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Adenosine Inhibits Activation-Induced T Cell Expression of CD2 and CD28 Co-Stimulatory Molecules: Role of Interleukin-2 and Cyclic AMP Signaling Pathways

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Abstract Adenosine is an immunosuppressive molecule that is associated with the microenvironment of solid tumors. Mouse T cells activated with anti-CD3 antibody in the presence of adenosine with or without coformycin (to prevent adenosine breakdown by adenosine deaminase) exhibited decreased tyrosine phosphorylation of some intracellular proteins and were inhibited in their ability to proliferate and synthesize interleukin (IL)-2. In addition, adenosine interfered with activation-induced expression of the co-stimulatory molecules CD2 and CD28. Activation-induced CD2 and CD28 expression was also diminished when T cells were activated in the presence of anti-IL-2 and anti-CD25 antibodies to neutralize IL-2 bioactivity. Collectively, these data suggest that CD2 and CD28 up-regulation following T cell activation is IL-2-dependent; and that adenosine inhibits activation-induced T cell expression of CD2 and CD28 by interfering with IL-2-dependent signaling. The inhibitory effect of adenosine on activation-induced CD2 and CD28 expression could not be attributed to cyclic AMP (cAMP) accumulation resulting from the stimulation of adenylyl cyclase-coupled adenosine receptors, even though cAMP at concentrations much higher than those generated following adenosine stimulation was inhibitory for both CD2 and CD28 expression. We conclude that adenosine interferes with IL-2-dependent T cell expression of co-stimulatory molecules via a mechanism that does not involve the accumulation of intracellular cAMP. J. Cell. Biochem. 89: 975–991, 2003.

Key words: adenosine; T lymphocytes; co-stimulation; interleukin-2; cyclic AMP

T cell activation requires independent biochemical signals originating from both the T cell receptor/CD3 complex and at least one of several co-stimulatory receptors [Croft and Dubey, 1997]. When combined with antigen-driven T cell receptor signaling, co-stimulation promotes high-level interleukin-2 (IL-2) production and provides a critical survival signal for T lymphocytes [Watts and DeBenedette, 1999]. Recent studies with CD2/CD28 double knock out mice have revealed that both CD2 and CD28 present on the surface of T cells act in concert to achieve optimal co-stimulation of T lymphocytes during interactions with antigen presenting cells [Green et al., 2000]. CD2 is a transmembrane glycoprotein that functions as both an adhesion molecule and a co-stimulatory molecule [Moingeon et al., 1989]. The major ligand of CD2 in the mouse is CD48, which is expressed predominantly by cells of the hematopoietic lineage [Kato et al., 1992]. CD28 is a disulfide-linked homodimeric glycoprotein that is generally believed to function as the principal

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co-stimulatory receptor for T lymphocyte activation [Lenschow et al., 1996]. The ligands for CD28 are CD80 (B7-1) and CD86 (B7-2), both of which are found on B cells, T cells, macrophages, and dendritic cells [Hathcock et al., 1994]. Although CD2 and CD28 are constitutively expressed on naive T cells, surface expression of both co-stimulatory molecules increases dramatically following T cell activation [Turka et al., 1990; Alberola-Ila et al., 1991]. It is noteworthy that oligonucleotide-mediated inhibition of activation-induced CD28 expression results in a state of T lymphocyte hyporesponsiveness, indicating that activation-induced expression of co-stimulatory molecules such as CD28 is required for efficient T cell activation [Tam et al., 1997].

Tumor cells elaborate various immunosuppressive molecules that have been implicated in the defective development and function of tumor-reactive T lymphocytes [Whiteside and Parmiani, 1994; Chouaib et al., 1997]. One important mediator of tumor-associated immunosuppression is adenosine, an endogenous purine nucleoside found at high concentrations (10^{-6}–10^{-5} M) in the extracellular fluid of solid tumors [Blay et al., 1997]. We and others have shown that low micromolar concentrations of adenosine have a potent inhibitory effect on various aspects of T cell function, including antigen-driven proliferation and cytotoxic T lymphocyte development, killer cell adhesion to tumor target cells, and granule exocytosis involved in cell-mediated cytotoxicity [MacKenzie et al., 1994; Koshiba et al., 1997; Williams et al., 1997; Hoskin et al., 2002; MacKenzie et al., 2002]. Adenosine mediates its effects through four distinct subtypes of G protein-coupled cell surface receptors designated A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R [Ralevic and Burnstock, 1998], as well as through an intracellular P-site [Marone et al., 1990]. We have recently demonstrated that mouse T lymphocytes express $A_{2A}R$, $A_{2B}R$, and A_3R subtypes but not the A_1R subtype [Hoskin et al., 2002]. The interaction of adenosine with adenylyl cyclase-coupled $A_{2A}R$ and/or $A_{2B}R$ has been suggested to inhibit T cell activation by bringing about a sustained increase in intracellular levels of the second messenger cyclic AMP (cAMP) [Huang et al., 1997; Koshiba et al., 1997; Mirabet et al., 1999]. Adenosine acting through A_3R also suppresses T cell-mediated immune responses, although the biochemical basis for this effect has not yet been elucidated [MacKenzie et al., 1994; Hoskin et al., 2002].

A recent report that the tumor microenvironment can suppress the function of tumor-infiltrating T cells specific for the Tag oncoprotein, despite abundant Tag and CD80 (a ligand for CD28) expression by the tumor cells [Ganss and Hanahan, 1998], prompted us to explore the effect of adenosine on the expression of co-stimulatory molecules by activated and resting T lymphocytes. Given the important role that activation-induced expression of CD28 plays in T cell activation [Tam et al., 1997], we hypothesized that adenosine, at concentrations typically present within the microenvironment of solid tumors, might interfere with the activation-induced expression of CD28, as well as additional T cell co-stimulatory molecules such as CD2. In this paper, we demonstrate that activation-induced expression of CD2 and CD28 is IL-2-dependent; and that adenosine inhibits activation-induced expression of CD2 and CD28 by interfering with IL-2 synthesis and utilization through a mechanism that is unrelated to adenosine-induced accumulation of intracellular cAMP.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice were purchased from Charles River Canada (Lasalle, PQ) and housed in the Carleton Animal Care Facility at Dalhousie University. Mice were maintained on standard laboratory chow and water supplied ad libitum. Mice were routinely used in experiments at 8–12 weeks of age.

Antibodies and Reagents

RPMI 1640 medium was supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, (all ICN Biomedicals Canada, Ltd., Mississauga, Ont.), 5 mM HEPES buffer (pH 7.4; Sigma-Aldrich, Oakville, Ont.); and 5% fetal calf serum (FCS; heat-inactivated at 56°C for 30 min; Invitrogen Life Technologies, Burlington, Ont.). Hybridoma clone 145-2C11, kindly provided by Dr. J. Bluestone (University of Chicago, IL) was the source of hamster anti-mouse CD3 mAb used in our studies. Hamster IgG, goat anti-hamster $F(ab')_2$ IgG, hamster anti-mouse CD28 mAb, rat IgG, rat anti-mouse CD25 mAb, and fluorescein isothiocyanate (FITC)-conjugated goat

anti-hamster IgG were purchased from Cedarlane Laboratories (Hornby, Ont.). Recombinant mouse IL-2 and rat anti-mouse IL-2 neutralizing mAb were obtained from Genzyme Diagnostics (Cambridge, MA). Rat anti-mouse CD2 mAb was from BD Pharmingen (Mississauga, Ont.) while FITC-conjugated mouse anti-rat F(ab′)2 IgG was from Jackson ImmunoResearch (West Grove, PA). Anti-asialoGM1 rabbit polyclonal antiserum was purchased from Wako Chemicals (Richmond, VA). Anti-phosphotyrosine mAb (clone 4-G10) was from Upstate Biotechnology (Lake Placid, NY) and horse radish peroxidase-conjugated anti-mouse IgG was from Bio-Rad (Hercules, CA). Adenosine, 8-Br-cAMP, CGS 21680, dilazep, dimethyl sulfoxide (DMSO), forskolin, 5′-*N*-ethylcaroxamidoadenosine (NECA), alloxazine, 8-(3-chlorostyryl)-caffeine (CSC), and the Rp isomer of adenosine 3′,5′-cyclic monophosphothioate (Rp-cAMPS) were purchased from Sigma-Aldrich. Coformycin was from Calbiochem (La Jolla, CA) and 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CSFE) was from Molecular Probes Inc. (Eugene, OR).

