoenzyme complex and releasing two free catalytic subunits, which then are able to phosphorylate serine and threonine residues on specific substrate proteins (26, 27).

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, PKAI and PKAII can be selectively activated (28). Thse, these University of Pittsburgh Cancer Institute Luminesce Core Facility as described (25). To generate a standard curve, 5-fold dilutions of appropriate standards were prepared in RPMI 1640. Standards and supernatant samples were metabolized by adenosine deaminase and, thus, the effect of adenosine and CADO is mediated via AdoRA2A. This conclusion is further supported by experiments in which ZM244385, an AdoRA 2-selective antagonist, was able to inhibited the cytotoxic activity of CD8+ T cells, and 8-PIP-cAMP which binds to RI A and RII B did not affect tumor cell killing. A combination of 8-PIP-cAMP and 6-Benz-cAMP (binds to A and B sites of RII subunits and therefore selectively activates PKAII) did not show any additional inhibition of cytotoxic activity (Table 1). 8-PA-cAMP that activates site B of RI did not inhibit cytotoxicity. A combination of 8-PA-cAMP with 6-Benz-cAMP that selectively activates PKAI had a more profound inhibitory effect than each modality used alone (Table 1). These data suggest that PKAI activation is predominantly responsible for the inhibition of the cytotoxic activity of antimelanoma specific CD8+ T cells.

Recently, it was found that CAMP might have an additional target that is independent of PKA (29, 30). This alternative cAMP target is exchange protein directly activated by cAMP (Epac1 and Epac2). Epac1 has an NH2-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 domain (29, 30). Therefore, we investigated whether Epac activation affects cytotoxic activity of CD8+ T cells. 8-PCPT-2′-O-methyl-cAMP is a potent activator of Epac1 and Epac2 (31). When CD8+ T cells were incubated with 8-PCPT-2′-O-methyl-cAMP (0.5 mmol/L), their ability to kill melanoma cells was not affected.

![Figure 1](image-url)
Stimulated CD8+ and CD4+ Th1 cells increased production of cytokines and chemokines (IFN-γ, RANTES, IL-13, MIP-1α, GM-CSF, IL-2, IL-4, IL-5, IL-12p40). Distribution of cytokines and chemokines basically corresponded to the phenotype of CD8+ and CD4+ Th1. CD8+ and CD4+ Th1 cells produced relatively high levels of IL-2, IL-10, IL-8, VEGF, FGF-β, and EGF that did not change following anti-CD3 stimulation. Low levels (9–60 pg/mL) of IL-7, IL-15, MCP-1, G-CSF, IFN-α, eotaxin, MIG, and HGF were found in the supernatants and anti-CD3 mAb did not stimulate production of these factors.

The results presented in Table 2 show that adenosine substantially inhibited production of IFN-γ, IL-2, TNF-α, GM-CSF, and MIP-1α in CD8+ and CD4+ Th1 cells. In addition, inhibition of MIP-1β, IL-13, and RANTES in these cells was observed (data not shown). Adenosine did not affect production of IL-1α, IL-10, IL-8, VEGF, FGF-β, EGF, IL-7, IL-15, MCP-1, G-CSF, IFN-α, eotaxin, MIG, and HGF, suggesting that these factors have a different pathway of regulation in comparison with those that were affected by adenosine.

Our studies with murine and human natural killer (NK) cells indicate that the ability of adenosine to inhibit cytokine production is mediated via AdoRA2A and AdoRA2B. This inhibitory effect can be blocked by ZM241385, an antagonist of these receptors (17, 22). Similarly, ZM241385 significantly (P < 0.05) blocked the inhibitory effects of adenosine on the cytokine production by human antimalanoma CD8+ and CD4+ Th1 cells (Table 2).

