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Inflammatory Cytokines Regulate Function and Expression of Adenosine A_{2A} Receptors in Human Monocytic THP-1 Cells[1] FREE

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Inflammatory Cytokines Regulate Function and Expression of Adenosine A_{2A} Receptors in Human Monocytic THP-1 Cells[1]

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Adenosine, acting at its receptors, particularly A_{2A} receptors, is a potent endogenous anti-inflammatory agent that modulates the functions and differentiation of inflammatory and immune cells. Because the inflammatory milieu abounds in proinflammatory cytokines, we investigated the effects of Th1-inflammatory cytokines on function and expression of adenosine A_{2A} receptors in the human monocytic cell line THP-1. We found that, consistent with previous reports, adenosine and 2-[*p*-(2-carnonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS-21680), a selective A_{2A} receptor agonist, suppress IL-12 production but increase IL-10 production in LPS-activated THP-1 cells. These effects were blocked by the A_{2A} receptor antagonist 4-{2-[7-amino-2-(2-furyl)[1,2,4-triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl}phenol (ZM-241385). More importantly, the suppressive effect of adenosine and CGS-21680 on IL-12 production was significantly enhanced in cells pretreated with either IL-1 (10 U/ml) or TNF-α (100 U/ml) but markedly attenuated in cells pretreated with IFN-γ (100 U/ml). Similarly, IL-1 and TNF-α treatment potentiated the stimulatory effect of adenosine and CGS-21680 on IL-10 production, whereas IFN-γ treatment almost completely abolished this effect. CGS-21680 stimulated an increase in intracellular cAMP in a time- and dose-dependent manner in IL-1- and TNF-α-treated cells but not in control or IFN-γ-treated cells. Both IL-1 and TNF-α increased A_{2A} receptor mRNA and protein. In parallel with its effect on A_{2A} receptor function, IFN-γ down-regulated A_{2A} receptor message and protein. Because adenosine mediates many of the antiinflammatory effects of drugs such as methotrexate, these observations suggest that local changes in the cytokine milieu may influence the therapeutic response to those drugs by altering the expression and function of adenosine receptors on inflammatory cells. ***The Journal of Immunology*****, 2001, 167: 4026–4032.**

Adenosine, released by cells and tissues, is a potent endogenous regulator of inflammation. Adenosine diminishes the proinflammatory actions of inflammatory and immune cells via interaction with specific cell surface receptors, of which there are four known subtypes, A_1, A_{2A}, A_{2B}, and A_3 (see reviews in Refs. 1 and 2). Recent reports also indicate that adenosine, acting at its receptors, modulates the anti-inflammatory action of antirheumatic drugs such as methotrexate (3).

Monocytes and macrophages synthesize and release into their environment a variety of cytokines and other proteins that play a central role in the development of acute and chronic inflammation. There have been some lines of evidence suggesting a regulatory connection between adenosine and its receptors and inflammatory cytokines. In human and murine monocytes/macrophages, the activation of adenosine receptors, particularly A_{2A} receptors, by adenosine or it analogues modulates the production of inflammatory cytokines including TNF-α, IL-10, and IL-12 (4–8). IL-12, a proinflammatory cytokine and a central inducer of Th1 responses and cell-mediated immunity, is suppressed by adenosine and its analogues, whereas secretion of IL-10, a protective cytokine that suppresses IL-12 and TNF-α release, is enhanced by adenosine and A_{2A} receptor agonists both in vitro and in vivo (6–9).

In contrast, other lines of evidence have suggested that the expression and functions of adenosine receptors may be regulated by numerous endogenous factors involved in inflammation and in cellular growth and differentiation such as glucocorticoids (10, 11), growth factors (12, 13), and other cytokines. For example, Xaus et al. (14) reported that IFN-γ up-regulates expression of A_{2B} receptors and promotes macrophage activation. The effects of IFN-γ and other prominent inflammatory cytokines such as IL-1 and TNF-α on expression and function of A_{2A} receptors, however, have not been documented.

We therefore investigated the effects of IL-1, TNF-α, and IFN-γ, the most prominent Th1-inflammatory cytokines in rheumatoid arthritis and other inflammatory diseases, on function and expression of adenosine A_{2A} receptors. We found, to our surprise, that IL-1, TNF-α, and IFN-γ all modulate the effects of A_{2A} receptor occupancy on secretion of IL-10 and IL-12 as well as cellular accumulation of cAMP in human monocytic THP-1 cells. In accord with their effects on A_{2A} receptor function, IL-1, TNF-α, and IFN-γ also regulate expression of A_{2A} receptors in THP-1 cells.

Materials and Methods

Reagents

Human rIL-1α, TNF-α, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). The A_{2A} receptor agonist 2-[*p*-(2-carnonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS-21680)[3] was purchased from Sigma (St. Louis, MO). The A_{2A} receptor antagonist 4-{2-[7-

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[3] Abbreviations used in this paper: CGS-21680, 2-[*p*-(2-carnonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; ZM-241385, 4-{2-[7-amino-2-(2-furyl)[1,2,4-triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl}phenol; MAP, mitogen-activated protein.

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amino-2-(2-furyl)[1,2,4-triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl} phenol (ZM-241385) was purchased from Tocris Cookson (Ballwin, MO). LPS (from *Escherichia coli*, serotype K-235) and adenosine were obtained from Sigma. The murine mAb (7F6-G5-A2) against human A_{2A} receptors with a high affinity and specificity (15) was a gift from Dr. J. Linden (University of Virginia School of Medicine, Charlottesville, VA).