T Lymphocyte Isolation and Activation

Mice were sacrificed by cervical dislocation. Spleens were removed using aseptic technique and spleen cell suspensions were prepared in cold phosphate buffered saline (PBS; pH 7.2). Following removal of erythrocytes by osmotic shock, the remaining lymphocytes were washed, resuspended in RPMI 1640 medium, and passaged through a nylon wool column (Cellular Products Inc., Buffalo, NY). Nylon wool-nonadherent spleen cells are highly enriched for T lymphocytes (typically >80% T cells). Natural killer cells were then depleted by anti-asialoGM1 antibody plus complement treatment. T cells were resuspended in RPMI 1640 medium and added to 96-well round-bottom microtiter plates or to 24-well flat-bottom culture plates. Each microtiter well contained 2.5×10^5 cells in a final volume of 0.2 ml while each flat-bottom well contained 8×10^6 cells in a final volume of 2 ml. T cells were activated by the addition of anti-CD3 mAb in the form of hybridoma culture supernatant at a final dilution of 1:20 (previously determined to yield optimal proliferative responses). All additional reagents were added at initiation of culture. After 48 h of culture at 37°C and 5% CO_2 in a 95% humidified atmosphere, T cells were harvested from flat-bottom plates, washed, and subjected to flow cytometric analysis. T cell proliferation in microtitre plates was measured by pulsing each well with 0.5 μCi of tritiated thymidine ([^{3}H]TdR; Sp. Act. 65 Ci/mmol; ICN Biomedicals Canada). After incubation for 6 h, cells were harvested with a Titer-Tek multiple sample harvester onto glass fiber mats and [^{3}H]TdR incorporation was measured by scintillation counting.

Flow Cytometry

T cells were washed with PBS, resuspended in PBS/1% BSA/ 0.2% sodium azide at 10^7 cells/ml, and 0.1 ml aliquots were placed on ice and treated for 30 min with 10 μg/ml goat anti-hamster IgG $F(ab')_2$ to block binding of the FITC-conjugated secondary antibody to any surface-bound anti-CD3 mAb. The cells were then washed and resuspended in PBS/1% BSA/ 0.2% sodium azide containing rat IgG, rat anti-mouse CD2, rat anti-mouse CD25, hamster IgG, or hamster anti-mouse CD28 mAb (all at 1 μg/ml). After a 30 min incubation on ice, the cells were washed and resuspended in PBS/1% BSA/ 0.2% sodium azide containing FITC-conjugated mouse anti-rat IgG $F(ab')_2$ for CD2 and CD25 detection or goat anti-hamster IgG $F(ab')_2$ for CD28 detection (both 10 μg/ml). Following a 45-min incubation on ice, the cells were washed and resuspended in PBS containing 1% paraformaldehyde. CD2, CD25, and CD28 expression were determined by analysis of 10^4 cells with a FACScan (Becton-Dickinson Canada, Mississauga, Ont.). For cell cycle analysis, T cells were stained for 10 min at 37°C with 2.5 μM CSFE. Immediately after staining, the cells were washed with FCS, followed by two washes with PBS. Stained T cells were then used in experiments. Fluorescence was measured by flow cytometry after 72 h of culture.

ELISA

IL-2 concentrations in supernatants from 24 h anti-CD3-activated T cell cultures were measured by sandwich ELISA using paired mAbs, recombinant cytokines, and protocols supplied by BD Pharmingen.

Measurement of Intracellular cAMP

Intracellular cAMP was determined by cAMP EIA according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Cell Lysate Preparation and Immunoblotting

T cells were aliquoted into sample tubes and stimulated for 5 and 20 min with anti-CD3 mAb in the absence or presence of adenosine with or without coformycin. At the end of the incubation period, samples were chilled on ice and centrifuged at 400*g* and 4°C for 5 min. The cell pellet was washed once with ice-cold PBS, followed by lysis in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 100 μM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin) for 30 min at 4°C. Insoluble cytoplasmic granules and nuclei were removed by centrifugation for 10 min at 12,000*g* and 4°C. Sample protein concentrations were determined by Bradford protein assay according to the manufacturer's instructions (Bio-Rad). Cell lysates were boiled in SDS sample buffer and 20 μg total protein was loaded into each well of an 8% SDS–polyacrylamide gel for separation by electrophoresis. Protein bands were then transferred onto nitrocellulose membranes and the resulting blots were blocked for 1 h with PBS-Tween (0.25 M Tris, pH 7.5, PBS, 150 mM NaCl, and 0.2% Tween 20) containing 5% powdered skim milk. Blots were probed overnight with 1 μg/ml anti-phosphotyrosine mAb. Blots were then washed three times with PBS-Tween and probed for 1 h with horse radish peroxidase-conjugated anti-mouse IgG. Following three additional washes with PBS-Tween, the protein bands were visualized using an ECL Western blot detection system (Bio-Rad).

RESULTS

Adenosine Inhibits Activation-Induced but not Constitutive CD2 and CD28 Expression by T Lymphocytes

We show here that exposure to adenosine (5, 10, or 25 μM) inhibited anti-CD3-induced T cell proliferation and IL-2 synthesis in a dose-dependent fashion (Table I). The addition of coformycin (2.5 μM) to prevent adenosine degradation by endogenous adenosine deaminase, which is normally present on the surface of activated T cells [Dong et al., 1996], dramatically enhanced the inhibitory effect of adenosine. Although coformycin alone did not affect T cell proliferation, there was a modest inhibitory effect of coformycin on IL-2 synthesis, suggesting an inhibition by endogenous adenosine that is known to be released within T cell cultures [Antonysamy et al., 1995]. Figure 1 demonstrates that the inhibitory effect of adenosine (with or without coformycin) was evident over the entire time course of the T cell proliferation assay, indicating that exposure to adenosine did not simply delay the kinetics of T cell activation. Impaired T cell activation in the presence of adenosine was associated with an altered pattern of tyrosine phosphorylation on some cytoplasmic proteins of T cells (Fig. 2), including a reduction in the phosphorylation of

TABLE I. Adenosine Inhibits IL-2 Synthesis and Proliferation of T Lymphocytes Stimulated With Anti-CD3 mAb

Additions to culture[a]	Coformycin	[^{3}H]TdR incorporation[b] (cpm ± SEM)	IL-2 synthesis[c] (pg/ml)
Medium	−	66,182 ± 1,732	19 ± 1
	+	70,360 ± 3,062	15 ± 1
5 μM adenosine	−	3,697 ± 168	8 ± 1
	+	144 ± 15	BLD
10 μM adenosine	−	2,690 ± 104	7 ± 1
	+	60 ± 9	BLD
25 μM adenosine	−	144 ± 17	4 ± 1
	+	274 ± 48	BLD

BLD, below limits of detection.
[a]T cells were activated with anti-CD3 mAb in the absence or presence of the indicated concentrations of adenosine with or without 2.5 μM coformycin.
[b]Cellular proliferation at the 48 h time point was determined by measuring [^{3}H]TdR incorporation. Data from a single experiment representative of 11 independent experiments are presented as mean cpm ± SEM from quadruplicate cultures.
[c]Cell-free supernatants were harvested after 24 h of culture for analysis of IL-2 content by ELISA. Data from a single experiment representative of five independent experiments are shown as mean pg/ml IL-2 ± SEM from triplicate samples.

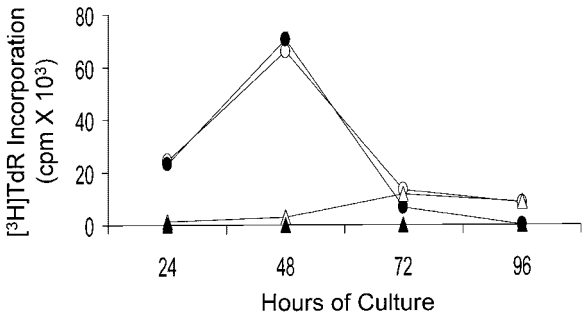

Fig. 1. Time course of adenosine-mediated inhibition of anti-CD3-induced T cell proliferation. T cells were activated with anti-CD3 mAb alone (open circles), anti-CD3 mAb plus 2.5 μM coformycin (closed circles), anti-CD3 mAb plus 10 μM adenosine (open triangles), or anti-CD3 mAb plus 2.5 μM coformycin and 10 μM adenosine (closed triangles). Cellular proliferation at 24, 48, 72, and 96 h time points was determined by measuring [^{3}H]TdR incorporation. Data are presented as mean cpm ± SEM from quadruplicate cultures.

tyrosine residues on proteins in the 50–60 and 70–75 kDa range (denoted by arrows). In addition, CSFE labeling was used to monitor T cell division following T cell activation in the presence of adenosine. As shown in Figure 3, T cells that were activated with anti-CD3 mAb in the presence of adenosine plus coformycin failed to progress through cell cycle. In comparison, control anti-CD3-activated T cells or T cell activated in the presence of coformycin alone progressed through several rounds of cell division.