To assess possible involvement of PKA isozyme I or II in the regulation of cytokine production by human CD8+, CD4+ T cells, we used various cAMP analogues specifically binding to A or B site of regulatory subunit I or II. Treatment of CD8+ cells with 6-Benz-cAMP or 8-PICP-cAMP separately significantly (P < 0.05) inhibited production of GM-CSF, TNF-α, MIP-1α, IFN-α, and IL-2. 6-Benz-cAMP had similar inhibitory effects in CD4+ Th1 cells. However, treatment with 8-PICP-cAMP significantly (P < 0.05) inhibited only TNF-α production in CD4+ Th1 cells (Table 3). When both A and B sites of RI were activated by a combination of 6-Benz-cAMP and 8-PICP-cAMP, there was no further increase in the inhibitory effects in comparison with each compound used separately. In contrast, a combination of 8-8A-cAMP with 6-Benz-cAMP that activates PKAI inhibited a more profound inhibitory effect than each compound used separately (Table 3). These results indicate that activation of PKAI, but not PKAII, mimics the inhibitory effects of adenosine on cytokine production in human CD8+ and CD4+ Th1 cells.

### Table 1. Effects of PKAI, PKAII, and Epac activation on CD8+ T-cell cytotoxic activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Binding site</th>
<th>% Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>RI A and RII A</td>
<td>58 ± 1</td>
</tr>
<tr>
<td>6-Benz-cAMP</td>
<td>RI A and RII A</td>
<td>47 ± 5*</td>
</tr>
<tr>
<td>8-PIP-cAMP</td>
<td>RI A and RII B</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>8-PIP-cAMP + 6-Benz-cAMP</td>
<td>PKAII (A + B)</td>
<td>47 ± 4*</td>
</tr>
<tr>
<td>8-HA-cAMP</td>
<td>RI B</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>8-HA-cAMP + 6-Benz-cAMP</td>
<td>PKAII (A + B)</td>
<td>25 ± 3*</td>
</tr>
<tr>
<td>8-pCPT-2’-O-methyl-cAMP</td>
<td>Epac</td>
<td>57 ± 1</td>
</tr>
</tbody>
</table>

### Table 2. Inhibitory effects of adenosine on cytokine production by anti-CD3–stimulated CD8+ and CD4+ Th1 cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>IL-2</th>
<th>GM-CSF</th>
<th>MIP-1α</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+</td>
<td>7,740 ± 96</td>
<td>462 ± 62</td>
<td>227 ± 19</td>
<td>437 ± 30</td>
<td>3,010 ± 86</td>
</tr>
<tr>
<td>+ADO</td>
<td>1,974 ± 80*</td>
<td>183 ± 14*</td>
<td>60 ± 5*</td>
<td>89 ± 7*</td>
<td>1,040 ± 19*</td>
</tr>
<tr>
<td>+ZM + ADO</td>
<td>4,358 ± 79*</td>
<td>296 ± 21</td>
<td>103 ± 9*</td>
<td>174 ± 13</td>
<td>1,593 ± 79*</td>
</tr>
<tr>
<td>CD4+ Th1</td>
<td>10,769 ± 370</td>
<td>2,138 ± 175</td>
<td>4,411 ± 215</td>
<td>3,497 ± 37</td>
<td>8,695 ± 280</td>
</tr>
<tr>
<td>+ADO</td>
<td>2,153 ± 157*</td>
<td>1,086 ± 94*</td>
<td>1,369 ± 87*</td>
<td>1,167 ± 87*</td>
<td>6,473 ± 412*</td>
</tr>
<tr>
<td>+ZM + ADO</td>
<td>6,175 ± 214*</td>
<td>1,443 ± 69*</td>
<td>1,682 ± 96</td>
<td>1,687 ± 108*</td>
<td>7,179 ± 199*</td>
</tr>
</tbody>
</table>

**NOTE:** CD8+ T cells were treated for 30 min with the cAMP analogues that specifically bind to A and B sites of RI or RII PKA subunits (0.5 mmol/L when analogues used alone or 0.25 ± 0.25 mmol/L when they were used in combination). Some cells were treated with the activator Epac (0.5 mmol/L). CTLs were then mixed with 51Cr-labeled targets specific for CD8+ (CD8+ 7,740, CD4+ Th1 10,769). Supernatants were collected, and percentage of cytotoxicity was determined (Table 1). Thus, these results indicate that activation of PKAI, but not PKAII or Epac, mimics the inhibitory effect of adenosine on the cytotoxic activity of antimalanoma specific CD8+ T cells.