Cell culture and cytokine treatment

THP-1 cells, a human monocytic leukemia cell line (16), were maintained in culture with RPMI 1640 medium supplemented with 10% FBS, penicillin (50 U/ml) and streptomycin (50 μl/ml) at 37°C in a humidified atmosphere consisting of 5% CO_2. At the onset of each experiment the cells were placed in fresh medium and then cultured in the presence of IL-1α (10 U/ml), TNF-α (100 U/ml), IFN-γ (100 U/ml), or medium alone for 3 h or overnight (18 h) followed by further analyses.

Assays for IL-10 and IL-12

THP-1 cells were pretreated overnight with IL-1, TNF-α, or IFN-γ and then placed in 24-well tissue culture plates at $\sim 10^6$ cell/ml with 1.5 ml medium/well. The cells were further cultured overnight in the presence or absence of LPS (5 μg/ml). The A_{2A} receptor agonist CGS-21680 or adenosine with or without the A_{2A} receptor antagonist ZM-241385 was added to cultures 20 min before the addition of LPS. Culture supernatants were collected and subjected to assays for human IL-12 p70 (the heterodimer form), IL-12 p40 subunit, and IL-10 using ELISA Quantikine kits purchased from R&D Systems.

cAMP assay

THP-1 cells were pretreated overnight with the cytokines. On the day of the cAMP assay, the cells were placed in fresh medium and incubated with CGS-21680 at different concentrations (0.01–100 μM) or 1 μM CGS-21680 for varying periods of time (0–25 min). Cells were then harvested, lysed, and assayed for cellular cAMP accumulation using the cAMP enzyme immunoassay system kit (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's instructions.

Isolation of mRNA and semiquantitative RT-PCR

mRNA was isolated from THP-1 cells using a MicroFastTrack kit (Invitrogen, Carlsbad, CA) and transcribed into cDNA (RT). Aliquots of RT, after serial dilutions, were subject to PCR using an RNA-PCR Core kit (PerkinElmer, Branchburg, NJ). The PCRs were performed using primers specific for adenosine A_{2A} receptors and *GAPDH*, a housekeeping gene. The cycle number was adjusted to allow the PCR to proceed in a linear range. Briefly, PCR conditions for A_{2A} receptors were 32–35 cycles of 45 s at 94ºC, 45 s at 62ºC, and 1 min at 72ºC with 1× and 2× diluted RT as templates. PCR for GAPDH was run for 22–25 cycles with RT of 100× and 200× dilutions as templates. The primer sequences were 5′-ACCTG CAGAACGTCACCAAC-3′ (forward) and 5′-TCTGCTTCAGCTGTCGT CGC-3′ (reverse) for A_{2A} receptor and 5′-ACCATCATCCCTGCCTCT AC-3′ (forward) and 5′-CCTGTTGCTGTAGCCAAAT-3′ (reverse) for GAPDH. All primers were designed to amplify cDNA that crossed an intron in the genomic DNA, and the amplicon was sequenced to confirm the identity of the cDNA amplified. Aliquots of PCR product were loaded onto ethidium bromide-stained agarose gel, visualized with an ultraviolet transilluminator, and digitally photographed. The amplicon was quantitated densitometrically using Kodak Digital Science software and all values were normalized to the GAPDH amplicon.

Membrane protein extraction and Western blot analysis

All Western blots were done on cell membrane preparations after overnight cytokine treatment. Crude membrane protein was isolated after sonication of the cells in a modification of a previously described technique (13, 17). Proteins ($\sim$10 μg/lane) were separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Nonspecific Ab binding to the membrane was blocked with 3% nonfat milk in Tris-buffered saline containing 0.3% Tween 20. The membranes were then incubated for 1–2 h with a mAb against adenosine A_{2A} receptors (15). After three washes of 5 min each in 0.3% Tween 20-TBS, the blots were incubated with an alkaline phosphatase-conjugated secondary Ab for 1 h. After extensive washes, the blots were exposed to a fluorescent enhanced chemifluorescence (diethanolamine) substrate (Amersham Pharmacia Biotech, Sunnyvale, CA) and scanned using the Storm PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). The band density was then directly quantitated using Image Quant software (Molecular Dynamics).

Data analysis

Data are presented as mean ± SEM. One-way and two-way ANOVAs were used to determine statistical significance between means or curves. Differences with a p value of <0.05 were considered significant. All statistical analysis was performed using the SigmaStat program (SPSS, Chicago, IL).

Results

Inflammatory cytokines modulate A_{2A} receptor-mediated suppression of IL-12 production

Without LPS stimulation, production of IL-12 in monocytic THP-1 cells (either cytokine-treated or control) was undetectable (data not shown). In control LPS-activated THP-1 cells (without cytokine pretreatment), CGS-21680, a selective A_{2A} agonist, suppressed production of IL-12 p70 and p40 in a dose-dependent manner ($IC_{50} \approx 100$ and 60 nM, respectively) as previously reported (7, 8). CGS-21680 itself, at concentrations up to 10 μM, inhibited LPS-induced IL-12 production by up to 40% (for IL-12p70) and 60% (for IL-12p40). In the cells pretreated with IFN-γ, LPS stimulated greater IL-12 p70 and p40 secretion than control, IL-1-treated, and TNF-α-treated cells, and the inhibitory effect of CGS-21680 on secretion of IL-12, especially IL-12 p40, was markedly attenuated ($p < 0.05$, IFN-γ vs control, for both IL-12 p70 and IL-12 p40). In contrast, pretreatment of the cells with either IL-1 or TNF-α significantly enhanced the effect of CGS-21680 on suppression of both active heterodimer IL-12 p70 ($p < 0.05$ and $p < 0.01$ vs control, respectively; $IC_{50} \approx 50$ nM) and IL-12 p40 subunit ($p < 0.01$ for both vs control, $IC_{50} \approx 10$ nM). Under all treatment conditions, the changes in levels of p40 subunit were more marked than those of p70 heterodimer (Fig. 1, *A* and *B*). The inhibitory effect of CGS-21680 on IL-12 production was reversed by the specific A_{2A} receptor antagonist ZM-241385 (10 μM; Fig. 1*C*), further supporting the role of A_{2A} receptors in modulation of IL-12 release.

