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IFN-γ Up-Regulates the A_{2B} Adenosine Receptor Expression in Macrophages: A Mechanism of Macrophage Deactivation[1]

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Adenosine is a potent endogenous anti-inflammatory agent released by cells in metabolically unfavorable conditions, such as hypoxia or ischemia. Adenosine modulates different functional activities in macrophages. Some of these activities are believed to be induced through the uptake of adenosine into the macrophages, while others are due to the interaction with specific cell surface receptors. In murine bone marrow-derived macrophages, the use of different radioligands for adenosine receptors suggests the presence of A_{2B} and A_3 adenosine receptor subtypes. The presence of A_{2B} receptors was confirmed by flow cytometry using specific Abs. The A_{2B} receptor is functional in murine macrophages, as indicated by the fact that agonists of A_{2B} receptors, but not agonists for A_1, A_{2A}, or A_3, lead to an increase in cAMP levels. IFN-γ up-regulates the surface protein and gene expression of the A_{2B} adenosine receptor by induction of de novo synthesis. The up-regulation of A_{2B} receptors correlates with an increase in cAMP production in macrophages treated with adenosine receptor agonist. The stimulation of A_{2B} receptors by adenosine or its analogues inhibits the IFN-γ-induced expression of MHC class II genes and also the IFN-γ-induced expression of nitric oxide synthase and of proinflammatory cytokines. Therefore, the up-regulation of the A_{2B} adenosine receptor expression induced by IFN-γ could be a feedback mechanism for macrophage deactivation. *The Journal of Immunology,* **1999, 162: 3607–3614.**

Adenosine is a purine nucleoside produced and secreted into the extracellular medium by cells in normoxic conditions. Nevertheless, in stress situations such as ischemia or hypoxia, massive ATP degradation increases the local adenosine concentration to a micromolar range (1, 2). Adenosine modulates several functions of macrophages, such as the regulation of nitrite production (3–7), the inhibition of LPS-induced TNF-α production (5, 8–10), the increase in IL-6 or IL-10 production (5, 10, 11), and the inhibition of proliferation induced by macrophage-CSF, IL-3, or PMA (12).

The intra- and extracellular pools of adenosine are regulated by nucleoside transporters (13, 14) and by the activity of enzymes responsible for the metabolism of adenosine expressed either inside the cells or associated with the cell surface (15, 16). To induce the synthesis of molecules such as nitric oxide or to inhibit the induction of TNF-α by LPS, adenosine must be transported into the cell. In contrast, for other functions, adenosine interacts with specific cell surface receptors that are coupled to G proteins. Several adenosine receptors have been described and named A_1, A_{2A}, A_{2B}, and A_3 according to their functional ability to modulate adenylate cyclase activity (17, 18). The number and type of receptors are characteristic of each cell type. Over the past years, all four adenosine receptor subtypes have been cloned in many organisms (19–22).

We have analyzed the expression of adenosine receptors in macrophages using murine bone marrow-derived macrophages (BMDM),[3] which are homogeneous populations of nontransformed primary cells. Our results suggest that BMDM mainly express A_{2B} and A_3 adenosine receptors. The interaction of the A_{2B} receptors with adenosine induces the production of cAMP. The A_{2B} adenosine receptor expression is up-regulated by IFN-γ. The stimulation of A_{2B} adenosine receptor inhibits the MHC class II expression induced by IFN-γ and also inhibits the IFN-γ-induced expression of iNOS and modulates the expression of IFN-γ-induced cytokines, such as TNF-α and IL-1β. Therefore, the up-regulation of A_{2B} adenosine receptors by IFN-γ could be a negative feedback mechanism to regulate macrophage activation at the inflammatory foci.

Materials and Methods

Reagents

Adenosine, 5′-*N*-ethyl-carboxamidoadenosine (NECA), N^6-(*R*)-phenylisopropyl-adenosine (R-PIA), and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were all obtained from Sigma (St. Louis, MO). 2-[*p*-(2-Carbonyl-ethyl)-phenylethylamino]-5′-*N*-ethylcarboxamido-adenosine (CGS 21680) and N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA) were purchased from Research Biochemicals International (Natick, MA). [^{3}H]NECA, [^{3}H]R-PIA, and [^{3}H]CGS21680 were obtained from New England Nuclear (Boston, MA). Recombinant murine IFN-γ was a gift from Genentech (San Francisco, CA). All other products were of the best grade available and were purchased from Sigma (St. Louis, MO). Deionized water further purified with a Millipore Milli-Q system (Bedford, MA) was used throughout.

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[3] Abbreviations used in this paper: BMDM, bone marrow-derived macrophages; iNOS, inducible nitric oxide synthase; NECA, adenosine 5′-*N*-ethyl-carboxamidoadenosine; R-PIA, N^6-(*R*)-phenylisopropyl-adenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; CGS 21680 2-[*p*-(2-carbonyl-ethyl)-phenyl-ethylamino]-5′-*N*-ethylcarboxamido-adenosine; IB-MECA, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide; EIA, enzyme immunoassay.

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Cell culture

BMDM were isolated as previously described (23). Six-week-old BALB/c mice (Charles River Laboratories, Wilmington, MA) were killed by cervical dislocation, and both femurs were dissected free of adherent tissue. The ends of the bones were cut off, and the marrow tissue was eluted by irrigation with medium. The marrow plugs were dispersed by passing through a 25-gauge needle, and the cells were suspended by vigorous pipetting and washed by centrifugation. The cells were cultured in plastic tissue culture dishes (150 mm) in 40 ml of DMEM containing 20% FBS and 30% L cell-conditioned medium as a source of macrophage-CSF. After 7 days of culture the macrophages are a homogeneous population of adherent cells. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Antibodies

