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Incubation of purified C57BL/6 murine CD4+ T lymphocytes with anti-CD3 mAb serves as a model of TCR-mediated activation and results in increased IFN-γ production and cell surface expression of CD25 and CD69. We demonstrate here that signaling through the TCR causes a rapid (4-h) 5-fold increase in A2A adenosine receptor (AR) mRNA, which is correlated with a significant increase in the efficacy of A2AAR-mediated cAMP accumulation in these cells. A2AAR activation reduces TCR-mediated production of IFN-γ by 98% with a potency order of 4-[3-(6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-2-yl)prop-2-ynyl]cyclohexanecarboxylic acid methyl ester (ATL146e; EC50 = 0.19 ± 0.03 nM) > 4-[3-(6-amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-2-yl)prop-2-ynyl]piperidine-1-carboxylic acid methyl ester (ATL313; 0.43 ± 0.06 nM) > 5′-N-ethylcarboxamidoadenosine (3.5 ± 0.77 nM) > 2-[4-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680; 7.2 ± 1.4 nM) > N6-cyclohexyladenosine (110 ± 33 nM) > 2-chloro-N6-(3-iodobenzy)-5′-N-methylcarboxamide (390 ± 160 nM), similar to the potency order to compete for radioligand binding to the recombinant murine A2AAR but not the A3AR. The selective A2AAR antagonist, 4-[2-[7-amino-2-[2-furyl]1,2,4]triazolo[2,3-α][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM241385), inhibits the effect of ATL146e with a pA2 of 0.34 nM and also inhibits the effects of N6-cyclohexyladenosine and 2-chloro-N6-(3-iodobenzy)-5′-N-methylcarboxamide. In CD4+ T cells derived from A2AAR−/− and A2AAR+/+ mice, the IFN-γ release response to ATL146e is reduced by 100 and 50%, respectively, indicative of a gene dose effect. The response of T cells to the phosphodiesterase inhibitor, 4-(3′-cyclohexyl-3′-methoxyphenyl)-2-pyrrolidone (rolipram), is not affected by A2AAR deletion. We conclude that the rapid induction of the A2AAR mRNA in T cells provides a mechanism for limiting T cell activation and secondary macrophage activation in inflamed tissues. The Journal of Immunology, 2005, 174: 1073–1080.

The purine nucleoside adenosine is released by various cells, including fibroblasts, epithelial cells, endothelial cells, platelets, and muscle cells (1–4) or is derived from the extracellular metabolism of released purine nucleotides (5). Adenosine levels are elevated during conditions of hypoxia (6), muscle exercise (7), inflammation, or adenosine deaminase (ADA)3 deficiency. Extracellular adenosine initiates transmembrane signaling through four subtypes of G protein-coupled adenosine receptors (AR), A1, A2A, A2B, and A3 (8). The expression of the A2AAR, A2BAR, and A3AR on human (9) and mouse (10) T lymphocytes has been demonstrated, and signaling through these three adenosine receptors has been implicated in the regulation of various TCR-mediated events.

Normally, the recognition of Ag by the TCR complex initiates a cascade of signaling events resulting ultimately in T cell activation, as manifested by the synthesis and secretion of cytokines such as IFN-γ and IL-2, cellular cytotoxicity, and T cell proliferation. However, TCR-mediated IL-2 production (11), CD25 and CD69 expression (12), granule exocytosis, Fas ligand up-regulation (13), and cell proliferation (11) are modulated by the activation of cell surface adenosine receptors. Extracellular adenosine-triggered cAMP accumulation (14) and inhibition of activation-induced CD25 expression (15) were reported to be mediated by the A2AAR. However, a conflicting report suggests that the A2BAR is responsible for the accumulation of cAMP, as well as the inhibition of TCR-triggered IL-2 production, in T lymphocytes (16). Inhibition of killer T cell activation by adenosine has been attributed to signaling through the A3AR (10). Signaling through ARs has multiple and varied effects on virtually all cells of the immune system, including neutrophils, monocytes, macrophages, T lymphocytes, and mast cells (reviewed in Refs. 17–20). Agents or physiological conditions that generate an inflammatory response from these cells have been found to influence AR expression. Exposure to LPS, IL-1, or TNF-α triggers an up-regulation of A2AAR mRNA and protein in the human monocytic cell line THP-1 (21, 22). Ischemia-reperfusion injury down-regulates A3AR, and induces A2BAR, transcript in 2- to 4-mo-old C57BL/6 mouse heart (23), and the reactive oxygen-generating agent, cisplatin, up-regulates A3AR expression in the testes of Sprague Dawley rats and in DDT1MF2 smooth muscle cells (24, 25). The time course and extent of effect on AR expression varies with cell type and condition.

