Adenosine Inhibits Macrophage Colony-Stimulating Factor-Dependent Proliferation of Macrophages Through the Induction of p27kip-1 Expression

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Adenosine Inhibits Macrophage Colony-Stimulating Factor-Dependent Proliferation of Macrophages Through the Induction of p27kip-1 Expression

Jordi Xaus,2* Annabel F. Valledor,2* Marina Cardó, Laura Marquès, Jorge Beleta, † José M. Palacios, † and Antonio Celada3*

Adenosine is produced during inflammation and modulates different functional activities in macrophages. In murine bone marrow-derived macrophages, adenosine inhibits M-CSF-dependent proliferation with an IC_{50} of 45 μM. Only specific agonists that can activate A_{2B} adenosine receptors such as 5'-N-ethylcarboxamidoadenosine, but not those active on A_{1} (N^6-(R)-phenylisopropyladenosine), A_{2A} ([p-(2-carbonylethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine), or A_{3} (N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide) receptors, induce the generation of cAMP and modulate macrophage proliferation. This suggests that adenosine regulates macrophage proliferation by interacting with the A_{2B} receptor and subsequently inducing the production of cAMP. In fact, both 8-Br-cAMP (IC_{50} 85 μM) and forskolin (IC_{50} 7 μM) inhibit macrophage proliferation. Moreover, the inhibition of adenylyl cyclase and protein kinase A blocks the inhibitory effect of adenosine and its analogues on macrophage proliferation. Adenosine causes an arrest of macrophages at the G_{1} phase of the cell cycle without altering the activation of the extracellular-regulated protein kinase pathway. The treatment of macrophages with adenosine induces the expression of p27kip-1, a G_{1} cyclin-dependent kinase inhibitor, in a protein kinase A-dependent way. Moreover, the involvement of p27kip-1 in the adenine inhibition of macrophage proliferation was confirmed using macrophages from mice with a disrupted p27kip-1 gene. These results demonstrate that adenosine inhibits macrophage proliferation through a mechanism that involves binding to A_{2B} adenosine receptor, the generation of cAMP, and the induction of p27kip-1 expression. The Journal of Immunology, 1999, 163: 4140–4149.

Macrophages are generated through a process of differentiation known as myelopoiesis. In the bone marrow, pluripotent stem cells differentiate into monocytes in the presence of M-CSF and other cytokines. Monocytes leave the bone marrow and, by circulating through blood vessels, reach different tissues where they terminally differentiate into macrophages and perform their specialized functions (1–3). Tissue macrophages are able to proliferate thanks to the autocrine production of M-CSF (4). Macrophages require M-CSF for proliferation, differentiation, and survival (5). M-CSF is the main growth factor for macrophages and also the only one specific for these cells. After interacting with the tyrosine kinase receptor c-fms, M-CSF triggers the activation of several signal transducing molecules in macrophages (5–8), such as some protein kinases of the Src family (9), the transcription factors Stat-1, Stat-3, and Stat-5 (10, 11), protein kinase C (12), and phosphatidylinositol 3-kinase (13, 14). M-CSF also activates the Raf/mitogen-activated protein/extracellular signal-related kinase (ERK)4 kinase pathway in macrophages (15). The activation of ERK-1/2 is required for macrophage proliferation in response to M-CSF.3 Active ERKs phosphorylate and regulate several cellular proteins (16), including other protein kinases, cytoskeletal components, phospholipase A_{2}, and nuclear transcription factors, such as Elk1/TCF and c-Jun, which regulate the expression of immediate early genes (17, 18).

Mitogen-activated protein kinase phosphatase-1 (MKP-1) is a member of a family of dual-specificity phosphatases (19, 20) that dephosphorylate both phosphotyrosine and phosphothreonine residues on target proteins. MKP-1 dephosphorylates and inactivates ERK-1 and -2 both in vitro and in vivo, suggesting that this phosphatase has a critical effect on maintaining the balance between ERK phosphorylation and dephosphorylation (21). The overexpression of MKP-1 inhibits ERK-regulated reporter gene expression, the synthesis of DNA induced by Ras and G_{1}-specific gene transcription, and entry of fibroblasts to the S phase in response to mitogenic stimuli (22, 23). The induction of the expression of MKP-1 is a mechanism used by the cell to control and attenuate proliferative signaling pathways.