The surface expression of adenosine receptor was analyzed using affinity-purified Abs anti-A_1 and anti-A_{2B} against human molecules developed by our group (24, 53). These Abs were developed against peptides of human adenosine receptors, which are highly conserved in murine cells. The peptide used for immunization corresponds to the deduced amino acid sequence from the putative third extracellular loop (FQPAQGKNKPKWA) of the cloned human A_{2B} adenosine receptor (25, 53). The specificity of these receptors has been assessed using CHO cells transfected with the cDNA encoding each of the adenosine receptors (24, 53). FITC-labeled rat anti-rabbit IgG Ab fluorescein-conjugated from Sigma was used as secondary Ab. For surface staining of MHC class II molecules we used Ab 34-5-3 (anti-I-A^d; PharMingen, San Diego, CA). FITC-labeled sheep anti-mouse IgG (Cappel, Turnhout, Belgium) was used as secondary Ab. To block Fc receptors we used anti-CD16/CD32 Ab (PharMingen). For Western blot analysis, we used rabbit anti-mouse iNOS Abs (Calbiochem, San Diego, CA) and, as a control, mouse anti-mouse β-actin Abs (Sigma). Peroxidase-conjugated rat anti-rabbit IgG or anti-mouse IgG were used as secondary Abs, respectively (Cappel).

Binding experiments

The experiments were performed in 24-well plates (2×10^5 cells/well) at 4°C in serum-free DMEM buffered with 20 mM HEPES and containing 2 U/ml adenosine deaminase (Boehringer Mannheim, Indianapolis, IN). Ligand binding was measured by incubation of the cells with the radiolabeled agonist in the presence or the absence of different displacers for 2 h at 4°C. Then, the cells were washed with ice-cold PBS (10 mM; pH 7.4) and disrupted with 0.2% SDS. Aliquots of the suspension were transferred to scintillation vials containing 10 ml of formula 989 scintillation mixture (New England Nuclear), and radioactivity was measured using a Packard 1600 Tri-Carb scintillation counter (Downers Grove, IL) with 50% efficiency. The amount of protein was determined by the bicinchonimic acid method as described previously (26).

Determination of Ag cell surface expression

Cell surface staining was conducted using specific Abs and cytofluorometric analysis as described previously (27). Cells (10^6) were harvested and washed in cold PBS. After fixation with 2% paraformaldehyde during 30 min at 4°C, the cells were resuspended in 50 μl of PBS containing 5% FBS. They were then incubated at 4°C with 1 μg/10^6 cells of anti-CD16/CD32 mAb to block the Fc receptors. After 15 min, the primary Ab was added, and the cells were further incubated for 1 h. The A_{2B}-specific Ab (MPE1) was used at 5 μg/10^6 cells, and the anti-I-A^d Ab was used at 1 μg/10^6 cells. Then, the cells were washed by centrifugation through an FBS cushion. Finally, they were incubated with FITC-conjugated secondary Ab for another hour at 4°C. Stained cell suspensions were analyzed using an EPICS XL flow cytometer (Coulter, Hialeah, FL). FITC excitation was obtained by a 488-nm argon laser lamp, and its fluorescence was collected using a 525-nm band-pass filter. The parameters used to select cell populations for analysis were forward and side light scatter. As a control, we used an unrelated Ab.

To analyze the level of expression induced by IFN-γ, 10^6 cells were cultured in plastic dishes (60 mm) and activated with 300 U/ml of IFN-γ for the indicated times; then, A_{2B} expression was measured as described.

To permeabilize macrophages, 10^6 cells were resuspended in 100 μl of PBS containing 0.5% saponin (Sigma) for 20 min at room temperature (28). Then, the cells were washed by centrifugation through an FBS cushion and resuspended in 50 μl of PBS containing 5% FBS to incubate with the appropriate Abs.

Determination of cAMP

Production of cAMP was evaluated using a standard procedure (cAMP EIA system, Amersham, Aylesbury, U.K.). Briefly, 10^6 macrophages were cultured in 24-well plates in normal medium. The cells were stimulated with the indicated adenosine agonists and antagonists for 15 min. The cAMP from the cells was extracted using a liquid phase extraction method. Ice-cold ethanol was added to the cell suspension to a final concentration of 65% (v/v) ethanol. After allowing the cells to settle, the supernatants were transferred to test tubes and centrifuged at $2000 \times g$ for 15 min at 4°C. The supernatants were transferred to fresh tubes and dried using a Speed-Vac (Bio-Rad, Hercules, CA). The dried extracts were dissolved in assay buffer, and the amount of cAMP was analyzed using a nonacetylation cAMP EIA system (Amersham). Each sample was made in triplicate, and the results are presented as the mean ± SEM.

Northern blot analysis

Total cellular RNA (20 μg), extracted by the acid guanidinium thiocyanate-phenol-chloroform method (29), was run in 1% agarose with 5 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS) (pH 7.0) and 1 M formaldehyde buffer. The RNA was transferred to a GeneScreen (Life Science Products, Boston, MA) nitrocellulose membrane overnight and fixed with 150 mJ UV. As a probe, we used either a randomly primed human A_{2B} adenosine receptor cDNA fragment (25) or the 18S ribosomal RNA probe (30) as a control for the amount of loaded RNA. To detect the A_{2B} receptor mRNA and to ensure the recognition of the mouse mRNA by the human probe, hybridization was performed at low stringency in a rotating incubator at 42°C and 10% formamide for 18 h. For the analysis of I-Aβ RNA, a fragment of I-Aβ^k cDNA ranging from positions 1–230 of the open reading frame was used. For I-Aα we used a fragment of the IAα^k cDNA covering positions 1–489 of the open reading frame. Both fragments were subcloned into the pGEM3 vector (Promega, Madison, WI) (27). The IFN-γ-induced cytokine expression was analyzed using cDNA probes for TNF-α and IL-1β, which were provided by Dr. M. Nabholz (Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges, Switzerland) and Dr. R. Wilson (Glaxo, Greenford, U.K.), respectively. The blots were sequentially washed in 1× SSC/0.2% SDS twice at room temperature and once at 65°C, exposed for 12–72 h at −80°C to Kodak film (Eastman Kodak, Rochester, NY) and analyzed using a Molecular Analyst (Bio-Rad).