To further understand the role of inflammatory cytokines in modulating adenosine receptor function, we examined the responsiveness of cytokine-treated THP-1 cells to adenosine. Like CGS-21680, adenosine itself inhibited LPS-induced IL-12 production in control, IL-1-treated, and TNF-α-treated cells but not in IFN-γ-treated cells, although it seemed that adenosine was less potent than CGS-21680 at the same concentration, probably due, in part, to prompt uptake and metabolism of adenosine by THP-1 cells (Fig. 2*A*). Moreover, the effect of adenosine was abrogated by ZM-241385 (Fig. 2*B*), providing further evidence that the A_{2A} receptor is primarily involved in regulation of IL-12 production.

Inflammatory cytokines modulate A_{2A} receptor-mediated stimulation of IL-10 production

Like IL-12, no detectable IL-10 was produced by THP-1 cells without LPS stimulation (not shown). The concentrations of IL-10 in supernatants of control LPS-activated THP-1 cells remained low but were increased in a dose-dependent manner by CGS-21680 ($EC_{50} \approx 100$ nM). The stimulatory effect of CGS-21680 on IL-10 production was significantly greater in THP-1 cells pretreated with IL-1 or TNF-α ($p < 0.05$ and $p < 0.01$ vs control, respectively; $EC_{50} \approx 50$ nM for both) and reached increases of 160 and 170%, respectively, in IL-10 secretion in response to 10 μM CGS-21680, compared with an increase of 141% in controls. In contrast, CGS-21680 did not increase IL-10 production in THP-1 cells that had been pretreated with IFN-γ ($p = 0.01$ vs control; Fig. 3*A*). ZM-241385 reversed the effect of CGS-21680 on IL-10 production by untreated THP-1 cells and THP-1 cells pretreated with IL-1 or TNF-α (Fig. 3*B*).

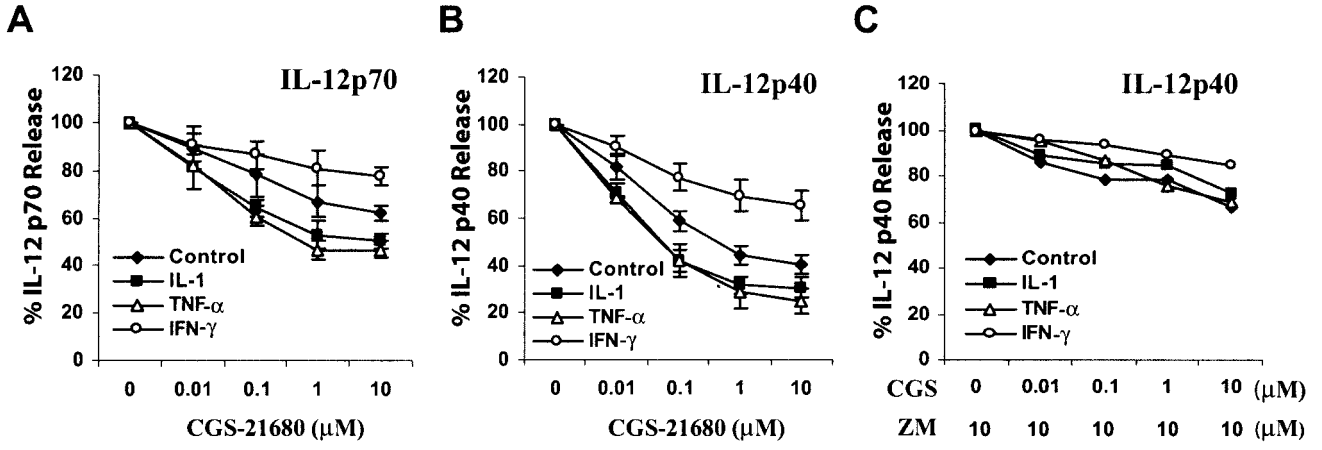

FIGURE 1. Effect of inflammatory cytokines on A_{2A} receptor-mediated production of IL-12 in LPS-activated THP-1 cells. THP-1 cells were pretreated with IL-1 (10 U/ml), TNF-α (100 U/ml), IFN-γ (100 U/ml), or medium alone (Control) and then stimulated with CGS-21680 and LPS (5 μg/ml) as described in *Materials and Methods*. Culture supernatants were collected and subject to ELISA for IL-12. *A*, Inhibition of IL-12 p70 secretion in LPS-treated cells in response to CGS-21680. The basal levels (without CGS-21680 stimulation) of IL-12 p70 were 29.4 ± 13.3 pg/ml (Control), 26.5 ± 12.4 pg/ml (IL-1), 24.3 ± 13.5 pg/ml (TNF-α), and 66.5 ± 27.5 pg/ml (IFN-γ). *B*, Inhibition of IL-12 p40 secretion in response to CGS-21680. The basal levels of IL-12 p40 were 237.8 ± 100.0 pg/ml (Control), 201.4 ± 73 pg/ml (IL-1), 136.8 ± 49.3 pg/ml (TNF-α), and 724.4 ± 267 pg/ml (IFN-γ). *C*, The inhibitory effect of CGS-21680 (CGS) on IL-12 p40 secretion was greatly blocked in the presence of the A_{2A} receptor antagonist ZM-241385 (ZM, 10 μM). Shown are representative results of a single experiment of two experiments with similar results. The basal levels of IL-12 p40 in the presence of ZM-241385 were 256.2 pg/ml (Control), 260 pg/ml (IL-1), 196.4 pg/ml (TNF-α), and 685.8 pg/ml (IFN-γ). For *A* and *B*, data are presented as mean ± SEM of three independent experiments.