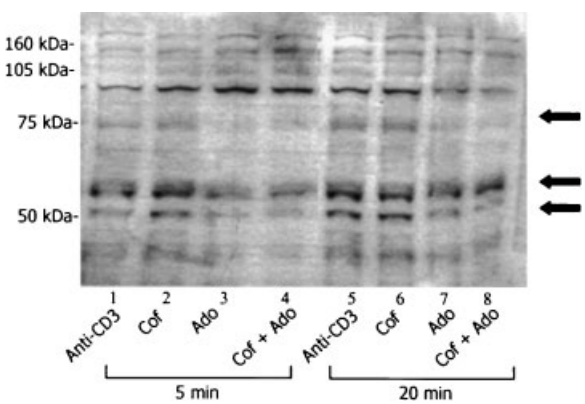

Fig. 2. Inhibitory effect of adenosine on tyrosine phosphorylation of intracellular proteins in T cells. Whole cell lysates were prepared from T cells stimulated for 5 and 20 min with anti-CD3 mAb in the absence or presence of 10 μM adenosine with or without 2.5 μM coformycin. Protein content of lysate samples was measured and equal amounts of protein from each treatment group were subjected to PAGE–SDS electrophoresis, transferred to nitrocellulose, and probed with anti-phosphotyrosine mAb as described in the Materials and Methods.

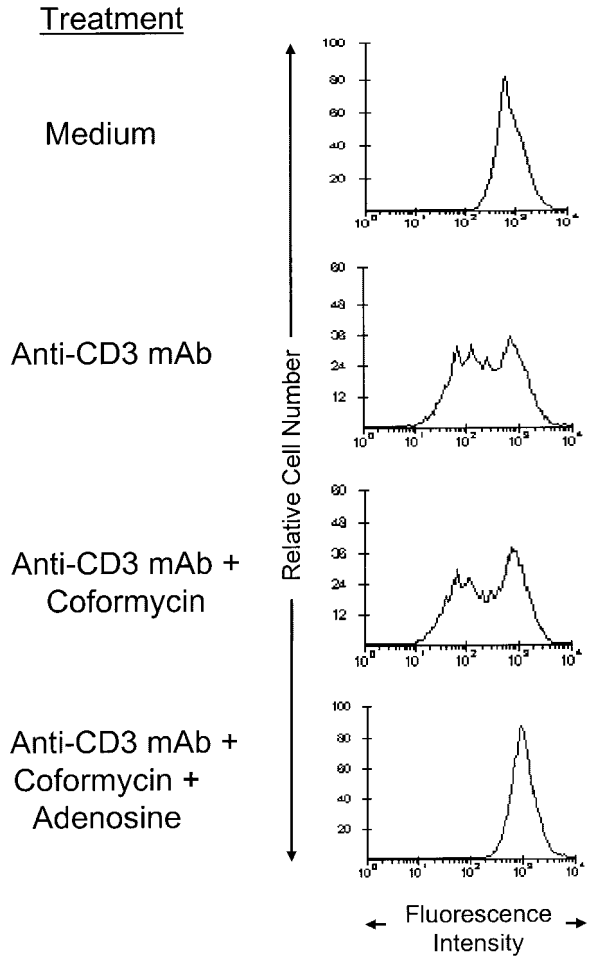

Fig. 3. Adenosine prevents cell cycle progression of activated T cells. Active T cell division was monitored by labeling T cells with CSFE prior to culture for 72 h in medium alone, anti-CD3 mAb alone, anti-CD3 mAb plus 2.5 μM coformycin, or anti-CD3 mAb plus 2.5 μM coformycin and 10 μM adenosine. Flow cytometric analysis was used to measure cell cycle progression of CSFE-labeled T cells.

Since T cell activation and sustained IL-2 synthesis is dependent on co-stimulatory signaling [Watts and DeBenedette, 1999], we next examined the effect of adenosine on CD2 and CD28 expression by T cells. We chose to examine CD2 and CD28 expression after 48 h of culture because DNA synthesis by T cells in response to stimulation with anti-CD3 mAb peaks at 48 h of culture (Fig. 1), as does activation-induced expression of CD2 and CD28 [Abraham et al., 1994; Butler et al., 2002]. Figure 4 shows that adenosine, in the absence or presence of coformycin, inhibited activation-induced expression of CD2 and CD28 by mouse T cells. The inhibitory effect of adenosine on inducible CD2 and CD28 expression was dose-dependent (data not

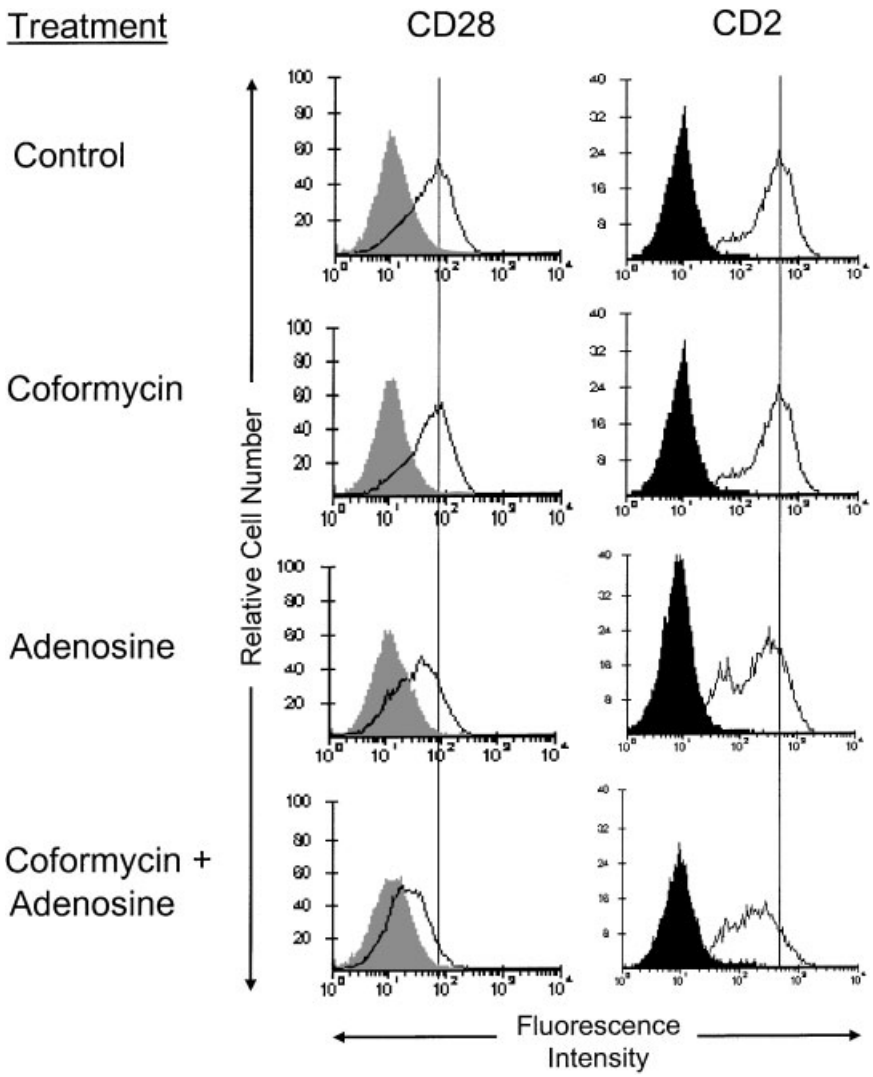

Fig. 4. Activation-induced expression of CD2 and CD28 is inhibited in the presence of adenosine. T lymphocytes were activated with anti-CD3 mAb in the presence of medium alone (control), 2.5 μM coformycin, 10 μM adenosine, or 10 μM adenosine plus 2.5 μM coformycin. Activation-induced CD28 and CD2 expression was determined by flow cytometry after 48 h of culture. Histograms depict anti-CD28 or anti-CD2 staining (solid black line) compared to background staining with an isotype control antibody (filled area). Results are representative of 11 independent experiments.

shown). Coformycin by itself had no detrimental effect on either CD28 or CD2 expression by anti-CD3-activated T cells. In contrast, constitutive expression of CD2 and CD28 by mouse T lymphocytes was unaffected following culture in the presence of adenosine for 24 h (data not shown). Adenosine plus coformycin, at the concentrations used in these studies, did not adversely affect T cell viability in comparison to untreated controls, as assessed by the ability of the T cells to exclude trypan blue dye at the end of the 48 h culture period (66 ± 9 % vs. 75 ± 9 % viability, $P > 0.05$ by Student's t-test).