All these signaling pathways allow macrophages to enter the cell cycle in response to M-CSF. Passage through the cell cycle is

*Departamento de Fisiologia (Biologia del Macrofág), Facultat de Biologia i Fundació August Pi i Sunyer, Campus de Bellvitge, Universitat de Barcelona, Barcelona, Spain; and 1Laboratorios Almirall Prodesfarma SA, Research Center, Barcelona, Barcelona, Spain

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2 J.X. and A.F.V. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Antonio Celada, Departamento de Fisiologia, Facultat de Biologia, Av. Diagonal 645, 08028 Barcelona, Spain. E-mail address: acelada@bio.ub.es

4 Abbreviations used in this paper: ERK, extracellular signal-related kinase; NECA, 5'-N-ethylcarboxamidoadenosine; R-PIA, N^6-(R)-phenylisopropyladenosine; DPCPX, 1,3-dipropyl-8-(carboxamidopropyl)adenine; CGS 21680, 2-[p-(2-carbonyl-ethyl)phenylamino]-5'- N-ethylcarboxamido adenosine; IB - MECA, N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide; BMDM, bone marrow-derived macrophages; PKA, protein kinase A; MKP, mitogen-activated protein kinase phosphatase; cdk, cyclin-dependent kinase; cki, cdk inhibitor; CADO, 2-chloroadenosine; DAPI, 4,6-diamidino-2-phenylindole.

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regulated through the interaction of a family of protein kinase complexes. Each complex is composed of at least a catalytic subunit, a cyclin-dependent kinase (cdk), and its essential activating partner, which is a cyclin (reviewed in Refs. 24 and 25). These complexes are activated at various checkpoints after specific intervals during the cell cycle, but they can also be modulated by exogenous factors. The cdks are inhibited when associated with a group of proteins known as cdk inhibitors (cki) (26). The cki's best described are p16\(^{INK-4}\), which belongs to the INK 4 family, p21\(^{waf-1}\), and p27\(^{kip-1}\), the latter belonging to the CIP/KIP family of cdk inhibitors (27–29). The presence of growth factors is only required during the G\(_1\) phase. Once the cell reaches the restriction point that appears late during the G\(_2\) phase and at the beginning of the S phase, the growth factors are no longer necessary and the cell is committed to complete the cell cycle (30, 31). This may explain why several regulatory steps of the cell cycle, such as the p53 checkpoint, pRb phosphorylation, and the activity of the cdk inhibitors, take place at this point (32–35).

Adenosine is a purine nucleoside produced and secreted to the extracellular media by cells during normal intracellular ATP metabolism and degradation. Nevertheless, in stress situations like ischemia or hypoxia, massive ATP degradation increases local adenosine concentration to micromolar values (36, 37). In these situations, adenosine modulates several physiological functions, acting mainly as an endogenous antiinflammatory agent (reviewed in Ref. 38).

Most functional activities of extracellular adenosine are mediated through binding to specific surface receptors. However, it has been reported that adenosine needs to be internalized to induce some functions such as NO production and inhibition of LPS-induced TNF-\(\alpha\) expression in some models (39). So far, four different adenosine receptors have been described and called A\(_1\), A\(_2A\), A\(_2B\), and A\(_3\) depending on their structural, functional, and pharmacological characteristics (40). Recently, all four adenosine receptor subtypes have been cloned from several species (41–43). All belong to the G-protein-coupled receptor superfamily. The type and density of adenosine receptors present on the cell surface are characteristic of each cell type.

The antiinflammatory role of adenosine has been associated to its effects on neutrophil activity. Additionally, adenosine may also play an important role in the attenuation of macrophage activity, as it modulates several functions of macrophages, such as the regulation of nitrite production (39, 44), the inhibition of LPS-induced TNF-\(\alpha\) expression (39, 45), and the induction of IL-6 (46) and IL-10 production (39, 47).

We have found that adenosine inhibits M-CSF-dependent proliferation of murine bone marrow–derived macrophages (BMDM). To do this, adenosine interacts with the A\(_2B\) receptor at the cell surface and induces a subsequent increase of cAMP levels. Treatment of macrophages with adenosine does not inhibit the activation of the ERK pathway. Instead, adenosine induces the expression of p27\(^{kip-1}\) in a protein kinase A (PKA)-dependent pathway, thus causing the growth arrest at the G\(_1\) phase of the cell cycle without inducing apoptosis. These results reveal the molecular mechanism involved in the adenosine-mediated inhibition of the M-CSF-dependent proliferation of macrophages and remark the relevance of this nucleoside as an immunosuppressor of macrophage activity and proliferation.