Like CGS-21680, adenosine enhanced LPS-induced secretion of IL-10 in untreated THP-1 cells, although to a lesser extend, and the effects of IL-1, TNF-α, and IFN-γ on IL-10 secretion were very similar to those observed with CGS-21680 (Fig. 4*A*). The enhancement in IL-10 levels by adenosine in control, IL-1-treated, and TNF-α-treated cells was completely abolished in the presence of ZM-241385 (Fig. 4*B*). These results indicate that, as with IL-12, the A_{2A} receptor is the adenosine receptor primarily responsible for modulation of IL-10 production.

Inflammatory cytokines modulate A_{2A} receptor-mediated accumulation of cAMP

Both A_{2A} and A_{2B} receptors signal via Gs_a protein-cAMP pathways. To further determine whether inflammatory cytokines modulate A_{2A} receptor function, we studied the cAMP response of cytokine-treated THP-1 cells after agonist ligation. Surprisingly, the basal levels of cAMP in IL-1- and TNF-α-treated cells were higher than those in either control or IFN-γ-treated cells ($p < 0.05$ vs control for TNF-α; Fig. 5).

The addition of 1 μM CGS-21680, a concentration at which CGS-21680 is still highly selective for A_{2A} receptors, to control cells did not significantly stimulate cAMP accumulation at any time point up to 25 min (Fig. 5*A*). Moreover, there was only a slight increase of cAMP production in control cells with a very high concentration (100 μM) of CGS-21680 (Fig. 5*B*). The CGS-21680-induced cAMP response of IFN-γ-treated cells was very similar to that of controls. Although the cAMP levels induced by high doses (>1 μM) of CGS-21680 in IFN-γ-treated cells were slightly lower than in control cells, the overall responses to CGS-21680 stimulation by IFN-γ-treated and control cells were not significantly different ($p > 0.05$, two-way ANOVA; Fig. 3). In contrast, CGS-21680 induced striking cAMP generation by IL-1- and TNF-α-treated cells, as much as 3- to 4-fold higher than basal levels, and this increment differed significantly from the response of control cells (two-way ANOVA, $p < 0.001$). The peak levels of cAMP generation in IL-1- and TNF-α-treated cells occurred as early as 2–5 min after CGS-21680 stimulation (Fig. 5*A*) and in a dose-dependent manner (EC_{50} ≈40 and 30 nM, respectively; Fig. 5*B*). These observations together with those on IL-10 and IL-12 production are consistent with the hypothesis that IL-1, TNF-α, and IFN-γ regulate A_{2A} receptor levels and/or function.

Inflammatory cytokines regulate A_{2A} receptor expression

To gain greater insight into the mechanism by which the Th1 cytokines IL-1, TNF-α, or IFN-γ modulate A_{2A} receptor sensitivity and function, we examined the effects of those cytokines on the expression of A_{2A} receptors in THP-1 cells. As shown in Fig. 6, the message for A_{2A} receptors was significantly altered on treatment of the cells with each of the cytokines studied. A 3-h incubation with IL-1 or TNF-α led to an increase in expression of A_{2A} message to 149 ± 16 and 159 ± 22% of control, respectively ($n = 9$, $p < 0.01$ vs control for both). In contrast, a 3-h treatment with IFN-γ decreased the A_{2A} message to 69 ± 5% of control ($n = 9$, $p < 0.01$). The effects of the cytokines on A_{2A} message remained consistent after overnight incubation; the message levels for A_{2A} receptors were 144 ± 12, 167 ± 16, and 65 ± 8% of control in IL-1-, TNF-α-, and IFN-γ-treated cells, respectively ($n = 9$, $p < 0.01$ vs control for IL-1 and IFN-γ, and $p < 0.001$ for TNF-α).

To determine the significance of the change in mRNA levels for the A_{2A} receptors, we examined protein expression by Western blot analysis using a mAb against A_{2A} receptors. The immunoblots of membrane preparations from THP-1 cells pretreated overnight with medium or cytokines revealed a single band of a 45-kDa protein, the size of A_{2A} receptors (15), under all treatment conditions (Fig. 7*A*). Compared with controls, protein levels of A_{2A} receptors were increased by IL-1 and TNF-α to 127 ± 9 and 136 ± 9% ($n = 5$, $p < 0.05$ vs control for IL-1, and $p < 0.01$ for TNF-α) and decreased by IFN-γ to 73 ± 4% of control ($n = 5$, $p < 0.05$; Fig. 7*B*).

