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Multidrug Resistance Protein 4 (MRP4/ABCC4) Regulates cAMP Cellular Levels and Controls Human Leukemia Cell Proliferation and Differentiation*

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Sabrina Copsel[‡§¶], Corina Garcia[‡¶], Federico Diez[‡], Monica Vermeulem[¶‖], Alberto Baldi[§¶], Liliana G. Bianciotti[¶], Frans G. M. Russel[‡‡], Carina Shayo[§¶], and Carlos Davio[‡¶1]**

*From the [‡]Laboratorio de Farmacología de Receptores, Cátedra de Química Medicinal, Departamento de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 1113 Buenos Aires, Argentina, the [§]Laboratorio de Patología y Farmacología Molecular, Instituto de Biología y Medicina Experimental, 1428 Buenos Aires, Argentina, the [¶]Consejo Nacional de Investigaciones Científicas y Técnicas and [‖]Departamento de Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, 1425 Buenos Aires, Argentina, the **Cátedra de Fisiopatología, Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 1113 Buenos Aires, Argentina, and the [‡‡]Department of Pharmacology and Toxicology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, 6525 HP Nijmegen, The Netherlands*

Increased intracellular cAMP concentration plays a well established role in leukemic cell maturation. We previously reported that U937 cells stimulated by H2 receptor agonists, despite a robust increase in cAMP, fail to mature because of rapid H2 receptor desensitization and phosphodiesterase (PDE) activation. Here we show that intracellular cAMP levels not only in U937 cells but also in other acute myeloid leukemia cell lines are also regulated by multidrug resistance-associated proteins (MRPs), particularly MRP4. U937, HL-60, and KG-1a cells, exposed to amthamine (H2-receptor agonist), augmented intracellular cAMP concentration with a concomitant increase in the efflux. Extrusion of cAMP was ATP-dependent and probenecid-sensitive, supporting that the transport was MRP-mediated. Cells exposed to amthamine and the PDE4 inhibitor showed enhanced cAMP extrusion, but this response was inhibited by MRP blockade. Amthamine stimulation, combined with PDE4 and MRP inhibition, induced maximal cell arrest proliferation. Knockdown strategy by shRNA revealed that this process was mediated by MRP4. Furthermore, blockade by probenecid or MRP4 knockdown showed that increased intracellular cAMP levels induce maturation in U937 cells. These findings confirm the key role of intracellular cAMP levels in leukemic cell maturation and provide the first evidence that MRP4 may represent a new potential target for leukemia differentiation therapy.

Over the last few decades, the concept of differentiation therapy, whereby immature cells may be stimulated to develop into their mature phenotype, aroused considerable interest. Many efforts are in progress to evaluate new differentiation drugs for the treatment of leukemia in which early hematopoietic progenitors appear to exhibit maturation arrest. Treatment of acute promyelocytic leukemia with the differentiation agents, vitamin A metabolite all-*trans*-retinoic acid (1) or arsenic trioxide (As_2O_3) (2), has been successfully applied. In addition, factors that increase cAMP-mediated signaling, such as cyclic nucleotide phosphodiesterase (PDE)[2] inhibitors, augment the ability of these approved therapies to induce differentiation in acute promyelocytic leukemia blast cells (3).

The first second messenger discovered, cAMP, plays a critical role in the cellular response to numerous extracellular stimuli. The cAMP signaling pathway controls a diverse range of cellular events such as cell proliferation, differentiation, and apoptosis, including hematopoietic development (4). Classically, the regulation of cAMP signaling involves the cyclic nucleotide degradation by PDEs and G-coupled receptor desensitization. Several reports highlight the importance of cAMP in leukemic cell maturation. In mouse myeloid leukemia cells as well as in the human promonocytic U937 cell line, dibutyryl cyclic AMP (Bt_2cAMP) induces monocytic differentiation (5, 6). We previously reported that the time course of cAMP signaling is critical for leukemia U937 cell differentiation (6, 7). Furthermore, despite a large increase in cAMP levels mediated by H2 receptor activation, U937 cells trigger PDE4 activation and G-protein-coupled receptor kinase 2 (GRK2)-mediated receptor desensitization. These mechanisms regulate the duration and intensity of cAMP signaling, thereby partially conditioning cell differentiation failure (8–10).

The recently discovered cyclic nucleotide efflux transporters have also been implicated in the temporal resolution of cAMP signal regulation. Multidrug resistance-associated proteins (MRPs), also known as ATP-binding cassette (ABC) trans-

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[1] To whom correspondence should be addressed: Junin 956 PP (1113), Buenos Aires, Argentina. Tel.: 54-11-4964-8233; Fax: 54-11-4786-2564; E-mail: cardavio@ffyb.uba.ar.

[2] The abbreviations and trivial names used are: PDE, cyclic nucleotide phosphodiesterase; MRP, multidrug resistance-associated protein; ABC, ATP-binding cassette; icAMP, intracellular cAMP; ecAMP, extracellular cAMP; Bt_2cAMP, dibutyryl cyclic AMP; amthamine, 2-amino-4-methylthiazole-5-ethanamine; AML, acute myeloid leukemia; Rp-cAMPS, adenosine 3′,5′-phosphorothioate; RNP II, RNA polymerase II.

porter subfamily C members, actively transport substrates out of the cells (11). In particular, MRP4/ABCC4, MRP5/ABCC5, and MRP8/ABCC11 are implicated in cGMP and cAMP efflux (12–14). In addition to PDE activity, this process may also modulate the intracellular concentration of these second messengers and further supply extracellular cyclic nucleotides, which have been proposed to function as primary messengers (15, 16). MRP4, MRP5, and MRP8 are widely expressed in several tissues and cell lines (17), including those of the hematopoietic lineage (18). It has been reported that these transporters are expressed in blast cells of adult patients with acute myeloid leukemia (AML) (19). Furthermore, it has been found that urinary excretion of cAMP and cGMP is 3–5-fold higher in patients with acute and chronic leukemias than in normal subjects (20).