Materials and Methods

Reagents

Adenosine, 2-chloroadenosine (CADO), 5'-N-ethylcarboxamidoadenosine (NECA), N\(^6\)-(R)-phenylisopropyladenosine (R-PIA), and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were obtained from Sigma (St. Louis, MO). 2-[\(\beta\)-(2-Carboxylethylphenylylaminio)-5'-N-ethylcarboxamido-adenosine (CGS 21680) and N\(^3\)-(3-iodobenzyl)adenosine 5'-N-methyluronamide (IB-MECA) were purchased from Research Biochemicals (Natick, MA). 8-Br-cAMP and forskolin were obtained from Fluka Biochemika (Buchs, Switzerland). \(^{3}H\)Thymidine was obtained from Amer sham (Buckinghamshire, U.K.). 4.6-Diamidino-2-phenylindole (DAPI), SQ 22536, and KT 5720 were purchased from Calbiochem (La Jolla, CA). All the other products were of the best grade available and were purchased from Sigma. Deionized water further purified with a Millipore Milli-Q system (Bedford, MA) was used.

Cell culture

BMDM were isolated from 8-wk-old BALB/c mice (Charles River Laboratories, Wilmington, MA) as previously described (49). The cells were cultured in plastic tissue culture dishes (150 mm) in 40 ml DMEM containing 20% FBS and 300 U/ml penicillin-streptomycin as a source of M-CSF. The cells were incubated at 37°C in a humidified 5% CO\(_2\) atmosphere. After 7 days of culture, an homogeneous population of adherent macrophages was obtained. To render the cells quiescent, when the macrophages were confluent they were deprived of L cell-conditioned medium for 14–16 h before the experiment. BMDM from p27\(^{kip-1}\)-knockout mice were isolated in the same conditions. These mice were kindly donated by Dr. J. Roberts from Howard Hughes Medical Institute (Seattle, WA) (49).

Antibodies

Surface expression of the M-CSF receptor (c-fms) was analyzed by using affinity-purified rabbit Abs anti-mouse c-fms (Upstate Biotechnology, Lake Placid, NY). Fluorescein-conjugated rat anti-rabbit IgG Ab from Sigma was used as a secondary Ab. To block Fc receptors, we used an anti-CD16/CD32 Ab (PharMingen, San Diego, CA). For the analysis of p27\(^{kip-1}\) expression by Western blotting, we used a monoclonal anti-mouse p27\(^{kip-1}\) Ab (PharMingen). The rabbit anti-mouse MKP-1 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For ERK mobility shift assays, we used an anti-ERK-1/2 Ab, which was a kind gift of Dr. M. J. Weber (University of Virginia School of Medicine, Charlottesville, VA). Peroxidase-conjugated anti-mouse and anti-rabbit IgGs (Cappel, Turnhout, Belgium) were used as secondary Abs. Primary Abs against mouse \(\beta\)-actin were purchased from Sigma.

Plasmids and constructs

The pMHI17 plasmid corresponds to the mouse p21\(^{waf-1}\) full-length cDNA cloned in pEx-lox and was kindly provided by Dr. Massague (Sloan Kettering Institute, Howard Hughes Medical Institute, New York, NY). The pET-3d plasmid corresponds to the D\(_1\) cyclin cDNA cloned in pET-12 as described (50). The pCMJ3/cdk-4 plasmid contains the mouse cdk-4 full-length cDNA cloned in pBlueScript K5 as described (51). The probe for the 18S rRNA was obtained as described (52).

Proliferation assay

Cell proliferation was measured as previously described (53, 54) with minor modifications. The cells were deprived of M-CSF for 18 h and then 10\(^5\) BMDM were incubated for 24 h in 24-well plates (3424 MARK II; Costar, Cambridge, MA) in 1 ml of complete medium in the presence or absence of the indicated adenosine analogues or derivatives. After this period of time, the medium was removed and replaced with 0.5 ml of medium containing \(^{3}H\)thymidine (1 \(\mu\)Ci/ml). After two additional h of incubation at 37°C, the medium was removed and the cells were fixed in ice-cold 70% methanol. After three washes in ice-cold 10% TCA, the cells were solubilized in 1% SDS, 0.3 N NaOH. Radioactivity was counted by liquid scintillation using a 1500 Packard Tri-Carb scintillation counter (Meriden, CT). Each experiment was performed three times, and the results were expressed as the mean \(\pm SD\).