Discussion

The effects of adenosine and its analogues on cytokine secretion have been studied using human and murine monocytes/macrophages and human and murine monocyte/macrophage cell lines

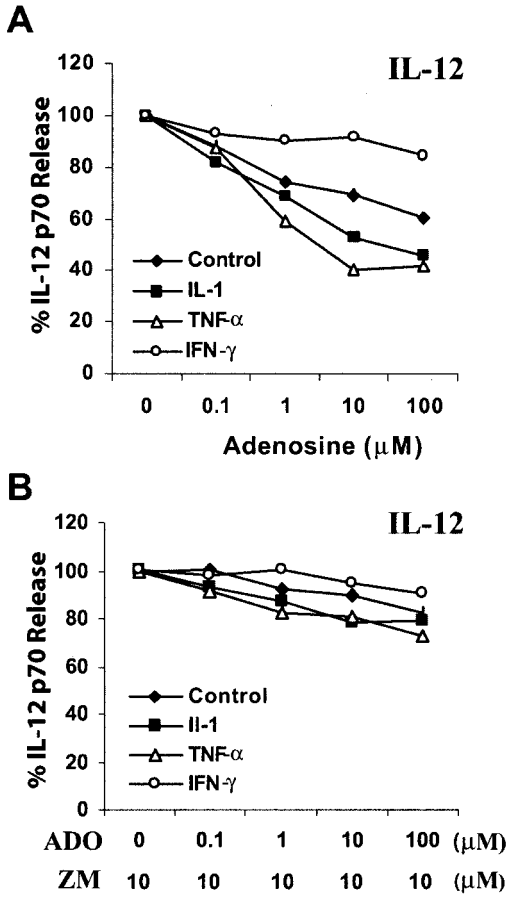

FIGURE 2. Effect of inflammatory cytokines on LPS-induced IL-12 production of THP-1 cells in response to adenosine. THP-1 cells were pretreated with IL-1, TNF-α, or IFN-γ and then stimulated with adenosine (ADO) and LPS in the absence (*A*) or presence (*B*) of ZM-241385 (ZM), as indicated. Culture supernatants were assayed for IL-12 p70. Shown are representative data from a single experiment of two independent experiments with similar results. The basal IL-12 p70 levels were 21.6 pg/ml (Control), 20 pg/ml (IL-1), 20.4 pg/ml (TNF-α), and 64.7 pg/ml (IFN-γ) in the absence of ZM-241385 and 24.6, 23.9, 25.3, 68.2 pg/ml, respectively, in the presence of ZM-241385.

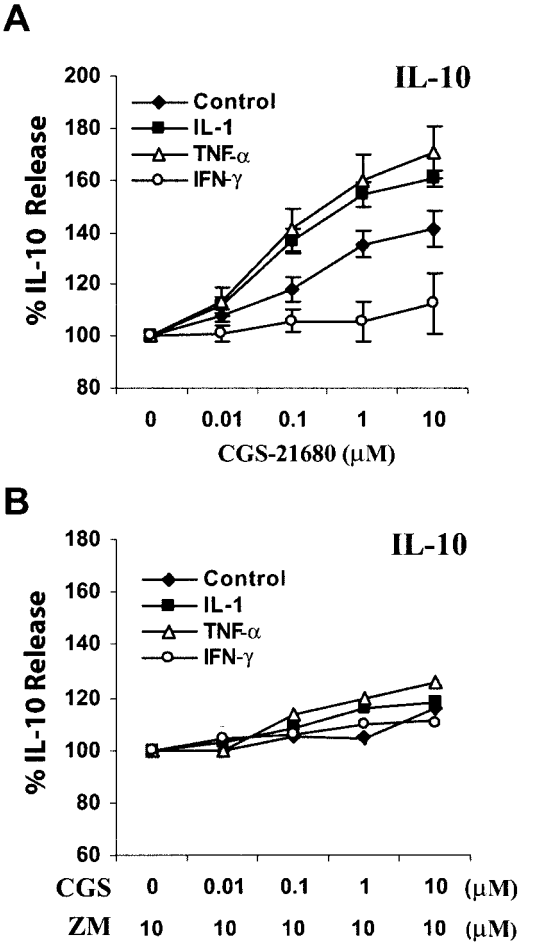

FIGURE 3. Effect of inflammatory cytokines on A_{2A} receptor-mediated production of IL-10 in LPS-activated THP-1 cells. IL-10 content of culture supernatants from THP-1 cells pretreated with cytokines and then stimulated with LPS and CGS-21680 (CGS) was analyzed by ELISA. *A,* Induction of LPS-induced production of IL-10 in response to CGS-21680. Basal IL-10 concentrations were 28.3 $\pm$ 11.7 pg/ml (Control), 30.7 $\pm$ 12.7 pg/ml (IL-1), 28.7 $\pm$ 11.6 pg/ml (TNF-α), and 25.8 $\pm$ 7.6 pg/ml (IFN-γ). Data are presented as mean $\pm$ SEM of three independent experiments. *B,* ZM-241385 (ZM, 10 μM) blocked the stimulatory effect of CGS-21680 (CGS) on IL-10 secretion. Shown is a single representative experiment of two experiments with similar results. The basal IL-10 levels in the presence of ZM-241385 were 24.4 pg/ml (Control), 23.3 pg/ml (IL-1), 23.4 pg/ml (TNF-α), and 27.6 pg/ml (IFN-γ).

