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ORIGINAL ARTICLE

Receptor desensitization and blockade of the suppressive effects of prostaglandin E_2 and adenosine on the cytotoxic activity of human melanoma-infiltrating T lymphocytes

Yunyun Su · Edwin K. Jackson · Elieser Gorelik

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Abstract Previous studies document that PGE_2 and adenosine suppress production of inflammatory cytokines. The present study demonstrates for the first time that (1) PGE_2 and 2-chloroadenosine (CADO; a stable analog of adenosine) directly inhibit the cytolytic function of human tumor-infiltrating lymphocytes (TILs); (2) the combination PGE_2 and CADO have additive suppressive effects; and (3) the cooperative immunosuppressive actions of PGE_2 and CADO are mediated via EP2 receptors (EP2Rs) and A_{2A} receptors (A_{2A}Rs) and are due to amplification of cAMP production, activation of protein kinase A (PKA) and T cell receptor (TCR) inhibitor Csk leading to inhibition of Lck, ZAP-70 and Akt phosphorylation. (4) During ex vivo expansion, TILs undergo three stages of differentiation converting from TILs with high cytotoxic activity and relative resistance to combined EP2R/A_{2A}R suppression (stage I) to TILs retaining high cytotoxicity and gaining sensitivity to combined suppression (stage II) and then to TILS that are less cytotoxic and very sensitive to combined suppression (stage III). (5) Finally, we find that pretreatment of TILs with non-inhibitory concentrations of EP2R agonists (such as PGE_2 or butaprost) or A_{2A}R agonists (such as CADO or CGS21680) increases their cytotoxic activity and induces resistance to EP2R and A_{2A}R inhibitory signaling (cross-resistance) due to homologous and heterologous desensitization and internalization of EP2Rs and A_{2A}Rs, thus preventing their inhibitory signaling. We conclude that inducing resistance of TILs to the suppressive effects of PGE_2 and adenosine in the tumor microenvironment could represent a novel strategy for improving the efficacy of adoptive immunotherapy.

Keywords Adenosine · PGE_2 · Human CTLs · Tumor immunology

Y. Su · E. Gorelik (✉)
Department of Pathology,
University of Pittsburgh Cancer Institute,
5117 Centre Ave, Pittsburgh, PA 15213, USA
e-mail: gorelik@pitt.edu

E. Gorelik
Department of Immunology,
University of Pittsburgh Cancer Institute,
5117 Centre Ave, Pittsburgh, PA 15213, USA

E. K. Jackson
Department of Pharmacology and Chemical Biology,
University of Pittsburgh Medical School,
Pittsburgh, PA, USA

Introduction

Adoptive therapy with anti-melanoma-specific cytotoxic T lymphocytes (CTLs) induces clinical responses in 50–72% of patients with advanced melanoma [1, 2]. This success is achieved by depleting the host's lymphocytes with chemotherapy or irradiation prior to adoptive transfer of ex vivo expanded melanoma infiltrated CTLs. This procedure eliminates T regulatory cells and increases the availability of cytokines essential for the survival and function of transfused CTLs [1, 2]. Nonetheless, as transferred CTLs enter the tumor, various microenvironmental factors, such as TGF-β, IL-10 and VEGF, inhibit CTL activity and compromise therapeutic efficacy [3–7].

Along these lines, emerging evidence suggests that tumor-derived adenosine and PGE_2 may also function as immunosuppressive molecules in the tumor microenvironment [8–13]. PGE_2 and adenosine have much in common. They both exert various biological effects by engaging

receptors (Rs) that couple to signal transduction systems via G-proteins: EP1Rs, EP2Rs, EP3Rs and EP4Rs for PGE_2; and A_1Rs, A_{2A}Rs, A_{2B}Rs and A_3Rs for adenosine [11, 14]. Moreover, both PGE_2 and adenosine can stimulate adenylyl cylcase via EP2Rs/EP4Rs and A_{2A}Rs/A_{2B}Rs, respectively [11, 14]. In lymphoid cells, cAMP signaling mediates a potent immunosuppressive effect and the magnitude of immunosuppression is a function of the amount of cAMP produced [12, 15, 16]. This information suggests that in combination PGE_2 and adenosine increase cAMP production and cause amplification of signaling via the cAMP–PKA pathway, yielding a more potent immunosuppressive effect than each modality acting separately [17]. This concept seems likely because intratumor inflammation and hypoxia cause over-production of both adenosine and PGE_2 [18–20].

The above considerations may have clinical significance. Adoptively transferred immune T cells upon tumor infiltration may be inactivated by the combined actions of adenosine and PGE_2. However, direct experimental support of this possibility is lacking because knowledge regarding the immunosuppressive properties of adenosine and PGE_2 is based mostly on results obtained from experimental models of inflammation and autoimmunity. Although these studies demonstrate the ability of adenosine and PGE_2 to suppress inflammatory cytokine production by various immune cells [10, 12, 13, 21, 22], only a few studies demonstrate that adenosine inhibits anti-tumor, T cell-mediated responses. Pharmacological or genetic blockade of cAMP-elevating, A_{2A}R signaling substantially improves the efficacy of anti-tumor immune responses and inhibits growth of local and metastatic tumors in mice [20]. However, it remains unknown whether adenosine and PGE_2 are able to inhibit the cytotoxic activity of human antitumor-specific CTLs. Therefore, in the present study, we investigated the effects of adenosine and PGE_2 on the cytotoxic activity of anti-melanoma-specific CTLs used for adoptive therapy of patients with metastatic melanoma. Moreover, we investigated the mechanisms of the suppressive effects of adenosine and PGE_2 and identified three transitional stages of tumor-infiltrating lymphocytes (TILs) differentiation during their in vitro expansion characterized by reduced cytolytic ability and increased sensitivity to the suppressive effects of EP2R and A_{2A}R agonists.

Because of the importance of developing approaches to increase TIL cytotoxic activity by inducing resistance to immunosuppression mediated by tumor-produced adenosine and PGE_2, we also examined the effects of brief pre-treatment of anti-melanoma-specific TILs during stages II and III with relatively non-inhibitory concentrations of EP2R and A_{2A}R agonists. Importantly, such treatments increased the cytotoxic activity of TILs, rendered them resistant to A_{2A}R and EP2R suppressive signaling and induced cross-resistance due to homologous and heterologous desensitization and internalization of EP2Rs and A_{2A}Rs.

Materials and methods

Reagents

2-Chloroadenosine (CADO), PGE_2, rolipram, forskolin and butaprost were purchased from Sigma-Aldrich (St. Louis, MO, USA). CGS21680 (CGS) was from Tocris (Ellisville, MO, USA). IL-2 was provided by the BRB Preclinical Repository, NCI, NIH.