Inhibitory effects of adenosine on cytokine production by human antimalanoma specific CD8+ and CD4+ T cells. We use Luminex Lab MAP multiplex technology to analyze the ability of adenosine to affect production of various cytokines and chemokines in CD8+ and CD4+ cell clones. Cells were rested for 2 h and then were activated with the plastic adherent anti-CD3 mAb. Stimulated CD8+ and CD4+ Th1 cells increased production of 13 cytokines and chemokines (IFN-γ, IL-2, IL-4, IL-5, IP-10, TNF-α, IL-6, RANTES, IL-13, MIP-1α, MIP-1β, GM-CSF, and IL-12p40). Distribution of cytokines and chemokines basically corresponded to the phenotype of CD8+ and CD4+ Th1. CD8+ and CD4+ Th1 cells produced relatively high levels of IL-1α, IL-10, IL-8, VEGF, FGF-β, and EGF that did not change following anti-CD3 stimulation. Low levels (9–60 pg/mL) of IL-7, IL-15, MCP-1, G-CSF, IFN-α, eotaxin, MIG, and HGF were found in the supernatants and anti-CD3 mAb did not stimulate production of these factors.

The results presented in Table 2 show that adenosine substantially inhibited production of IFN-γ, IL-2, TNF-α, GM-CSF, and MIP-1α in CD8+ and CD4+ Th1 cells. In addition, inhibition of MIP-1β, IL-13, and RANTES in these cells was observed (data not shown). Adenosine did not affect production of IL-1α, IL-10, IL-8, VEGF, FGF-β, EGF, IL-7, IL-15, MCP-1, G-CSF, IFN-α, eotaxin, MIG, and HGF, suggesting that these factors have a different pathway of regulation in comparison with those that were affected by adenosine.

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To assess possible involvement of PKA isozyme I or II in the regulation of cytokine production by human CD8+, CD4+ T cells, we used various cAMP analogues specifically binding to A or B site of regulatory subunit I or II. Treatment of CD8+ cells with 6-Benz-cAMP or 8-PICP-cAMP separately significantly (P < 0.05) inhibited production of GM-CSF, TNF-α, MIP-1α, IFN-α, and IL-2. 6-Benz-cAMP had similar inhibitory effects in CD4+ Th1 cells. However, treatment with 8-PICP-cAMP significantly (P < 0.05) inhibited only TNF-α production in CD4+ Th1 cells (Table 3). When both A and B sites of RI were activated by a combination of 6-Benz-cAMP and 8-PICP-cAMP, there was no further increase in the inhibitory effects in comparison with each compound used separately. In contrast, a combination of 8-8A-cAMP with 6-Benz-cAMP that activates PKAI showed a more profound inhibitory effect than each compound used separately (Table 3). These results indicate that activation of PKAI, but not PKAII, mimics the inhibitory effects of adenosine on cytokine production in human CD8+ and CD4+ Th1 cells.