Activation-Induced CD2 and CD28 Expression is Dependent on IL-2 Signaling

Because adenosine inhibited IL-2 synthesis, as well as CD2 and CD28 expression by activated T cells, we considered the possibility that activation-induced CD2 and CD28 expression might be IL-2-dependent. To determine the effect of reduced IL-2 bioactivity on activation-induced expression of CD2 and CD28, T cells were activated in the absence or presence of anti-IL-2 neutralizing mAb in combination with anti-CD25 mAb (to block the high affinity IL-2

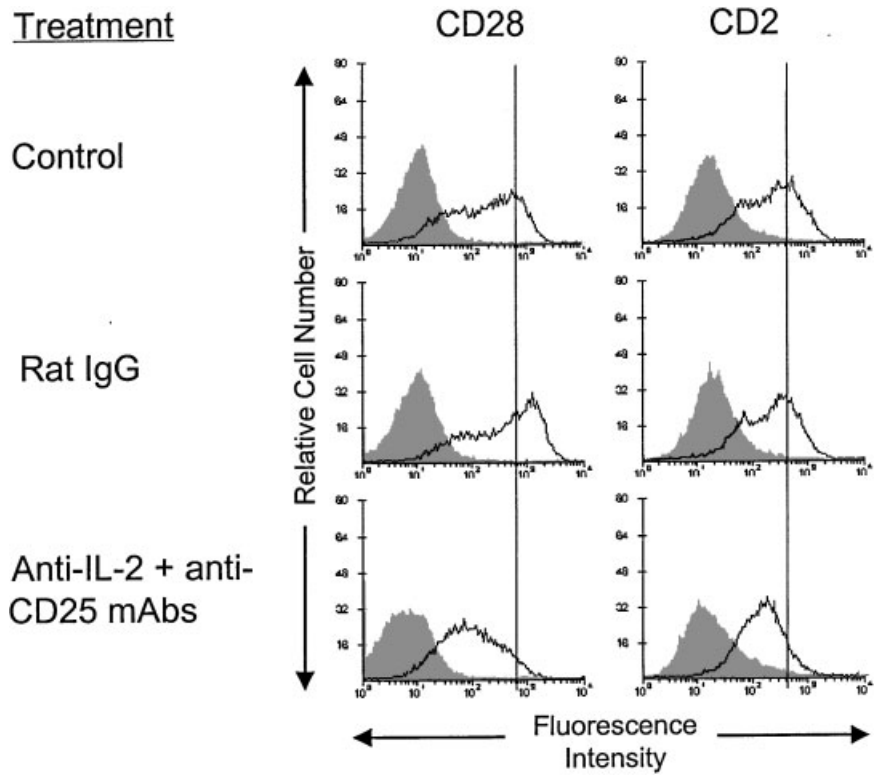

Fig. 5. Neutralization of interleukin-2 (IL-2) bioactivity inhibits activation-induced CD2 and CD28 expression. T lymphocytes were activated with anti-CD3 mAb in the presence of medium alone (control), an irrelevant rat IgG (20 μg/ml), or anti-IL-2 plus anti-CD25 mAbs (both 10 μg/ml). Activation-induced CD28 and CD2 expression was determined by flow cytometry after 48 h of culture. Histograms depict anti-CD28 or anti-CD2 staining (solid black line) compared to background staining with an isotype control antibody (shaded grey area). Results are representative of five independent experiments.

receptor). Figure 5 shows that inhibition of IL-2 bioactivity in activated T cell cultures resulted in decreased CD2 and CD28 expression, indicating that inducible CD2 and CD28 expression was regulated by IL-2 signaling pathways. Moreover, the decrease in activation-induced CD2 and CD28 expression was very similar to that observed in the presence of adenosine, suggesting that adenosine-mediated inhibition of IL-2 synthesis was the cause of reduced CD2 and CD28 expression by T cells activated in the presence of adenosine.

Exogenous IL-2 Fails to Restore Inducible Expression of CD2 and CD28 by T cells Activated in the Presence of Adenosine

Since activation-induced expression of CD2 and CD28 appeared to depend on signaling through the IL-2 receptor, we next attempted to reverse the inhibitory effect of adenosine on inducible CD2 and CD28 expression by adding exogenous IL-2 (at a concentration typically present in anti-CD3-activated T cell cultures [Kaiser et al., 1993]) at the initiation of culture. As shown in Figure 6, the addition of exogenous IL-2 (50 U/ml) did not substantially up-regulate either CD2 or CD28 expression by T cells activated in the presence of adenosine. We, therefore, considered the possibility that adenosine might inhibit expression of the high affinity IL-2 receptor (CD25), which is required for effective IL-2 utilization by T lymphocytes. Figure 6 also shows that T cell expression of CD25 was reduced in presence of adenosine whether or not exogenous IL-2 was also present. This accounts for the failure of exogenous IL-2 to reverse the inhibitory effect of adenosine on activation-induced CD2 and CD28 expression.

cAMP Regulates Activation-Induced CD2 and CD28 Expression

The adenosine impairment of IL-2 production in activated T cells has been linked to increased cAMP production via adenylyl cyclase-coupled $A_{2A}R$ and/or$A_{2B}R$ [Koshiba et al., 1997; Mirabet et al., 1999]. We, therefore, examined the