Protein extraction and Western blot analysis

The cells were washed twice in ice-cold PBS and lysed on ice with lysis solution (1% Triton X-100, 10% glycerol, 50 mM HEPES (pH 7.5), 150 mM NaCl, and protease inhibitors). The protein concentration of the samples was determined by the Bio-Rad protein assay. One hundred micrograms of the cell lysates were boiled at 95°C in Laemmli SDS loading buffer, separated by 7.5% SDS-PAGE, and electrotransferred to nitrocellulose membranes (Hybond-ECL, Amersham, Arlington Heights, IL). The membranes were blocked for at least 1 h at room temperature in Tris-buffered saline/0.1% Tween-20 (TBS-T) containing 5% nonfat dry milk and then incubated with TBS-T containing the primary Ab. For iNOS and β-actin immunoblotting, incubation was performed for 1 h at room temperature. After three washes of 15 min each in TBS-T, the membranes were incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG Abs, respectively (Cappel), for 1 h. After three washes of 15 min with TBS-T, enhanced chemiluminescence detection was performed (Amersham), and the membranes were exposed to x-ray films (Amersham). The blots were quantified by densitometric analysis.

Results

We used macrophages obtained from bone marrow cultures, since they represent a normal population of resting macrophages. Although BMDM were incubated with L cell-conditioned medium, they were washed extensively and incubated in the absence of L cell-conditioned medium for 24 h before any treatment or measurement was performed. This procedure was designed to minimize any possible effects of the L cell-conditioned medium, which contained CSF-1, on the regulation of A_{2B} adenosine receptors.

To characterize adenosine receptors in BMDM we measured the binding of adenosine analogues specific to each type of receptor. BMDM were incubated with NECA, a nonselective adenosine receptor agonist (31); CGS 21680, a specific A_{2A} adenosine receptor agonist (32); R-PIA, a specific A_1 adenosine receptor agonist (33);

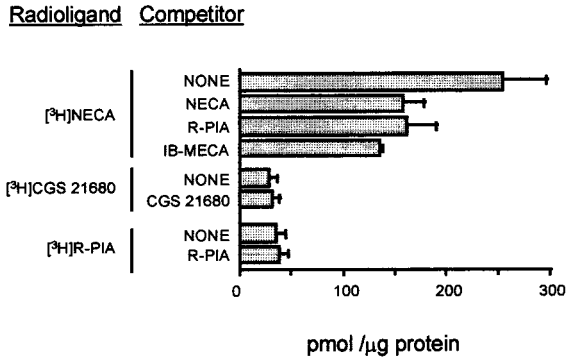

FIGURE 1. Displacement of [^{3}H]NECA binding by different adenosine analogues. Macrophages were incubated at 4°C with 50 nM [^{3}H]NECA, 3 nM [^{3}H]R-PIA, and 50 nM [^{3}H]CGS 21680 in serum-free DMEM in the absence or the presence of 750 μM of the indicated reagents as a competitors. After 2 h of incubation, free radioligand was separated from bound radioligand as indicated in *Materials and Methods*. The data are the mean of triplicate determinations from a representative experiment. Higher concentrations of some of the compounds could not be achieved due to solubility problems.

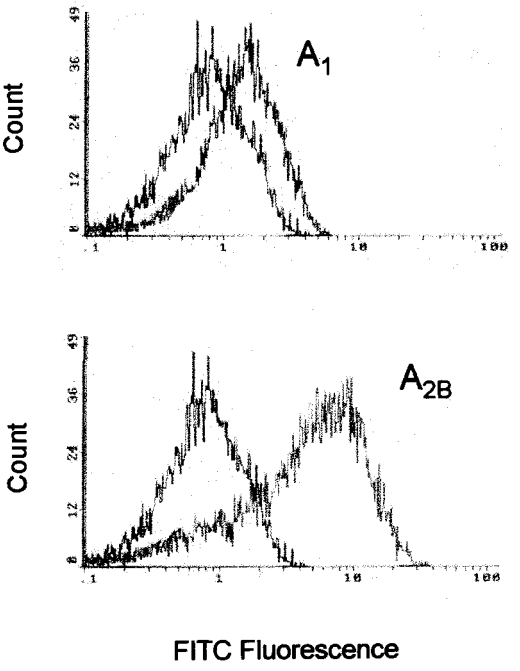

FIGURE 2. Macrophages express A_{2B} adenosine receptors. The analysis of expression of the receptor in BMDM was conducted using flow cytometry and affinity-purified Abs against human A_1 and A_{2B} adenosine receptors. As secondary Abs we used FITC-labeled anti-rabbit IgG Abs. The control histogram corresponds to macrophages labeled with a nonrelevant Ab.

and IB-MECA, a specific A_3 adenosine receptor agonist (34). The competition experiments between radiolabeled and cold ligands showed that macrophages bound [^{3}H]NECA, a synthetic adenosine analogue recognized by all four subtypes of adenosine receptors. Due to the low affinity of the A_{2B} receptors for any of the adenosine analogues, there was a considerable amount of nonspecific [^{3}H]NECA binding. For this reason the binding competition analysis with 750 μM cold NECA only showed a competition of 45%. The specific binding of either [^{3}H]R-PIA at low concentrations (3 nM) or [^{3}H]CGS21680 was negligible, thus suggesting the absence of A_1 or A_{2A} receptors (Fig. 1). The binding of [^{3}H]NECA was competed by high amounts (750 μM) of R-PIA and IB-MECA. At high concentrations, R-PIA bound to the A_{2B} receptors, and IB-MECA bound to the A_3 receptors. The displacement of [^{3}H]NECA binding induced by high concentrations of R-PIA indicated that NECA binding was not due to adetonine-like molecules (35). Therefore, these results suggest the presence of both A_{2B} and A_3 receptors in macrophages.

