Accumulation of extracellular and intracellular adenosine (Ado) under hypoxic conditions or in the absence of adenosine deaminase results in lymphocyte depletion and in severe combined immunodeficiency, which are currently explained by direct intracellular lymphotoxicity of Ado metabolites. In support of the alternative, “signaling” mechanism, we show that extracellular Ado (extAdo) suppresses all tested T cell receptor (TCR)-triggered effector functions of T lymphocytes including the TCR-triggered FasL mRNA up-regulation in cytotoxic T lymphocytes. Strong evidence against the intracellular lymphotoxicity of Ado (and in support of the signaling model) is provided by abrogation of TCR-triggered growth inhibition in Ado-exposed T cells. The brief exposure to Ado was sufficient to observe inhibition of TCR-triggered effector functions. The “memory” of T cells to exposure to extAdo is best explained by sustained increases in cAMP. Selective agonist (CGS21680) and antagonist (ZM241385) of A2A adenosine receptor were used in functional assays and cDNA probes for different subtypes of adenosine receptors were used in Northern blot studies. A2A receptors are identified as the predominantly expressed subtype of Gs-coupled Ado receptors in T cells. The demonstration of cross-talk between the A2A receptors and TCR in both directions support the possible role of A2A receptors in mechanisms of extAdo-mediated immunosuppression in vivo under adenosine deaminase deficiency and hypoxic conditions in, e.g., solid tumors.

Adenosine (Ado) has been implicated in pathogenesis of diseases (1–5) and in normal T cell immunity (6, 7) and is considered to be an endogenous anti-inflammatory agent (8). Ado analogs are also used as pharmacological agents (9–11).

Increased concentrations of Ado have been detected in the absence of adenosine deaminase activity (ADA) (4) or under hypoxic conditions (e.g. large solid tumors) (12). Accumulated Ado is believed to be lymphotoxic, and humans with inherited ADA deficiency (undetectable ADA activity) are ill and have severe combined immunodeficiency (SCID) (2–4). ADA SCID is currently explained by the accumulation of lymphotoxic intracellular deoxyATP (dATP) and/or of S-adenosyl homocysteine as the cause of direct lymphotoxicity leading to the depletion of lymphocytes (2–4, 13).

Alternative mechanism(s) of effects of Ado on lymphocytes, as well as the possibility that their inhibition may be due to extracellular Ado-mediated transmembrane signaling through Ado (purinergic) receptors (5, 6, 14–16) on thymocytes and peripheral T cells, have not yet been carefully explored.

According to this “signaling” model (5, 6), the accumulation of extAdo due to insufficient or absent ADA or under hypoxic conditions results in increased signaling through Ado receptors on T cells and causes immunosuppression. This signaling mechanism is attractive inasmuch as, if correct, it could provide a novel molecular target for immunomodulation; however, studies about the effect(s) of extAdo-triggered signaling on the effector functions of T cells and about the repertoire of expressed Ado receptors on T cells need to be performed.

Clarification of the cellular and biochemical mechanisms of ADA SCID is important for the understanding of the pathogenesis of this disease. In addition, studies of the effects of extAdo on T cells are valuable in devising strategies based on the use of adoptively transferred cytotoxic T lymphocytes (CTLs) to patients with solid tumors where hypoxic conditions may lead to Ado accumulation (12). Rational predictions of the side effects of Ado-based pharmacological agents also require this knowledge.

Recent advances in understanding of mechanisms of T cell-mediated effector functions, together with the availability of molecular tools to study Ado receptors, will allow more detailed and precise evaluation of the effects of extAdo under well controlled conditions. In this study, we asked (i) whether Ado-induced immunosuppression of peripheral T lymphocytes in vivo may be at least partially explained by extracellular versus intracellular Ado-mediated inhibition of antigen/T cell receptor (TCR)-driven, perforin- and Fas-based, T lymphocyte-mediated cytotoxicity and lymphokine secretion and (ii) whether the effects of Ado could be explained by extAdo receptor-mediated signaling. Here we present data that demonstrate that even short term exposure of T cells to extAdo results in strong inhibition of TCR-triggered functions, thereby providing evidence of a “memory” of Ado effects in surrounding T cells. The ability of extAdo to rescue T cells from apoptosis strongly supports signaling versus an intracellular toxicity mechanism of Ado action. TCR-signaling antagonizing A2A receptors are identified as predominantly expressed and are probably functional transducers of the T cell-inhibiting effects of extAdo.
Adenosine A<sub>2A</sub> Receptor-mediated Signaling in T Cells

MA). Selective A<sub>2A</sub> receptor antagonist ZM241385 was a kind gift from Dr. Joel Linden (University of Virginia, Charlottesville, VA) and Dr. Kenneth Jacobson (NIH, Bethesda, MD).

Anti-CD3 e-chain monoclonal antibody (mAb) 145-2C11 (2C11) was prepared as described (17). Anti-Thy-1 mAb (anti-Thy-1.2, clone 59-2.1) and anti-omas IgG (OMA) mAb (clone 44/2) were obtained from PharMingen (San Diego, CA) and Alexis (San Diego, CA), respectively.