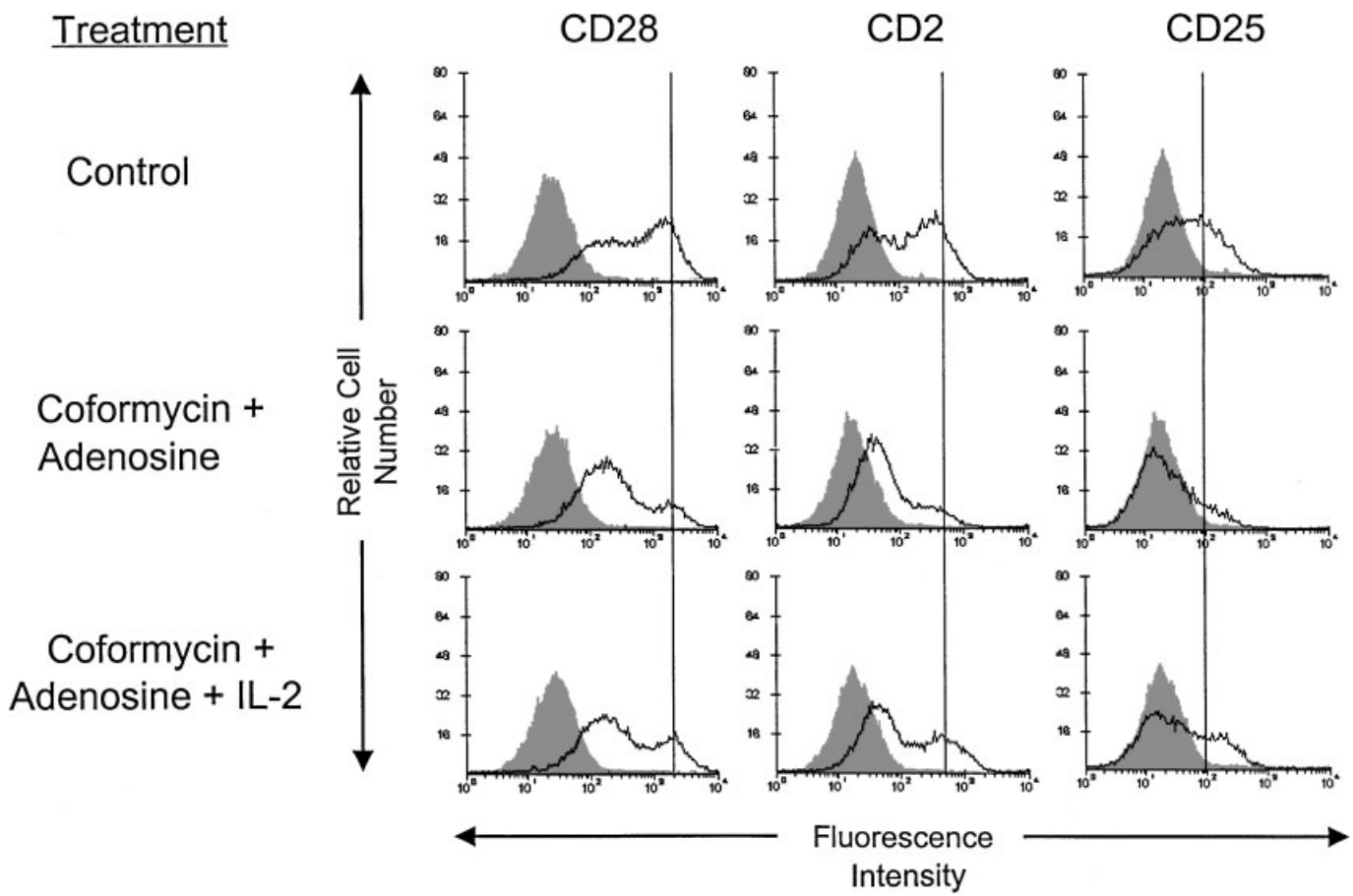

Fig. 6. Adenosine-mediated inhibition of inducible CD2 and CD28 expression is not reversed by exogenous IL-2 due to an inhibitory effect of adenosine on CD25 expression. T lymphocytes were activated with anti-CD3 mAb in the presence of medium plus 50 U/ml IL-2 (control), 10 μM adenosine plus 2.5 μM coformycin, or 10 μM adenosine plus 2.5 μM coformycin and 50 U/ml IL-2. CD2, CD25, and CD28 expression was determined by flow cytometry after 48 h of culture. Histograms depict anti-CD2, anti-CD25, or anti-CD28 staining (solid black line) compared to background staining with an isotype control antibody (shaded grey area). Results are representative of three independent experiments.

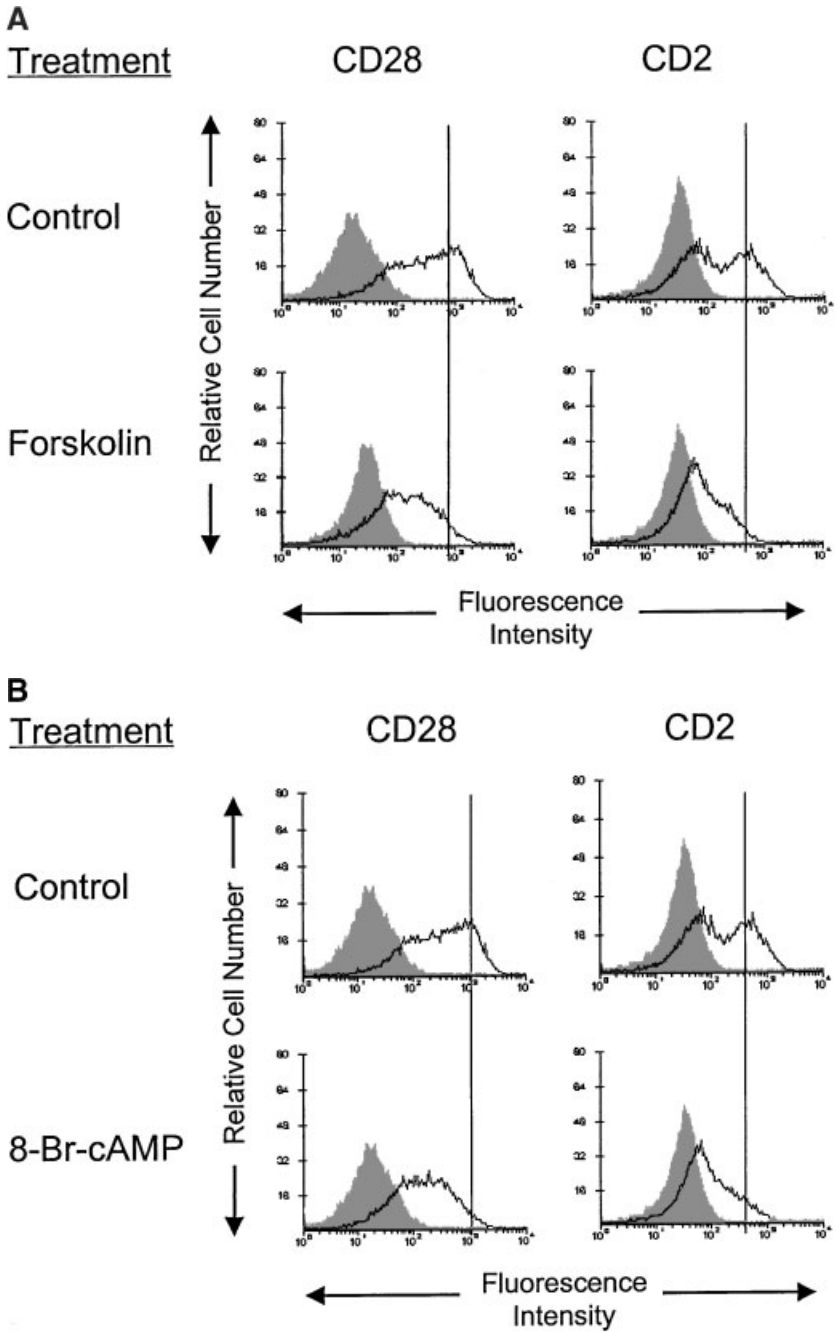

Fig. 7. Stimulation of cAMP-dependent pathways inhibits activation-induced CD2 and CD28 expression. T lymphocytes were activated with anti-CD3 mAb in the absence or presence of 25 μM forskolin (**Panel A**) or 1 mM 8-Br-cAMP (**Panel B**). Activation-induced CD28 and CD2 expression was determined by flow cytometry after 48 h of culture. Histograms depict anti-CD28 or anti-CD2 staining (solid black line) compared to background staining with an isotype control antibody (shaded grey area). Results are representative of four independent experiments.

effect of stimulating cAMP-dependent pathways on CD2 and CD28 expression by activated T lymphocytes. Figure 7 shows that stimulation of adenylyl cyclase activity with forskolin (25 μM) or exposure to the stable, cell-permeable cAMP analogue 8-Br-cAMP (1 mM) had a marked inhibitory effect on anti-CD3-induced CD2 and CD28 expression, indicating that cAMP was able to modulate CD2 and CD28 gene expression.

Adenosine-Induced Accumulation of Intracellular cAMP is not Sufficient to Inhibit CD2 and CD28 Expression by Activated T cells

The effect of the $A_{2A}R$-selective agonist CGS 21680 on activation-induced expression of CD2 and CD28 was investigated to determine whether increased cAMP production induced by stimulation of adenylyl cyclase-coupled $A_{2A}R$ might be responsible for the inhibitory effect of adenosine on the expression of co-stimulatory molecules by T cells. CGS 21680 (5 μM) consistently failed to inhibit activation-induced CD2 and CD28 expression (Fig. 8), ruling out an effect mediated through $A_{2A}R$. The adenosine analogue NECA at a concentration (5 μM) able to stimulate both $A_{2A}R$ and $A_{2B}R$ also failed to inhibit CD2 and CD28 expression (data not shown), arguing against a role for adenylyl cyclase-coupled $A_{2B}R$. Moreover, neither the $A_{2A}R$-selective antagonist CSC nor the $A_{2B}R$-selective antagonist alloxazine (both at 10 μM), alone or in combination, ameliorated the inhibitory effect of adenosine on activation-induced expression of CD2 (Fig. 9A) or CD28 (Fig. 9B). The same concentrations of CSC and alloxazine blocked signaling through $A_{2A}R$ and $A_{2B}R$, respectively, in T cells in an unrelated series of experiments (data not shown), thereby confirming the activity of these reagents in our experimental system. Surprisingly, despite the failure of CGS 21680 or NECA to inhibit inducible CD2 and CD28 expression, both adenosine agonists were as effective as adenosine (10 μM) in the presence of coformycin in causing intracellular cAMP to accumulate in anti-CD3-activated T lymphocytes (Table II). Interestingly, intracellular cAMP accumulation in response to forskolin (25 μM) treatment greatly exceeded (approximately fivefold) that caused by adenosine or adenosine receptor agonists. Moreover, the apparent level of immunoreactive cAMP in anti-CD3-activated T cells treated with 8-Br-cAMP (1 mM) was $1{,}639 \pm 509$ fmol/10^6 cells, approximately 19-fold that caused by adenosine.