Tumor-infiltrating lymphocytes

Cryopreserved samples of TILs that were used for adoptive therapy of human metastatic melanomas were provided by Steven Rosenberg (Surgery Branch, NCI, NIH). TILs were propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine, nonessential aminoacids, 2-ME, antibiotics and IL-2 (5,000 IU/ml). In total, we tested the cytotoxic activity of nine individual HLA $A2^+$ MART-1-specific TIL lines. Their sensitivity to suppressive effects of PGE_2 and/or CADO was also analyzed.

Cytotoxic activity of TILs

The cytotoxic activity of TILs was tested against ^{51}Cr-labeled T2 cells that were loaded with MART-$1_{27–35}$ peptide (10 μg/ml) in the presence of the tested agents. After 4 h of incubation, supernatants (25 μl) were transferred into yttrium silicate scintillator-coated white microplates (LumaPlate™-96, PerkinElmer, Boston, MA, USA) and the level of β-emission by released ^{51}Cr was measured in a β-counter. The percentage of cytotoxicity was calculated as previously described [22]. In some experiments, HLA-A*0201$^+$/DRB1*0401$^+$ UPCI-MEL-136 melanoma cells isolated from a primary human melanoma were used as targets (gift from Dr. Hassan Zarour, University of Pittsburgh).

Western blot analysis

Tumor-infiltrating lymphocytes were equilibrated for 1 day without IL-2 and then stimulated with plate bound anti-CD3 (5 μg/ml) in the presence of CADO and PGE_2 for 30 min. TILs were lysed and protein extracts (50 μg) were resolved using 10% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with the primary antibodies recognizing phosphorylated cAMP responding element-binding protein (CREB) (anti-phospho

S129 + S133; 1:1,000, Abcam, England), total CREB (1:2,000, Abcam), phosphorylated Csk (anti-phospho S364 1:200, Abcam), total Csk (1:200, Santa Cruz), phosphorylated Lck (anti-phospho Tyr505, 1:1,000 or anti-phospho Tyr394, 1:1,000, Cell Signaling), phosphorylated Akt (anti-phospho Ser473, 1:100, Cell Signaling), total Akt (1:500, Cell Signaling) and β-actin (1:500, Santa Cruz). The horseradish peroxidase-labeled polyclonals were used as secondary antibodies (Santa Cruz). The signals were detected by ECL (Amersham). Films were scanned and analyzed by ImageQuanT data analysis software (Molecular Dynamics) [17].

Flow cytometry

TILs #2009 were stimulated with anti-CD3 in the absence or presence of CADO (C; 50 μM) and PGE_2 (P; 0.5 μM) for 30 min. Unstimulated cells served as a control. Cells were fixed, permeabilized and stained with rabbit anti-phospho-Zap-70 (Tyr493) and then with the secondary anti-rabbit IgG-PE (Santa Cruz). Cell fluorescent intensity was analyzed by flow cytometry.

Real-time PCR

RNA was isolated from human TILs #2009 using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was produced from 500 ng to 1 μg of RNA by using the RT2 First Strand kit (SABioscience, Frederick, MD, USA). Real-time PCR was carried out as described previously [17]. Primers were: EP2R-forward, 5′-caaacatttaagctgtggtcaagg-3′; EP2R-reverse, 5′-caaaaccacatactttgtcattcaa-3′; EP4R-forward, 5′-ccatgtcaggccactctcgc-3′; EP4R-reverse, 5′-ccagcctcatccaccagtaag-3′; A_{2A}R-forward, 5′-cttgggttctgaggaagcag-3′; A_{2A}R-reverse, 5′-cagcagctcctgaaccctag-3′; A_{2B}R-forward, 5′-gacacggctggttttcattg-3′; A_{2B}R-reverse, 5′-gctgttggcataatccacac-3′; EP1R-forward, 5′-accagatcctggaccttg-3′, EP1R-reverse, 5′-tagaagtggctgaggccg-3′; EP3R-forward, 5′-agcccacccccaaatataac-3′, EP3R-reverse, 5′-tgctccttgtcatgatgagtg-3′. Relative quantitative expression of those receptors was calculated by the comparative cycle cross-threshold method. Differences in gene expression were calculated using the $\Delta\Delta C_t$ method according to the manual from SA Bioscience.

Analysis of 3′, 5′-cAMP (cAMP)

cAMP levels were measured by high-performance liquid chromatography–tandem mass spectrometry using a triple quadrupole mass spectrometer (TSQ Quantum-Ultra, ThermoFisher Scientific, San Jose, CA, USA) operating in the selected reaction monitoring mode with a heated electrospray ionization source as previously described [17, 22].

Statistics

Statistical analyses were performed with *t* tests using the GraphPad PRISM software program. All data were expressed as mean ± SD. The significance level was set at $p < 0.05$. All experiments were repeated 2–5 times and representative data are presented.

Results

Inhibitory effects of PGE_2 and CADO on the cytotoxic activity of TILs

The cytotoxic activity of anti-melanoma-specific TILs #2009 was tested against T2 cells pulsed with MART-$1_{27–35}$ peptide in the absence or presence of PGE_2 or 2-chloroadenosine (CADO), a stable analog of adenosine that is resistant to metabolism by adenosine deaminase. PGE_2 or CADO substantially inhibited the cytotoxic activity of TILs, and in combination their inhibitory effects were more profound compared with each agent used individually (Fig. 1a). Treatment of target cells with CADO and/or PGE_2 did not increase ^{51}Cr release or affect their sensitivity to TIL-mediated cytotoxicity. CADO and PGE_2 at the concentrations employed did not reduce the viability of TILs.

CGS (selective A_{2A}R agonist) or butaprost (selective EP2R agonist) also inhibited the cytotoxic activity of TILs (Fig. 1a). In combination, CGS and butaprost showed substantially higher inhibitory effects than each agent used alone, indicating that this increase was mediated via amplification of A_{2A}R and EP2R inhibitory signaling. In contrast, the immunosuppressive factors TGF-β and IL-10 at 10, 30 and 100 (not shown) ng/ml and even at 300 ng/ml did not inhibit the cytolytic activity of TILs (Fig. 1a). These data indicate that adenosine and PGE_2 have a unique ability to inhibit the cytolytic function of TILs via A_{2A}R and EP2R signaling, respectively.

TILs #2009 were also highly cytotoxic against HLA $A2^+$ UPCI-MEL-136 melanoma cells, and in the presence of CGS (25–100 μM), the cytotoxic activity of TILs was inhibited in a concentration-dependent manner. In combination, CGS and butaprost showed a substantially higher inhibitory effect than each agent used separately (Fig. 1b).