However, in the last few years, the extrusion of cAMP through MRP4 has been well documented in various cell types, suggesting that this transporter plays a relevant role in the regulation of cAMP signaling (21). Therefore the aim of the present study was to characterize cAMP extrusion, particularly that mediated by MRP4, in three different AML cell lines to assess the role of this transporter in cell proliferation and differentiation. Here we report that cAMP extrusion is directly involved in the regulation of cAMP intracellular levels. Blockade of MPR4 induced cell arrest and increased cell differentiation. Because intracellular cAMP levels are critical for leukemic cell differentiation, its regulation by MRP4 is to our knowledge the first direct evidence that these proteins may represent new potential targets for leukemia differentiation therapy.

EXPERIMENTAL PROCEDURES

Materials

RPMI medium 1640, antibiotics, phosphate-buffered saline (PBS), bovine serum albumin (BSA), 3-isobutyl-1-methylxanthine, cAMP, Bt_2cAMP, forskolin, isoproterenol, rolipram, probenecid, Fura 2 acetoxymethyl ester (Fura 2-AM), and rhC5a were obtained from Sigma. Rp-cAMPS was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Fetal calf serum (FCS) was purchased from Natocor. L-Glutamine was purchased from Invitrogen. Amthamine was from Tocris Cookson (Ballwin, MO). MK571 (3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{[3-dimethylamino-3-oxopropyl)-thio}-methyl]thio) propanoic acid) was obtained from Calbiochem. [^{3}H]cAMP and [^{3}H]methyl-thymidine were purchased from PerkinElmer Life Sciences. All other chemicals used were of analytical grade and obtained from standard sources.

Culture of Human Leukemic Cell Lines

U937, HL-60, and KG-1a cells were purchased from ATCC (American Type Culture Collection, Manassas, VA) and were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS and 50 μg/ml gentamicin. HL-60 cells medium was further supplemented with 2 mM L-glutamine. All cell lines were maintained at a density of 0.2–1 $\times$ 10^6/ml and were diluted 1 day before each experiment.

cAMP Assay

Cells were resuspended in RPMI 1640 medium at a density of 1 $\times$ 10^6 cells/ml and exposed to various agents at different concentrations and time points as indicated in the corresponding figure legends. Following centrifugation for 3 min at 3000 $\times$ *g* at 4 °C, 1 ml of ethanol was added to supernatants (extracellular cAMP) and pellets (intracellular cAMP). Ethanol was dried, and residues were resuspended in 50 mM Tris-HCl, pH 7.4, 0.1% BSA for further cAMP determination. Cyclic AMP content was determined by competitive radio-binding assay for PKA using [^{3}H]cAMP, as described previously (22). The standard curve was performed using eight cAMP concentrations ranging from 0.1 to 90 pmol. Duplicate samples in at least three independent experiments were analyzed.

Isolation of Membrane Vesicles from Leukemic Cell Lines

According to El-Sheikh *et al.* (23), cells were harvested by centrifugation, and the pellets were resuspended in ice-cold homogenization buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.4) supplemented with protease inhibitors and shaken at 4 °C for 60 min. Lysed cells were centrifuged at 4 °C at 100,000 $\times$ *g* for 30 min, and the pellets were homogenized in ice-cold TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) using a tight fitting Dounce homogenizer for 30 strokes. After centrifugation at 500 $\times$ *g* at 4 °C for 20 min, the supernatant was centrifuged 4 °C at 100,000 $\times$ *g* for 60 min. The resulting pellet was resuspended in TS buffer and passed through a 27-gauge needle 30 times. Aliquots of crude membrane vesicles were frozen in liquid nitrogen and stored at −80 °C until assayed. Protein concentration was determined by a Bio-Rad protein assay kit following the manufacturer's instructions.

Vesicular Transport Assays

The uptake of [^{3}H]cAMP into membrane vesicles was performed using a rapid filtration technique as described previously (24). The preparation consists of a mixture of an unknown ratio of inside-out and right-side-out vesicles. Only inside-out vesicles can transport the substrate in an ATP-dependent fashion. Briefly, TSB buffer (TS buffer with 0.2 mg/ml BSA) containing 83 μM [^{3}H]cAMP, 100 mM ATP, and 500 mM $MgCl_2$ was added to 15 μg of membrane vesicles, and cAMP uptake was measured in the presence or absence of 50 μM MK571 (MRP inhibitor) (17) in a 30-μl final volume and incubated at 37 °C. In control experiments, ATP was replaced by 5′-AMP. Samples were withdrawn at indicated time points, diluted in 150 μl of ice-cold TSB buffer to stop the reaction, and filtered by a vacuum filtration device through 0.45-μm-pore nitrocellulose filters. Tritium activity was determined in the filters by the usual scintillation counting methods. Net ATP-dependent transport was calculated by subtracting values obtained in the presence of 5′-AMP from those in the presence of ATP. Triplicate samples in at least three independent experiments were analyzed.

RT-PCR and Quantitative Real-time PCR

Total RNA was isolated from U937, HL-60, and KG-1a cells using TRIzol reagent following the manufacturer's instructions (Invitrogen). For the first-strand cDNA synthesis, 3 μg of total

RNA were reverse-transcribed using M-MLV reverse transcriptase (Promega) with random primers. 2 μl of the resulting cDNA were amplified at 30 cycles for 30 s at 94 °C, 30 s at melting temperature (55 °C), and 1 min at 72 °C followed by a final amplification step for 10 min using 1.6 units of *Taq* DNA polymerase and 200 μM of the following primers: human MRP4 forward, 5′-GGACAAAGACAAC-TGGTGTGCC-3′ and reverse, 5′-AATGGTTAGCACGGTGCAGTGG-3′; and human RNA polymerase II (RNP II) forward, 5′-GCTGTGTCTGCTTCTTCTG-3′ and reverse, 5′-CGAACTTGTTGTCCATCTCC-3′. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide. Reactions without reverse transcriptase served as negative controls.