Determination of cAMP

The production of cAMP was measured using a standard procedure. Briefly, 10^5 macrophages were cultured in 24-well plates in complete media. The cells were stimulated with the indicated adenosine agonists for 15 min. Extraction of cAMP from the cells was conducted 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 being allowed 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 new tubes and dried using a speed-vac system (Bio-Rad, Hercules, CA). The dried extracts were dissolved in assay buffer, and the amount of cAMP was analyzed using a nonacetylation cAMP enzyme immunoassay system (Amersham). Each
sample was analyzed in triplicate, and the results were represented as the mean ± SD.

**Determination of c-fms cell-surface expression**

Cell-surface staining was conducted using specific Abs and cytofluorometric analysis. After treatment with adenosine or its analogues for 24 h, 10^6 cells were harvested and washed in cold PBS. After fixing with 2% paraformaldehyde during 30 min at 4°C, the cells were resuspended in 50 μl PBS containing 5% FBS and then incubated at 4°C for 15 min with 1 μg/10^6 cells of anti-CD16/CD32 mAb to block Fc receptors. Then, the cells were incubated for 1 h at room temperature with murine c-fms-specific Ab (1 μg/10^6 cells). The cells were then washed by centrifugation through a FBS cushion. Finally, cells were incubated with FITC-conjugated anti-mouse IgG Ab for 1 h at 4°C. Stained cell suspensions were washed in ice-cold PBS. The cells were lysed in 0.5 ml of lysis buffer (50 mM sodium orthovanadate, 90 mM citric acid, and 2 mM 3-[N-morpholino]propanesulfonic acid (pH 7.0)/1 M formaldehyde buffer. The DNA was transferred overnight to a GeneScreen nitrocellulose membrane (Life Science Products, Boston, MA) and fixed by UV irradiation (150 mJ). For p21\(^{\text{kip-1}}\) mRNA detection, we obtained the full-length cDNA of p21\(^{\text{kip-1}}\) by digesting pMH117 with Eco\(_R\)I/HindIII and used it as a probe. The D\(_1\) cyclin probe was prepared by digesting the pET-3d construct with Bgl2/EcoRV. PC3M3/cdk-4 digestion with EcoRI allowed us to obtain a cdk-4 probe. To check for differences in RNA loading, we analyzed the expression of the 18S rRNA transcript. All probes were labeled with [\(\alpha\text{-}^{32}\text{P}\)]dCTP (ICN Pharmaceuticals) with the oligolabeling kit method (Pharmacia Biotech, Uppsala, Sweden). After incubating the membranes for 18 h at 65°C in hybridization solution (20% formamide, 5× SSC, 10 mM EDTA, 1% SDS, 25 mM Na\(_2\)HPO\(_4\), 25 mM NaH\(_2\)PO\(_4\), 0.2 mg/ml salmon sperm DNA, and 10^6 cpm/ml of \(32\text{P}\)-labeled probe), they were exposed to Kodak X-AR films. The bands of interest were quantified with a molecular analyst system (Bio-Rad).

**Protein extraction and Western blot analysis**

Cells were washed twice in cold PBS and lysed on ice with lysis solution (1% Triton X-100, 10% glycerol, 50 mM HEPES, pH 7.5, 150 mM NaCl, protease inhibitors). Then, 100 μM sodium orthovanadate was added to inhibit the activity of tyrosine phosphatases when necessary. The protein concentration of the samples was determined by the Bio-Rad protein assay. The proteins from the cell lysates (100–150 μg) were boiled at 95°C in Laemmli SDS-loading buffer, separated in 12% SDS-PAGE, and electro-transferred to nitrocellulose membranes (Hybond-ECL; Amersham). The membranes were blocked for at least 1 h at room temperature in TBS containing 5% nonfat dry milk and then incubated with TBS-T containing the primary Ab. For p2\(^{\text{Thr}}\) 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-mouse or anti-rabbit IgG Abs (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). Quantification of the blot was conducted by densitometric analysis.