PGE_2 and butaprost manifested more potent suppressive effects and were able to inhibit the cytotoxic activity of TILs at lower concentrations than CADO and CGS. These potency differences were likely due to differences in expression of EP2Rs and A_{2A}Rs. Because the density of adenosine and PGE_2 receptors was too low for detection by flow cytometry, we used real-time PCR to approximate the relative expression of these receptors. TILs expressed low levels of EP1R mRNA, whereas expression of EP2R mRNA

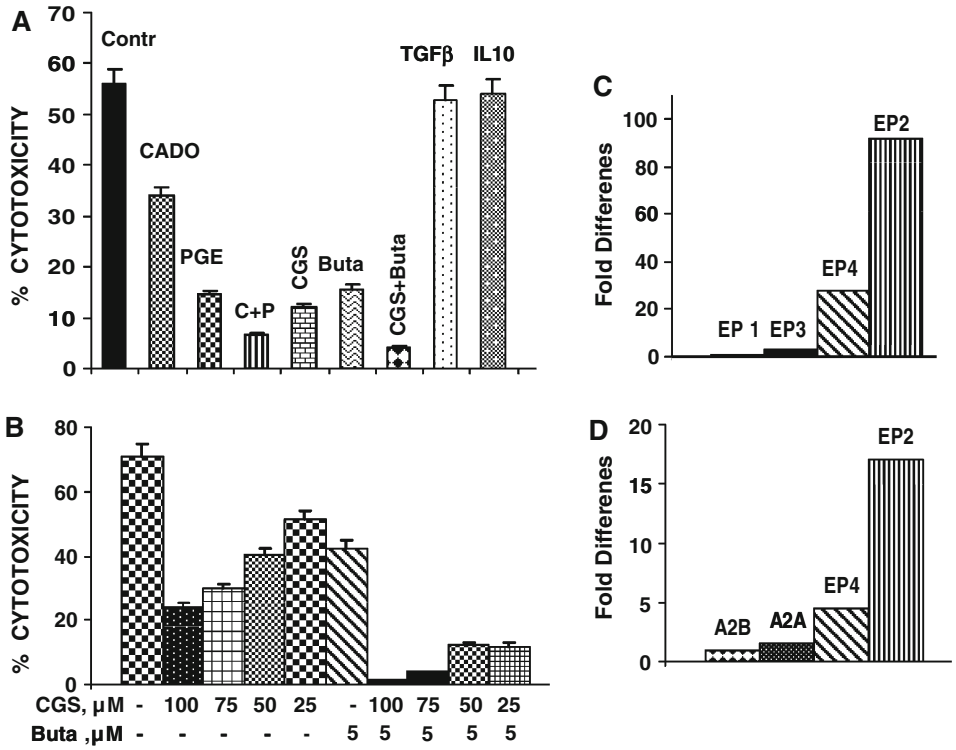

Fig. 1 Combined inhibitory effects of agonists on the cytotoxic activity of TILs. **a** TILs #2009 were mixed with CADO (50 μM), PGE_2 (50 nM), CGS (50 μM), butaprost (5 μM), TGF-β or IL-10 (300 ng/ml) and then ^{51}Cr-labeled T2 cells pulsed with MART-$1_{27–35}$ peptide were added at E:T ratio 5:1. After 4 h of incubation, the level of released radioactivity was determined and percent of cytotoxicity was calculated. Control group significantly ($p < 0.01$) differs from other groups, except TGF-β or IL-10 treated groups. **b** TILs #2009 were mixed with ^{51}Cr-labeled UPMCI-Mel-136 melanoma cells at E:T ratio 5:1 in the absence or presence of CGS (25–100 μM) and/or butaprost (5 μM) in a 16 h ^{51}Cr-release assay. Groups with combined CGS + butaprost treatment significantly ($p < 0.01$) differ from other groups. **c** Real-time PCR analysis of PGE_2 receptors expression in TILs #2009. **d** Real-time PCR analysis expression of A_{2A}, A_{2B} and EP2, EP4 receptors in TILs #2009

was 92-fold higher. EP2R mRNA expression was 3.3-fold higher than EP4R mRNA expression (Fig. 1c). TILs did not express A_1R or A_3R mRNA, expressed very low levels of A_{2B}R mRNA and expressed slightly higher levels of A_{2A}R mRNA. EP2R mRNA expression in TILs was 11.7-fold higher than A_{2A}R mRNA expression (Fig. 1d). Low receptor density requires higher concentrations of ligands to trigger signaling. Indeed, in T cells of mice with only one A_{2A}R allele, concentrations of ligand must be substantially increased to provide sufficient signaling and induce cAMP production [23].

Tumor-infiltrating lymphocytes from different melanoma patients showed wide-ranging cytotoxic activity that varied from 20 to 90%. TILs also differed in their sensitivity to the inhibitory effects of CADO and PGE_2. Some TILs were highly sensitive, whereas others showed only slight, if any, changes in their cytotoxicity in the presence of CADO and PGE_2. We initially attributed these differences to the properties of individual TIL cell lines. Later, it became apparent that the same TILs tested at different time periods in culture varied in their cytotoxic activity and sensitivity to CADO and PGE_2 that could be attributed to differences in their stage of differentiation during their ex vivo expansion. In total, we analyzed the cytotoxic activity of nine individual TIL lines. Five TIL lines had limited proliferative ability, and consequently supplies of these were exhausted during testing. Four TIL lines (#2009, #2044, #3072 and #2113) were highly proliferative and their cytotoxic activity and sensitivity to CGS and butaprost were analyzed at different time periods in culture (Fig. 2). Initially, these TIL lines showed high cytotoxic activity and resistance to the inhibitory effects of CGS or butaprost, even in combination (Fig. 2, stage I). After 3–4 weeks in culture, TILs exhibited reduced proliferation, yet still were highly cytotoxic but became sensitive to CGS and butaprost, that in combination manifested a remarkable inhibitory effect (Fig. 2, stage II). After an additional 1–2 weeks in culture, TILs showed a substantial reduction in their proliferation and cytotoxic activity but maintained high sensitivity to CGS and butaprost (Fig. 2, stage III).