Quantitative real-time PCR was performed in triplicate using the ABI PRISM 7500 sequence detection system (Applied Biosystems) with the specific MRP4 and RNP II primers detailed above. The PCR mixture contained 7.5 μl of 2× SYBR Green PCR master mix (Applied Biosystems) in a 15-μl final volume. The specificity of each primer set was monitored by analyzing the dissociation curve. The relative MRP4 mRNA levels were calculated using the calibration curve method and normalized by RNP II (housekeeping gene).

Western Blot Assay

Membrane vesicles (2–10 μg of protein) were diluted with sample buffer and boiled for 5 min. Proteins were separated by 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The residual binding sites were blocked with 5% nonfat powdered milk in PBS containing 0.05% Tween 20, and membranes were incubated with 3 μg/ml rat monoclonal anti-MRP4 M4I-10 (Alexis Biochemicals); 1 μg/ml rabbit monoclonal anti-PP2A (Epitomics); 1 μg/ml goat anti-CD88 (Santa Cruz Biotechnology); or 1 μg/ml goat anti-actin antibody (Santa Cruz Biotechnology) in PBS containing 0.05% Tween 20. All subsequent washes were performed with the same buffer. Reactivity was developed using an anti-rat, anti-mouse, or anti-rabbit polyclonal antibody linked to horseradish peroxidase (Santa Cruz Biotechnology) and enhanced chemiluminescence reagents following the manufacturer's instructions (Amersham Biosciences).

Assessment of Cell Viability

Cell viability was monitored by the trypan blue exclusion test. Cells growing in exponential phase exposed to different treatments were incubated for 48 or 72 h. An aliquot was then withdrawn and mixed with an equal volume of 0.4% trypan blue. After 5 min, the number of viable cells was estimated in a hemocytometer chamber. The assays were carried out by triplicate in at least three independent experiments.

Proliferation Assay

Cells growing in exponential phase were seeded at 1×10^4 cells in 150 μl of RPMI 1640 in a 96-well culture plate and incubated in a 5% CO_2 atmosphere. Cells were exposed to different agents for 24 or 48 h, but 12 h before the end of the experiment, 0.5 mCi of [^{3}H]methyl-thymidine (specific activity 740.0 GBq/mmol) (PerkinElmer Life Sciences) was added to the medium. Cells were then harvested in an automatic cell harvester (Nunc). The incorporation of the radioactive nucleotide was measured in an Amersham Biosciences Wallac 1410 liquid scintillation counter and expressed as incorporation percentage with respect to the control group (cells seeded only with culture medium). Assays were performed by quadruplicate in at least four independent experiments. Cell number was validated with a cellular meter Coulter Z-1 (Coulter).

Down-regulation of MRP4 by shRNA

Short Hairpin RNA (shRNA) Expression Plasmids

Circular pSUPER.retro.puro vector (OligoEngine, Seattle, WA) was linearized with BglII and HindIII restriction enzymes according to standard protocols. MRP4 shRNA sequence was designed according to Sassi *et al.* (25) and synthesized as an oligonucleotide to be used in pSUPER plasmids. DNA oligonucleotides (see below) specifically containing either MRP4 shRNA sequence or a scramble shRNA sequence were annealed, ligated to the pSUPER plasmid, and then transformed into bacteria. The newly created pSUPER-MRP4 shRNA and pSUPER-scramble shRNA vectors were prepared from individual bacterial colonies. Correct orientation and location of the oligonucleotide cloning were confirmed by using the following sequencing primer: 5′-GGAAGCCTTGGCTTTTG-3′.

MRP4 shRNA oligonucleotides contained a region specific to MRP4 mRNA (bold), a hairpin loop region (italic), and linker sequences for subcloning into BglII and HindIII sites of the pSUPER vector. Primers are: sense, 5′-GATCCCC**CAGTGTTCTTACACTTCCT***TTCAAGAGA***AGGAAGTGTAAGAACCTG**TTTTTA-3′; antisense, 5′-AGCTTAAAAA**CAGTGTTCTTACACTTCCT***TCTCTTGAA***AGGAAGTGTAAGAACACTG**GGG-3′). The scramble shRNA oligonucleotides contained a sequence of DNA that was not complementary to any human gene (GAAACTGCTGACCGTTAAT) and an identical hairpin loop and linker sequence.

Generation of Stable U937 Cells Expressing shRNA Targeting MRP4

To generate retroviruses, HEK293T cells were plated at 2×10^6/10-cm plate 1 day prior to calcium phosphate-mediated transfection with either pSUPER-MRP4 shRNA or scramble shRNA. The medium was changed 24 h after transfection, and 48 h after transfection, viral supernatant was collected and used for the infection of U937 cells after the addition of 4 μg/ml Polybrene (Sigma). After 48 h, U937 cells were resuspended in fresh medium containing 3 μg/ml puromycin and selected during 3 weeks. Puromycin-resistant U937 cells were maintained in media containing 1 μg/ml puromycin.

Determination of U937 Cell Differentiation Markers

Following the exposure of U937 cells to various agents for 72 h, the following cell differentiation markers were assessed.

Surface Myeloid CD11b and CD14 Antigen Assay

The expression of CD11b and CD14 was detected by direct immunofluorescence staining. Cells were washed twice in PBS and incubated with a saturated concentration of phycoerythrin anti-CD11b or anti-CD14 antibodies (Coulter-Immunotech)

or an equivalent concentration of isotype-matched control at 48 °C for 30 min. Cells were washed twice with PBS supplemented with 1% FCS and immediately analyzed in a FACScan flow cytometer (BD Biosciences). A minimum of 5000 events was acquired for each sample. The percentage of positive cells between specific CD-immunolabeled cells and their corresponding negative control was established for both untreated and treated cells.

Expression and Functionality of the C5a Receptor (CD88)

Expression—CD88 expression was evaluated by Western blot assay as detailed previously using a goat anti-CD88 antibody.