Cells—Splenocytes and lymphocytes were isolated from 4–6-week-old DBA2 mice (H-2<sup>d</sup>). Mouse CD8<sup>+</sup> CTL clone OE4 (anti-H-2<sup>d</sup>) (18) was stimulated every 4 weeks with irradiated splenocytes from DBA2 mice in EL4-conditioned medium, expanded 4 days after stimulation, and harvested at day 8 (4 days after expansion) (19). Mouse B cell lymphoma line A20.2J (H-2<sup>d</sup>) and mouse T cell leukemia line EL4 (H-2<sup>b</sup>) were used as specific and nonspecific target cells for OEt, respectively. The perforin-deficient, FasL-expressing CD8<sup>+</sup> CTL line PO.K.3.A8 (anti-H-2<sup>d</sup>) was maintained as described previously (20). The perforin-deficient, FasL-expressing CD4<sup>+</sup> CTL line PO.P.C was maintained by biweekly stimulation and expansion with irradiated spleen cells. They were usually used 12 days after the last stimulation and were cultured in complete RPMI medium supplemented with 10% FCS and 2.5% supernatant from concanavalin A-stimulated rat spleen cells as source of T cell growth factors. B cell hybridoma LB 27.4 (LB; H-2<sup>a</sup>-<sup>b</sup><sup>d</sup>) (21) was sensitive to Fas-mediated cytotoxicity and used as a target of CTL PO.P.C. Mouse T helper hybridoma 2B4.11 (2B4) was prepared as described previously (22).

Granule Exocytosis Assay—Secreted benzoxycarbonyl-L-lysine thiobenzyl ester (BLT; Calbiochem) esterase activity was measured as described previously (23). Typically, the amount of secreted BLT esterase was determined after the incubation of 1 × 10<sup>5</sup> CTLs in wells coated with immobilized 2C11 mAb (5 μg/ml) with or without Ado analogs. After 4 h of incubation at 37 °C under 5% CO<sub>2</sub>, cells were centrifuged at 200 × g for 5 min. To assay BLT esterase activity, 50 μl of culture supernatant was mixed with 950 μl of BLT solution (0.2 mM BLT, 0.22 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (Calbiochem) in phosphate-buffered saline, pH 7.2). The mixture was incubated at 37 °C for 20 min, and the reaction was stopped by adding 10 μl of 0.1 M phenylmethylsulfonyl fluoride, which was dissolved in MeSO<sub>2</sub>. The solution was diluted 1:10 with phosphate-buffered saline, and the absorbance at 412 nm was measured in comparison to a blank solution (RPMI 1640, 10 mM HEPES, 5% FCS) that was treated exactly the same as the experimental solution. Inasmuch as the culture medium does not react with 5,5′-dithio-bis(2-nitrobenzoic acid), the experimental absorbance value minus the blank absorbance value is proportional to the BLT esterase activity. The total cellular content of the enzyme was determined using 0.1% Triton X-100-solubilized cells. Determinations were carried out in triplicates, and data are presented as the specific percent of enzymatic activity released, which was calculated from the following equation.

\[
\text{% release} = 100 \times \frac{(E - S)}{(T - S)} \quad \text{(Eq. 1)}
\]

The representative absorbance value in the supernatants of the experimental wells, S is the mean absorbance value in the supernatants of the wells containing no stimuli, and T represents the total enzyme content of BLT esterase in the CTLs.

Cytotoxicity Assay—CTL cytotoxicity was measured using a standard 3<sup>11</sup>Cr release assay (24). Briefly, target cells were labeled with 50 μCi of Na<sup>31</sup>CrO<sub>4</sub> for 1 h at 37 °C and then washed three times. 3<sup>11</sup>Cr-Labeled target cells (2.5 × 10<sup>4</sup>) were incubated with different numbers of CTLs as indicated according to effector/target (E/T) ratio. With or without Ado or Ado analogs in 200 μl of complete RPMI 1640 medium with 5% FCS, cells were incubated for 24 h at 37 °C in V-bottomed 96-well plates. Culture supernatants were harvested using the Skatron harvesting system (Skatron, Lier, Norway). Cytolytic activity, i.e. percentage of specific 3<sup>11</sup>Cr release, was calculated as follows.

\[
\text{% of specific } {3^{11}\text{Cr}} \text{ release} = 100 \times \frac{(E - S)}{(T - S)} \quad \text{(Eq. 2)}
\]

E, S, and T indicate experimental release, spontaneous release, and total incorporation, respectively.

Retargeting Assay—In this assay, the A20.2J cells (H-2<sup>d</sup>) were used as targets. Cells are normally not recognized and therefore not killed by POK.3.A8 CTLs (anti-H-2<sup>d</sup>); however, the binding of anti-CD3 mAb to Fc receptors on A20.2J cells results in CTL/A20.2J conjugation and TCR triggering of the CTLs, which is followed by activation of the CTLs and the lethal hit delivery to targets, so that the target cells become susceptible to lysis (25, 26).