In this study, we sought to investigate the effect of TCR-mediated activation of CD4+ T cells on AR expression and to further evaluate the role of AR signaling in the regulation of CD4+ T cell activity. We demonstrate that signaling through the TCR causes a rapid increase in A2AAR but not A2BAR mRNA, which is accompanied by an increased efficacy of the A2AAR agonist, ATL146e, to

3 Abbreviations used in this paper: ADA, adenosine deaminase; AR, adenosine receptor; NECA, 5′-N-ethylcarboxamidoadenosine; CI-IB-MECA, 2-chloro-N6-(3-iodobenzy)-5′-N-methylcarboxamide; CPA, N6-cyclohexyladenosine.
mediate cAMP accumulation. Furthermore, activation of the A2A receptor, but not the A3 receptor, on CD4+ T cells counteracts the ability of TCR activation to stimulate IFN-γ production, an integral event in CD4+ T cell-driven inflammatory responses.

Materials and Methods

Reagents

ADCA was purchased from Roche. 5′-N-Ethylcarboxamidoadenosine (NECA), 2-[(4-carboxyethyl)phosphonamido]-5′-N-ethylcarboxamido-adenosine (CGS21680), 2-chloro-N(3-iodobenzyl)-5′-N-ethylcarboxamidoadenosine (CI-IB-MECA), and 5′-N-cyclohexyladenosine (CPA) were purchased from Sigma-Aldrich. 4-[3-(Cyclopentonyloxy)-4-methoxyphenyl]-2-pyridinethione (rolipram) was a gift from Berlex. 4-[3-(6-Amino-9-(5-ethylcarbamoyl)-3,4-dihydroxybutyryl-a-2y1-9H-purin-2-yl)-2-propyl]-1-2-2-propyl-1-y1]cylohexaneacarbonyl acid methyl ester (ATL146e) and 4-[3-(6-Amino-9-(5-cyclopentylcarbamoyl)-3,4-dihydroxybutyryl-a-2y1-9H-purin-2-yl)-2-propyl]-1-2-2-propyl-1-y1]piperidine-1-carboxylic acid methyl ester (ATL313) were gifts from Adenosine Therapeutics. 4-[2-[7-Amino-2-[2-furyl][1,2,4]triazolo[2,3-b]pyridin-5-yl]amino]ethyl)phenol (ZM241385) was purchased from Torc. A2AR knockout mice were a gift from Dr. J.-F. Chen of Boston University (Boston, MA).

Creating A2AAR knockout mice congenic to C57BL/6

The knockout locus of B6;129P-adora2a+/- mice with an a2AAR gene on a mixed genetic background (26) was moved to a C57BL/6 background by monitoring 96 microsatellites for five generations of marker-assisted breeding. In the resulting mouse line, DNA derived from the 129 strain can be detected only in an 8-cM region between D10Mit31 and D10Mit42 surrounding the adaora2a locus on chromosome 10.

T lymphocyte isolation and activation

Purified CD4+ T cells were washed and resuspended in PBS at 5 × 10^6 cells/ml. Aliquots (0.1 ml) were placed in ice and labeled for 30 min in the dark with PE-conjugated anti-CD4 (BD Biosciences) or FITC-conjugated anti-CD4 (eBioscience). After 30 min, the samples were washed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic (In VitroGen). The fluorescence intensity was measured with a BD Biosciences FACS-Vantage SE Turbo Sorter (BD Biosciences) to produce a cell population (92%) that was >99.8% pure CD4+ T cells. Stained T cells were washed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic (In VitroGen). T cells were activated by incubation in 96-well plates coated with 2–20 μg/ml immobilized anti-CD3 mAb (BD Biosciences) at 37°C in 5% CO2.