Intracellular Ca^{2+} Measurements—Fura 2-AM was used as a fluorescent indicator. Cells from each experimental group were washed, resuspended, and incubated in a buffered saline solution (140 mM NaCl, 3.9 mM KCl, 0.7 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM Hepes, pH 7.5, 10 mM glucose, and 0.1% BSA) in the presence of 2 μM Fura 2-AM. Cells were incubated for 60 min at 37 °C in an atmosphere of 5% CO_2 to allow Fura 2 to be intracellularly trapped by esterase cleavage. Cells were then washed twice in buffered saline solution without Fura 2-AM and brought to a density of 2×10^6 cells/ml buffered saline solution. Fluorescence was measured in a spectrofluorometer (Jasco, Tokyo, Japan) provided with the CA-261 accessory to measure Ca^{2+} under continuous stirring, with the thermostat adjusted to 37 °C and an injection chamber. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels were registered every second by exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured. Under these conditions, light intensities and the F_{340}/F_{380} ratios were tracked. Different agents were injected (5 μl) into the chamber as a 100-fold concentrated solution without interrupting recording. The preparation was calibrated by determining the maximal fluorescence induced by 0.1% Triton X-100 and the minimal fluorescence in the presence of 6 mM EGTA (pH 8.3). $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al.* (26).

Chemotaxis Assay—The "*in vitro*" migration of both control and treated U937 cells was assayed using the micropore filter technique. Briefly, 1×10^5 control or 72-h treated cells were seeded onto the top compartment of the chemotactic chambers in 100 μl of RPMI 1640 and placed in a 24-well tissue culture plate. A polyvinylpyrrolidone-free polycarbonate filter with a pore size of 5 μm separated the top and bottom compartments. The bottom compartment was filled with 0.6 ml of medium with or without 5×10^{-9} M recombinant human C5a. Chambers were incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. Migrated cells were collected and counted using a flow cytometer (BD Biosciences).

Cell Morphology Evaluation

Treated and control cells were washed twice in PBS, and cytospin slide preparations of cell suspensions were fixed and stained with May Grünwald-Giemsa (Biopur) following standard protocols. Cell morphology was determined by light microscopy (Zeiss), 10 randomly selected fields were acquired from each group, and 100 cells were minimally counted. Assays were carried out by triplicate in at least three independent experiments.

Data Analysis

Results are expressed as mean ± S.E. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni's multiple comparison test. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Characterization of cAMP Efflux Mediated by MRPs in U937 Cells—To study the efflux and intracellular accumulation of cAMP, U937 cells were exposed to 10 μM amthamine, a selective H2 receptor agonist, in the presence or absence of PDE inhibitors. In the absence of PDE inhibitors, amthamine rapidly increased icAMP levels, peaking at 5 min and thereafter declining to control values. The accumulation of ecAMP gradually augmented, reaching the highest level at 60 min. Moreover, at that time point, cAMP was found predominantly in the extracellular compartment (Fig. 1*A*). These results indicate that cAMP extrusion is independent of PDE activity. In the presence of 10 μM rolipram, a PDE4-selective inhibitor, accumulation of icAMP and ecAMP levels increased at all time points examined (Fig. 1*B*), suggesting that cAMP extrusion tends to compensate the inhibition of cAMP degradation, maintaining certain levels of cAMP within the cell.

To further characterize cAMP efflux, U937 cells were incubated with other cAMP-stimulating agents such as 100 μM forskolin (direct adenylyl cyclase activator) or 1 μM isoproterenol (β-adrenergic agonist) (data not shown). Both isoproterenol and forskolin showed a kinetic pattern similar to that of amthamine (Fig. 1*C*).

Concentration-response curves to amthamine at different times showed that icAMP was evident at 5 min, without a significant increase in ecAMP (Fig. 1, *D* and *E*). Conversely, a typical concentration-response curve for ecAMP was observed at 30, 60, and 120 min without significant changes in the EC_{50} (Fig. 1*E*). These findings indicate that cAMP efflux is a time- and icAMP-dependent process. Furthermore, there was a direct correlation among amthamine concentration, icAMP, and efflux.

To determine whether cAMP transport was ATP-dependent, we determined [^{3}H]cAMP uptake in normal and ATP-depleting conditions using U937 membrane vesicles. As shown in Fig. 1*F*, cAMP transport was time- and ATP-dependent, displaying a typical linear pattern. Furthermore, cAMP transport was inhibited by MK571 (MRP inhibitor), supporting that cAMP transport in U937 cells was mediated by MRPs.

Role of MRPs in cAMP Transport— cAMP transport in intact U937 cells was further characterized by exposing the cells to a subtoxic concentration of the MRP inhibitor probenecid (500 μM). Blockade of MRPs caused a significant inhibition of amthamine-induced cAMP efflux, and a higher increase in icAMP was evoked by the H2 agonist. Furthermore, when PDE4 was inhibited by 10 μM rolipram, similar results were obtained, but considerably higher levels of icAMP were achieved (Fig. 2). These findings clearly show that combined