Determination of FasL mRNA Expression by Competitive RT-PCR—

1 × 10<sup>5</sup> OE4 cells/sample were incubated with plate-bound anti-CD3 mAb 2C11 (5 μg/ml) alone or together with 50 μM extAdo with or without 1 μM ZM241385. Total RNA was prepared by the single-step method of Chomczynski and Sacchi (RNA STAT-60; Tel-Test “B”, Friendswood, TX). After DNase I treatment the first-strand cDNA was synthesized by the SuperScript preamplification system (Life Technologies, Inc.) according to the manufacturer’s instructions. The mouse FasL sequence was amplified with primers corresponding to 9–25 and 741–722 nucleotides of open reading frame (ORF) of mouse FasL cDNA (27). For primer usings were synthesized by Genosys (The Woodlands, TX). The double-strand competitor DNA for quantitation of FasL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by RT-PCR were prepared using the PCR MIMIC Construction Kit (CLONTECH Laboratories, Palo Alto, CA) according to the manufacturer’s instructions. The amplification efficiency of each competitor sequence did not show any significant difference from that of the corresponding target cDNA sequence (data not shown).

The competitive PCR was performed under standard conditions in a 30-μl reaction volume that included 3 μl of the diluted cDNA and 1.2 μl of competitor DNA. Ten μl of PCR product were electrophoresed on a 4% (w/v) NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) and examined by ethidium bromide staining. The density of each band was determined (ImageQuant version 3.3, Molecular Dynamics, Sunnyvale, CA) from the negative picture of the gel using the NIH Image software (no. 55/4 × 5 instant sheet film, Polaroid, Cambridge, MA). The densities of RT-PCR products were normalized for differences in cDNA quantity between samples using PCR quantitation of GAPDH mRNA. Primers for mouse GAPDH cDNA amplification were 290–309 and 1010–989 nucleotides of ORF.

Northern Blot Analysis of Ado Receptor mRNA Expression—The total RNA from murine lymphoid cells was extracted as above and poly(A) RNA was subsequently isolated using oligotex mini mRNA Mini Kit (Qiagen, Chatsworth, CA). The poly(A) RNA from 250 μg of total RNA was analyzed by standard Northern blotting procedure described previously (5).

RT-PCR Analysis of A<sub>2A</sub> Receptor—The first-strand cDNA was prepared from OE4 and 2B4 cells as described above. The mouse A<sub>2A</sub> receptor sequence was amplified with primers corresponding to 556–575 and 927–910 nucleotides of ORF of mouse A<sub>2A</sub> cDNA (28). A<sub>2A</sub> cDNA was a kind gift from Dr. D. L. Marquardt (University of California, San Diego) and used as a positive control.

CAMP Measurements—The effects of different treatments of lymphocytes on their CAMP content were tested by incubating 0.5–1.0 × 10<sup>5</sup> cells in triplicates with different combinations of Ado analogs and/or immobilized antibodies to CD3 or Thy-1 surface antigens. Levels of CAMP were measured using the CAMP EIA System (Amersham) according to the manufacturer’s protocol. Measurements were done in triplicate with standard deviations calculated.

Studies of T Cell Memory of Exposure to extAdo—The effect of extAdo pretreatment on anti-CD3 mAb (2C11)-induced CTL granule exocytosis was tested by preincubating cells for 30, 20, 10, 5, 1, or 0 min with 50 μM 2-CADO in 5% FCS-containing medium. Cells were washed and incubated for 4 h in triplicate in a V-bottomed 96-well plate with 2C11 mAb immobilized at 5 μg/ml, and granule exocytosis was calculated as described.

Effect of 2-CADO pretreatment on anti-CD3 mAb (2C11)-induced cell growth inhibition and IL-2 production of 2B4 cells (22) was tested by preincubating cells for 30, 20, 10, 5, 1, or 0 min with 50 μM 2-CADO in 5% FCS-containing medium in triplicate. Then cells were washed, incubated for 21 h with immobilized 2C11, and pulsed with 1 μCi of [H]thymidine, followed by an additional 6-h incubation at 37 °C, 5% CO<sub>2</sub> in the presence of 2C11. The amount of secreted IL-2 in supernatants of 2B4 was estimated by sandwich ELISA (Intertest-2X mouse IL-2 ELISA kit; Genzyme, Cambridge, MA).

RESULTS

extAdo Suppresses TCR-triggered Effector Functions of T Lymphocytes—CTL-mediated cytotoxicity involves several steps including early biochemical events of TCR-mediated signaling, conjugate formation, and lethal hit delivery (29). CTL-mediated cytotoxicity is explained by two complementary mechanisms. CTLs kill target cells (i) due to the exocytosis of perforin- and granzyme-containing cytotoxic granules (30) and (ii) due to FasL/Fas receptor interactions (31). These are two
completely different mechanisms that have in common only the requirement for TCR triggering. It was important to determine which of these pathways (or both) was susceptible to effects of extAdo. Different combinations of effector CTLs and target cells and the use of a granule exocytosis assay allowed us to use genetic controls to discriminate between Fas-mediated and granule exocytosis-based cytotoxicity. We demonstrated earlier that [cAMP]-elevating treatments of CTLs result in inhibition of both early and late biochemical events leading to CTL-mediated cytotoxicity and to exocytosis of cytolytic granules (32). However, in our earlier model studies, we did not use relatively low intensity extracellular signaling as a method of raising cAMP levels. Therefore, it was interesting to test whether extAdo-induced levels of cAMP will be sufficient to cause the inhibition of CTL functions if signaling through Ado receptors in peripheral T cells involves Gs protein-coupled and cAMP-raising A2A or the A2B subtypes of receptors (11).