Blocking the CADO-induced inhibition of cytokine production and cytotoxic activity of T cells by PKA inhibitors. Our
finding that activation of PKAI mimics the inhibitory effects of adenosine on cytokine production by T cells might suggest that the inhibitory effect of adenosine is mediated via the PKA pathway. If this hypothesis is correct, one would expect that inhibition of PKA activity would block the inhibitory effects of adenosine. To test this prediction, we examined the ability of the PKA inhibitors H89 and myristoylated PKI14–22 peptide to abrogate the inhibitory effect of CADO on cytokine production by CD4+ Th1 cells. Using PKA activity kit (Stressgen Bioreagents), we found that H89 and PKI14–22 peptide failed to abrogate the inhibitory effects of CADO on cytokine and chemokine production (Fig. 2). Similarly, treatment of CD8+ cells with CADO resulted in a profound inhibition of cytokine production (Fig. 2A). Pretreatment of CD8+ cells with Rp-8-Br-cAMPS completely blocked the inhibitory effects of CADO, and the production of IL-2, IFN-γ, MIP-1α, IL-13, and GM-CSF was similar to that found in control untreated CD8+ cells. Production of TNF-α was partially blocked by Rp-8-Br-cAMPS. In contrast, blocking PKA activity with PKI14–22 peptide failed to abrogate the inhibitory effects of CADO on cytokine and chemokine production (Fig. 2A).

Next, we evaluated the activity of PKA inhibitors to block the suppressive effects of CADO on the cytoxic activity of CD8+ cells. CTLs were pretreated with H89, myristoylated PKI14–22 peptide, or Rp-8-Br-cAMPS and incubated with 51Cr-labeled T2 cells pulsed with MART-127–35 peptide in the presence or absence of CADO. CADO significantly (P < 0.05) inhibited the cytoxic activity of antimalanoma specific CTLs. H89, even at 5 μmol/L, inhibited cytokine and chemokine production (Fig. 2A). In contrast, antagonist of RI subunits Rp-8-Br-cAMPS completely prevented the inhibitory effect of CADO on the cytoxic activity of CTLs (Fig. 2B).

Discussion

Our studies indicate that adenosine could be a potent immunoregulatory factor affecting both cytokine production and cytoxic activity of antimalanoma specific T cells. Adenosine inhibits production of various cytokines and chemokines (IFN-γ, IL-2, TNF-α, GM-CSF, MIP-1α, MIP-1β, IL-13, and RANTES) in CD8+ and CD4+ Th1 cells, indicating that adenosine affects some common regulatory mechanism in the production of these cytokines and chemokines. However, adenosine did not affect production of IL-1α, IL-8, VEGF, FGF-β, EGF, IL-7, IL-15, IL-6, IL-10, GM-CSF, and MIP-1α.