Flow cytometry of T cell activation markers

For some experiments, the column-purified cells were then stained for 30 min with FITC-conjugated anti-mouse CD4 (eBioscience) and then sorted using a FACSVantage SE Turbo Sorter (BD Biosciences). For some experiments, the column-purified cells were then stained for 30 min with FITC-conjugated anti-mouse CD4 (eBioscience) and then sorted using a FACSVantage SE Turbo Sorter (BD Biosciences) to produce a cell population (>99.8% pure CD4+ T lymphocytes). Unlabeled purified CD4+ T cells were activated by incubation in 96-well plates coated with 2–10 μg/ml immobilized anti-CD3 mAb (BD Biosciences) at 37°C in 5% CO2.

Radioligand binding assays

Membranes from HEK-293 cells stably expressing the mouse A1AR, A2AR, or A3AR were used for competition binding assays with [3H]-NECA, [3H]-IB-MECA, and [3H]-IB-MECA. The membranes were washed and incubated with a 100 nM NECA buffer (10 μM HEPES and 1 mM EDTA (pH 7.4)) supplemented with 2 U/ml ADA and 5 mM MgCl2. Nonspecific binding was measured in the presence of 100 μM NECA. The incubation time was 120 min at room temperature. Membranes were filtered on Milipore MultiScreen assay system 96-well filtration plates and washed three times with ice-cold buffer (10 mM Tris, 1 mM MgCl2. (pH 7.4)) using a Brandel 96-well plate washer. IC50 values were calculated using GraphPad Prism, and Kd values were determined as described by Scatchard plots.

Radioimmunoassay for intracellular cAMP

The A2AR transcript in a column-purified population of CD4+ T lymphocytes was measured using the chemiluminescent immunoassay system for the quantitation of A2AR from mammalian cells, CAMP-Screen System, according to the manufacturer’s protocol (Applied Biosystems).

Results

CD4+ T lymphocyte activation induces A2AR mRNA expression

As a model of TCR-mediated activation, purified murine CD4+ T cells were incubated on immobilized anti-CD3 mAb for 24 h. This resulted in an approximate 150-fold increase in IFN-γ production, an integral event in CD4+ T cell-driven inflammatory responses (26). When a highly purified population of CD4+ T lymphocytes (>92% CD4+ T cells) peaked after 4 h of incubation on immobilized anti-CD3 mAb, with an approximate 10-fold increase in transcript over controls, and returned to baseline after 16 h (Fig. 1a). When a highly purified population of CD4+ T lymphocytes (>99.8% CD4+ T cells) was used, the induction of A2AR transcript was again found to peak after 4 h of activation, with a 5-fold increase over resting levels. A2AR mRNA levels in this cell population also returned to baseline by 16 h of activation (Fig. 1a).

Although A2AR mRNA was also detected in both populations of purified CD4+ T cells, no induction of transcript was observed.
experiments performed in triplicate.

Figure 1. Effect of TCR-mediated T lymphocyte activation on A2AAR expression. Murine splenocytes were column purified to result in cell populations of \( \geq 99.8\% \) pure CD4\(^+\) T cells. As indicated, column-purified populations of CD4\(^+\) T cells were then further purified using a FACSVantage SE Turbo Sorter to result in cell populations of \( \geq 99.8\% \) pure CD4\(^+\) T cells. Cells were either collected immediately after purification or incubated at the indicated time points for 10 min with 1 U/ml ADA in the presence of vehicle or 1 \( \mu M \) rolipram. Intracellular cAMP accumulation was measured using a chemiluminescent immunoassay system. Data shown are the mean ± SEM from three independent experiments performed in triplicate.

A2AAR expression. Murine splenocytes were column purified to result in cell populations of \( \geq 99.8\% \) pure CD4\(^+\) T cells. As indicated, column-purified populations of CD4\(^+\) T cells were then further purified using a FACSVantage SE Turbo Sorter to result in cell populations of \( \geq 99.8\% \) pure CD4\(^+\) T cells. Cells were either collected immediately after purification or incubated at the indicated time points for 10 min with 1 U/ml ADA in the presence of vehicle or 1 \( \mu M \) rolipram. Intracellular cAMP accumulation was measured using a chemiluminescent immunoassay system. Data shown are the mean ± SEM from three independent experiments performed in triplicate.