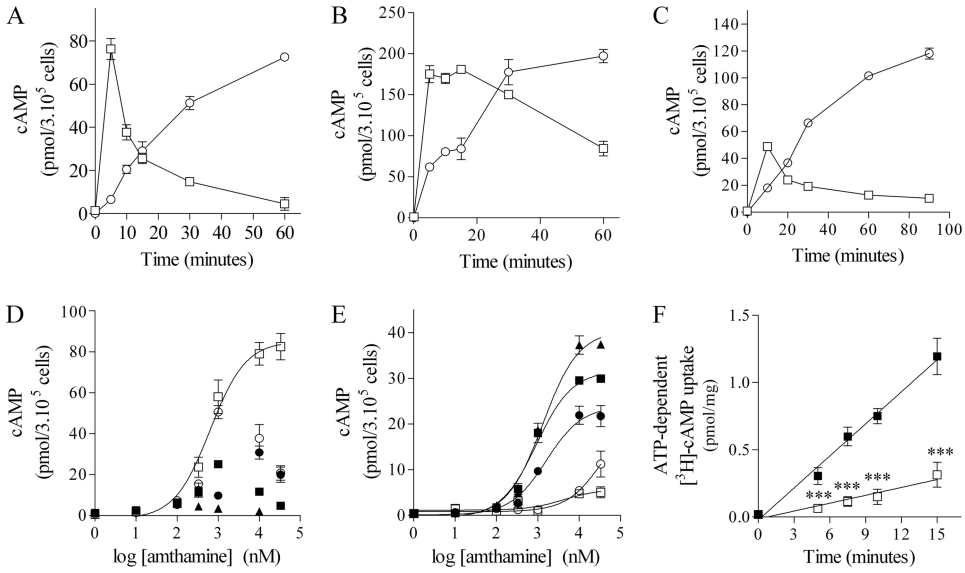

FIGURE 1. **Efflux of cAMP in U937 cells.** *A–C*, time course of icAMP (□) and ecAMP (○) levels in U937 cells in the presence of 10 μM amthamine (*A*), 10 μM amthamine + 10 μM rolipram (*B*), or 100 μM forskolin (*C*). The cyclic nucleotide was excluded in the presence or absence of PDE inhibitors in a time-dependent fashion. *D* and *E*, icAMP (*D*) and ecAMP (*E*) concentration-response curves to amthamine in U937 cells at different time points: 5 (□), 10 (○), 30 (●), 60 (■), and 120 (▲) min. A decrease in icAMP levels (*D*) was observed in parallel with its extracellular accumulation (*E*). *F*, ATP-dependent cAMP uptake in U937 cell vesicles is MRP-mediated. Membrane vesicles (15 μg) were incubated with 83 μM [^{3}H]cAMP in the absence or presence of 4 mM ATP. The rate of net ATP-dependent transport was calculated by subtracting the uptake with 100 mM 5′-AMP from that in the presence of 4 mM ATP. The transport was analyzed in control (■) and 50 μM MK571 (MRPs selective inhibitor)-treated vesicles (□) at different time points. ***, $p < 0.001$ respect to control. Data represent mean ± S.E. ($n = 3$).

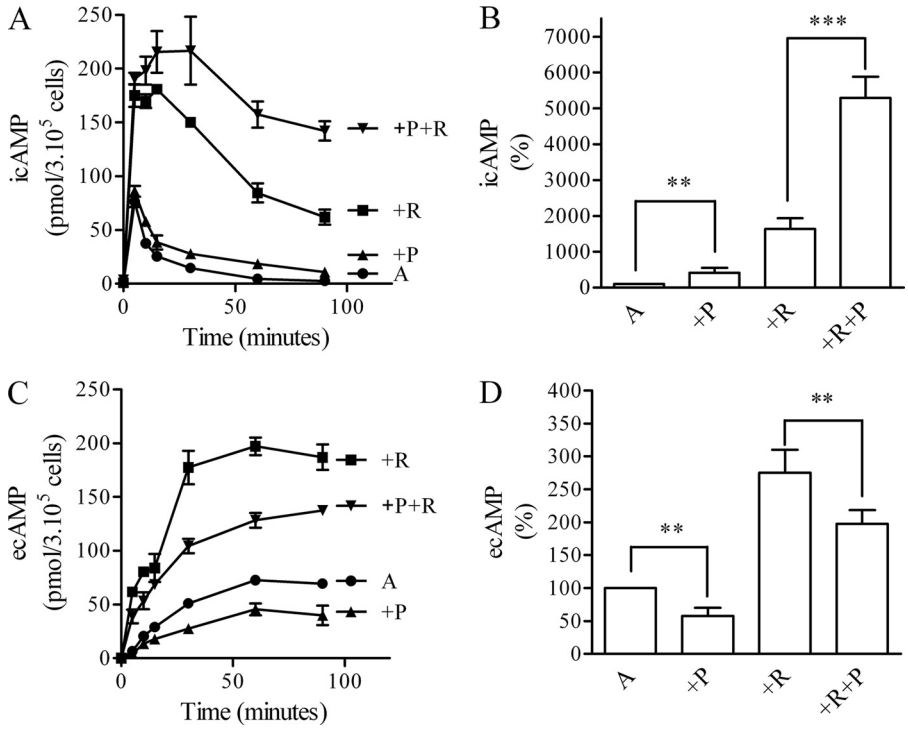

FIGURE 2. **MRPs are involved in cAMP transport in intact U937 cells.** *A* and *C*, time course study of icAMP (*A*) and ecAMP (*C*) levels in U937 cells exposed to non-cytotoxic concentrations of different agents: 10 μM amthamine (*A*) alone or in combination with 500 μM probenecid (+*P*) and/or 10 μM rolipram (+*R*, +*P*+*R*). Data shown are representative of three independent experiments. *B* and *D*, percentage of icAMP (*B*) and ecAMP (*D*) levels at 90 min in U937 cells exposed to the same agents. Combined PDE4 and MRPs inhibition results in significant enhancement of icAMP in amthamine-stimulated U937 cells. **, $p < 0.01$, ***, $p < 0.001$. Data represent mean ± S.E. ($n = 3$).

PDE4 and MRP inhibition resulted in a significant enhancement of icAMP in amthamine-stimulated U937 cells.

Effect of MRP Inhibition on Cell Proliferation—Cyclic AMP levels are critical for leukemic cell proliferation, so this process was evaluated in the presence of non-cytotoxic concentrations of cAMP modulators and/or MRP inhibitor. Cell proliferation was assessed in U937 cells exposed for 2 days to 0.4 mM Bt_2cAMP (positive control), 10 μM amthamine, 10 μM rolipram, or 500 μM probenecid, either alone or combined. Neither amthamine nor rolipram alone inhibited cell division; however,

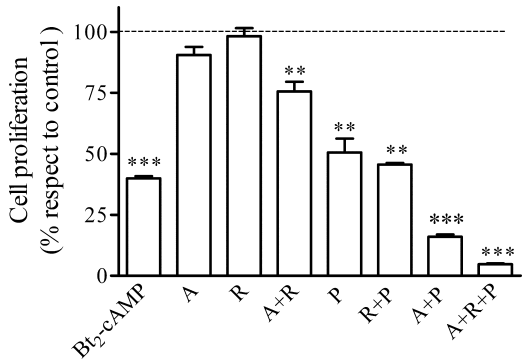

FIGURE 3. **Blockade of MRPs enhances the inhibitory effect of icAMP on U937 cell proliferation.** Cell proliferation was assessed in U937 cells exposed to 0.4 mM Bt_2cAMP, 10 μM amthamine (*A*), 500 μM probenecid (*P*), and 10 μM rolipram (*R*). The maximal inhibition in cell proliferation was observed with the combined blockade of MRPs and PDE4. **, $p < 0.01$, ***, $p < 0.001$ *versus* control. Data represent mean ± S.E. ($n = 3$).