**Determination of the ERK phosphorylation state by mobility shift assay**

This assay was performed as described for the Western blot analysis with slight modifications (56). Proteins from cell lysates (50–100 μg) were subjected to 7.5% SDS-PAGE to allow efficient separation of the phosphorylated and dephosphorylated forms of ERK. The incubation of the membranes with anti-ERK-1/2 primary Ab or peroxidase-conjugated anti-mouse IgG Ab were done sequentially in TBS-T for 1 h at room temperature.
Adenosine inhibits M-CSF-dependent proliferation of BMDM. A, BMDM were obtained after 7 days of culture in the presence of M-CSF. A total of $10^5$ macrophages were incubated in 24-well plates in the presence of the indicated amounts of M-CSF either alone (Control) or with $10^{-4}$ M adenosine. Proliferation was determined as indicated in Materials and Methods. B, Adenosine inhibits macrophage proliferation in a dose-dependent manner. A total of $10^5$ macrophages were incubated in 24-well plates in the presence of 1000 U/ml of M-CSF and the indicated amounts of adenosine. Control cells were incubated with M-CSF alone. Each determination was made in triplicate, and the values represented correspond to the mean ± SD of one representative of four independent experiments.

The natural ligand adenosine induced a lower macrophage response than NECA, probably due to the surface expression of ectoadenosine deaminase, a molecule that degrades adenosine before it can be metabolized (68). Therefore, we used NECA in the following experiments. The NECA-induced production of intracellular

pH 7.4

proliferation. Treatment of macrophages with $10^{-5}$ M CGS 21680, $10^{-6}$ M R-PIA, or $10^{-5}$ M IB-MECA did not induce a detectable increase of intracellular cAMP levels. To date, there are no specific agonists for $A_{2B}$ receptors. The results, obtained with adenosine, CADO, or NECA, three nonspecific agonists, indicate that the effect of adenosine is mediated through their interaction with the $A_{2B}$ adenosine receptor, because specific agonists for the other adenosine receptor subtypes did not have any effect on cAMP production at those concentrations specific for their receptors, while at higher concentrations they could bind to $A_{2B}$ receptors and induce macrophage proliferation. This is the case of R-PIA, an inhibitor of $A_1$ receptors (data not shown). Furthermore, $10^{-6}$ M DPCPX inhibited completely the cAMP increase induced by $10^{-5}$ M NECA (data not shown). Although we cannot exclude the presence of other adenosine receptors, all our results suggest that the $A_{2B}$ adenosine receptor is the main responsible for the cAMP increases found in response to adenosine in BMDM.

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cAMP in BMDM was both dose- and time-dependent (Fig. 3, B and C), with an EC_{50} of 5 \mu M similar to that observed in previous reports (69).

So far we have shown that the adenosine-induced inhibition of M-CSF-dependent proliferation is mediated through the A2B receptors, and that the interaction of adenosine with these receptors

**FIGURE 3.** Induction of cAMP production by adenosine agonists. A, A total of 10^6 BMDM were stimulated for 15 min with 5 \times 10^{-5} M adenosine (Ado), 10^{-3} M NECA, 10^{-3} M CADO, 10^{-3} M IB-MECA, 10^{-3} M CGS 21680, or 10^{-6} M R-PIA. The cultures were liquid phase extracted (see Materials and Methods), and the cAMP content was measured with an enzyme immunoassay system (Amersham). B, The production of cAMP in BMDM stimulated with NECA is dose-dependent. The cells were treated for 15 min with the indicated concentrations of NECA. C, Time-course of NECA-induced cAMP production in macrophages. BMDM were treated with 10^{-3} M NECA for the indicated periods of times. Each sample was analyzed in triplicate, and the data are represented as the mean \pm SD of triplicate determinations of two independent experiments.