We found that the presence of Ado analogs significantly inhibits TCR-triggered granule exocytosis (Fig. 1A), thereby providing the first demonstration of direct effects of extAdo analogs on cytotoxicity-mediating granule exocytosis in CTLs. As little as 1.0 mM NECA was able to inhibit granule exocytosis (Fig. 1A), and both NECA and 2-CADO were efficient in inhibition. Inhibition of exocytosis of cytotoxic granules in CTLs by Ado (Fig. 1A) was expected to result in the inhibition of target cell lysis, and this was demonstrated by testing the effect of extAdo on target cell lysis by CTLs (Fig. 1B). The CTL clone used was unable to lyse the nonspecific targets, as

**Fig. 1.** Effects of extAdo and nonhydrolyzable Ado analogs on T cell receptor-triggered granule exocytosis (A) and cytotoxicity against antigen-bearing target cells (B). A, dose-dependent inhibition of TCR-triggered granule exocytosis by 2-CADO (top left) and by NECA (top right). OE4 CTLs (1 x 10⁶ cells; 4th day after expansion) were incubated in triplicate for 4 h at 37 °C, 5% CO₂, with immobilized anti-CD3 mAb 2C11 (5 μg/ml) and indicated concentrations of Ado analogs. The percent of secretion of granule-located enzyme BLT-esterase was calculated as described under “Materials and Methods” according to a routinely used CTL activation granule exocytosis assay (23). The effects of most selective to date (34) A₂A adenosine receptor antagonist ZM241385 at 1 μM are presented in lower panel. These effects of ZM241384 on TCR-triggered granule exocytosis are statistically significant (p < 0.05) according to one-way analysis of variance with pairwise comparison by the Bonferroni method. In the control experiment, 1 μM ZM241386 alone did not have effect on exocytosis of TCR-triggered CTL. B, dose-dependent inhibition of CTL-mediated cytotoxicity by Ado and Ado analogs 2-CADO and NECA. OE4 cells (anti-H-2d) were incubated with ⁵¹Cr-labeled specific target A20.2J (H-2b) or nonspecific target EL4 (H-2b) for 4 h. The upper left graph presents the dependence of cytotoxicity on CTL (effector):target ratio. The upper right graph presents effects of different concentrations of Ado, 2-CADO, and NECA on cytotoxicity at a CTL:target cell ratio of 9:1. The inset in the upper right panel demonstrates the control for the antigen-specific nature of cytotoxicity by the CTL clone used here; CTL OE4 killed the antigen-bearing specific target A20.2J (open squares) but did not lyse antigen-nonbearing target EL4 (dark squares). Effects of selective A₂A agonist CGS21680 (0.1 and 50 μM) on lysis of antigen-specific target cells (A20.2J) by CTL clone OE4 are presented in the lower left panel. The degree of inhibition by 50 μM CGS21680 was compared with that of extAdo and 2-CADO in the lower right panel.
Adenosine A2A Receptor-mediated Signaling in T Cells

**Fig. 2.** extAdo inhibits T cell receptor-triggered FasL-mediated cytotoxicity (A) and FasL mRNA up-regulation (B). A, extAdo inhibits T cell receptor-triggered FasL-mediated cytotoxicity. Effects of extAdo and nonhydrolyzable Ado analog 2-CADO on cytotoxicity of perforin-deficient CTL were tested using CTL line P0P.C and Fas death pathway-susceptible target LB at a 7:1 ratio as described under "Materials and Methods." The combination of CTLs and targets used here allowed us to evaluate the effects of extAdo on Fas-mediated cytotoxicity, inasmuch as no perforin-mediated cytotoxicity is involved. Inset, 51Cr-labeled LB cells were incubated with 100 ng/ml anti-Fas mAb 2C11 with or without 5 mM Ado or 2-CADO for 4 h at 37°C, 5% CO2. The effect of extAdo and 2-CADO on anti-Fas mAb-induced death of LB cells was tested by incubation of Fas-sensitive cells with high concentrations of extAdo and 2-CADO to exclude the alternative explanation of the results as being due to a block in programmed cell death. B, extAdo inhibits T cell receptor-triggered FasL mRNA up-regulation. The competitive RT-PCR procedure for Fasl mRNA detection (see "Materials and Methods") was used to test the effects of extAdo on anti-CD3 mAb (2C11)-triggered FasL mRNA up-regulation. OE4 CTL clones were incubated with plate-bound 2C11 (5 μg/ml) alone (2C11), together with 50 μM Ado (2C11 + Ado) or with Ado and 1 μM ZM241385 (2C11 + Ado + ZM) for 2 and 4 h, and FasL expression was determined by competitive RT-PCR. Results of the competitive RT-PCR were calculated by measuring density of DNA bands in gels described under demonstrated by the control experiment (Fig. 1B, inset), thereby confirming that target cell lysis is antigen-specific and TCR-triggered. It was found that inclusion of Ado in the assay significantly inhibited target cell death. Of interest, not only Ado but also Ado analogs (2-CADO, NECA) were inhibitory, and up to 500 μM Ado was as efficient as nonhydrolyzable 2-CADO and NECA, suggesting that signaling, rather than Ado degradation (metabolism) products, is responsible for these effects. Selective A2A agonist CGS21680 at concentrations as low as 0.1 μM (Fig. 1B, lower left panel) also was able to inhibit the lysis of antigen-specific target cells (A20.2J) by CTL clone OE4. At such low concentrations, this agonist was shown to be highly selective for A2A receptor (33). The degree of inhibition by CGS21680 was comparable to that of extracellular adenosine and of 2-CADO even at higher concentrations of 50 μM (Fig. 1B, lower right panel).