The increase in A2AAR mRNA expression observed in the \( \geq 99.8\% \) pure CD4\(^+\) T cell population after 4 h of activation was correlated with an approximate 2-fold increase in the efficacy of the selective A2AAR agonist, ATL146e, to stimulate cAMP accumulation. This increased efficacy was maintained for up to 24 h of activation (with a \( \sim 1.5\)-fold increase over control cells at 24 h), indicating that the up-regulation of the expression of functional receptor is relatively stable, compared with the more transient increase in A2AAR transcript. cAMP accumulation in response to 1 \( \mu M \) of the type IV phosphodiesterase inhibitor, rolipram, in the absence of ATL146e, did not change in cells as a result of TCR activation (Fig. 1c).

TCR-mediated IFN-\( \gamma \) production is inhibited by adenosine analogs

The activation of CD4\(^+\) T cells in the presence of various adenosine analogs resulted in a dose-dependent inhibition of TCR-mediated IFN-\( \gamma \) production (Fig. 2a). AR agonists that were found to be selective for recombinant murine A1AR, A2AAR, or A3AR (Table I) were used to inhibit IFN-\( \gamma \) production with a rank order of potency consistent with a response mediated by the A2AAR. The A2AAR-selective agonists, ATL146e, ATL313, and CGS21680, inhibited IFN-\( \gamma \) production with EC\(_{50}\) values of 0.19 ± 0.02, 0.43 ± 0.06, and 7.2 ± 1.4 nM, respectively. NECA, a nonselective agonist, inhibited IFN-\( \gamma \) production with an EC\(_{50}\) value of 3.5 ± 7.7 nM, whereas the A1AR- and A3AR-selective agonists, CPA and CI-IB-MECA, demonstrated inhibitory effects only at substantially higher doses (Table II). As shown in Fig. 2b, the potency of agonists to inhibit IFN-\( \gamma \) accumulation in T cells was well correlated with binding affinity to murine A2AAR but not A1AR, A3AR. In addition to inhibiting activation-induced IFN-\( \gamma \) production, treatment with 100 nM A2AAR agonist ATL146e was shown to attenuate the TCR-triggered CD25 (Fig. 3a) and CD69 (b) expression. This effect was mimicked by 10 \( \mu M \) rolipram.

Selective A2AAR antagonist ZM241385 inhibits the effect of ATL146e on IFN-\( \gamma \) production

To further confirm that adenosine acts through the A2AAR to inhibit TCR-mediated IFN-\( \gamma \) production, CD4\(^+\) T cells were activated in the presence of ATL146e with or without 2.5 nM selective A2AAR antagonist ZM241385. Based upon the right shift in the ATL146e dose-response curve for inhibition of IFN-\( \gamma \) production, the \( K_i \) for ZM241385 was calculated to be 0.34 ± 0.08 nM (Fig. 4a), which is similar to the \( K_i \) value of 0.50 ± 0.25 nM for binding of \(^{125}\)I-radiolabeled ZM241385 to recombinant murine A2AAR (Fig. 4b). Additionally, the effects of 5 \( \mu M \) IB-MECA or 5 \( \mu M \) CPA to inhibit IFN-\( \gamma \) production in activated T cells was blocked competently by 100 nM ZM241385 (Fig. 4c), indicating that, at high concentrations, these agonists exert an inhibitory effect via signaling through A2AAR, and not through A1AR or A3AR.

There is a gene-dose effect in A2AAR-mediated inhibition of IFN-\( \gamma \) production

Purified CD4\(^+\) T cells were collected from age-matched C57BL/6 mice with A2AAR\(^{-/-}\), A2AAR\(^{+/+}\), or A2AAR\(^{+/+}\) genotypes. The inhibitory effect of ATL146e on IFN-\( \gamma \) production was abolished in A2AAR\(^{-/-}\) cells and attenuated by \( \sim 50\% \) in A2AAR\(^{+/+}\) cells compared with the A2AAR\(^{+/+}\) wild-type controls (Fig. 5).

cAMP-elevating agent, rolipram, mimics the inhibitory effect of A2AAR agonists on TCR-mediated IFN-\( \gamma \) production

By initiating signaling through the G\(_i\)-coupled A2AAR, ATL146e triggers intracellular cAMP accumulation. The type IV phosphodiesterase inhibitor, rolipram, also elevates intracellular cAMP.
A3AR (data were derived from Tables I and II).