when combined, they significantly inhibited cell proliferation. Probenecid alone caused almost 50% inhibition of cell proliferation, and it further enhanced the antiproliferative response evoked by amthamine and rolipram (Fig. 3).

cAMP Extrusion, Cell Proliferation, and MRP4 Expression in Myeloid Leukemia Cell Lines—To further confirm the findings in U937 cells, two other leukemic cell lines, KG-1a and HL-60, were subjected to the same experimental approach. In both cell lines, blockade of MRPs by probenecid also regulated cAMP extrusion. Following amthamine, rolipram, and probenecid cell exposure for 60 min, amthamine-induced cAMP efflux was inhibited, and icAMP was increased (Fig. 4*A*). Similarly as shown in U937 cells, icAMP accumulation induced by PDE4 and MRP inhibition significantly decreased cell proliferation in HL-60 and KG1-a cells. In addition, treatment of both cell lines with probenecid alone caused a reduction in cell proliferation in accordance with the result obtained in U937 cells (Fig. 4*B*).

Because MRP4 was reported to function as a high affinity efflux pump for cAMP, the molecular identification of this transporter was assessed. In the three cell lines, MRP4 mRNA and protein expression was revealed by RT-PCR and Western blot, respectively (Fig. 4, *C* and *D*).

Effect of MRP4 shRNA on cAMP Cellular Levels and Cell Proliferation in U937 Cells—To investigate the specific role of MRP4 in cAMP efflux and in the regulation of human leukemia cell proliferation, MRP4 was knocked down by a retroviral vector encoding shRNA against MRP4 (pSUPER-MRP4 shRNA). A significant reduction of MRP4 mRNA (61 ± 5%) and protein levels (63 ± 7%) in MRP4 shRNA with respect to scramble shRNA transduced cells was observed (Fig. 5, *A* and *B*), with no significant changes in the expression of the subfamily-related transporter MRP5 (data not shown). In accordance with decreased expression of MRP4, cAMP efflux was significantly lower in U937 cells transduced with MRP4 shRNA with respect to scramble shRNA. When these cells were exposed to amthamine and rolipram for 60 min, icAMP was increased, and ecAMP was decreased. These findings were similar to those observed in scramble cells treated with probenecid (Fig. 5*C*). The accumulation of icAMP in MRP4 shRNA cells induced by amthamine and rolipram resulted in the inhibition of cell proliferation (Fig. 5*D*).

To confirm that cAMP was responsible for the inhibition of cell proliferation, scramble or MRP4 shRNA cells were pretreated with Rp-cAMPS, a potent and specific inhibitor of cAMP-dependent protein kinases. As shown in Fig. 5*E*, in both cases, the inhibition of cell proliferation was significantly reverted in the presence of the inhibitor.

Effects of MRP4 Inhibition on Cell Differentiation—U937 cell differentiation to monocytes was assessed by CD11b, CD14, and CD88 expression as well as CD88 functionality in the presence of the cAMP modulators described above for the proliferation assay. CD88 (C5a receptor, rhC5a) is a monocytic marker generally used to assess terminal maturation; it is associated with Ca^{2+} released from intracellular stores and with chemotactic responses (27, 28). As shown in Fig. 6*A*, co-incubation with amthamine and rolipram induced CD88 expression; moreover, the addition of probenecid yielded higher levels of this marker. Consistent with CD88 expression, cells treated with the three agents displayed a higher $[Ca^{2+}]_i$ peak and an enhanced rhC5a-induced chemotaxis as compared with cells treated with amthamine and rolipram (Fig. 6, *B* and *C*). The expression of CD11b and CD14 (myelomonocytic lineage markers) was assessed by flow cytometry analysis. Cells treated with amthamine and rolipram showed enhanced CD11b and CD14 expression, and probenecid further enhanced their expression (Fig. 6, *D* and *E*). Fig. 6, *F* and *G*, show that MRP4, by regulating cAMP cellular levels, controls human leukemia cell differentiation. In MRP4 shRNA transduced cells, the treatment with amthamine plus rolipram resulted in an increase of the differentiation marker CD11b and in the capacity of cell migration. Again these findings were similar to those observed in scramble cells treated with probenecid. Morphological changes were next evaluated using May Grünwald-Giemsa staining. Probenecid treatment or MRP4 knockdown in the presence of amthamine plus rolipram resulted in characteristic monocytic morphology with a high cytoplasm/nucleus ratio and eccentrically located kidney-shaped nuclei (Fig. 6*H*).

DISCUSSION

The study of MRPs was classically focused on their role in cancer chemotherapy, particularly on their ability to confer clinical drug resistance (29). The physiological actions of these proteins are quite diverse, and drug transport appears not to be the most important evolutionarily conserved function. Diverse studies show that MRP4, MRP5, and MRP8 induce the extrusion of cyclic nucleotides in various cell types; however, MRP4 has emerged as the main transporter for cAMP (30, 31). The discovery of MRP4 as cyclic nucleotide transporter has important implications in the homeostasis of these signaling molecules inside the cells. It was reported that MRP4 modulates cAMP-mediated signaling in mouse embryonic fibroblasts (32) and in human arterial smooth muscle cells (25). Furthermore, this transporter acts as an endogenous regulator of intracellular cyclic nucleotide levels and in turn as a mediator of related signaling pathways (21). Intracellular cAMP content is governed by two independent mechanisms, efflux by MRP4 and degradation by PDEs. It is well established that cAMP plays a