Due to the very low affinity of A_{2B} receptors and the lack of a specific agonist for this receptor type, the expression of A_{2B} adenosine receptors was confirmed using flow cytometry and a specific Ab (MPE1) against an extracellular epitope of human A_{2B} adenosine receptor that is highly conserved in human and mouse cells. The specificity of these receptors has been assessed by using CHO cells transfected with the cDNA encoding for human A_{2B} adenosine receptor (see Footnote 4). This Ab was able to detect the murine A_{2B} adenosine receptor at the cell surface (Fig. 2). BMDM stained with the MPE-1 Ab contained two cell populations, one lacking and the other (76.4 $\pm$ 4.8%) expressing the A_{2B} adenosine receptors. As a control we used an Ab against the A_1 adenosine receptor (24), which did not result in cell surface staining (Fig. 2).

The biological relevance of the receptor was confirmed by assessing the relationship between the ligand-receptor interaction and the induction of a functional response to adenosine mediated through the receptor. Since adenosine receptors are linked to adenylate cyclase (36, 37), we used this property to stimulate macrophages with different ligands and measure the intracellular production of cAMP. The treatment of BMDM for 15 min with 5×10^{-5} M adenosine or 10^{-5} M NECA, resulted in a marked increase in intracellular cAMP levels (Fig. 3*A*). The treatment of macrophages with 10^{-5} M CGS 21680 did not increase cAMP production. Similar results were obtained when we used 10^{-6} M R-PIA or 10^{-5} M IB-MECA. As selective agonists for A_1, A_{2A}, and A_3 adenosine receptors did not induce cAMP production, the increase in cAMP production obtained with adenosine or NECA may be mediated through interaction with the A_{2B} adenosine receptor. Furthermore, DPCPX at a concentration (10^{-6} M) that blocked A_{2B} receptor (38) inhibited completely the increase in cAMP induced by 10^{-5} M NECA. Although we could not exclude the presence of A_3 adenosine receptors, the cAMP-mediated responses in macrophages seemed to be mediated through A_{2B} receptors.

The natural ligand adenosine gave a lower response than NECA, probably due to the presence of adenosine-degrading ecto adenosine deaminase at the surface of macrophages (39, 40). Therefore, we used NECA in all subsequent experiments. The production of intracellular cAMP in BMDM induced by NECA was dose- and time-dependent (Fig. 3*B*) with an ED_{50} of 5 μM, which is similar to that reported for other model systems (17). The induction of cAMP by NECA showed biphasic kinetics (Fig. 3*C*); it increased quickly during the first 30 min of stimulation and then it decreased, reaching basal levels 5 h later.

Macrophage activation has been defined as a series of functional and biochemical modifications in macrophage populations that are induced by several lymphokines. IFN-γ can activate macrophages and may be the major macrophage activator produced by T cells (41). The treatment of macrophages with saturating amounts of IFN-γ (300 U/ml) induced an up-regulation of the expression of A_{2B} adenosine receptors at the cell surface (Fig. 4*A*). This increase was a late event and was not measurable until 24–48 h after the treatment. The permeabilization of the macrophages with 0.5% saponin also allowed measurement of the intracellular receptors. We observed that in these conditions the up-regulation of the A_{2B} adenosine receptors took place not only at the cell surface but also

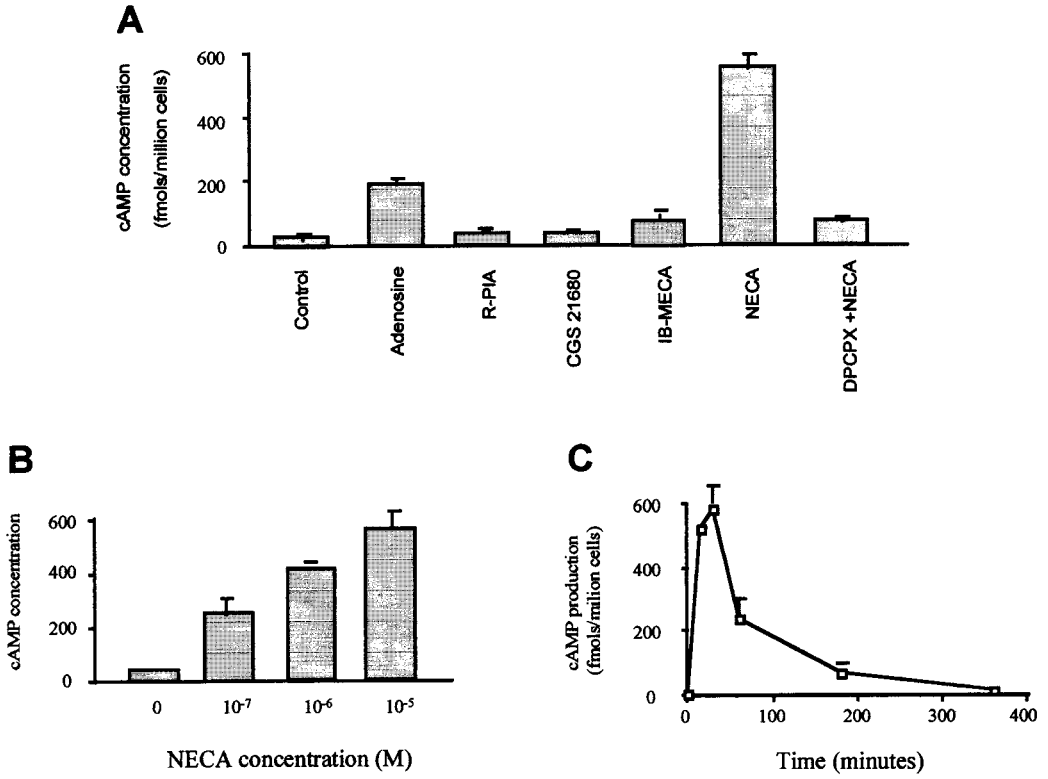

FIGURE 3. Measurement of cAMP production. *A*, BMDM (10^6) were stimulated for 15 min with 5×10^{-5} M adenosine, 10^{-5} M NECA, 10^{-6} M R-PIA, 10^{-5} M IB-MECA, 10^{-5} M CGS 21680, or 10^{-6} M DPCPX plus 10^{-5} M NECA. The cultures were liquid phase extracted (see *Materials and Methods*), and the cAMP content was measured with an EIA system (Amersham, Aylesbury, U.K.). *B*, The production of cAMP in BMDM stimulated with NECA is dose dependent. The cells were treated at the indicated concentrations for 15 min. *C*, Time course of cAMP production in macrophages treated with 10^{-5} M NECA at the indicated times. All data points are represented as the mean ± SEM of triplicate determinations from one representative experiment.