The most selective to date (34) A2A adenosine receptor antagonist ZM241385 was also used to identify the A2A receptor in mediating effects of adenosine on T cells. It is shown (Fig. 1A, lower panel) that the presence of ZM241385 at 1 μM was sufficient to almost completely protect CTL even from the exposure to the highest tested (50 μM) concentration of 2-CADO. In a control experiment, ZM241385 alone at 1 μM did not have an effect on exocytosis of TCR-triggered CTL.

Thus, both antigen receptor-triggered exocytosis of cytotoxic granules and specific antigen-triggered T cell lysis were inhibited by extAdo. Although the effects of extAdo on processes in target cells undergoing perforin-induced death are still to be established, these data allow us to conclude that granule exocytosis-based CTL-mediated cytotoxicity is inhibited by extAdo. The inhibition of exocytosis was highly reproducible, but the degree of inhibition varied depending on the extent of TCR-triggered exocytosis in the particular assay and on the preparation of expanded CTL clones. Both nonhydrolyzable analogs 2-CADO and NECA were effective in inhibition of the protein synthesis-independent short term process of granule exocytosis, suggesting that these effects are due to signaling rather than to the interference into DNA synthetic pathways and dATP-triggered apoptosis (13).

Control experiments were performed to consider the possibility that the decrease in CTL viability by Ado and anti-CD3 mAb may account for the Ado-mediated inhibition of granule exocytosis and cytotoxicity. In controls, no changes in viability of CTLs were detected using trypan blue (data not shown). In addition, this possibility is discounted by results of flow cytometry (forward scatter versus side scatter) measurements of CTL viability, inasmuch as even at very high concentrations (e.g. 5 mM) of extAdo more than 70% of CTLs were viable and not apoptotic.

extAdo Inhibits Fas-mediated Cytotoxicity and TCR-triggered FasL Up-regulation—To study the susceptibility of Fas-mediated cytotoxicity and FasL/Fas cytotoxicity pathways to inhibition by Ado, we took advantage of the availability of Fas-expressing and Fas-mediated death pathway-sensitive targets and tested the lysis of such targets with a perforin-deficient CTLs that possesses only the FasL-based mechanism of cytotoxicity.

The CD4+ CTL line P0P.C cells used in the experiment described in Fig. 2A were derived from perforin-deficient mice; thus, the cytotoxicity of these CTLs depends only on the Fas-mediated pathway. It is shown (Fig. 2A) that extAdo and Ado analog 2-CADO were able to inhibit the cytotoxicity of P0P.C

"Materials and Methods." The level of Fasl mRNA expression is represented as percent of Fasl expression detected at time zero (right graph).
the CTL surface, and antibody/TCR links alone are sufficient for both the stable conjugate formation and CTL activation (25, 26). In contrast to normal conjugate formation, cell adhesion proteins are not needed for target lysis in a retargeting assay. Indeed, we established that extAdo inhibited FasL-mediated cytotoxicity of CTLs in a retargeting assay (data not shown), thus eliminating the first possibility.

It was also important to exclude the possibility that inhibition of Fas-mediated cytotoxicity was due to the interference of extAdo into Fas-triggered processes of programmed death of target cells. This was done in an experiment presented as an inset to Fig. 2A. The Fas-sensitive cells, the same LB target cells that were used as targets with CTLs in Fig. 2A, were incubated with apoptosis-inducing anti-Fas Jo2 mAb (20) in the presence or absence of extAdo or 2-CADO. We found that both extAdo and 2-CADO were unable to have an effect on the Fas-mediated death pathway in LB target cells, even at the very high concentration (5 mM). It is also interesting that even at these high concentrations, extAdo and 2-CADO did not have any lymphotoxic effect on their own (data not shown). Thus, the data suggest that the inhibition of Fas-mediated cytotoxicity is due to the inhibition of TCR-triggered FasL up-regulation.

Our attempts to test the expression of FasL on CTLs by flow cytometry using available anti-FasL antibody failed due to insufficient sensitivity of the assay (data not shown). Therefore, we developed the competitive RT-PCR procedure for FasL mRNA detection to test the effect of extAdo on FasL expression in CTLs (see “Materials and Methods”). The effects of extAdo on anti-CD3 mAb-triggered FasL mRNA expression in CTL OE4 (Fig. 2B) were tested by incubating these cells with CTL activating plate-bound anti-CD3 (2C11) alone, together with Ado (2C11 + Ado) or Ado and ZM214385 (2C11 + Ado + ZM) for 2 or 4 h (Fig. 2B). In these assays, we used two different competitive RT-PCR procedures to detect changes in expression of both FasL mRNA and of housekeeping GAPDH gene in the same samples (Fig. 2B). Measurement of this housekeeping gene mRNA expression was necessary, inasmuch as we found recently that the mRNA expression of the so-called housekeeping gene β-actin could be less stable than that of GAPDH during time-course studies of TCR-induced processes in T lymphocytes (35). Accordingly, the data obtained by competitive
RT-PCR of FasL mRNA were normalized and calculated using determinations of GAPDH in the same samples.