We also examined the effect of the cAMP antagonist Rp-cAMPS [Rothermel et al., 1984] on the ability of adenosine to interfere with activation-induced CD2 and CD28 expression. Figure 10 shows that Rp-cAMPS (100 μM) failed to alleviate the inhibitory effect of adenosine on CD2 and CD28 expression by activated T cells. The same concentration of Rp-cAMPS inhibited NECA-induced phosphorylation of ERK1/2 in T cells (data not shown), confirming that this reagent is active in our experimental system. Taken together, these data indicate that adenosine-induced cAMP accumulation was not

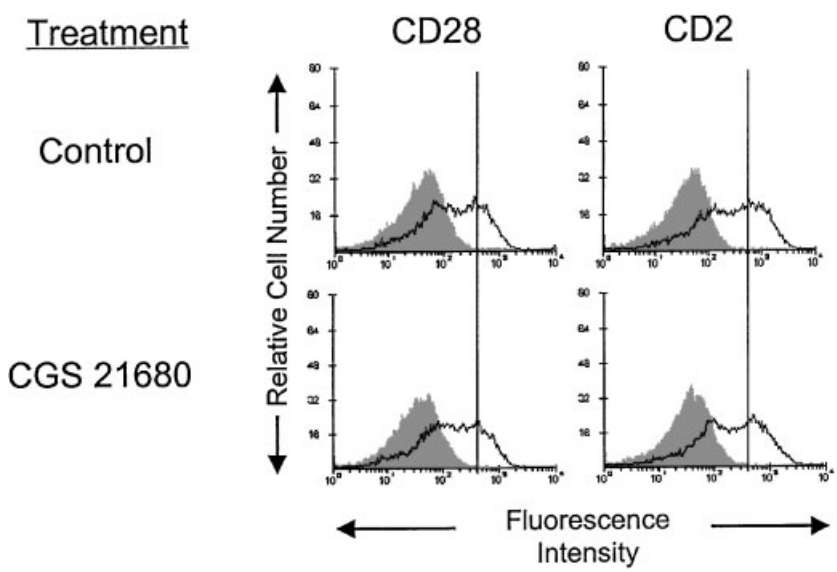

Fig. 8. CGS 21680 fails to affect activation-induced CD2 and CD28 expression. T lymphocytes were activated with anti-CD3 mAb in the presence of medium alone (control) or 5 μM CGS 21680. Activation-induced CD28 and CD2 expression was determined by flow cytometry after 48 h of culture. Histograms depict anti-CD28 or anti-CD2 staining (solid black line) compared to background staining with an isotype control antibody (shaded grey area). Results are representative of five independent experiments.

responsible for the inhibitory effect of adenosine on activation-induced CD2 and CD28 expression. Although activation-induced CD2 and CD28 expression can be inhibited by intracellular cAMP such as seen with forskolin (Fig. 7A), this effect was associated with the accumulation of higher levels of intracellular cAMP than can be induced by adenosine.

DISCUSSION

Many solid tumors evade cell-mediated immune responses by generating a state of progressive systemic and localized immune suppression in cancer patients or tumor-bearing animals [Kiessling et al., 1999]. Tumor-associated immune suppression has been linked to multiple factors including tumor cell expression of Fas ligand that triggers apoptosis in tumor-infiltrating lymphocytes [Bennett et al., 1998], the induction of immunoregulatory cells [Kusmartsev et al., 2000], and the presence of immunosuppressive factors such as transforming growth factor β [Vánky et al., 1997] and adenosine [Blay et al., 1997] within the tumor microenvironment. We show here that adenosine, at low micromolar concentrations typically present within the tumor microenvironment [Blay et al., 1997], had a dose-dependent inhibitory effect on T cell proliferation and IL-2 synthesis in response to mitogenic anti-CD3 mAb. Moreover, CFSE staining revealed that

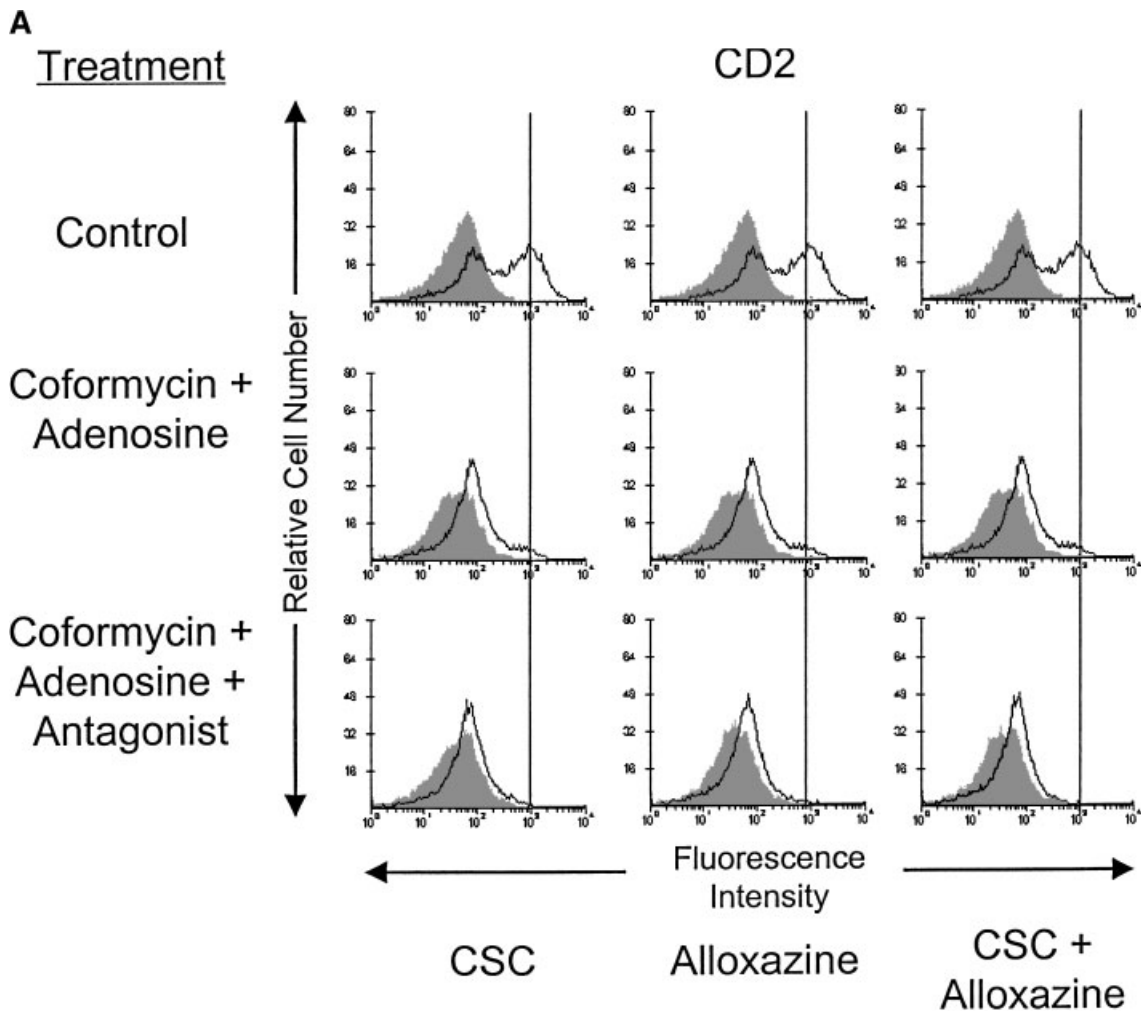

Fig. 9. CSC and/or alloxazine fail to diminish the inhibitory effect of adenosine on inducible CD2 and CD28 expression. T lymphocytes were activated with anti-CD3 mAb in the presence of medium alone (control), or 10 μM adenosine plus 2.5 μM coformycin without or with 10 μM CSC or alloxazine. Activation-induced CD2 (**Panel A**) and CD28 (**Panel B**) expression was determined by flow cytometry after 48 h of culture. Histograms depict anti-CD2 or anti-CD28 staining (solid black line) compared to background staining with an isotype control antibody (shaded grey area). Results are representative of three independent experiments.