inside the cell (Fig. 4*A*). This suggested that the up-regulation of the A_{2B} adenosine receptors was not caused by the expression of an internal pool of receptors, but probably by de novo synthesis of receptors. This was confirmed by Northern blotting; BMDM initially expressed little A_{2B} receptor mRNA, which suggested that the half-life of the A_{2B} receptor protein is very high in resting macrophages (Fig. 4*B*). After treatment with IFN-γ, an increase in the mRNA levels of A_{2B} adenosine receptor became evident after 24 h and especially after 48 h. This demonstrated that the up-regulation of A_{2B} adenosine receptors both inside and at the cell surface was due to the induction of de novo synthesis by IFN-γ. This is the first time that a heterologous regulation of adenosine receptors by a cytokine has been demonstrated, and it is crucial to understand the relationship between the anti-inflammatory effect of adenosine on the different components of the immune system and the resolution of the immune response.

To study the functional consequences of the up-regulation of A_{2B} adenosine receptors by IFN-γ, we measured the production of cAMP induced by agonists. The IFN-γ-induced increase in A_{2B} receptor expression is time-dependent (Fig. 5*A*). After the addition of adenosine or NECA to IFN-γ-activated macrophages, we observed that production of cAMP increased in a time-dependent manner (Fig. 5*B*). The increase in cAMP correlated with the up-regulation by IFN-γ of the A_{2B} adenosine receptors. This suggested that up-regulation of A_{2B} adenosine receptors in activated macrophages is physiologically relevant.

The expression of MHC class II molecules is necessary for Ag presentation to $CD4^+$ T cells by macrophages. The expression of MHC class II molecules is very slow even with saturating amounts of IFN-γ, and these proteins are expressed at the macrophage cell surface only 24–72 h after stimulation (27). cAMP represses at the transcriptional level the expression of MHC class II induced by IFN-γ on macrophages (42–44). The activation of the A_{2B} adenosine receptor by NECA produced enough cAMP to inhibit by 66% the expression of class II molecules at the surface of macrophages (Fig. 6). The inhibition was higher when the macrophages were treated for a long time with IFN-γ, i.e., when the up-regulation of A_{2B} adenosine receptors was higher. Therefore, it seems that the up-regulation of adenosine receptors is a mechanism of deactivation of macrophages. The inhibitory effect of NECA was receptor mediated, since it was blocked by the antagonist DPCPX.

After 24 h of treatment, IFN-γ induced the expression of IAα and IAβ mRNA (Fig. 6*C*). The addition of the adenosine agonist NECA inhibited almost completely the expression of IAα and IAβ mRNA induced by IFN-γ (Fig. 6*C*). As IAα and IAβ mRNAs are very stable (45), this suggested that inhibition by cAMP of MHC class II production takes place at the transcriptional level. The inhibition by adenosine analogues of class II mRNA expression induced by IFN-γ took place through the production of cAMP, since only NECA, forskolin, or 8-bromo-cAMP, but not the control drug R-PIA, repressed the induction of mRNA (Fig. 7*A*). Finally, only drugs that activate A_{2B} receptors inhibited the induction of IAα or IAβ mRNA by IFN-γ (Fig. 7*B*). Therefore, the inhibition of class II expression is mediated through the production of cAMP by the interaction of adenosine and its analogues with the A_{2B} receptors. Thus, the activation of up-regulated A_{2B} receptors would lead to an inhibition of the IFN-γ induction of MHC class II molecules.

Regarding the effect of adenosine on the IFN-γ-induced expression of class II MHC genes, we wanted to analyze the effects of

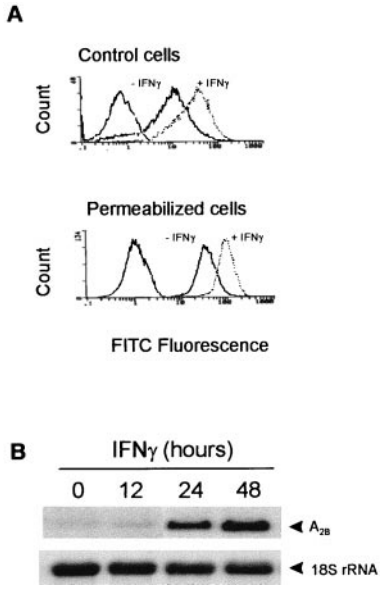

FIGURE 4. IFN-γ up-regulates the A_{2B} adenosine receptor expression. *A*, BMDM (10^6) cells were treated with 300 U/ml IFN-γ for 48 h, and A_{2B} adenosine receptor surface expression was analyzed by flow cytometry in nonpermeabilized and permeabilized cells. The continuous line histogram shows basal A_{2B} expression in nonstimulated cells, whereas the noncontinuous line histogram corresponds to A_{2B} receptor expression in cells treated with IFN-γ. The Fc receptors were blocked using a specific Ab (anti-CD16/CD32). *B*, Northern blot analysis of A_{2B} adenosine receptor. Twenty micrograms of total RNA from BMDM treated with 300 U/ml IFN-γ at the indicated times was probed with a randomly primed human A_{2B} adenosine receptor cDNA fragment. A unique 1.6-kb transcript was detected in BMDM. The amount of loaded RNA was corrected by ribosomal 18S RNA gene expression.