We considered that these changes represented three distinct stages of TIL differentiation and classified TILs into stages I and II when their cytotoxic activity was above 50% (at an E:T ratio 5:1) and below 50% at stage III. TILs at

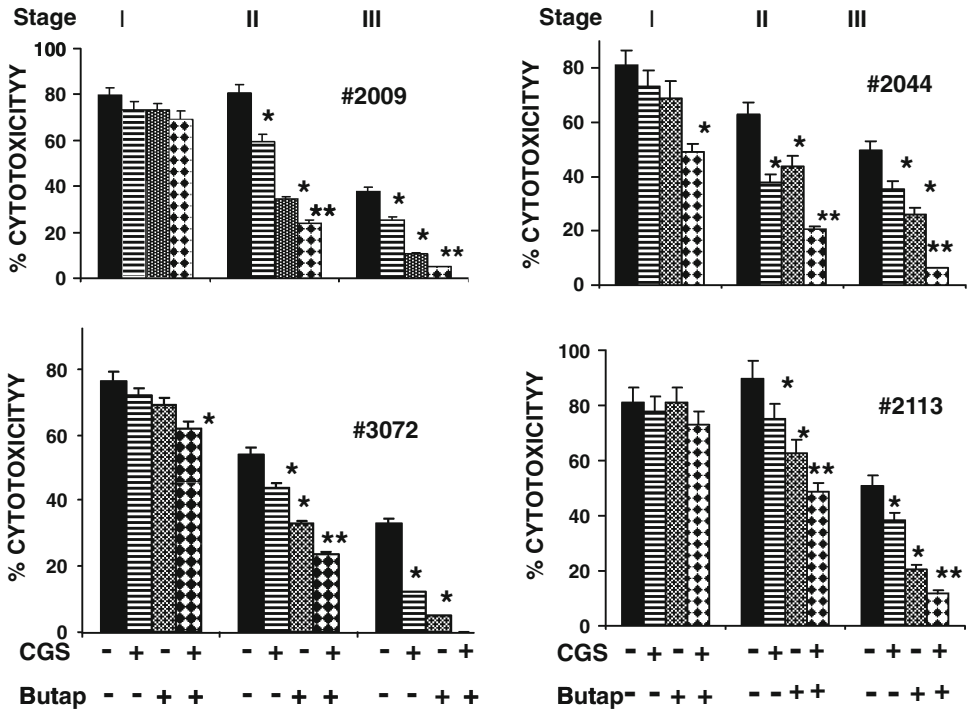

Fig. 2 Stage-dependent cytotoxic activity of TILs and their sensitivity to the suppressive effects of CGS and butaprost**.** The cytotoxic activities of four TIL lines (#2009, #2133, #2044, and #3072) were tested against T2 cells pulsed with MART-$1_{27–35}$ peptide in the presence of CGS (50 μM) and/or butaprost (5 μM) at 2–4 weeks intervals during TILs culture with IL-2. *Significantly ($p < 0.01$) different from control group. **Significantly ($p < 0.01$) different from other groups

stages II and III were distinguished from stage I by their sensitivity to suppression by EP2R and $A_{2A}R$ agonists.

Desensitization of A_{2A}Rs and EP2Rs and blockade of the suppressive effects of EP2R and $A_{2A}R$ agonists

Our data indicate that in vitro expansion of TILs increases their sensitivity to the suppressive effects of adenosine and PGE_2 and decreases their cytotoxic activity, which could reduce their efficacy as adoptively transferred TILs in a tumor environment enriched with adenosine and PGE_2. Therefore, it is important to develop an approach that could preserve or restore TIL cytotoxic activity and maintain their resistance to adenosine and PGE_2. Based on the findings that ligand binding to A_{2A}Rs and EP2Rs causes receptor desensitization and internalization [24–26], we hypothesized that desensitization of A_{2A}Rs and EP2Rs could block the suppressive effects of PGE_2 and adenosine on the cytotoxic activity of TILs. To test this possibility, TILs #2679 or TILs #2009 were pretreated with CADO (5 μM) and/or PGE_2 (5–25 nM) at concentrations that did not suppress TIL cytotoxicity. After overnight incubation, TILs were washed and the ability of CGS or butaprost to affect the cytotoxic activity of non-pretreated or pretreated TILs was tested. CGS at 50 μM and butaprost at 5 μM substantially inhibited the cytotoxic activity of control, non-pretreated TILs #2679, and in combination they had higher suppressive effects and reduced the cytotoxicity from 59 to 18.4% (Table 1). Importantly, pretreatment with CADO or PGE_2 substantially increased the cytotoxic activity of TILs #2679 from 59 to 78.6%. Moreover, pretreated TILs #2679 became resistant to the inhibitory effects of CGS and butaprost. In combination, CGS and butaprost only slightly inhibited TIL cytotoxic activity and actually their residual cytotoxicity was almost threefold higher than non-pretreated control TILs #2679 (Table 1). Interestingly, TILs #2679 pretreated with PGE_2 were resistant to inhibitory signaling by both butaprost, an agonist of EP2Rs, as well as by CGS, an agonist of A_{2A}Rs. Likewise, pretreatment with CADO rendered TILs resistant to CGS as well as butaprost (Table 1).

Retesting of TILs #2679 after 5 days in culture with IL-2 showed that their cytotoxic activity was reduced by twofold, signifying that TILs #2679 had entered stage III. They retained high sensitivity to the suppressive effects of CADO and PGE_2 that in combination almost completely inhibited TIL cytotoxicity (Table 1). Pretreatment with CADO or PGE_2 increased cytotoxic activity of TILs #2679 at stage III and induced cross-resistance to the suppressive effects of butaprost and CGS (Table 1).

The experiments were repeated with TILs #2009 and similar results were obtained. TILs #2009 were highly cytotoxic but their activity was substantially reduced by CGS and butaprost, and in combination they reduced TIL cytotoxicity from 80.9 to 24.0%, indicating that these TILs were at stage II (Table 1). After 10 days in culture, their cytotoxicity was reduced from 80.9 to 34% and they were highly sensitive to the inhibitory effects of CGS and butaprost (Table 1). Pretreatment of TILs #2009 at stage II or III with CADO or PGE_2 increased their cytotoxic activity

Table 1 Effects of CADO or PGE_2 pretreatment on TIL cytotoxic activity and sensitivity to the suppressive effects of A_{2A} and EP2 receptor signaling