**FIGURE 4.** cAMP inhibits M-CSF-dependent proliferation of macrophages. Macrophages were treated with 8-Br-cAMP (A) or forskolin (B) at the indicated concentrations, and thymidine incorporation was measured as described in Materials and Methods. Control cells were grown in the presence of 1000 U/ml of M-CSF with no other treatment. C, NECA inhibits macrophage proliferation through an adenylyl cyclase- and PKA-dependent pathway. A total of 10^6 macrophages grown in the presence of the indicated concentrations of M-CSF (Control) were treated with 10^{-5} M NECA alone or combined with either 10^{-4} M SQ 22536 or 10^{-7} M KT 5720. D, Dose-dependent effect of PKA and adenylyl cyclase inhibitors on NECA-induced inhibition of macrophage proliferation. Macrophages were treated with 1000 U/ml of M-CSF alone or combined with 10^{-5} M NECA and the indicated concentrations of SQ 22536 or KT 5720. Each determination was made in triplicate, and the values represented correspond to the mean \pm SD of one representative of three independent experiments.
induces the production of cAMP. We were also interested in studying the role of cAMP in the inhibition of M-CSF-dependent proliferation of macrophages. 8-Br-cAMP, a cell membrane-permeable and nonmetabolizable cAMP analogue, inhibited macrophage proliferation in response to 1200 U/ml of M-CSF. The IC50 for this inhibition was 85 μM (Fig. 4A). Treatment of macrophages with forskolin, a drug that directly activates adenylyl cyclase and induces the generation of cAMP, also inhibited M-CSF-dependent macrophage proliferation in a dose-dependent manner (Fig. 4B). This suggested that the production of cAMP was sufficient to inhibit macrophage proliferation. To confirm that the activation of adenylyl cyclase and the production of cAMP was responsible for the inhibitory effect of NECA on macrophage proliferation, we analyzed the effect of the inhibitor of adenylyl cyclase SQ 22536. This drug blocked the inhibitory effect on macrophage proliferation induced by NECA (Fig. 4, C and D), thus indicating that NECA inhibits M-CSF-dependent proliferation through activating the adenylyl cyclase and the subsequent production of cAMP. Moreover, treatment of macrophages with KT 5720, a PKA inhibitor, also blocked the anti-proliferative effect of NECA (Fig. 4, C and D). This confirms that NECA inhibits macrophage proliferation through a PKA-dependent pathway.

We next determined the molecular mechanism used by adenosine and its analogues to inhibit macrophage proliferation. First, we studied the effect of adenosine on the modulation of M-CSF receptors. An 18-h starvation of M-CSF induced a 4- to 5-fold increase in the level of expression of M-CSF receptors compared with that observed in cells growing in the presence of M-CSF (control) (Fig. 5). In contrast, no modification in the number of M-CSF receptors was detected in macrophages incubated for 24 h with adenosine, NECA, forskolin, or 8-Br-cAMP (Fig. 5). Therefore, the adenosine-induced inhibition of M-CSF-dependent proliferation was not due to an altered expression of M-CSF receptors.

Bone marrow macrophage cultures grown in the presence of M-CSF are not cell cycle-synchronized and showed a random distribution, with 51% of cells in G0/G1, 30% in S, and 17% in G2/M (Fig. 6). In response to adenosine, macrophages appeared to be distributed homogeneously (88% of total cells) in a peak corresponding to the G0/G1 phase of the cell cycle (Fig. 6A). We did not find any subdiploid peak corresponding to apoptotic cells. Besides, cells treated with adenosine, NECA, or forskolin did not show DNA fragmentation in comparison to cells in which apoptosis had been induced by treatment with actinomycin D (70) (Fig. 6B). These results indicated that adenosine-mediated inhibition of proliferation was not due to a massive induction of apoptosis. Instead, the cell cycle stop induced by adenosine explains the inhibition of proliferation. Therefore, we were interested in studying the mechanisms used by adenosine to stop the cell cycle at the G0 phase.

In an initial approach, we first analyzed the effects of adenosine on the activation of the ERK pathway. The activation of ERK-1/2 is required for M-CSF-dependent proliferation of macrophages (5). Phosphorylation of ERK-2 was analyzed by a mobility shift assay.
ERK-2 phosphorylation can be used as an indicator of ERK activation in macrophages, because there is a close correlation between the phosphorylation state of ERK-1/2 and their activity in an in-gel-kinase assay (data not shown). M-CSF induced ERK-2 phosphorylation in bone marrow macrophages (Fig. 7A). No differences in ERK-2 phosphorylation were observed when M-CSF-treated macrophages were incubated in the presence of adenosine, NECA, or 8-Br-cAMP (Fig. 7A). We also analyzed the effect of NECA on the kinetics of ERK activation by in-gel-kinase assays (data not shown). The induction of ERK-2 phosphorylation by M-CSF was assessed with a mobility shift assay. Then, 80 µg of total protein were loaded per lane. The positions of phosphorylated and dephosphorylated forms of ERK-2 are indicated with arrows. B, cAMP does not modify the kinetics of the M-CSF-induced ERK activation. Quiescent macrophages were either left untreated or preincubated with 10−5 M NECA for 15 min and then stimulated with M-CSF (1200 U/ml) for the indicated times. ERK activity was analyzed with an in-gel-kinase assay.