The presence of extAdo (50 μM) resulted in a dramatic (about 50%) inhibition of FasL mRNA expression in TCR-stimulated OE4 at 2 h (Fig. 2B). It is shown that highly selective A2A receptor antagonist ZM241385 (1 μM) completely prevented the extAdo-mediated inhibition of T cell receptor-triggered FasL mRNA up-regulation (Fig. 2B), thereby confirming the role of A2A receptors in these effects of extAdo. Interestingly, the expression of FasL mRNA in restimulated CTLs was about 3 times higher than in resting CTLs (data not shown).

extAdo Inhibits TCR-triggered IL-2 Secretion—Ado does not solely inhibit short term, protein synthesis-independent effector functions, inasmuch as a decrease in IL-2 secretion was observed after incubation of T cells with anti-TCR mAb (Fig. 3B and data not shown). The IL-2 measurements in these experiments were performed using ELISA instead of the bioassay to avoid possible artifacts due to the effects of “carried-over” extAdo and Ado analogs on detector CTLL cells. No effect of Ado on viability of cells were observed (data not shown), supporting the view that Ado interferes with IL-2 secretion due to Ado-mediated signaling and not because of the toxicity of Ado. In all of these experiments, the effects of Ado were mimicked by addition of dbcAMP.

Thus, practically all tested TCR-triggered effector functions, but not the Fas-triggered process of programmed cell death of targets, were inhibited by extAdo. These results are in agreement with our hypothesis that Ado receptor signaling could be the underlying mechanism of the immunosuppressive effects of increased concentrations of Ado.

T Cell Memory of Exposure to extAdo and to Ado Receptor-mediated Signaling—Even short term exposure to Ado was sufficient to inhibit TCR-triggered effector functions of T killer and T helper cells. These experiments were done to address conceptual difficulties with extrapolating the effects of extAdo on lymphocytes in vitro to the effects in vivo due to possible enzymatic degradation of extAdo by ADA activity. ADA-mediated enzymatic degradation could significantly shorten the half-life of extAdo and could thereby diminish its effect as an autocrine/paracrine lymphocyte regulator. To be of physiologic consequence, the effects of extAdo should be executed swiftly and be long-lasting. Our time-course studies of Ado receptor signaling through increases in [cAMP] suggested that increased levels of second messengers could persist after exposure to Ado (Fig. 5A and data not shown; see below). We therefore considered the possible existence of memory of exposure to extAdo. This prompted experiments that involved preincubation of cells with extAdo or Ado analogs, followed by removal of Ado and incubation of T cells in granule exocytosis and in lymphokine secretion assays. The question was whether brief exposure to extAdo would affect subsequent TCR-triggered T cell activation. It was found that preincubation of T cells with 2-CADO for 20 min resulted in approximately 50% inhibition of TCR-triggered granule exocytosis (Fig. 3A) as compared with preincubation in medium alone. Ten minutes preincubation was sufficient to observe significant inhibition. Similarly, as little as 20 min of exposure to extAdo resulted in significant inhibition of TCR-triggered IL-2 secretion after 21 h of incubation (Fig. 3B).

"Rescue" of extAdo-exposed T Cells from TCR-triggered Inhi...
bition of Proliferation—Fig. 4 describes results of an experiment that extends the effects of extAdo to include not only inhibition of different effector functions but also the rescue of T cells from TCR-triggered apoptosis and inhibition of proliferation. In these experiments, we took advantage of the well documented ability of TCR-triggered signaling to cause the inhibition of T hybridoma proliferation (36). These results illustrate two important properties of the effects of extAdo. First, memory of exposure to extAdo is confirmed here in yet another assay; as little as 10 min of exposure was sufficient to rescue more than 50% of T cells as compared with control (i.e. without anti-CD3 mAb). Second, the overall effect of extAdo (maintenance of T cell ability to proliferate) is not consistent with the widely accepted intracellular lymphocyte toxicity model of Ado action (2–4). Indeed, it is unlikely that extAdo can reverse TCR signaling and inhibition of proliferation by being toxic and/or by inhibiting DNA synthesis. Thus, the data presented above suggest that inhibition of TCR-triggered effector functions of T cells is probably due to Ado receptor-mediated signaling and resulting cAMP accumulation. Indeed, we have shown previously that the increases in cAMP are sufficient to inhibit CTL functions, including granule exocytosis (32), and it is likely that the memory of exposure to extAdo is due to a sustained increase in intracellular levels of cAMP.