TILs #	Pretreatment[a]	% Cytotoxicity			
		Treatments			
		None	CGS (50 μM)	Butaprost (5 μM)	CGS + Butaprost
TILs #2679, Stage II	None	59.0 ± 4.9*	37.1 ± 4.2*	25.7 ± 3.8*	18.4 ± 3.2*
	PGE_2 (25 nM)	78.6 ± 4.0	74.1 ± 4.1	73.7 ± 3.2	69.3 ± 6.7
	CADO (5 μM)	78.0 ± 6.7	68.1 ± 6.3	65.1 ± 1.6	45.5 ± 5.0
	CADO + PGE_2	68.9 ± 5.2	53.8 ± 4.0	57.9 ± 3.9	34.5 ± 5.2
TILs #2679, Stage III	None	28.9 ± 3.6*	10.7 ± 0.8*	12.6 ± 0.9*	4.3 ± 0.9*
	PGE_2 (5 nM)	49.3 ± 8.3	19.9 ± 5.4	28.0 ± 9.4	19.0 ± 5.1
	CADO (5 μM)	34.5 ± 7.5	16.7 ± 0.9	17.0 ± 0.6	8.8 ± 0.4
	CADO + PGE_2	45.7 ± 10.2	27.8 ± 3.6	22.6 ± 2.4	18.9 ± 3.1
	Butaprost (1 μM)	46.2 ± 4.2	34.7 ± 3.6	26.6 ± 9.8	16.3 ± 8.7
TILs #2009, Stage II	None	80.9 ± 7.0*	59.6 ± 6.4*	34.3 ± 8.4*	24.0 ± 7.3*
	PGE_2 (25 nM)	90.0 ± 6.6	86.2 ± 7.0	75.5 ± 1.9	69.9 ± 3.3
	CADO (5 μM)	85.1 ± 5.9	84.5 ± 1.1	82.8 ± 5.6	76.6 ± 0.9
	CADO + PGE_2	86.4 ± 5.9	78.5 ± 4.4	70.2 ± 1.3	67.3 ± 4.2
TILs #2009, Stage III	None	34.0 ± 2.8*	25.4 ± 3.2*	8.5 ± 7.2*	5.0 ± 4.4*
	PGE_2 (5 nM)	61.4 ± 3.9	47.2 ± 2.9	35.7 ± 5.3	23.1 ± 0.9
	CADO (5 μM)	44.5 ± 4.7	33.7 ± 5.5	17.9 ± 1.9	10.2 ± 3.0
	CGS (5 μM)	44.9 ± 3.0	41.7 ± 1.4	27.2 ± 4.2	18.6 ± 9.4
	Butaprost (2 μM)	53.2 ± 0.9	51.1 ± 2.2	37.6 ± 2.0	21.5 ± 6.5

[a] TILs #2679 or #2009 were pretreated overnight with CADO and/or PGE_2 or butaprost at the indicated concentrations. After washing, the cytotoxic activity of pretreated TILs was tested in the 4-h ^{51}Cr release assay against ^{51}Cr-labeled T2 cells pulsed with MART-$1_{27–35}$ peptide at E:T ratio 5:1 in the presence of CGS (50 μM) and/or butaprost (5 μM). The experiments with TILs #2679 and TILs #2009 were retested after 5 and 10 days of culture with IL-2, respectively

* Significantly ($p < 0.05$) different from other groups

and their resistance to the inhibitory effects of CGS and butaprost. However, pretreatment of TILs with a combination of CADO and PGE_2 was not superior when compared with each modality used individually (Table 1). These effects were likely mediated via cAMP-elevating receptors because pretreatment of TILs with a specific agonist of EP2Rs, butaprost (1–2 μM), had the same effects as pretreatment with PGE_2. Pretreatment of TILs with CGS (5–10 μM) had similar but less efficient effects than butaprost. In general, PGE_2 was more efficient than CADO with regard to stimulating TIL cytotoxicity and inducing resistance (Table 1).

Mechanism of resistance and sensitivity to the suppressive effects of EP2R and $A_{2A}R$ agonists

Tumor-infiltrating lymphocytes resistance to the inhibitory effects of EP2R and $A_{2A}R$ agonists at stage I could be due to inefficient receptor signaling to produce cAMP or to high activity of phosphodiesterases (PDEs) that hydrolyze cAMP. In this regard, PDE4 is a major PDE form in T lymphocytes [27]. Therefore, if PDE4, by metabolizing cAMP induced by EP2R and $A_{2A}R$ agonists, restricts their inhibitory effects, rolipram, a specific inhibitor of PDE4, should increase intracellular concentrations of cAMP and inhibit the cytotoxic activity of TILs. In contrast to this prediction, rolipram alone did not inhibit the cytotoxic activity of TILs, and in combination with CADO and/or PGE_2, rolipram only marginal inhibited cytotoxic activity of TILs (Fig. 3a). These findings suggest that resistance of TILs is not solely based on high activity of PDE4.

Next, we tested whether high concentrations of the $A_{2A}R$ agonist CGS could overcome the resistance of TILs. CGS, even at 100 μM, failed to inhibit TIL cytotoxicity. When CGS was used in combination with butaprost (5 μM) a slight but significant ($p < 0.05$) inhibition of TIL cytotoxicity was observed (Fig. 3b). Similar levels of inhibition were found when either CGS or butaprost were used in combination with rolipram, an inhibitor of PDE4. Further inhibition of TIL cytotoxicity was achieved only when all three agents were combined (Fig. 3b).

The resistance of TILs to EP2 and A_{2A} agonists could be due to insufficiency of receptor-mediated signaling and activation of adenylyl cyclase. If so, receptor-independent