Table 3. Comparative effects of PKAI and PKAII activation on cytokine production by anti-CD3–stimulated CD8+ and CD4+ Th1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PKA binding site</th>
<th>GM-CSF</th>
<th>TNF-α</th>
<th>MIP-1α</th>
<th>IFN-γ</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+6-Benz-cAMP</td>
<td>RI A/RI A</td>
<td>2,485 ± 359</td>
<td>12,323 ± 1,862</td>
<td>16,813 ± 3,126</td>
<td>805 ± 84</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>+8-PIP-cAMP</td>
<td>RI A/RII B</td>
<td>1,655 ± 13</td>
<td>6,460 ± 440</td>
<td>9,604 ± 8</td>
<td>539 ± 4</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>+8-PIP + 6-Benz</td>
<td>PKIA (A + B)</td>
<td>1,722 ± 28</td>
<td>7,231 ± 490</td>
<td>9,160 ± 254</td>
<td>616 ± 9</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>+8-HA-cAMP</td>
<td>RI B</td>
<td>1,570 ± 38</td>
<td>4,017 ± 352</td>
<td>8,931 ± 1,108</td>
<td>555 ± 31</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>+8-HA + 6-Benz</td>
<td>PKIA (A + B)</td>
<td>2,224 ± 189</td>
<td>8,446 ± 108</td>
<td>12,996 ± 452</td>
<td>825 ± 119</td>
<td>72 ± 7</td>
</tr>
<tr>
<td>+8-HA + 6-Ben</td>
<td>PKIA (A + B)</td>
<td>991 ± 117*</td>
<td>1,482 ± 381*</td>
<td>5,306 ± 149*</td>
<td>405 ± 64*</td>
<td>16 ± 2*</td>
</tr>
<tr>
<td>CD4+ Th1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+6-Benz-cAMP</td>
<td>RI A/RI A</td>
<td>18,947 ± 929</td>
<td>35,871 ± 1,233</td>
<td>34,178 ± 1,847</td>
<td>1,090 ± 84</td>
<td>564 ± 5</td>
</tr>
<tr>
<td>+8-PIP-cAMP</td>
<td>RI A/RII B</td>
<td>7,210 ± 283</td>
<td>5,741 ± 240</td>
<td>15,407 ± 1,356</td>
<td>498 ± 23</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>+8-PIP + 6-Ben</td>
<td>PKIA (A + B)</td>
<td>17,155 ± 490</td>
<td>28,872 ± 1,565</td>
<td>33,415 ± 2,273</td>
<td>987 ± 108</td>
<td>497 ± 33</td>
</tr>
<tr>
<td>+8-HA-cAMP</td>
<td>RI B</td>
<td>9,586 ± 44</td>
<td>9,401 ± 521</td>
<td>17,509 ± 1,807</td>
<td>738 ± 21</td>
<td>148 ± 12</td>
</tr>
<tr>
<td>+8-HA + 6-Ben</td>
<td>PKIA (A + B)</td>
<td>16,311 ± 381</td>
<td>24,117 ± 1,505</td>
<td>30,170 ± 197</td>
<td>1,043 ± 8</td>
<td>437 ± 35</td>
</tr>
<tr>
<td>+8-HA + 6-Ben</td>
<td>PKIA (A + B)</td>
<td>4,497 ± 150*</td>
<td>1,431 ± 267*</td>
<td>10,040 ± 1,601*</td>
<td>362 ± 3*</td>
<td>32 ± 4*</td>
</tr>
</tbody>
</table>

NOTE: CD8+ and CD4+ Th1 cells were rested for 2 h and then were activated with plate-bound anti-CD3 mAb and were incubated for 16 h in the presence of cAMP analogues that specifically bind to A and B sites of RI or RII PKA subunits (0.5 mmol/L when analogues used alone or 0.25 + 0.25 mmol/L when they were used in combination). The levels of cytokine in supernatants (pg/mL) were determined by Luminex LabMAP technology. *Significantly different from other groups (P < 0.01). The experiment was repeated twice with similar results.
IL-17, MCP-1, G-CSF, IFN-α, eotaxin, MIG, and HGF, suggesting that these factors have a different pathway of regulation.

The ability of CGS21680, a selective agonist of AdoRA2A, to inhibit the cytotoxic activity and cytokine production by human T cells suggests the involvement of the cAMP-elevating A2A receptor in these effects. This conclusion is further supported by the finding that ZM241385, a selective antagonist of AdoRA2, was able to block the inhibitory effects of adenosine.

Binding of adenosine to AdoR2A stimulates cAMP production and activates PKA (5, 9, 22); therefore, it is conceivable that the cAMP/PKA pathway transmits the signal leading to inhibition of T-cell cytotoxicity and cytokine production. Using isozyme-selective cAMP analogue pairs that preferentially activate PKAI or PKAII, we found that activation of PKAI, but not PKAII, mimics the suppressive effects of adenosine in human T cells. Similarly, our previous studies showed that activation of PKAI, but not PKAII, inhibited the cytotoxic activity and cytokine production by murine and human activated NK cells (21, 22).