Because effects of Ado on thymocytes and on functions of CTLs and T helper cells were mimicked by 3-isobutyl-1-methylxanthine, an inhibitor of cAMP phosphodiesterase, or by the addition of dbcAMP (32), we considered the effects of extAdo on CTLs and T helper cells as due to increases in intracellular cAMP that are triggered by Ado receptors. Exposure to extAdo on T Lymphocytes Results in A2A Purinergic Receptor-mediated Transmembrane Signaling and cAMP Accumulation—Exposure of thymocytes or of cloned CTLs to extAdo or to nonhydrolyzable Ado receptor agonists resulted in a rapid and sustained increase of intracellular cAMP (Figs. 5A and 6), although the degree of cAMP induction varied (see below). The increased levels were maintained for as long as 2 h and then decreased to control levels between the 2nd and 4th h of incubation. Extracellularly added Ado, 2-CADO, and NECA were all efficient in triggering cAMP accumulation (Figs. 5A and 6 and data not shown). These data exclude cAMP-level-decreasing A1 and A3 receptors as responsible for the cAMP-raising effects of extAdo in T cells. We also show that, according to pharmacologic criteria (37), A2A purinergic receptors are predominantly expressed and functionally coupled in T cells (Fig. 5A). OE4 CTLs were incubated with 10 μM CGS21680 in the presence or absence of 1 μM CSC, and cAMP levels were measured. The selective A2A receptor agonist CGS21680 (37) was found to induce, whereas selective A2A receptor antagonist CSC inhibited, cAMP accumulation in CTL clones. Similar data were obtained with mouse thymocytes, thymocyte clones, and T cells from spleen and lymph nodes (Fig. 5 and data not shown). These experiments suggest that a potential role for cAMP-level-raising A2B receptors in T cells is unlikely. The predominant expression of A2A receptors, which has been established in functional assays using selective agonists and antagonists of A2A receptors, is confirmed by Northern blot studies of A1, A2A, and A3 mRNA expression in 2B4 T helper cells (Fig. 5B). We show (Fig. 5B) that A2A receptor mRNA is predominantly expressed in T helper cells, whereas A1 and A3 mRNA are not detectable at all. The expression of A1 and A3 was, however, detectable in mRNA extracted from tissues used for positive controls (Fig. 5B, lanes 2 and 3). A2A receptor mRNA, but not A1 and A3 receptor mRNA, was detected by Northern blot studies in mouse thymocytes and T cells from spleen and lymph nodes (data not shown). The Northern blot procedure requires substantially more material than is available from the CTL clones used here. Therefore, RT-PCR of A2A mRNA expression was established to enable characterization of Ado receptors in samples from a limited number of cells and to study the regulation of A2A receptor mRNA expression in future. The RT-PCR data confirm the Northern blot detection of A2A receptor mRNA in T helper cells and demonstrate its expression in cloned CTLs (Fig. 5C).

Cross-talk between TCR and A2A-triggered Biochemical Pathways—Although the extAdo-triggered accumulation of cAMP was highly reproducible, the degree of cAMP induction strongly varied with individual preparations of CTLs. This is illustrated by the results of two independent time-course studies with different preparations of CTLs (Fig. 5A). We explain
this by the nature of the protocol: maintenance and expansion of CTL clones in vitro by periodic restimulation of their TCRs with an excess of antigen-bearing cells from irradiated spleens (18). This procedure did not allow us to reproducibly control the state of “residual” TCR activation in preparations of CTLs, thereby suggesting that the variability is due to cross-talk between TCR and Ado receptors in T cells. This view was supported by the results of experiments described in Fig. 6. We treated different T cells with nonhydrolyzable Ado analog 2-CADO alone or together with immobilized anti-CD3 mAb 2C11 or anti-Thy-1 to detect changes in cAMP. As shown, treatment of normal, untransformed T cells with anti-TCR/CD3 complex mAb resulted in the decrease of extAdo-triggered cAMP accumulation. Such effects of T cell treatment with anti-CD3 mAb were most pronounced in ex vivo splenocytes (Fig. 6A) and thymocytes (Fig. 6B), but a statistically significant decrease was also detected with CTL clones (Fig. 6C). Inhibition by anti-TCR/CD3 mAb was dose-dependent (data not shown). Interesting differences between splenocytes, thymocytes, CTL clone OE4, and transformed 2B4 tumor cells were revealed in this experiment. First, anti-Thy-1 mAb did not affect the outcome of subsequent extAdo signaling in thymocytes and OE4 CTLs, but anti-Thy-1 mAb was similar to anti-CD3 mAb in its effect on splenocytes, where the inhibition by anti-Thy-1 mAb was as effective as that of 5 μg/ml of 2C11; however, although inhibition by anti-CD3 mAb was dose dependent, the inhibiting effects of anti-Thy-1 antibody were not (data not shown). To date, there are no report of the anti-Thy-1 mAb used here promoting any T cell function. Second, in contrast to other tested cells, only OE4 CTLs increased their basal cAMP levels significantly during prolonged incubation in vitro. Third, at 5 μg/ml, 2C11 anti-CD3 mAb, but not anti-Thy-1 antibody, seemed to inhibit 2-CADO-induced cAMP accumulation on T cells but splenocytes. Finally, 2B4 tumor cells (Fig. 6D) were very different from all normal, untransformed T cells, because the 2-CADO-induced cAMP accumulation was significantly enhanced instead of being inhibited by anti-CD3 mAb in a dose-dependent manner (data not shown), with anti-Thy-1 mAb having no effect. These data present evidence of cross-talk between TCR-triggered and extAdo/A2A receptor-triggered biochemical pathways.