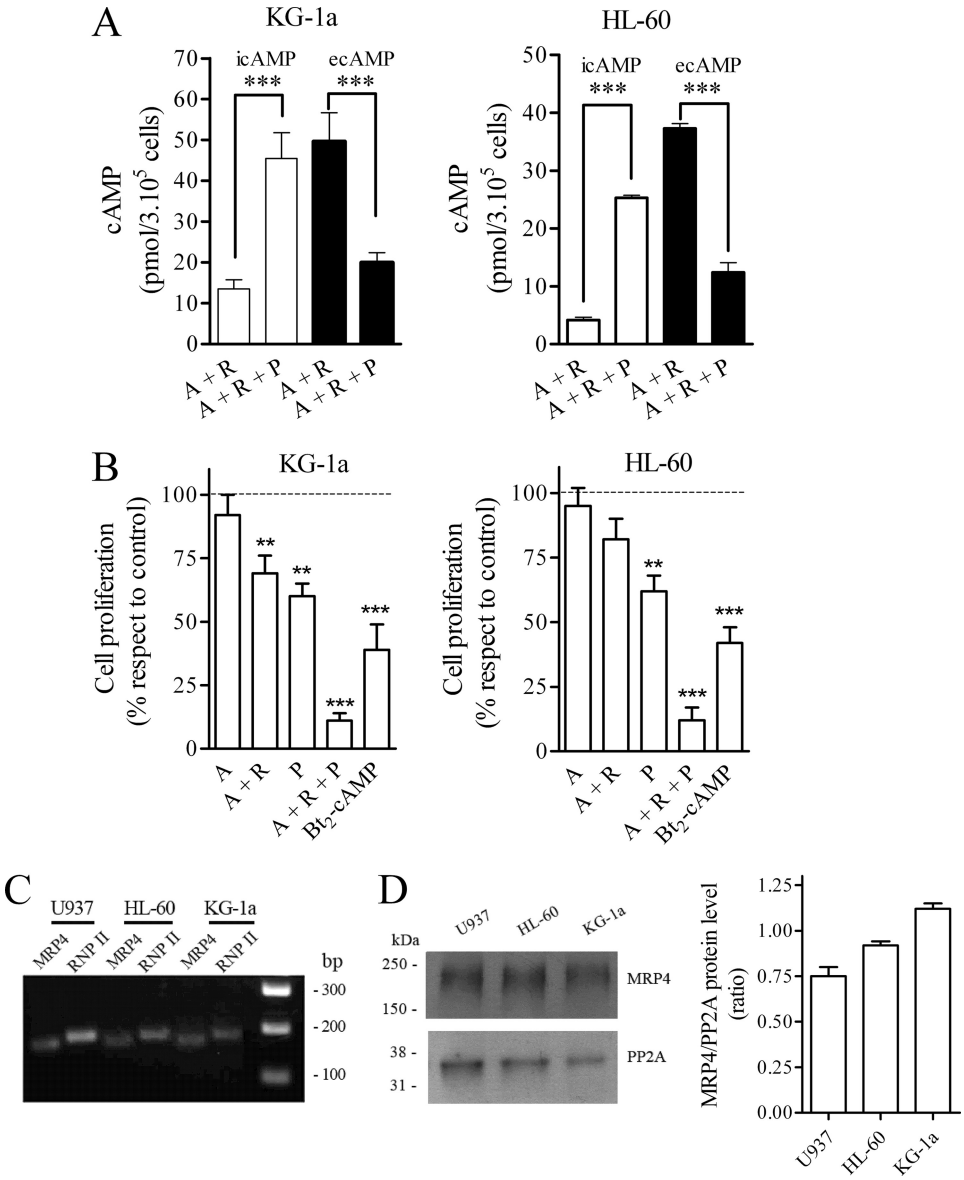

FIGURE 4. **cAMP extrusion, cell proliferation, and MRP4 expression in myeloid leukemia cell lines.** *A*, KG-1a (*left*) and HL-60 (*right*) cells were exposed for 60 min to 10 μM amthamine and 10 μM rolipram ($A + R$) or 10 μM amthamine, 10 μM rolipram, and 500 μM probenecid ($A + R + P$), and icAMP and ecAMP were evaluated. *B*, cell proliferation was assessed in KG-1a (*left*) and HL-60 (*right*) cells exposed to 10 μM amthamine (*A*), 500 μM probenecid (*P*), 10 μM amthamine and 10 μM rolipram ($A + R$), or 10 μM amthamine, 10 μM rolipram, and 500 μM probenecid ($A + R + P$); 0.4 mM Bt_2cAMP was used as a positive control. The maximal inhibition in cell proliferation was observed with combined blockade of MRPs and PDE4. **, $p < 0.01$, ***, $p < 0.001$ *versus* control. Data represent mean ± S.E. ($n = 3$). *C*, representative RT-PCR showing detection of MRP4 and RNP II mRNA in U937, HL-60, and KG-1a cells. *D*, Western blot analysis of MRP4 in U937, HL-60, and KG-1a cells. PP2A was used as a loading control.

key role in hematopoietic cell differentiation (4). Patients with leukemia exhibit enhanced cAMP urinary excretion and cyclic nucleotide transporter expression in their blast cells (19, 20). The present work was carried out to further characterize cAMP efflux by MRPs, particularly MRP4, in human myeloid leukemia cells and the effect of MRP blockade on cell proliferation and differentiation to a non-neoplastic phenotype. To our knowledge, this is the first study to link MRP4, cAMP efflux, as well as cell proliferation and differentiation in human myeloid leukemia cells.