with very similar results. Based on the published data, the A_{2A} receptor is the most prominent regulator of cytokine secretion. We observed similar effects of the A_{2A} receptor agonist CGS-21680 on LPS-induced production of IL-12 and IL-10 in resting (control) THP-1 cells as had previously been reported for human peripheral blood mononuclear cells and isolated monocytes by Link et al. (8). More importantly, in the current study we found that IL-1, TNF-α, and IFN-γ, the most prominent cytokines secreted by monocyte/macrophages and Th1 cells, modulated the capacity of A_{2A} receptor occupancy to regulate secretion of IL-10 and IL-12 in LPS-activated THP-1 cells. Whereas IL-1 and TNF-α clearly potentiate the A_{2A} receptor function, IFN-γ attenuates it. These data also extend the previous observation that the suppressive effect of adenosine on IL-12 production by murine macrophages was much less pronounced in cells activated with both LPS and IFN-γ than in those activated with LPS alone (7). It is well recognized that cytokines stimulate or inhibit the production of other cytokines in an autocrine and paracrine manner, and our findings suggest that adenosine receptors may play a role in mediating this mutual interaction among cytokines. The functional modulation of adenosine receptors is likely a key factor in the regulation of inflammatory conditions involving numerous cytokines.

It has been shown previously that adenosine suppresses IL-12 production in murine macrophages by A_{2A}-dependent and A_3-dependent mechanisms (7). However, our finding that adenosine and CGS-21680 regulate cytokine secretion by THP-1 cells, both untreated and cytokine treated, and that the effects of both agents were blocked by ZM-241385 is most consistent with regulation via the A_{2A} receptor alone. Absence of a role for A_3 receptor in regulating cytokine production by human monocytes is consistent with the previous findings of Link et al. (8).

The A_{2A} receptor-mediated increase in intracellular cAMP accumulation provides further evidence for the regulatory effects of inflammatory cytokines on A_{2A} receptor function and A_{2A}-mediated signaling. Although some cell types respond to A_{2A} receptor occupancy with a brisk cAMP response (18), we observed a minimal cAMP response of resting THP-1 cells to CGS-21680, consistent with previous findings in the same cell line by Munro et al.

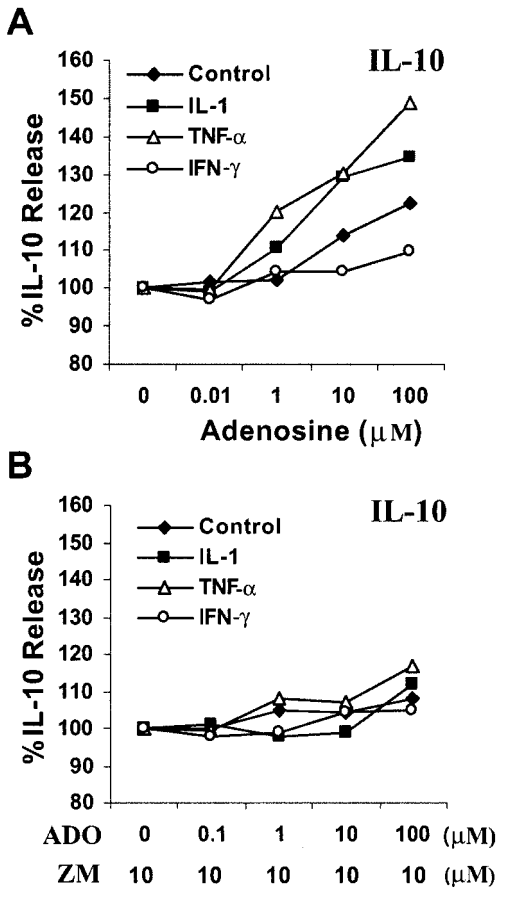

FIGURE 4. Effect of inflammatory cytokines on LPS-induced IL-10 production of THP-1 cells in response to adenosine. Cytokine-treated THP-1 cells were stimulated with adenosine (ADO) and LPS in the absence (*A*) or presence (*B*) of ZM-241385 (ZM), and culture supernatants were assayed for IL-10 as described. Shown are representative data from a single experiments of two independent experiments with similar results. The IL-10 basal levels were 26 pg/ml (Control), 27.8 pg/ml (IL-1), 28.5 pg/ml (TNF-α), and 20.1 pg/ml (IFN-γ) in the absence of ZM-241385 and 24.2, 25.2, 25.5, and 20.8 pg/ml, respectively, in the presence of ZM-241385.