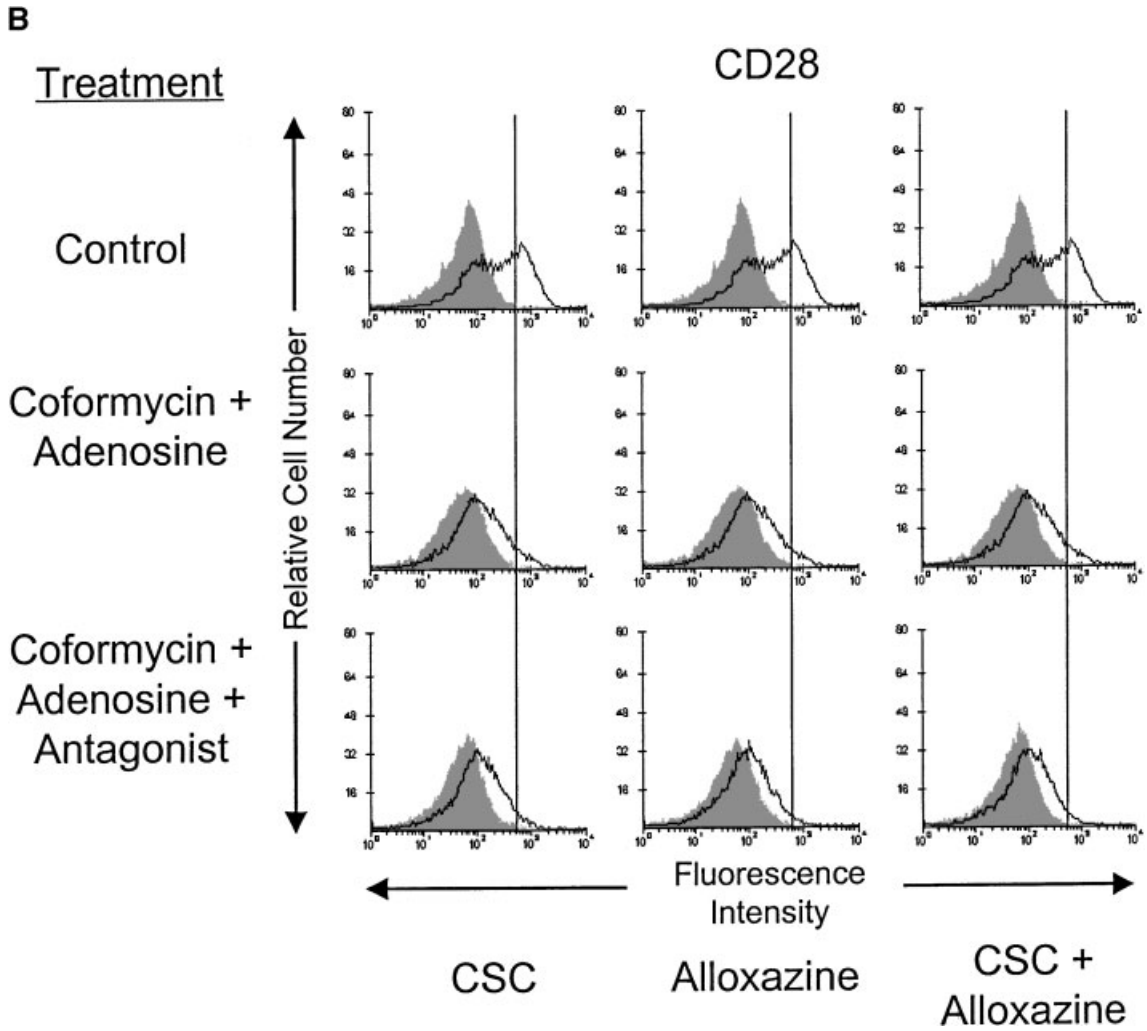

Fig. 9. (*Continued*)

TABLE II. Effects of Adenosine, CGS 21680, NECA, and Forskolin on Intracellular cAMP Accumulation in Anti-CD3-Activated T Lymphocytes

Treatment[a]	Intracellular cAMP (fmol/10^6 cells ± SEM)
None	20 ± 3
10 μM adenosine + coformycin	85 ± 30
5 μM CGS 21680	82 ± 14
5 μM NECA	91 ± 10
25 μM forskolin	401 ± 19

[a]T cells were activated with anti-CD3 mAb in the absence or presence of the indicated agents and intracellular cAMP accumulation after 30 min was measured by EIA. Data from a single experiment representative of two independent experiments are presented as mean fmol cAMP/10^6 cells ± SEM from triplicate samples. The apparent level of immunoreactive cAMP in cells treated with 1 mM 8-Br-cAMP was 1,639 ± 509 fmol/10^6 cells.

T cells that were activated in the presence of adenosine failed to progress through cell cycle. In addition, a reduction in the phosphorylation of tyrosine residues on some cytoplasmic proteins was observed when T cells were activated in the presence of adenosine. These observations are consistent with an inhibitory effect of adenosine at the level of T cell receptor signaling, which may account for the ability of adenosine to suppress antigen-driven T cell proliferation, cytotoxic T lymphocyte development, T cell adhesion to tumor target cells, and cytotoxic effector function [MacKenzie et al., 1994; Koshiba et al., 1997; Williams et al., 1997; Hoskin et al., 2002; MacKenzie et al., 2002]. Similar inhibitory effects by tumor-associated adenosine may contribute to T cell dysfunction within the tumor microenvironment [Kiessling et al., 1999].

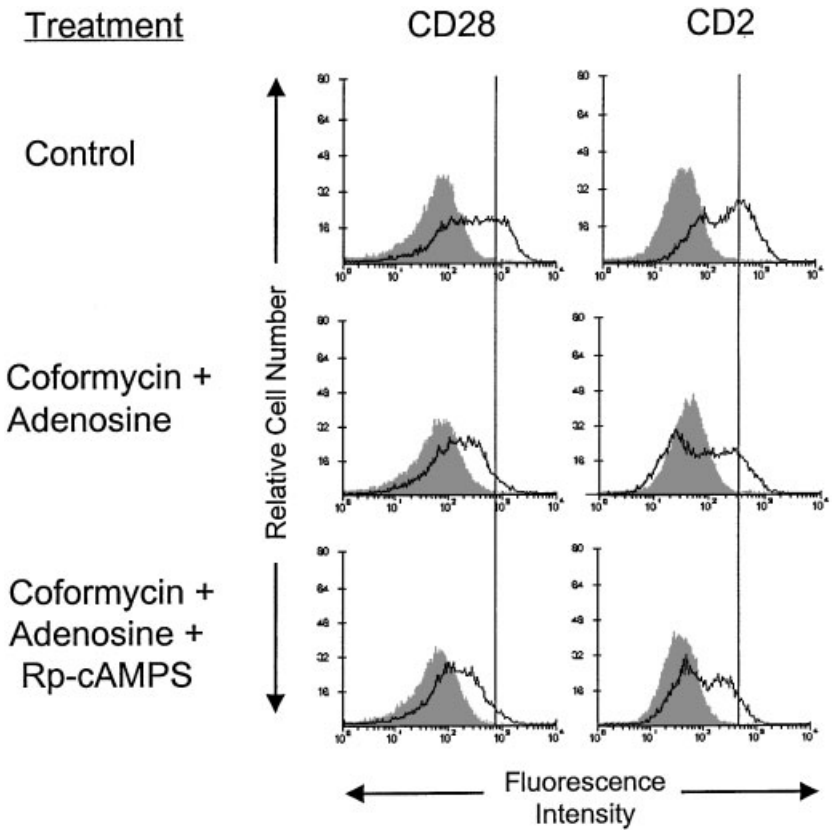

Fig. 10. Adenosine-mediated inhibition of activation-induced CD2 and CD28 expression is not affected by Rp-cAMPS. T lymphocytes were activated with anti-CD3 mAb in the presence of medium alone (control), or 10 μM adenosine plus 2.5 μM coformycin without or with 100 μM Rp-cAMPS. Activation-induced CD28 and CD2 expression was determined by flow cytometry after 48 h of culture. Histograms depict anti-CD28 or anti-CD2 staining (solid black line) compared to background staining with an isotype control antibody (shaded grey area). Results are representative of three independent experiments.