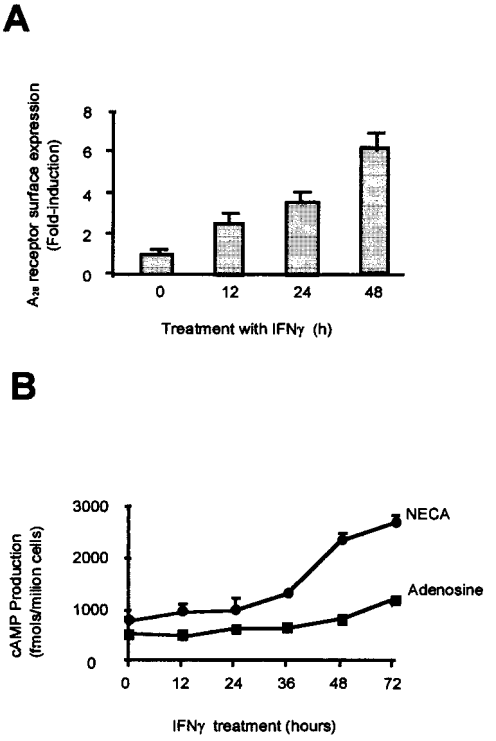

FIGURE 5. The number of A_{2B} adenosine receptors correlates with cAMP production. *A*, Time course of IFN-γ-induced A_{2B} adenosine receptor surface expression. Macrophages (10^6) were treated with 300 U/ml of IFN-γ for the indicated times, and the IFN-γ-induced surface expression of the A_{2B} adenosine receptor was analyzed by flow cytometry. *B*, Cells (10^6) pretreated with 300 U/ml of IFN-γ for the indicated times were stimulated with 10^{-5} M NECA or 5×10^{-5} M adenosine for 15 min, and cAMP production was measured with an EIA system (Amersham, Aylesbury, U.K.).

adenosine analogues on other aspects of IFN-γ-induced macrophage activation. IFN-γ induces the expression of NOS, regulates the production of nitric oxide in macrophages (4), and modulates the expression of several proinflammatory cytokines, such as TNF-α and IL-1β (41). The treatment of macrophages with NECA inhibited the IFN-γ-induced expression of iNOS (Fig. 8*A*). Moreover, NECA also inhibits the expression of TNF-α and IL-1β induced by IFN-γ (Fig. 8*B*). Thus, the inhibitory effect of adenosine and its analogues is not specific for IFN-γ-induced class II MHC expression, but is also present for several aspects of macrophage activation induced by IFN-γ.

Discussion

This report shows that A_{2B} adenosine receptors are preferentially expressed on murine macrophages. It also demonstrates that receptor engagement is a necessary first step in the modulation of at least some of the functional activities of macrophages.

The role of the adenosine A_{2B} receptors was demonstrated using BMDM, which are primarily quiescent nontransformed cells (23), and was supported by several observations. First, the low affinity binding of radiolabeled NECA, an agonist of the four types of adenosine receptors that is not displaced by low concentrations of agonists specific for either A_1 or A_{2A} adenosine receptors; this is consistent with the presence of the A_{2B} adenosine receptor. Second, a specific Ab against A_{2B} receptors, but not against A_1 receptors, stained a high percentage of the macrophages; this is the first time that specific Abs against the A_{2B} receptors have been used. Third, the effects of NECA on cAMP levels are probably mediated by A_{2B} receptors, since CGS 21680, which is a selective agonist for A_{2A} receptors, was ineffective. The activation of A_3 receptors does not seem to be involved in cAMP production, since A_3 receptors are negatively coupled to the adenylate cyclase, and DPCPX, which does not interact with A_3 receptors, blocks the NECA-induced response. These results are consistent with the presence of the A_{2B} and A_3 subtypes, but not A_1 or A_{2A} receptors, in macrophages. The presence of A_3 receptors in cells of the monocyte/macrophage lineage has been previously reported (8, 9). In contrast, this is the first report demonstrating the presence of A_{2B} receptors in macrophages.

IFN-γ is the major activator of macrophages, and over 200 genes are now known to be regulated by this cytokine (46, 47). IFN-γ modulates the expression of many receptors. In some cases, IFN-γ induces the expression of receptors such as the high affinity IgG receptor (Fcγ-RI) (48) and also down-regulates the expression of other receptors, such as the CSF-1 receptor (c-*fms*) (49). A time-course analysis showed that IFN-γ induced an increase in the number of A_{2B} adenosine receptors on the cell surface. This is the first time that a modulation of the adenosine receptors by a cytokine has been described. This progressive increase was due to de novo synthesis of the receptor, and it correlated with the higher capacity of adenosine to induce an increase in cAMP levels, thus suggesting that the newly synthesized receptors were functional.

It has been shown that the expression of MHC class II molecules induced by IFN-γ is down-regulated by several mediators, such as LPS, PGs, and glucocorticoids (42). The mechanism of repression by which some of these agents operate has been described recently.