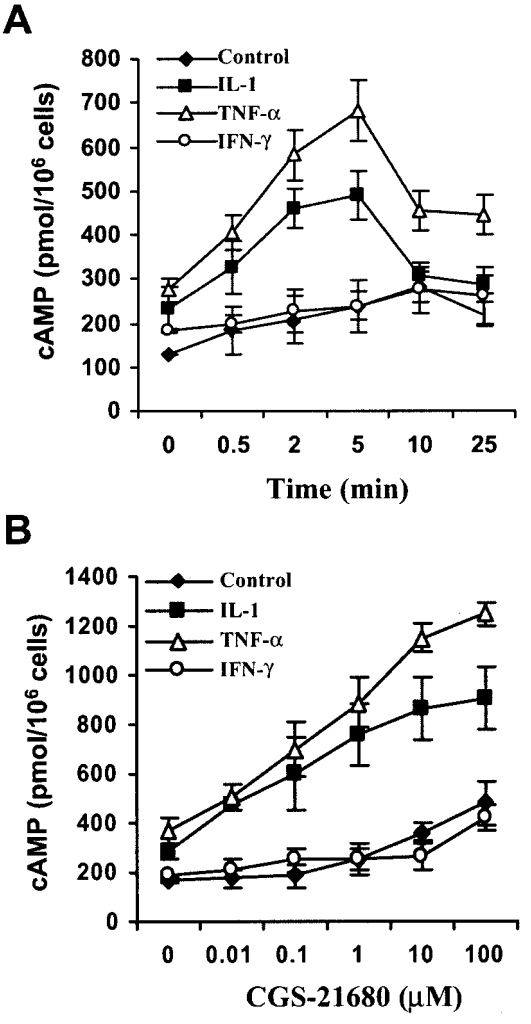

FIGURE 5. A_{2A} receptor-mediated accumulation of intracellular cAMP in cytokine-treated THP-1 cells. THP-1 cells were treated overnight with IL-1, TNF-α, or IFN-γ, as described in *Materials and Methods*, and thereafter stimulated with the A_{2A} adenosine receptor agonist CGS-21680. The intracellular cAMP content of the cells was assayed using the enzyme immunoassay kit, as described. *A*, Cytokine-treated cells were stimulated with 1 μM CGS-21680 for various periods of time. *B*, Cytokine-treated cells were stimulated for 5 min with increasing doses of CGS-21680. Values are expressed as the mean $\pm$ SEM of three independent experiments for each point.

(19). The minimal accumulation of cAMP in resting and IFN-γ-treated THP-1 cells reported here may have resulted from a reduction in functional A_{2A} receptor expression, desensitization of A_{2A} receptors on THP-1 cells, or diminished signal transduction machinery. The in vitro up-regulation of A_{2A} receptor function by IL-1 and TNF-α treatment parallels the observation that in vivo cAMP production is enhanced in ciliary epithelial bilayers from IL-1 or TNF-α-inflamed eyes (20). Our observation that there was an increased cAMP response after treatment with an A_{2A} receptor agonist in IL-1- and TNF-α-treated THP-1 cells suggests that the elevated cAMP production in these models may have resulted, at least in part, from the activation of an increased number and/or sensitivity of A_{2A} receptors. Because elevated intracellular levels of cAMP are generally associated with suppression of inflammatory responses, it is likely that the up-regulation of A_{2A} receptors along with endogenous adenosine release at inflamed sites constitutes a feedback loop to diminish or terminate the inflammatory response.

In parallel with the observed functional changes, expression of A_{2A} receptor message and protein was found to be regulated on cytokine treatment. The Th1 cytokines IL-1, TNF-α, and IFN-γ play a critical role in the pathogenesis of many severe inflammatory conditions such as rheumatoid arthritis (see review in Refs. 21 and 22). Although they are all regarded as proinflammatory cytokines, the differences in their actions on adenosine A_{2A} receptor expression may reflect a complex mechanism of receptor regulation, especially at inflamed sites (e.g., inflamed synovium) at which numerous cytokines are secreted. It has been reported that IFN-γ up-regulates A_{2B} receptor expression in murine bone marrow-derived macrophages (14). Our data indicate that IFN-γ has an opposing effect on A_{2A} receptors. This difference in the regulatory action of IFN-γ on the two receptors may be significant in modulating adenosine-mediated functions in cells and tissues where subtypes of adenosine receptors have different patterns of expression and distribution.

It is still not clear how the inflammatory cytokines studied here regulate adenosine A_{2A} receptor expression. Direct regulation of transcription or altered mRNA stability after cytokine stimulation may explain our observations. Computerized analysis of upstream

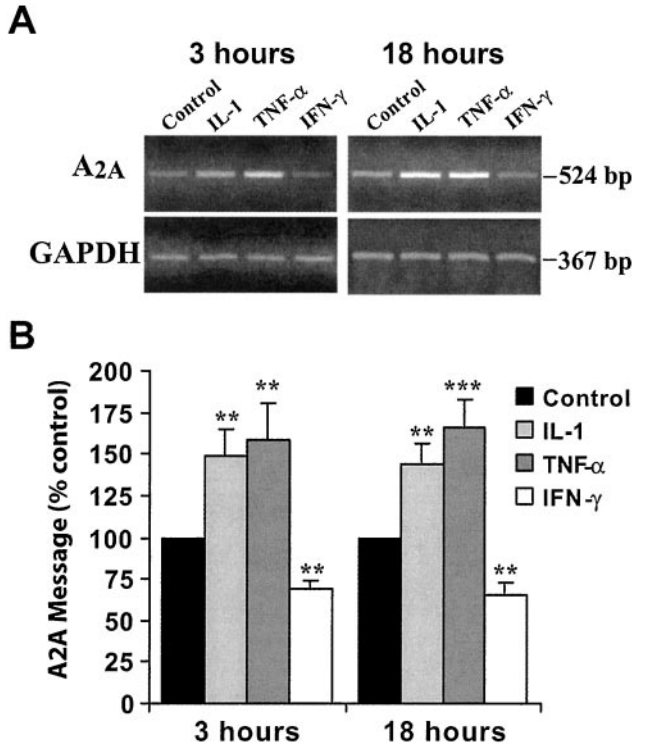

FIGURE 6. Semiquantitative RT-PCR analysis of A_{2A} receptor expression in cytokine-treated THP-1 cells. mRNA isolated from cells treated with IL-1 (10 U/ml), TNF-α (100 U/ml), IFN-γ (100 U/ml), or medium alone (Control) for 3 h or overnight (18 h) was subject to RT-PCR using primers specific for A_{2A} receptors and GAPDH with serially diluted RT as templates. *A,* Agarose gel electrophoresis of RT-PCR product from a representative experiment. *B,* The level of message amplification for A_{2A} receptors was densitometrically quantitated and normalized to GAPDH. Data shown are the means ± SEM of the percentages of control from nine independent experiments. **, $p < 0.01$; ***, $p < 0.001$ vs control.