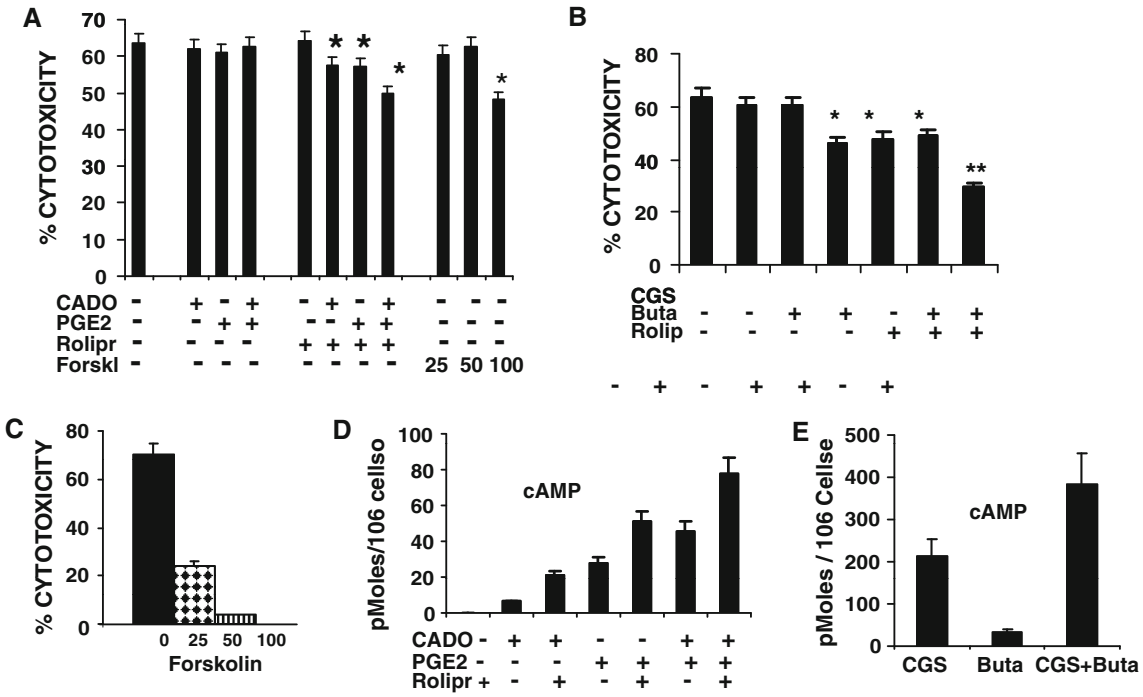

Fig. 3 Analysis of signaling and cAMP production by TILs at stage I. **a** The cytotoxic activity of TILs #2009 at stage I was tested against T2 cells pulsed with MART-$1_{27–35}$ peptide in the presence of CADO (50 μM), PGE_2 (50 nM), PDE4 inhibitor rolipram (rolipr; 3 μM) or cAMP inducer forskolin (forskl; 25–100 μM). *Significantly ($p < 0.05$) different from control group. **b** Effect of CGS (100 μM), butaprost (Buta; 5 μM) and/or rolipram (3 μM) on the cytotoxic activity of TILs #2009 against T2 cells pulsed with MART-$1_{27–35}$ peptide. *Significantly ($p < 0.05$) different from control group. **c** The cytotoxic activity of TILs #2009 at stage II was tested against T2 cells pulsed with MART-$1_{27–35}$ peptide in the presence of forskolin (25–100 μM) on the cytotoxic activity of stage II TILs #2009 at E:T ratio 5:1. Inhibitory effect of forskolin was significant ($p < 0.01$). **d**, **e** Analysis of cAMP production. TILs #2009 at stage I were incubated for 10 min with CADO (50 μM), PGE_2 (50 nM) and/or, rolipram (3 μM) (**d**) or with CGS (100 μM), butaprost (5 μM) and/or rolipram (3 μM) (**e**), and the levels of cAMP in cell extracts were analyzed using mass spectrometry. Combined treatment significantly ($p < 0.01$) differs from a single agent treatment

direct stimulation of adenylyl cyclase by forskolin should cause a substantial inhibition of TIL cytotoxicity. In contrast to this expectation, forskolin at 25 and 50 μM failed to inhibit TIL cytotoxic activity. Even a very high concentration (100 μM) forskolin caused only a slight inhibition (Fig. 3a). In contrast, when TILs #2009 were tested at stage II they showed high sensitivity to the inhibitory effects of forskolin. In this case, foskolin at 25 and 50 μM reduced their cytotoxicity from 70.9 to 24.4% and 4.1%, respectively. Moreover, 100 μM of forskolin completely inhibited TIL cytotoxicity at stage II (Fig. 3c).

It is possible that adenylyl cyclase of TILs at stage I fails to respond to $A_{2A}R$ and EP2R signaling and to produce cAMP. However, TILs #2009 at stage I treated with CADO or PGE_2 were able to produce cAMP that was higher when combined treatment was applied (Fig. 3d). The PDE4 inhibitor rolipram further increased levels of cAMP induced by CADO and/or PGE_2 (Fig. 3d). Although CGS at 100 μM failed to inhibit TIL cytotoxic activity (Fig. 3b), this same concentration of CGS induced very high levels of cAMP in the same TILs. cAMP production further increased when CGS was used in combination with butaprost (Fig. 3e).

Similarly, cAMP production was observed when TILs #2009 at stage II were treated with CADO or PGE_2 as well as CGS or butaprost, and in combination they induced much higher levels of cAMP (Fig. 4a). In general, no differences in cAMP induction were observed between TILs at stages I and II, but TILs at stage II were sensitive to the immunosuppressive effects of the agonists. These data support the concept that the resistance of stage I TILs to adenosine and PGE_2 is not due to inefficiencies in EP2R or $A_{2A}R$ signaling and cAMP production or cAMP metabolism by PDE4 but rather to changes in signaling downstream of cAMP.

We next analyzed downstream mechanisms of cAMP signaling following treatment of stage II TILs with CADO or PGE_2. Because cAMP activates PKA resulting in phosphorylation of CREB [14], we examined whether treatment of TILs #2009 at stage II with CADO or PGE_2 triggers PKA activation and phosphorylation of CREB. Stimulation of TILs with anti-CD3 caused CREB phosphorylation, and

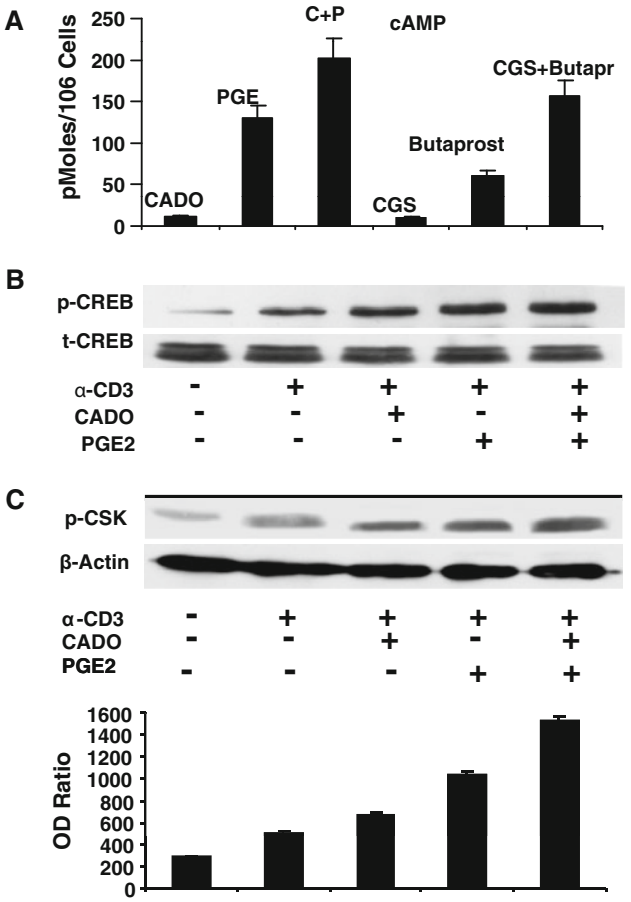

Fig. 4 Effect of CADO and PGE_2 on cAMP production (**a**) and CREB (**b**) and Csk (**c**) phosphorylation. **a** TILs #2009 at stage II were incubated with CADO (50 μM) and/or PGE_2 (0.5 μM) or CGS (50 μM) and/or butaprost (5 μM) for 10 min. The levels of cAMP in cellular extracts were analyzed using mass spectrometry. **b**, **c** TILs #2009 at stage II were stimulated with anti-CD3 in the presence of CADO (50 μM) and/or PGE_2 (0.5 μM) for 30 min. TIL protein extracts (50 μg) were resolved using 10% SDS-PAGE and transferred to PVDF membranes. The level of CREB (**b**) and Csk (**c**) phosphorylation was analyzed using specific antibodies. The optic density (OD) ratio of the bands was analyzed using ImageQuanT data analysis software (Molecular Dynamics)

CADO or PGE_2 further increased the levels of CREB phosphorylation, and in combination they induced higher levels of CREB phosphorylation than each modality used alone (Fig. 4b). These results demonstrate that the increased immunosuppressive effects of adenosine and PGE_2 in combination are associated with amplification of PKA activation.