The rapid induction of A2AAR transcript in CD4+ T cells collected from wild-type C57BL/6 mice (Fig. 6A). The inhibitory effect of rolipram on TCR-mediated IFN-γ production was retained in CD4+ T cells deficient for the A2AAR (Fig. 6B). This demonstrates that deletion of the A2AAR gene did not modify their phenotype to alter their response to cAMP accumulation. Additionally, the inhibitory effect of a suboptimal dose of ATL146e (0.01 nM) on IFN-γ production was markedly enhanced by the addition of a suboptimal dose of rolipram (data not shown), which is consistent with previous findings that ATL146e acts synergistically with rolipram to mediate intracellular cAMP accumulation.

Discussion

This study demonstrates that the A2AAR is rapidly induced in CD4+ T cells upon TCR activation, and that activation of the A2AAR on CD4+ T cells inhibits IFN-γ release. Macrophages require two signals for activation, IFN-γ and a secondary signal (such as CD40L-CD40 interaction) that sensitizes the cells to IFN-γ. CD4+ Th1 cells can deliver both of these signals. The activation of macrophages is the primary effector function of armed Th1 cells, and therefore IFN-γ is often characterized as the most important effector molecule synthesized by Th1 cells (28–30). Although the activity of macrophages is vital for host response to pathogens, such activity results also in localized tissue destruction and large energy consumption. It is therefore necessary that the activation of macrophages be tightly regulated. This is accomplished by mechanisms controlling the synthesis and secretion of IFN-γ. Two such mechanisms are the rapid destruction of IFN-γ mRNA and the targeted focal delivery of IFN-γ by Th1 cells (31, 32). The results of this study suggest that the activation of A2AAR in CD4+ T cells controls the production of IFN-γ, and this response contributes to the regulation of macrophage activation.

Extracellular adenosine initiates signaling through four subtypes of G protein-coupled ARs, the Gs-coupled A2AAR and A2BAR and the Gi-coupled A1AR and A3AR. Through the use of quantitative RT-PCR, we demonstrate both the presence of A2AAR and A2BAR mRNA in purified resting murine CD4+ T cells, as well as the rapid induction of A2AAR transcript in CD4+ T cells that have been activated via signaling through the TCR. Because the A2AAR is expressed by, and up-regulated in, various cell types (21, 22, 33), it is significant that in our experiments we used a highly purified population of CD4+ T cells (≥99.8% pure). This, coupled with the fact that our activating stimulus (immobilized anti-CD3 mAb) is presumed to be specific for T lymphocytes, supports our assertion that the increase in A2AAR transcript as measured by RT-PCR is indeed a result of the TCR-mediated activation of CD4+ T cells rather than a contaminating population of cells. We hypothesize that the increased induction of A2AAR mRNA observed when a less pure (≥92%) population of CD4+ T cells is used may be due to the fact that these cells are in an enhanced state of activation due to interactions with other inflammatory cells (e.g., macrophages) or inflammatory cell-secreted cytokines in the culture. It is notable that TCR activation caused induction of A2AAR mRNA but failed to induce A2BAR mRNA. Both AR subtypes are coupled to Gs and cAMP accumulation and may participate in regulating T cell activation. The present study does not address the possible role of A2BAR activation in regulating CD4+ T cell function in response to adenosine, but the results clearly demonstrate that the responses to ATL146e are mediated entirely by activation of the A2AAR because they are blocked by ZM241385 and are absent in A2AAR knockout mice.