We next determined the effects of adenosine on different elements involved in cell cycle control. Because adenosine and its analogues cause an arrest of macrophages at the G1 phase of the cell cycle, we studied the expression of G1 cdks. The expression of the mRNA for the components of the cyclin D/cdk-4 complex was not modified by treatment with adenosine and its analogues. Moreover, the analysis of the immunoprecipitates and blotting of the cyclin D/cdk-4 complex formation showed no differences between control and NECA-treated cells (data not shown). We also analyzed the expression of cdk inhibitors. The expression of p21Waf-1, a dual inhibitor of cdk required for the passage through the cell cycle (28), was not modified by adenosine or analogues (Fig. 8).

In contrast, adenosine, NECA, forskolin, and 8-Br-cAMP induced a 3- to 8-fold increase of p27kip−1 protein levels (Fig. 9). p27kip−1 is another G1 cki of the CIP/KIP family that binds to cyclin/cdk complexes and inhibits their activity (29). NECA induced the expression of p27kip−1 in a time- and dose-dependent manner (Fig. 9, B and C). The induction of p27kip−1 by NECA was observed after 6 h of treatment and it was maintained during the whole time-course of 24 h. Besides, the expression of p27kip−1 induced by NECA was inhibited by the treatment with either QS 22536 or KT 5720 (Fig. 9D); therefore, the expression of p27kip−1 depended on the production of cAMP by activated adenylyl cyclase and on the activation of the PKA pathway. Thus, the induction of p27kip−1 expression mediated by adenosine and its analogues may be responsible for the adenosine-induced arrest of macrophages at the G1 phase of the cell cycle.

To confirm this hypothesis, we analyzed the inhibitory effect of NECA on macrophage proliferation using macrophages from mice with the p27kip−1 gene disrupted. In these macrophages, in contrast with that observed in macrophages from normal mice, NECA, but not IFN-γ, did not inhibit cell proliferation (Fig. 10), demonstrating that the expression of p27kip−1 is necessary for the inhibition by NECA of the M-CSF-dependent proliferation of macrophages.
Discussion

Most experiments on proliferation and cell cycling have been conducted using transformed cell lines. In this report, we have used primary cultures of BMDM, which is an homogeneous population that responds to physiological proliferative or activating stimuli (54).

We have found that adenosine blocks M-CSF-dependent proliferation of macrophages. The effect of adenosine seems to be mediated through the engagement of the A2B adenosine receptor. This is supported by the use of specific agonists and antagonists for the different types of adenosine receptors. NECA, an agonist for the four types of adenosine receptors so far described, induces a stronger inhibition of M-CSF-dependent proliferation than the agonists specific for A1, A2, and A2a adenosine receptors. The NECA-induced increase of the intracellular levels of cAMP is likely mediated by A2B receptors, because DPCPX, an inhibitor of this type of receptors, inhibits the production of cAMP in response to NECA. This confirms our previous observations about the expression of adenosine receptors in macrophages (59). By using binding assays with radiolabeled NECA in competition with different agonists and immunoblotting with specific Abs, we have identified the presence of A2B and A3 adenosine receptors on the cell surface of macrophages, whereas A1 and A2a receptors are poorly represented in these cells.

The inhibition of M-CSF-dependent proliferation by adenosine may be caused by an increase in the production of cAMP. Both cAMP and PKA activators inhibit the proliferation of macrophages. Treatment of BMDM with adenosine arrests them in the G1 phase of the cell cycle, as it has been already demonstrated with cAMP analogues (71–73). Moreover, the inhibition of adenyl cyclase and PKA blocks the antiproliferative effect of NECA in macrophages.

The first step in the induction of macrophage proliferation in response to M-CSF is the interaction of this growth factor with its specific cell-surface receptor, c-fms. IFN-γ, the major activator of macrophages, inhibits M-CSF-induced proliferation and also down-modulates the expression of c-fms (74). However, neither adenosine nor cAMP-increasing agents modulated the expression of c-fms, thus indicating that growth arrest was not due to a reduction in M-CSF recognition at the cell surface.