Because Csk is a downstream target of PKAI and is a physiological inhibitor of TCRs [28–32], it is possible that CADO or PGE_2 induce Csk phosphorylation in TILs. Indeed, our western blot analysis showed that CADO or PGE_2-induced phosphorylation of Csk that was more profound when CADO and PGE_2 were used in combination (Fig. 4c).

Motivated by the fact that Csk regulates TCR signaling by inhibiting Lck [28–32], we next analyzed Lck phosphorylation in TILs treated with CADO or PGE_2. Lck can be phosphorylated on Y394 and Y505, leading to activation and inhibition of Lck, respectively [33]. In TILs, Lck was consistently phosphorylated on Y505. Treatment with CADO or PGE_2 further increased phosphorylation of the Lck Y505 inhibitory site, and this was more profound when combined treatment was applied (Fig. 5a). Stimulation of TILs with anti-CD3-induced phosphorylation of Lck Y394 that was inhibited by CADO or PGE_2 (Fig. 5a). Thus, CADO and PGE_2 increased Lck phosphorylation on inhibitory Y505 site, yet inhibited phosphorylation of the stimulatory Y394 site.

Using flow cytometric analysis, we found that anti-CD3 stimulation induced phosphorylation of ZAP-70, and this response was inhibited by CADO and PGE_2 (Fig. 5b). Because phosphorylation and activation of AKT is an important step in TCR-mediated activation and the cytotoxic activity of CTLs [34, 35], it is conceivable that CADO and PGE_2 inhibit phosphorylation of Akt. In this regard, we observed that stimulation of TILs with anti-CD3-induced Akt phosphorylation that was substantially inhibited by CADO or PGE_2 (Fig. 5c). Thus, it appears that the immunosuppressive effects of CADO and PGE_2 in TILs are mediated via A_{2A}Rs and EP2Rs, which trigger a common pathway: elevated cAMP → activation of PKA → activation of Csk → inhibition of Lck → inhibition of ZAP-70 and TCR signaling.

Discussion

Although several studies clearly document the ability of PGE_2 to suppress inflammatory cytokine production by various immune cells [10, 12, 13, 21, 22], our study for the first time demonstrates that PGE_2 inhibits the cytolytic activity of human tumor-specific CTLs. CADO, a stable analog of adenosine has similar effects, whereas other tested immunosuppressive factors, such as TGF-β and IL-10, fail to directly inhibit the cytolytic function of TILs. In combination, CADO and PGE_2 have additive suppressive effects.

Although the ability of PGE_2 to inhibit the cytolytic function of CTLs was not demonstrated the inhibitory effect of adenosine was extensively investigated showing that adenosine attenuates the cytotoxic activity of allospecific murine CTLs via inhibiting the exocytosis of cytotoxic granules and by reducing FasL expression [13, 36, 37]. Our analysis of the mechanisms of immunosuppressive signaling indicates that adenosine and PGE_2 by binding to A_{2A}Rs and EP2Rs trigger cAMP production, leading to activation of PKA and CREB phosphorylation that is higher when

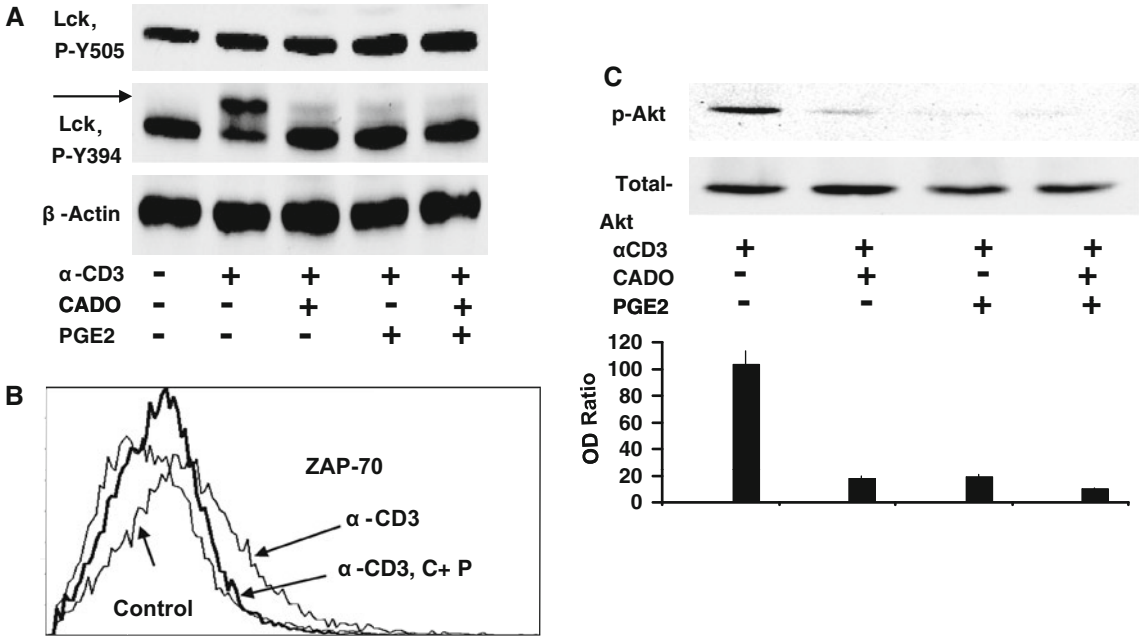

Fig. 5 Effects of CADO and PGE_2 on Lck, ZAP-70 and Akt phosphorylation. **a** TILs #2009 at stage II were stimulated with anti-CD3 in the presence of CADO (50 μM) and/or PGE_2 (0.5 μM) for 30 min. Western blot analysis of Lck phosphorylation was performed using anti-phospho Tyr505 or Tyr394 antibodies. **b** To analyze Zap-70 phosphorylation, stage II TILs #2009 were stimulated with anti-CD3 Ab. Some TILs were stimulated in the presence of CADO (C 50 μM) and PGE_2 (P 0.5 μM) (group αCD3, C + P). Control group: unstimulated TILs. After 30 min of stimulation, all cells were fixed, permeabilized and stained with rabbit anti-phospho-Zap-70 (Tyr493) and then with the secondary anti-rabbit IgG-PE and cell fluorescent intensity was analyzed by flow cytometry. **c** TILs #2009 were stimulated with anti-CD3 in the presence of CADO (50 μM) and/or PGE_2 (0.5 μM) for 30 min. Western blot analysis of Akt phosphorylation was performed using anti-phosphorylated-Akt (Ser473) or anti-total Akt antibody. In non-stimulated TILs, Akt was not phosphorylated (not shown)

these agents are used in combination. Our previous studies show that activation of PKA type I (PKAI), but not of PKA type II, mimics the inhibitory effect of CADO on cytotoxic activity and cytokine production by human melanoma-specific CTLs. The importance of PKAI activation in the inhibitory signaling is further supported by finding that Rp-8-BrAMPS, a specific inhibitor of PKA type I, blocks this inhibitory effect [22].