In this study, we report an induction of A2AAR transcript that is relatively rapid and transient (peaking at 4 h of activation and 0.48).
returning to baseline by 16 h). We also demonstrate that the selective A2AAR agonist ATL146e mediates intracellular cAMP accumulation in murine T cells, as has been shown previously with the less potent A2AAR agonist CGS21680 (14), and that an increase in A2AAR mRNA expression after TCR activation is correlated with an increase in the efficacy of ATL146e to stimulate cAMP in these cells. Furthermore, this increased efficacy is maintained through 24 h of activation, indicating that, although A2AAR transcript levels have returned to baseline levels at this point, there still exists an increased expression of functional receptor compared with control cells. We hypothesize that extracellular adenosine acts upon these up-regulated receptors to inhibit IFN-γ production by CD4+ T cells in conditions of inflammation. Although the local concentrations of adenosine, and the kinetics of adenosine accumulation, at sites of inflammation have not been thoroughly investigated, it has been observed that, under conditions of hypoxia in the rat brain (34) and heart (35), extracellular adenosine concentrations increase to the 10–20 μM range within a matter of minutes, with the extent of accumulation increasing with the duration of hypoxic event. Thus, the early peak in A2AAR expression may correspond with a rapid accumulation of extracellular adenosine in inflamed tissues. Correspondingly, the sustained increase in A2AAR function (~1.5-fold above baseline levels at 24 h) allows for activation of the A2AAR to function as a mechanism by which to regulate T lymphocyte responses during situations in which adenosine concentrations at sites of inflammation may not become elevated until after more considerable tissue damage has occurred.

Our results are distinct from previous reports of A2AAR induction in several ways. The peak magnitude of induction that we observe is greater than the ~2-fold increase in receptor expression observed in monocyte THP-1 cells after exposure to IL-1, TNF-α (22), or LPS (21). Additionally, the time course of mRNA induction that we report in TCR-activated murine CD4+ T cells is different from that of THP-1 cells exposed to IL-1 or TNF-α; we observe that transcript levels return to baseline by 16 h, whereas Khoa et al. (22) observe a sustained induction through 18 h of cell treatment. Similarly, it has been demonstrated that hypoxia induces a ~3-fold increase in A2AAR mRNA (and a 2-fold increase in A2AAR protein) in pheochromocytoma cells, and this induction is maintained through 18 h of hypoxia (33). The data from our experiments and others (21, 33, 36) indicate that the modulation of AR expression varies with cell type and stimuli.

The binding of bacterial or viral Ag or superantigen to the TCR on CD4+ T cells initiates a signaling cascade that leads ultimately to the activation of various transcription factors, such as NF-AT, AP-1, and NF-κB, which are known to induce the transcription of genes encoding cytokines, chemokines, and adhesion molecules, as well as multiple other genes that are involved in cell proliferation and differentiation (37–39). Although TCR-mediated activation of and cytokine production by CD4+ T cells is a vital component of the adaptive immune response, it is imperative that this response be rigorously controlled because unregulated activation may result in tissue damage. Our results suggest that the up-regulation of the A2A AR in response to TCR-mediated activation may act as an endogenous negative feedback mechanism for CD4+ T lymphocyte-driven inflammatory responses. There is a combined NF-AT/AP-1 binding site in the promoter of the IFN-γ gene (40–42), and although the regulation of A2A AR transcription by this transcriptional element complex has not been clearly demonstrated, there are putative regulatory elements for AP-1 as well as NF1 and AP-4 (43). It can therefore reasonably be hypothesized that, upon CD4+ T cell activation, common transcription factors may mediate the coordinated transcription of proinflammatory cytokines (such as IFN-γ) and the A2A AR as a homeostatic mechanism for controlling cytokine production.

Consistent with this hypothesis and through the use of AR subtype-selective agonists and antagonists, we show that adenosine

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<th>EC50 values of agonists to inhibit IFN-γ production in CD4+ T cellsa</th>
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<td>Mean EC50 (nM ± SEM)</td>
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<tr>
<td>ATL146e</td>
<td>0.19 ± 0.03</td>
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<td>ATL313</td>
<td>0.43 ± 0.06</td>
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<tr>
<td>NECA</td>
<td>3.5 ± 0.77</td>
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<tr>
<td>CGS21680</td>
<td>7.2 ± 14</td>
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<tr>
<td>CFA</td>
<td>110 ± 33</td>
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<tr>
<td>CI-IB-MECA</td>
<td>390 ± 160</td>
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a Data are the mean ± SEM of three independent experiments performed in triplicate.