Numerous reports showed that PKAI and PKAII have differential effects on the functional activity of B and T cells (27, 34–36). The very early events of T-cell activation are associated with activation of PKAII, but not PKAI (35). In addition, activation of PKAI, but not PKAII, is responsible for inhibition of receptor-induced B-cell and T-cell proliferation induced by cAMP-activating agents (36). It was found that lymphoid cells contain about 70% of PKAI and 30% of PKAII (27). Direct analysis of the phosphotransferase activity of PKAI and PKAII in human T cells revealed that a ratio of PKAI to PKAII isozyme activity is 3:9:1. However, the differences in their intracellular concentrations could not be solely responsible for the observed differences in PKAI and PKAII regulation of T-cell function. PKAI has higher affinity to cAMP and is predominantly located in the cytoplasm, whereas PKAII is associated with A-kinase–anchoring proteins that target PKAIII to different subcellular sites (centrosomes, actin, endoplasmic reticulum, mitochondria, nuclear matrix, etc.). Although they have the same catalytic subunits, they are functionally different (27, 34–36). The experiments with knockout of regulatory subunits of PKAI and PKAII in mice showed very distinct phenotypes, suggesting their involvement in different biological processes (26).

Several lines of evidence indicate that the inhibitory effects of PKAI are mediated via activation of the COOH-terminal Src kinase that phosphorylates Lck and inhibits T-cell receptor signaling (27, 36).

Although adenosine induces cAMP, activates PKA, and inhibits the cytotoxic activity and cytokine production by T cells similar to the PKAI agonists, this does not necessarily imply that these effects of adenosine are mediated via the PKA pathway. Some studies argue against the involvement of PKA in the inhibitory effects of cAMP inducers (37, 38). It was shown that various cAMP inducing agents, such as forskolin, dibutyryl cAMP, Sp-cAMPS, cholera toxin, rolipram, prostaglandin E2, albuterol, and isoproterenol, were able to activate PKA as well as inhibit T-cell proliferation and IL-2 and IL-5 production. However, it was concluded that the inhibitory effects of cAMP-activating agents on T-cell function are PKA independent (37, 38). This conclusion is based on the failure of PKA inhibitors (H89 and PKI14–22 peptide) to block the inhibitory effects of cAMP-elevating agents on human T-cell proliferation and IL-2 and IL-5 production (37, 38).

In our previous experiments with murine and human NK cells (21, 22) as well as current studies with human T-cell PKA inhibitors, H89 and PKI14–22 peptide also failed to attenuate the inhibitory effects of adenosine. On the other hand, Rp-8-Br-cAMPS, which prevents binding of cAMP and the release of RI subunits, blocked the inhibitory effects of adenosine. The mechanisms responsible for the differences in the effects of PKA inhibitors PKI14–22 peptide and Rp-8-Br-cAMPS remain unclear. These findings suggest that the inhibitory effect of adenosine on T-cell functional activity is mediated via cAMP-mediated release of regulatory RI but not catalytic subunits from PKAI.
In some studies, the role of catalytic and regulatory subunits of PKA in the regulation of murine CTLs functional activity was previously investigated and rather controversial results were obtained (39, 40). Pretreatment of CTLs with antisense oligomers complementary to mRNA for the α catalytic subunit of PKA enhanced the cytotoxic activity of CTLs but inhibited T-cell receptor–triggered IFN-γ production (39). When CD4+ hybridoma clone was transfected with cDNA encoding R and cytokine production of antimelanoma specific CD8+ and CD4+ T cells as well as activated NK cells was shown only in vitro, there are reasons to believe that adenosine exerts similar effects in vivo. Tumors have elevated levels of adenosine due to hypoxia and inflammation (7, 13, 15). Therefore, adenosine could inhibit the effector functions of immune cells and thus protect malignant tissues from immune-mediated destruction. Blocking adenosine receptor signaling might increase efficacy of immunotherapy, and recent studies strongly support such a possibility. Pharmacologic blockade or genetic disruption of A2A receptors increased the efficacy of T-cell–mediated immunity, resulting in inhibition or complete rejection of CL-8-1 melanoma and other tumors (15).