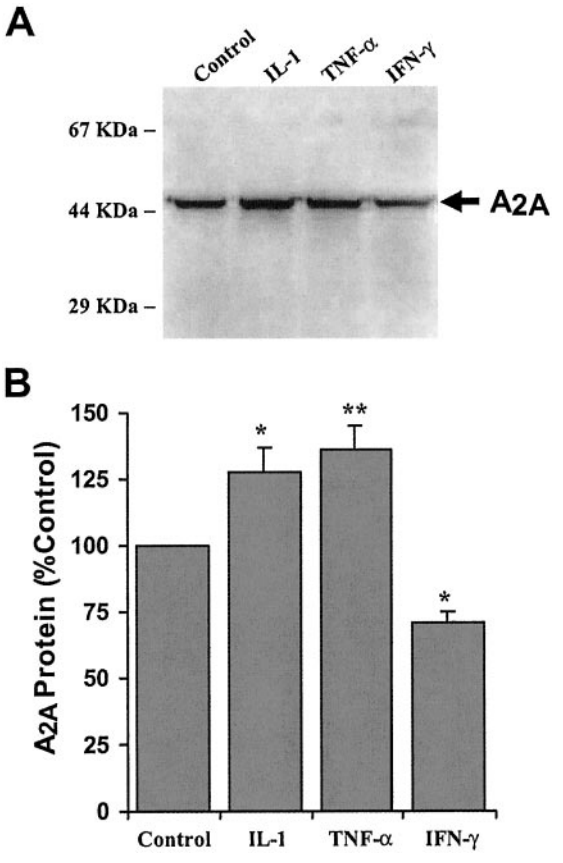

FIGURE 7. Western blot analysis of protein expression of A_{2A} receptors in cytokine-treated THP-1 cells. Crude membrane protein was isolated from treated cells, as described in *Materials and Methods*, and separated by SDS-PAGE. A_{2A} receptor expression was assessed by Western blot using a mAb against A_{2A} receptors, as described. *A,* Representative Western blot for A_{2A} receptors. *B,* Semiquantitation of protein expression in treated cells performed by densitometric analysis of Western blots. Data are expressed as mean ± SEM percentage of control ($n = 5$). *, $p < 0.05$; **, $p < 0.01$ vs control.

regions of the A_{2A} gene obtained from chromosome sequence databases demonstrates numerous potential regulatory elements including several NF-κB-binding sites. The presence of NF-κB-binding sites likely explains, at least in part, the effect of IL-1 and TNF-α on increased receptor expression, although which of these binding sites is involved is not yet known. Nevertheless, the effect of inflammatory cytokines on expression of A_{2A} receptors cannot entirely explain the observed changes in receptor function with respect to either cAMP generation or regulation of cytokine secretion. It has been reported that elevated cAMP inhibits NF-κB-mediated transcription of numerous genes (23). Thus, the increase in cAMP levels, although it seems transient, in IL-1- and TNF-α-treated cells observed here may have a feedback control on the increased expression of A_{2A} receptors by those cytokines. We were surprised to find that, in contrast to IL-1- or TNF-α-treated cells, A_{2A} occupancy did not alter intracellular cAMP concentration in untreated cells. Although an increase in the intracellular levels of cAMP itself can further influence the synthesis and release of IL-10 and IL-12 (24), our results suggest that, at least in control cells, cAMP is not the primary intracellular regulator of IL-10 and IL-12 production. In other words, the absence of a detectable increase in cAMP in these cells suggests that A_{2A} occupancy modulates cytokine production by a cAMP-independent mechanism which may be further enhanced by increased intracellular cAMP. Indeed, our results suggest that enhanced cAMP response adds to the regulatory effect in IL-1- and TNF-α-stimulated cells. In contrast, it has also previously been reported that the mechanism of action of adenosine and its analogues on IL-12 and IL-10 production was not associated with alterations in the activation of the p38 and p42/p44 mitogen-activated protein (MAP) kinases (7). However, other evidence suggests that in human endothelial cells stimulation of A_{2A} receptors by its agonists activates MAP kinases (25). In short, it cannot be ruled out that IL-1, TNF-α, or IFN-γ may also regulate the activation of MAP kinases or other A_{2A} receptor downstream transduction signals including a cAMP/protein kinase A pathway (8) leading to significant changes of A_{2A} receptor functions.

We and others have demonstrated that adenosine, acting at one or more of its receptors, mediates the anti-inflammatory effects of drugs such as methotrexate and sulfasalazine, commonly used and effective disease-modifying antirheumatic agents, in in vitro and in vivo models of acute inflammation and chronic arthritis (26–31). Despite its proven efficacy in the treatment of rheumatoid arthritis, many patients do not respond or only partially respond to methotrexate therapy. Higher levels of or greater sensitivity to IFN-γ in methotrexate-resistant patients may be an explanation for the diminished therapeutic response.

Our study demonstrates that function and expression of adenosine A_{2A} receptors are differentially regulated by the inflammatory cytokines IL-1, TNF-α, and IFN-γ. This finding suggests that the effects of adenosine and its analogues at inflamed sites may be

modified in the short and long term by inflammatory mediators. We speculate that the activation or inhibition of adenosine receptors by inflammatory cytokines may partly account for the variability in response to adenosine-mediated anti-inflammatory agents used in the treatment of inflammatory diseases such as rheumatoid arthritis.

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