Importantly, low micromolar concentrations of adenosine inhibited activation-induced but not constitutive expression of the T cell co-stimulatory molecules CD2 and CD28. Expression of α4 integrin by activated T cells was not affected by adenosine [W. MacKenzie and J. Blay, unpublished data], indicating that adenosine was not exerting a general inhibitory effect on the expression of T cell surface molecules. It is widely believed that co-stimulatory signaling intersects directly with signaling through the T cell receptor during T cell activation [Croft and Dubey, 1997]. However, Zhang et al. [1997] have proposed that the primary role of co-stimulatory signaling through CD28 is to prolong IL-2 production, rather than to provide a critical second signal at the same time as signaling through the T cell receptor. Consistent with this, blockade of CD28 and CD2 interactions with their respective ligands up to 12 h after stimulation through the T cell receptor has an inhibitory effect on T cell proliferation, which is approximately equivalent to that obtained when co-stimulatory signaling is blocked at the same time as T cell receptor triggering [Makrigiannis et al., 1999; Musgrave et al., 2003]. Activation-induced expression of co-stimulatory molecules is clearly important for optimal T cell activation since oligonucleotide-mediated inhibition of inducible CD28 expression leads to T cell hyporesponsiveness and reduced IL-2 synthesis [Tam et al., 1997]. In this regard, we took note that adenosine strongly inhibited IL-2 synthesis by activated T lymphocytes; and that inducible CD2 and CD28 expression was dependent on IL-2 signaling pathways since antibody-mediated neutralization of IL-2 bioactivity together with blockade of the IL-2 receptor inhibited the expression of CD2 and CD28 by activated T cells. This is in line with our finding that activation-induced expression of CD2 and CD28 is inhibited by

cyclosporine A [Butler et al., 2002; N. Ward and D. Hoskin, unpublished data], which is a potent inhibitor of IL-2 synthesis by T cells [Andersson et al., 1992]. Moreover, the magnitude of the inhibitory effect of adenosine on inducible CD2 and CD28 expression was comparable to that of IL-2 bioactivity neutralization, suggesting that adenosine inhibited activation-induced expression of co-stimulatory molecules by interfering with IL-2 signaling. However, the addition of exogenous IL-2 to cultures of T cells activated in the presence of adenosine failed to restore normal levels of CD2 and CD28 expression, pointing to the limiting step being diminished CD25 expression by T cells activated in the presence of adenosine. Taken together, our findings lead us to propose that adenosine-mediated inhibition of IL-2 synthesis and signaling by T cells within the tumor microenvironment leads to a reduction in the co-stimulatory capacity of CD2 and CD28 and subsequent impaired activation of tumoricidal T lymphocytes.

One question that arises is the degree to which tumor-infiltrating T cells might be resistant to the immunosuppressive effect of tumor-associated adenosine as a result of T-cell-surface expression of adenosine deaminase in association with CD26 [Dong et al., 1996]. However, adenosine deaminase activity is reported to be significantly lower in the peripheral blood T lymphocytes of cancer patients [Dasmahapatra et al., 1986; Murray et al., 1986], implying increased susceptibility of T cells to adenosine-mediated inhibition within the tumor microenvironment. Moreover, cytotoxic T lymphocytes possess very low levels of adenosine deaminase activity in comparison to thymocytes and bulk T cell cultures [Minkowski et al., 1984; Minkowski and Bandeira, 1985]. It is, therefore, reasonable to expect that tumor-infiltrating T cells of cancer patients, and in particular cytotoxic T lymphocytes, would be quite sensitive to the immune inhibitory effects of tumor-associated adenosine.

Intracellular cAMP is an important regulator of T lymphocyte proliferation and the production of type 1 cytokines such as IL-2 by activated T cells [Skalhegg et al., 1992; Benbernou et al., 1997]. The inhibitory effect of cAMP on T cell function has been attributed to cAMP-dependent protein kinase A that interferes with extracellular signal-related kinase and c-Jun N-terminal kinase activity associated with T cell signal transduction pathways [Tamir et al., 1996]. Inhibition of activation-induced CD2 and CD28 expression by forskolin and 8-Br-cAMP, which are potent stimulators of cAMP-dependent pathways, was consistent with the ability of intracellular cAMP to interfere with signal transduction downstream of the T cell receptor. However, adenosine did not appear to regulate inducible CD2 and CD28 expression via a cAMP-dependent mechanism since CGS 21680, which selectively stimulates the adenylyl cyclase-coupled $A_{2A}R$, and NECA, at concentrations that stimulate both $A_{2A}R$ and $A_{2B}R$, failed to inhibit CD2 and CD28 expression by activated T cells, despite inducing an intracellular cAMP accumulation that was comparable to that induced by adenosine. In addition, Rp-cAMPS, an inhibitor of protein kinase A [Rothermel et al., 1984], had no effect on the adenosine inhibition of activation-induced CD2 and CD28 expression. The apparent discrepancy with the anticipated effect of cAMP is explained by the finding that in comparison to more physiologically relevant elevations of intracellular cAMP concentrations achieved using externally applied adenosine or adenosine analogues, intracellular cAMP accumulation was at least fivefold greater when adenylyl cyclase was directly stimulated with forskolin or by substitution with 8-Br-cAMP. This observation is in line with a recent report that intracellular cAMP accumulation by equine chondrocytes is approximately 20 times higher in the presence of forskolin than in response to adenosine receptor agonists [Tesch et al., 2002]. We think it likely that the magnitude of intracellular cAMP accumulation induced by adenosine was simply not sufficient to dampen activation-induced T cell expression of CD2 and CD28 through protein kinase A-mediated pathways. Our findings indicate that caution is warranted when interpreting the results of studies employing cells that have been genetically engineered to overexpress adenylyl cyclase-coupled adenosine receptors since levels of intracellular cAMP that might accumulate in such cells may not be physiologically relevant.

Although the nature of the cAMP-independent mechanism by which adenosine interferes with activation-induced CD2 and CD28 expression is not clear at the present time, one possible mechanism might involve the observed inhibitory effect of adenosine on tyrosine phosphorylation of T cell cytoplasmic proteins in the

50–60 and 70–75 kDa range. Protein tyrosine kinases in this size range, including $p56^{lck}$ and ZAP-70, play an essential role in the signaling pathways that lead to T cell activation [Nel, 2002]. Since the function of protein tyrosine kinases such as $p56^{lck}$ is dependent on autophosphorylation of tyrosine residues [Xu and Littman, 1995; Pawson, 2002], adenosine-mediated inhibition of tyrosine phosphorylation events might interfere with the function of key signaling intermediates in the T cell receptor signal transduction pathway. Indeed, adenosine has previously been reported to inhibit the activity of the protein tyrosine kinase $p56^{lck}$ [Berger et al., 1996]. Impaired $p56^{lck}$ function in the presence of adenosine would be expected to lead to a reduction in T cell receptor signaling and subsequent IL-2 synthesis, which would, in turn, prevent activation-induced expression of CD2 and CD28.

The fact that neither the $A_{2A}R$ agonist CGS 21680 nor the $A_{2A}R/A_{2B}R$ agonist NECA were able to inhibit CD2 and CD28 expression by activated T cells argues that adenosine was not mediating its inhibitory effect on activation-induced expression of co-stimulatory molecules through $A_{2A}R$ and/or $A_{2B}R$. This was confirmed by the failure of the $A_{2A}R$-selective antagonist CSC and/or the $A_{2B}R$-selective antagonist alloxazine to prevent adenosine-mediated inhibition of CD2 and CD28 expression by activated T cells. Although adenosine is able to mediate effects through an intracellular P-site that regulates cAMP accumulation, such effects only occur at much higher adenosine concentrations (IC_{50} of ~80 μM) [Marone et al., 1990]. Moreover, we were not able to block the inhibitory effect of adenosine on inducible CD2 and CD28 expression with the nucleoside transport inhibitor dialazep [J. Butler and D. Hoskin, unpublished data] at a concentration (10 μM) that is inhibitory for both nitrobenzylthioguanosine-sensitive and -resistant nucleoside transporters [Hammond, 1991]. Furthermore, we have consistently been unable to block the negative effects of adenosine on T cell function using other inhibitors of nucleoside transport such as NBTI and dipyridamole [MacKenzie et al., 1994]. Collectively, our data lead us to conclude that adenosine must be acting through a cell-surface receptor that is coupled to signaling pathways other than those involving stimulation of adenylyl cyclase. In a previous study, we demonstrated by RT-PCR analysis that mouse T lymphocytes express mRNAs coding for $A_{2A}R$, $A_{2B}R$, and A_3R subtypes but not the A_1R subtype [Hoskin et al., 2002]. The failure of mouse T cells to express A_1R rules out any possible role for this adenosine receptor subtype in mediating the inhibitory effect of adenosine on activation-induced CD2 and CD28 expression. Our previous work has established that adenosine is able to regulate T cell-mediated immune responses by acting through the A_3R [MacKenzie et al., 1994; Hoskin et al., 2002]. A_3R stimulation may also inhibit inducible CD2 and CD28 expression by T lymphocytes, possibly through phospholipase C that is known to be linked to G protein-coupled A_3R [Abbracchio et al., 1995]. Ongoing studies in our laboratory seek to determine the role of A_3R and the biochemical basis of the inhibitory effect of adenosine on T cell signaling pathways, including the function of protein tyrosine kinases associated with T cell receptor signal transduction.

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