PKAI phosphorylates and activates Csk, a well-known physiological inhibitor of TCR signaling [29]. Csk is anchored by Cbp/PAG to lipid rafts and constitutively inhibits Lck and Fyn. Activation of TCR leads to dissociation of Csk from Cbp/PAG and releases Csk from lipid rafts thus allowing TCR signaling [28–32, 38, 39]. Our current studies show that treatment of human TILs with CADO or PGE_2 results in Csk phosporylation that is more profound when these agents are applied in combination. Csk phosphorylation is associated with inhibition of Lck, ZAP-70 and Akt phosphorylation. These results support the hypothesis that adenosine and PGE_2 trigger a common pathway involving PKA activation followed by Csk activation leading to inhibition TCR signaling. Combined CADO and PGE_2 treatment of TILs results in the amplification of this inhibitory pathway signaling and increases its immunosuppressive effects.

Adoptive immunotherapy necessitates isolation of small numbers of immune T lymphocytes from resected melanomas and their expansion to approximately 10^{11} cells, which requires an extended period of in vitro simulation with IL-2. Our studies demonstrate that prolonged TIL stimulation with IL-2 exhausts their ability to proliferate, renders them sensitive to the inhibitory effects of PGE_2 and adenosine and reduces their cytolytic activity. Based on these changes we identify three major transitional stages of TIL differentiation during their ex vivo expansion. Previous studies characterize stages of TIL differentiation during their culture with IL-2 based on their ability to proliferate and express PD-1, CD27 and its ligand CD70 [3, 40, 41]. However, we do not find an association between changes in CD27/CD70 expression and TIL resistance/sensitivity to CADO and PGE_2 (data not shown).

Tumors contain elevated levels of PGE_2 and adenosine [18–20]. However, precise intratumor concentrations of adenosine are difficult to determine due to adenosine's rapid hydrolysis by adenosine deaminase. An important issue is whether in vivo concentrations of adenosine are sufficiently high to exert suppressive effects. Findings that pharmacological blockade of $A_{2A}Rs$ and genetic knockout of $A_{2A}Rs$ substantially increase the efficacy of antitumor T cell-mediated immunity in mice indicates that in vivo con-

centrations of adenosine are sufficiently immunosuppressive [20].

Taking into consideration the elevated intratumor levels of adenosine and PGE_2 and their highly suppressive activity, it is of great importance to develop an approach to increase resistance of TILs to the suppressive effects of these factors. The immunosuppressive effects of CADO and PGE_2 can be blocked by specific antagonists of A_{2A}Rs and EP2Rs [11, 17, 42] or by blocking PKAI activation with a specific inhibitor Rp-8-BrAMPS [17, 22, 38]. Also, treatment of murine allospecific CTLs with NECA, an agonist of A_{2A}Rs, for 2 days renders them resistant to the suppressive effects of NECA that was attributed to selection of CTL variants with lower levels of A_{2A}Rs [43].

We find that overnight pretreatment of TILs at stages II and III with non-suppressive concentrations of CADO or PGE_2 increases TIL cytotoxic activity and their resistance to inhibitory signaling mediated via A_{2A}Rs and EP2Rs. These agents induce cross-resistance such that pretreatment with PGE_2 induces TIL resistance to inhibition by agonists of either EP2Rs (butaprost) or A_{2A}Rs (CGS). Similar effects are induced by pretreatment with CADO. These effects are probably mediated via desensitization and internalization of the inhibitory receptors. Due to the low density of adenosine and PGE2 receptors and to the lack of appropriate antibodies that recognize ligand bound and unbound receptors, flow cytometric analysis or immunofluorescence microscopy is not applicable for testing expression and internalization of these receptors. However, the mechanisms of desensitization and internalization of GPCRs, including adenosine and PGE_2 receptors, can be investigated using fibroblast or malignant cells overexpressing these receptors following transfection with a plasmid encoding the HA-epitope tagged receptors. Indeed, internalization of receptors was then determined by loss of cell-surface immunoreactivity of HA-tagged receptors [24–26]. Such studies show that phosphorylation of serine and/or threonine residues in the third intracellular loop and C terminus of receptors leads to homologous or heterologous desensitization. Homologous desensitization is specific for agonist-occupied receptors that are phosphorylated by one of the GPCR kinases (GRKs 1–7). Heterologous desensitization is induced by cAMP activated PKA leading to phosphorylation of receptors that are not necessarily occupied by ligand. Phosphorylated receptors bind β-arrestin that inhibits GPCR coupling and targets receptors to clathrin-coated pits for internalization [24–26]. Thus, it is likely that the increased resistance of TILs to the immunosuppressive effects of CADO and PGE_2 is due to internalization and desensitization of A_{2A}Rs and EP2Rs. Cross-resistance induced by TIL pretreatment with PGE_2 or CADO is likely based on their ability to activate PKA that leads to phosphorylation of A_{2A}Rs and EP2Rs and their internalization independent of the ligand binding.

It is of interest that pretreatment with CADO and PGE_2 stimulates TIL cytotoxicity, but the mechanisms of this stimulation are not clear. It is conceivable that pretreatment abolishes some controlling mechanisms. We consider that this pretreatment affects some mechanisms controlling CTL activity. Accumulated evidence indicates that Csk is a physiological inhibitor of TCR signaling. It is possible that pretreatment of TILs with low concentrations of PGE2 or CADO provides low level of signaling triggering dissociation of Csk from Cbp/PAG anchoring complexes, releasing Csk from lipid rafts and liberating its inhibitory effect on TCR and thus augmenting TIL cytotoxic activity.

Recent success in adoptive therapy of advanced melanoma is attributed to better understanding of conditions that provide prolonged survival of CTLs in the circulation [1, 2, 44]. Even so, transfused CTLs appear to lose their activity in the tumor microenvironment [3–7]. It is possible that this phenomenon is due to the presence of high intratumor concentrations of adenosine and PGE_2. Thus, using TILs that have high cytotoxic activity and resistance to the suppressive effects of adenosine and PGE_2 could be important for therapeutic efficacy. Based on our finding that desensitization of A_{2A}R and EP2R increases TIL cytotoxic activity and their resistance to A_{2A}R and EP2R agonists, pretreatment of TILs with low concentrations of PGE_2 or butaprost before their adoptive transfer may be beneficial and help to maintain therapeutic efficacy of TILs even in the presence of high intratumor concentrations of adenosine and PGE_2.

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Conflict of interest No potential conflicts of interest were disclosed.

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