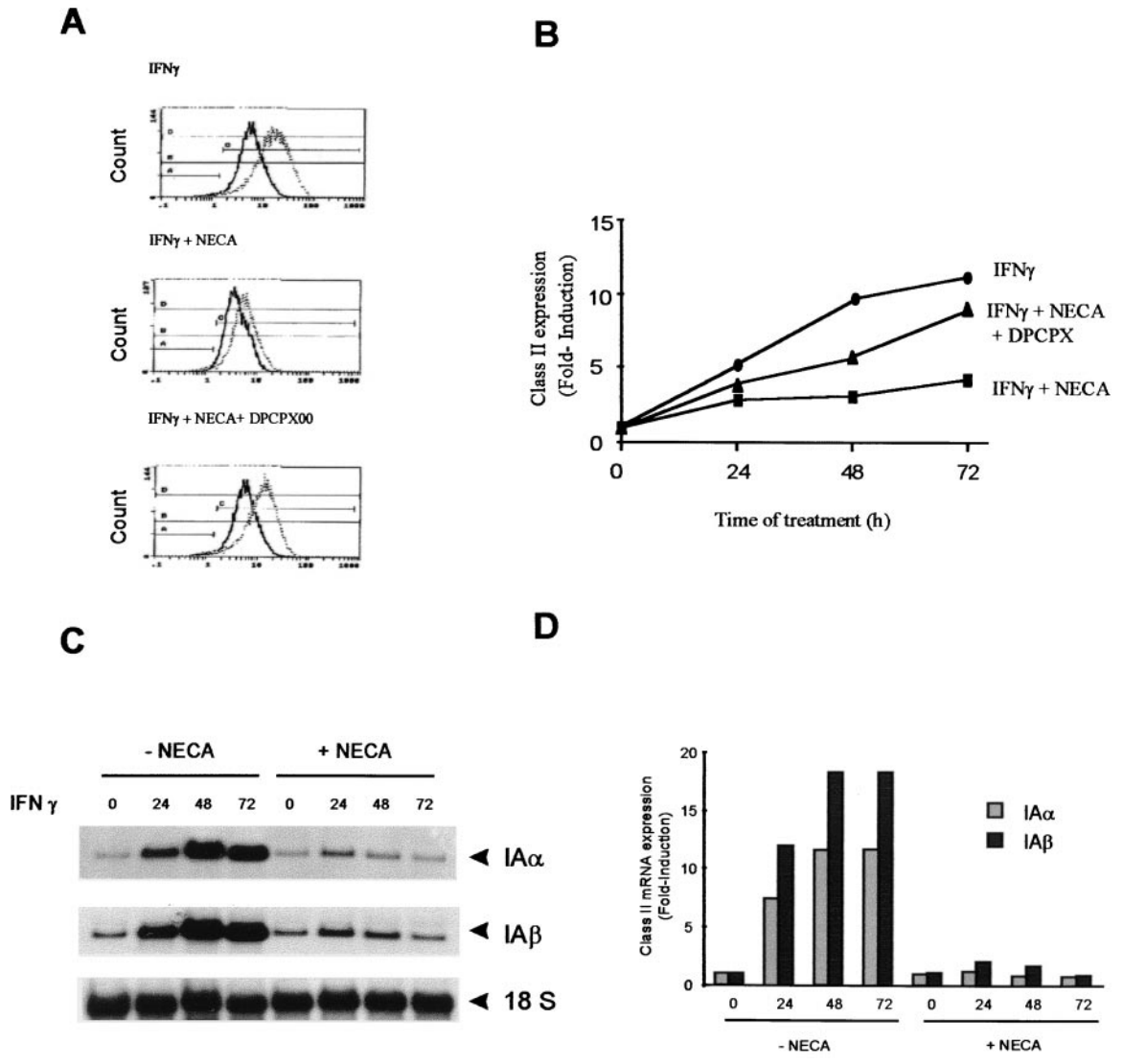

FIGURE 6. Inhibition of IFN-γ induction of MHC class II expression by adenosine analogues. *A*, Macrophages (10^6) were treated with IFN-γ for 72 h in the presence or the absence of 10^{-5} M NECA and 10^{-6} M DPCPX plus 10^{-5} M NECA, and the cell surface expression of MHC II was analyzed by flow cytometry using mAbs against I-A^d as primary Abs and detection with a FITC-labeled secondary Ab as described in *Materials and Methods*. The continuous line histogram corresponds to untreated cells, whereas the noncontinuous line histogram shows samples treated with IFN-γ in the presence or the absence of adenosine analogues, as indicated. *B*, Quantification of MHC class II surface expression after 24, 48, and 72 h in the same conditions as those described in *A*. The quantification of MHC class II molecule expression was conducted using the Immuno4 computer program. *C*, Inhibition of IFN-γ-induced MHC class II mRNA expression by NECA. For the Northern blotting analysis of IAα and IAβ mRNA, 20 μg of total RNA from BMDM was used. The amount of loaded RNA was corrected by the ribosomal 18S RNA gene expression. BMDM were treated with 30 U/ml of IFN-γ for the indicated times in the presence or the absence of 10^{-5} M NECA. *D*, Quantification of Northern blotting analysis using a Molecular Analyst system.

Dexamethasone decreases MHC class II expression in macrophages at the level of transcription by inhibiting the transcription factors that bind to the X box of the promoter (50). The addition of cAMP to the culture medium reduces the expression of MHC class II. It was originally found that the effect of cAMP was mediated at the transcriptional level by conserved promoter elements (43, 44). More recently, it has also been described that an increase in intracellular cAMP levels inhibits STAT1 activity in mononuclear cells (51), which is necessary for MHC class II gene expression (52). Our results show that adenosine, interacting through the A_{2B} receptor and probably through the production of cAMP, inhibits the IFN-γ induction of MHC class II molecules. The mechanism of inhibition probably acts at the transcriptional level.

Adenosine and its analogues inhibited the MHC class II expression induced by IFN-γ as well as other activities induced by IFN-γ such as the induction of iNOS and the regulation of the expression of proinflammatory cytokines such as TNF-α or IL-1β. Moreover, adenosine inhibits some LPS-induced functions in macrophage activation (3, 5, 9).

The induction of adenosine receptors by IFN-γ could be clinically relevant. The expression of adenosine receptors is very slow, requiring the synthesis of new receptors that seem to be very stable. This period of time correlates with the time course of expression of MHC class II molecules, which begins 12 h after IFN-γ treatment, reaches a maximum after 48 h, and then begins to decrease (27). The release of adenosine during the inflammatory process would lead to the activation of A_{2B} receptors and the production of cAMP. It is likely that the IFN-γ-activated macrophages at the inflammation sites express more A_{2B} receptors, and then activation by adenosine would lead to the cAMP-mediated down-regulation of MHC class II molecules and