It was shown that adoptive transfer of anti-melanoma specific T cells caused clinical responses in 50% of melanoma patients (43). Our findings that adenosine is able to impair function of human anti-melanoma T lymphocytes might suggest that the therapeutic efficacy of the adoptively transferred immune T cells can be further increased by blocking the immunosuppressive effects of adenosine. Decreasing the intratumor levels of adenosine or blocking adenosine-mediated immunosuppressive signaling might represent a new strategy for improvement of cancer immunotherapy.

### Acknowledgments


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We thank Adele Marrangoi and Liudmila Velikokhatnaya (Luminx Facility, University of Pittsburgh Cancer Institute) for their assistance with this work.

### Table 4. Comparative effects of PKA inhibitors H89, PKI, and Rp-8-Br-cAMP on cytokine production by CD4+ Th1 cells treated with CADO

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GM-CSF</th>
<th>TNF-α</th>
<th>MIP-1α</th>
<th>IFN-γ</th>
<th>IL-13</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.947 ± 929</td>
<td>35.871 ± 1.233</td>
<td>34.178 ± 1.847</td>
<td>1,090 ± 84</td>
<td>13,671 ± 377</td>
<td>564 ± 34</td>
</tr>
<tr>
<td>CADO</td>
<td>2.02 ± 150</td>
<td>1,508 ± 516</td>
<td>3,630 ± 174</td>
<td>126 ± 16</td>
<td>1,561 ± 101</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>H89</td>
<td>1,098 ± 54</td>
<td>1,090 ± 11</td>
<td>2,961 ± 140</td>
<td>116 ± 6</td>
<td>1,525 ± 271</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>H89 + CADO</td>
<td>151 ± 97</td>
<td>61 ± 11</td>
<td>830 ± 17</td>
<td>45 ± 1</td>
<td>375 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>PKI</td>
<td>19,627 ± 2,729</td>
<td>33,666 ± 3,737</td>
<td>34,696 ± 457</td>
<td>1,301 ± 131</td>
<td>15,728 ± 668</td>
<td>733 ± 67</td>
</tr>
<tr>
<td>PKI + CADO</td>
<td>1,256 ± 43</td>
<td>1,611 ± 314</td>
<td>3,862 ± 110</td>
<td>156 ± 12</td>
<td>1,715 ± 22</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Rp-8-Br-AMPS</td>
<td>17,132 ± 826</td>
<td>32,534 ± 2,543</td>
<td>31,543 ± 3,993</td>
<td>1,211 ± 93</td>
<td>14,143 ± 1,243</td>
<td>574 ± 61</td>
</tr>
<tr>
<td>Rp-8-Br-cAMP + CADO</td>
<td>3,335 ± 26*</td>
<td>5,152 ± 720*</td>
<td>7,072 ± 524*</td>
<td>302 ± 21*</td>
<td>3,324 ± 256*</td>
<td>104 ± 11*</td>
</tr>
</tbody>
</table>

NOTE: Restered CD4+ Th1 cells were transferred onto 96-well plates (1 × 10^4 per well) precoated with anti-CD3 mAb and were preincubated with PKA inhibitor H-89 (10 μmol/L), myristoylated PKI14–22 peptide (10 μmol/L), or Rp-8-Br-cAMPs (1 mmol/L) for 30 min before CADO (20 μmol/L) was added. After 12-h incubation at 37°C, supernatants (0.1 mL) were collected and concentrations of various cytokines and chemokines (pg/mL) were analyzed using a multiplex kit and Luminex LabMAP technology. All groups, except PKI and Rp-8-Br-cAMPs alone, were significantly (P < 0.05) lower than control group.

*Significantly higher than CADO-treated group (P < 0.05).

### References

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Inhibition of Cytokine Production and Cytotoxic Activity of Human Antimelanoma Specific CD8 + and CD4+ T Lymphocytes by Adenosine-Protein Kinase A Type I Signaling

Tatiana Raskovalova, Anna Lokshin, Xiaojun Huang, et al.


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