One of the earliest events in the signal transduction of M-CSF is the activation of ERK-1/2 (15, 75). The inhibition of ERK activation by using PD98059, a specific inhibitor of the mitogen-activated protein/ERK kinase, blocked macrophage proliferation in response to M-CSF. Recently, several reports have shown that the capability of Ras to activate Raf-1 was impaired in cells treated with cAMP-elevating agents, leading to a loss in the capability to activate ERKs (76). Therefore, the activation of PKA may prevent growth factor-mediated cell division by interfering with the activation of ERK-1/2. However, cAMP enhances the M-CSF-induced ERK activity in macrophages (77). In our experiments, neither adenosine nor cAMP altered the phosphorylation state or the activation kinetics of ERK-2 in response to M-CSF. Besides, the expression of the phosphatase MKP-1 was not modified by cAMP-elevating agents. Therefore, cAMP does not seem to inhibit macrophage proliferation by reducing the capability of M-CSF to activate the ERK pathway.

Macrophages incubated with cAMP are blocked at the G1 phase despite synthesizing normal amounts of cyclin D1 and cdk-4. The phosphorylation of cdk4 at threonine 172 is necessary for cdk-4 activation. The cdk-activating kinase precipitated from cAMP-treated cells was as active as that obtained from nontreated proliferating cells. This suggested the presence of an inhibitory activity present in cell lysates of cAMP-treated cells. Recently p27kip-1, an inhibitor of cdk-4, has been identified (72, 78). We have studied the effects of adenosine analogues on the expression of two cdk-4 inhibitors, p21waf-1 and p27kip-1. We detected p27kip-1 expression only in those cells treated with cAMP-increasing agents, which may account for the cAMP-mediated arrest of the cell cycle at the
ERK1/2 Abs. We also thank Dr. J. Massagué (Sloan Kettering Institute, Howard Hughes Medical Institute, New York, NY) for the pH11117 Fcs-1 knockout mice. We especially thank Dr. Gabriel Gil (Institut Municipal d’Investigaciones Biomédicas, Barcelona, Spain) for his help with the pH11117 Fcs-1 knockout mice. We also thank Martin Culliel-Youn for the revision of the manuscript.

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35rophages from mice with the p27kip-1 gene disrupted (49), which are unresponsive to the inhibitory effect of NECA on macrophage proliferation.

36. These data presented in this report allow us to suggest a model for adenosine-mediated inhibition of macrophage proliferation. Aden- 

37 osine binds to Aαβ receptors on the surface of macrophages and subsequently induces the production of cAMP by activating adeny- 

38lyl cyclase. As a consequence of the cAMP increase, PKA is activated and the expression of p27kip-1 is induced, thus blocking the activity of G1 cyclin/cdk complexes. As a result, macrophages cannot progress through the G1 phase of the cell cycle and their proliferation is blocked even in the presence of M-CSF.

39. These results could have clinical relevance. In fact, the inhibit- 

40 ion of macrophage proliferation could be part of the immunosup- 

41 pressive effect of adenosine. The inhibition of macrophage prolif- 

42 eration and activation could result in the modulation and the resolu- 

43 tion of the inflammatory process.

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49. FIGURE 10. NECA did not inhibit M-CSF-dependent proliferation of 

50 BMDM from p27kip-1 knockout mice. BMDM were obtained after 7 days 

51 of culture in the presence of M-CSF. A total of 10^5 macrophages from 

52 control (B) mice were incubated in 24-well plates in the presence of the indicated amounts of M-CSF alone or with 10^-7 M NECA or 300 U/ml IFN-γ. Proliferation was determined as indicated in Materials and Methods. Each determination was made in triplicate, and the values represented correspond to the mean ± SD of one representative of two independent experiments.

53. ERK1/2 Abs. We also thank Dr. J. Massagué (Sloan Kettering Institute, Howard Hughes Medical Institute, New York, NY) for the pH11117 Fcs-1 knockout mice. We especially thank Dr. Gabriel Gil (Institut Municipal d’Investigaciones Biomédicas, Barcelona, Spain) for his help with the pH11117 Fcs-1 knockout mice. We also thank Martin Culliel-Young for the revision of the manuscript.