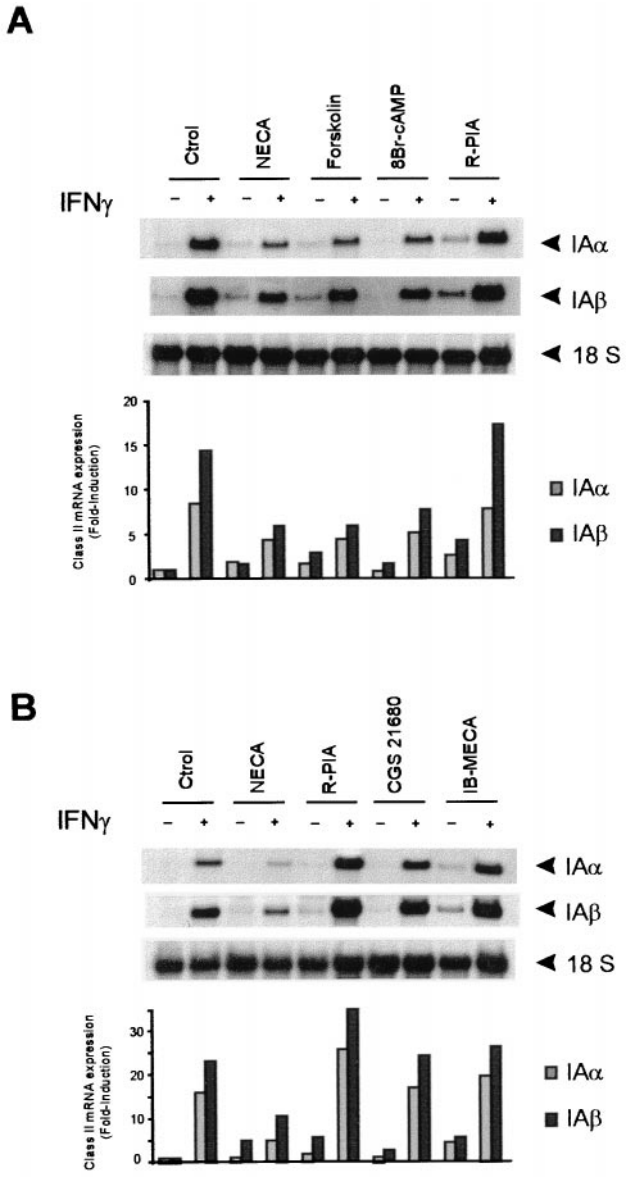

FIGURE 7. Inhibition of IFN-γ-induced MHC class II mRNA expression by adenosine. *A*, The inhibition of IFN-γ-induced mRNA of IAα or IAβ is mediated through the production of cAMP. BMDM were treated with 30 U/ml of IFN-γ for 24 h in the presence or the absence of NECA (10^{-5} M), forskolin (10^{-5} M), 8-bromo-cAMP (10^{-4} M), or R-PIA (10^{-6} M). *B*, The inhibition of IFN-γ-induced IAα or IAβ mRNA is specific for drugs that activate A_{2B} receptors. BMDM were treated with 30 U/ml of IFN-γ for 24 h in the presence or the absence of NECA (10^{-5} M), R-PIA (10^{-6} M), CGS 21680 (10^{-5} M), or IB-MECA (10^{-5} M).

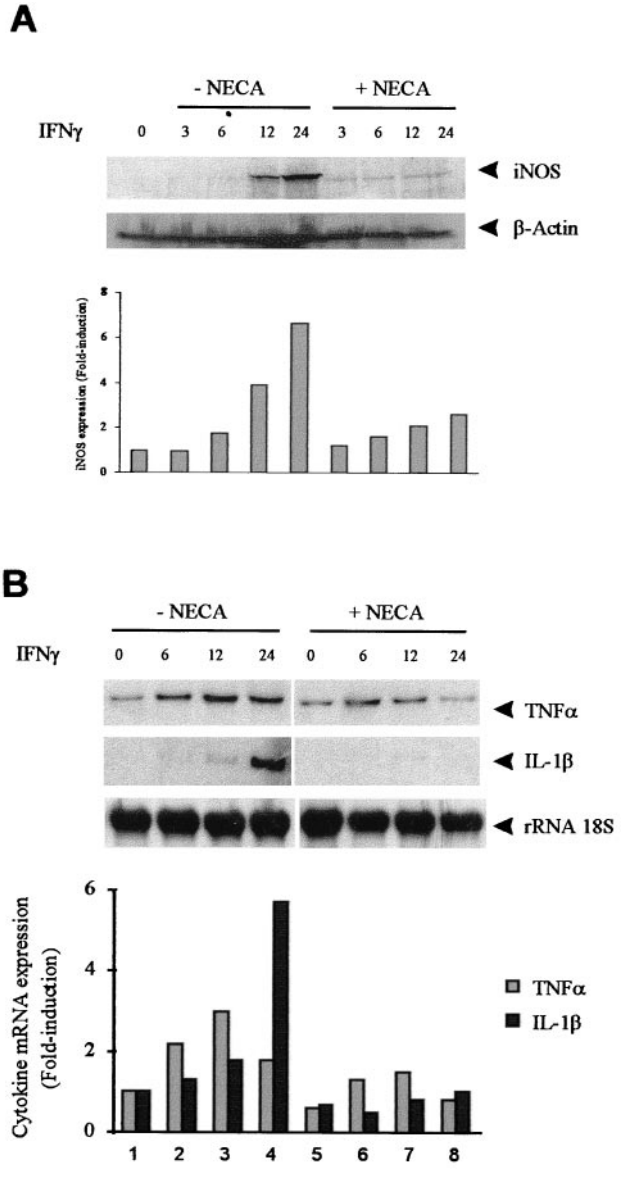

FIGURE 8. Inhibition of the IFN-γ-induced expression of iNOS and proinflammatory cytokines by adenosine. *A*, NECA inhibits the iNOS expression induced by IFN-γ. BMDM were treated with 30 U/ml of IFN-γ for the indicated times in the presence or the absence of NECA (10^{-5} M). Western blot analysis was performed with 100 μg of total protein extracts. *B*, The IFN-γ-induced mRNA expression of TNF-α and IL-1β was inhibited by NECA. For the Northern blotting analysis of these cytokines, 20 μg of total RNA from BMDM was used. The amount of loaded RNA was corrected by the ribosomal 18S RNA gene expression. BMDM were treated with 30 U/ml of IFN-γ for the indicated times in the presence or the absence of 10^{-5} M NECA.

other macrophage activities. This may constitute an important mechanism of macrophage deactivation.

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