FIGURE 3. Expression of CD25 and CD69 on murine CD4+ T cells. Column-purified murine CD4+ T cells were incubated for 24 h in the presence or absence of 2–10 μg/ml immobilized anti-CD3 mAb, 100 nM ATL146e, and/or 1 μM rolipram. Cell surface expression of CD25 (a) and CD69 (b) was assessed by FACS using PE-conjugated anti-CD25 mAb and FITC-conjugated anti-CD69. Data are shown as mean ± SEM from three independent experiments performed in duplicate. *p < 0.05 vs activated control.
FIGURE 4. Blockade by ZM241385 of the inhibitory effect of adenosine agonists on IFN-γ production. a. Column-purified murine CD4+ T cells were incubated on immobilized anti-CD3 mAb with 1 U/ml ADA and ATL146e in the presence or absence of 2.5 nM ZM241385. Supernatants were collected after 24 h, and IFN-γ concentrations were determined by ELISA. Data shown are from a single experiment performed in triplicate, representative of three independent experiments.

analogs inhibit TCR-mediated IFN-γ production in murine CD4+ T cells via the activation of the A2AAR. The selectivity of each analog for a given murine AR subtype was characterized by competition binding assays, which revealed that CPA and CI-IB-MECA are highly specific for the mouse A1AR and A3AR, respectively, whereas ATL313 and CG21680 demonstrate 10- to 100-fold greater selectivity for the A2AAR than for the A1AR or A3AR subtypes. However, the binding affinities of ATL146e at the mouse A2AAR and A3AR were found to be approximately equal.

To address this issue, the competitive and selective A2AAR antagonist ZM241385 was used to block the inhibitory effect of ATL146e and provide further substantiation that the agonist acts through the A2AAR to inhibit IFN-γ production. Additionally, the inhibitory effect exerted by high concentrations of CPA and CI-IB-MECA was blocked by 100 μM ZM241385 (a concentration that selectively blocks the A2AAR), ruling against the involvement of the A1AR or A3AR as important acute regulators of IFN-γ release from CD4+ T cells. Furthermore, it was determined that ATL146e has no inhibitory effect on IFN-γ production by CD4+ T cells collected from A2AAR-deficient mice. The observations that adenosine analogs inhibit IFN-γ production with a rank order of potency that is characteristic of a response mediated by the A2AAR along with the ability of ZM241385 to attenuate this effect are consistent with the assertion that signaling through the A2AAR mediates an inhibitory effect on TCR-triggered IFN-γ production. Moreover, the presence of a gene dose effect indicates that there is no receptor reserve for A2AAR-mediated inhibition of IFN-γ production, consistent with an earlier finding that the decrease in the functional cAMP response of T cells to adenosine (44).

We show that A2AAR activation mediates cAMP accumulation in murine CD4+ T cells, and it is known that cAMP-elevating agents have an inhibitory effect on several TCR-mediated events, including the production of the Th1 cytokine IFN-γ. Correspondingly, we show that adenosine analogs inhibit TCR-mediated IFN-γ production in murine CD4+ T cells via activation of the A2AAR. Three additional pieces of evidence support the hypothesis that it is the cAMP-elevating activity of adenosine that inhibits IFN-γ production. The inhibitory effect of A2AAR activation is mimicked by rolipram, the inhibitory effect of a suboptimal dose of ATL146e is significantly enhanced by the addition of a suboptimal dose of rolipram, and the inhibitory effect...
of rolipram on IFN-γ production is retained in A2AAR-deficient cells. In fact, A2AAR agonists are known to act through cAMP-mediated pathways to decrease the oxidative burst, inhibit the re-regulator of CD4 macrophage-damaged tissue serves to inhibit further CD4 T lymphocytes driven inflammatory responses, which may progress through a similar signaling pathway. The results of this study indicate that the activation of CD4+ T cells via signaling through the TCR results in the rapid up-regulation of A2AAR expression. Furthermore, signaling by extracellular adenosine through the A2AAR may act as an endogenous regulator of CD4+ T lymphocyte-driven inflammatory responses, forming a feedback loop wherein the adenosine released from macrophage-damaged tissue serves to inhibit further CD4+ T lymphocyte activity and ultimately macrophage activity.

Acknowledgments

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