**DISCUSSION**

We show here that even short term exposure of T lymphocytes to extAdo has profound effects and results in the inhibition of T cell effector functions (Figs. 1–3). These immunosuppressive effects of extAdo in short term CTL activation assays are best explained by extAdo-mediated signaling through A2A Ado receptors and sustained increases in cAMP that, in turn, antagonize TCR-triggered signaling (Fig. 4). The ability of selective A2A agonist CGS21680 (Fig. 1B) to inhibit the lysis of antigen-specific target cells by CTL strongly supports the identification of A2A receptor as the major predominantly expressed receptor in T lymphocytes. Similarly, the ability of A2A adenosine receptor agonist ZM241385 to almost completely protect CTL from the inhibition by 2CADO (Fig. 1A) and to completely prevent extAdo-mediated inhibition of Fas Ligand up-regulation (Fig. 2C) provides strong pharmacological evidence that these effects of adenosine on CTL functions are mediated by signaling through A2A receptors.

In some experiments (e.g. Fig. 1A, NECA) adenosine analogs were efficient even at very low concentrations. The strong inhibition of CTL response (more than 50%) by 1 μM NECA is in agreement with signaling versus metabolic effects of extAdo on T cells. It appears that extAdo-triggered accumulation of cAMP is much more efficient in antagonizing the relatively low levels of TCR-triggered responses. This may explain the variability of inhibiting effects of adenosine on cloned T cells by differences in preparations of CTL during their in vitro maintenance and expansion.

The existence of cross-talk between A2A receptor- and TCR-triggered pathways is demonstrated in both directions, inasmuch as pretreatment of T cells with extAdo inhibited the subsequent TCR-triggered processes (Figs. 3 and 4), whereas treatment or pretreatment of T cells with TCR cross-link resulted in inhibition of extAdo-triggered signaling and cAMP accumulation (Fig. 6). The inhibitory effects of extAdo/A2A receptor signaling on T cells described above are in agreement with the large body of literature on cAMP-dependent protein kinase in T cells (38). Indeed, it is well documented by us and others that many processes in CTLs (22, 32) and in other T cells (38) are susceptible to inhibition by the cAMP-mediated intracellular pathway. It should be pointed out that the degree of A2A receptor cross-talk in TCR-activated T cells varied depending on the origin of the T cells. In addition, only the ex vivo splenocytes were susceptible to inhibition by anti-Thy-1 mAb (Fig. 6A).

It would be of interest to analyze these differences in anti-Thy-1 mAb effects between different tested T cells in future studies. 2B4 T hybridomas provided another notable exception, inasmuch as pretreatment of these cells with anti-CD3 mAb resulted in more than two times higher accumulation of cAMP (Fig. 6D). Thus, these experiments illustrate the differences between ex vivo and in vitro cultured T cells (Fig. 6, A–C) and between normal, untransformed and tumor T cells (Fig. 6D). These effects of TCR signaling on extAdo signaling may explain the variations in intensity of cAMP response between different preparations of CTLs that were observed in the time-course experiments described in Fig. 5A. It remains to be determined whether pretreatment with anti-TCR mAb causes down-regulation of A2A extAdo receptors or affects the coupling of A2A receptors with transmembrane signaling pathways in T cells. The TCR-triggered changes in A2A receptor expression would provide an attractive mechanism of T cell feedback regulation. In agreement with this hypothesis, we demonstrated recently that expression of G protein-coupled, P2Y2 purinergic receptor mRNA in murine thymocytes follows the pattern of immediate early genes (35). Because the expression of P2Y2 mRNA could be rapidly induced by steroid hormones or anti-TCR mAb in a protein synthesis-independent manner, this led to a proposal that purinergic receptors could serve in a mechanism of feedback regulation of T cell responses (35).

Even short term exposure to extAdo was sufficient to inhibit subsequent TCR-triggered granule exocytosis and IL-2 production by T cells (Fig. 3). The memory of exposure to extAdo by T cells is probably maintained by sustained increases in cAMP levels after exposure to A2A receptor-binding ligands and may effectively counteract the effects of ADA by prolonging the effects of extAdo. These experiments reconcile conceptual difficulties of extrapolating the effects of extAdo on lymphocytes in vitro to effects in vivo due to possible enzymatic degradation of extAdo by ADA activity.

The partial rescue of T cells from TCR-induced growth inhibition by 10-min pretreatment, as well as the complete rescue after 20- or 30-min pretreatment with extAdo, were observed in the experiment described in Fig. 4. These results pose a strong challenge to a prevailing model in which Ado action on T cells is due to intracellular lymphotoxicity. Indeed, it is more likely that the transmembrane signaling by extAdo and A2A receptor, rather than lymphotoxicity of deoxyadenosine or dATP, are responsible for these growth inhibition-reversing effects of extAdo. The rescue of T cells is most logically explained by an antagonism between TCR and extAdo-triggered, cAMP-medi-
Adenosine A2A Receptor-mediated Signaling in T Cells

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