The present findings show that icAMP levels not only in U937 cells but also in other AML cell lines are also regulated by MRPs, particularly MRP4. In U937 cells, cAMP extrusion was independent of the stimuli that increased cAMP concentration but dependent on time and the agonist concentration. By using two well characterized MRP inhibitors such as probenecid and MK571 in intact cells and membrane vesicles, time course studies revealed that MRP inhibition further enhanced icAMP concentration, suggesting that cAMP extrusion was an MRP-mediated transport. It has been proposed that cyclic nucleotide efflux does not play an important role in the control of icAMP signaling (16) and that MRP4 is unlikely to regulate icAMP levels because of its relatively low affinity for the cyclic nucleotide (33). Nevertheless, and in accordance with other authors (25, 32), our results in AML cells demonstrate that cAMP transport modulates icAMP levels independently of PDE activity. In the hematopoietic lineage, the major PDE subfamily is PDE4

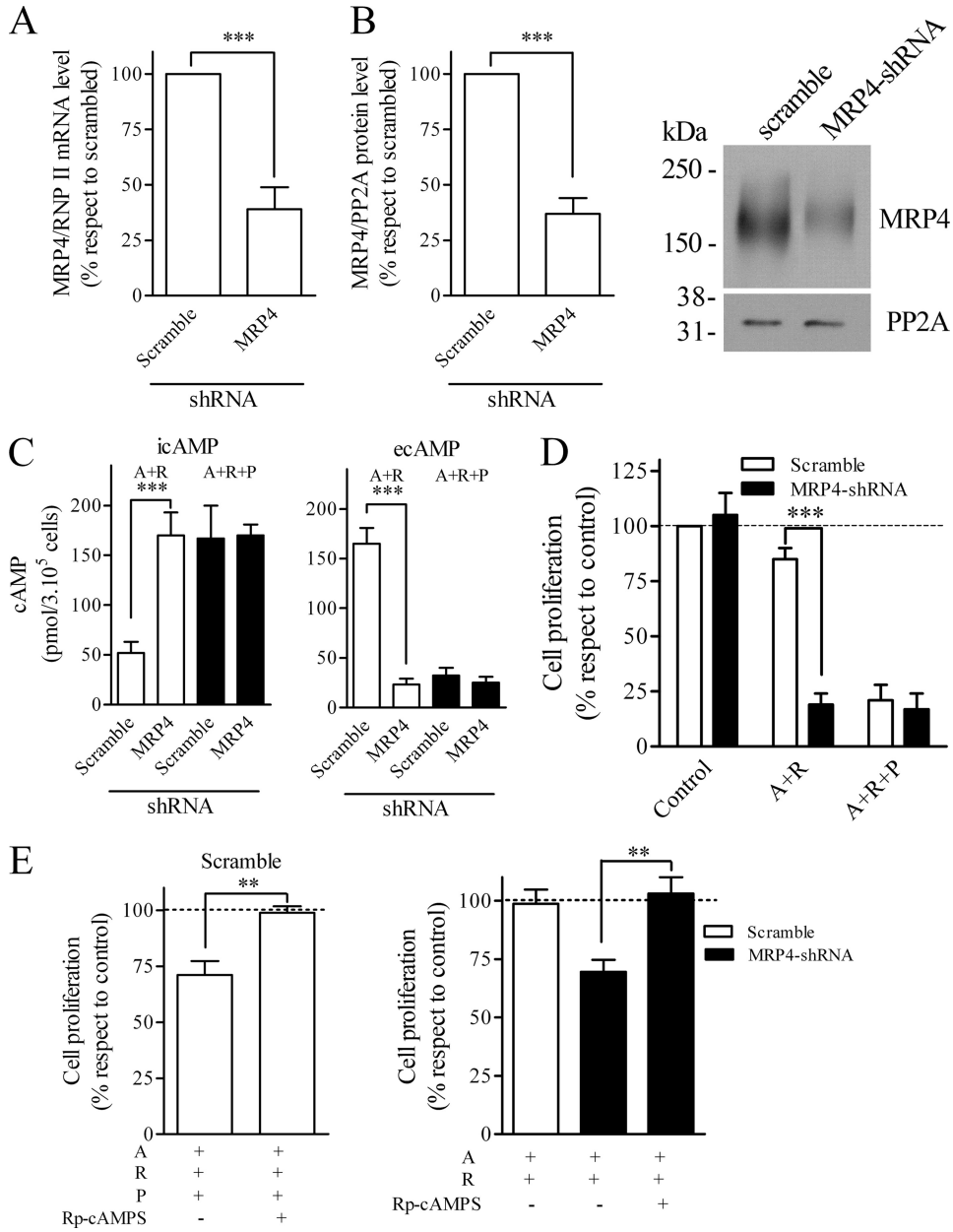

FIGURE 5. **Effect of MRP4 shRNA on cAMP cellular levels and proliferation in U937 cells.** *A* and *B*, quantitative real-time PCR (*A*) and Western blot (*B*) of U937 cells transfected with MRP4 shRNA or scramble shRNA. PP2A was used as a loading control. *C*, U937 cells transfected with MRP4 shRNA or scramble shRNA were exposed to 10 μM amthamine and 10 μM rolipram (*A*+*R*) or 10 μM amthamine, 10 μM rolipram, and 500 μM probenecid (*A*+*R*+*P*) for 60 min and icAMP (*left*) and ecAMP (*right*) were assessed. *D*, cell proliferation was evaluated after 48 h in scramble or MRP4 shRNA untreated cells (control) or exposed to 10 μM amthamine and 10 μM rolipram (*A*+*R*) or 10 μM amthamine, 10 μM rolipram, and 500 μM probenecid (*A*+*R*+*P*). *E*, cell proliferation was evaluated in scramble or MRP4 shRNA cells pretreated with 50 μM Rp-cAMPS and exposed 24 h to 10 μM amthamine, 10 μM rolipram, and 500 μM probenecid (*A*+*R*+*P*) or 10 μM amthamine and 10 μM rolipram (*A*+*R*). Control corresponds to scramble or MRP4 shRNA untreated cells. Data represent mean ± S.E. ($n = 3$). **, $p < 0.01$.

(34), and its blockade by rolipram augmented both icAMP and cAMP efflux, supporting that the inhibition of cAMP degradation has a significant impact on cAMP-mediated transport. The signaling pathway mediated by cAMP has emerged as a key regulator of blood cell proliferation, differentiation, and apoptosis in malignant cell populations (35–38). Several reports suggest that elevated icAMP levels promote hematopoietic cell maturation (5, 6). In the present work, MRP inhibition modified the intracellular content of cAMP concomitantly with an accentuated decrease in the proliferative rate of U937 cells. This inhibition was even more pronounced when MRP inhibitors were combined with cAMP-stimulating agents.

To determine whether cAMP MRP-mediated transport, as well as its implication in cell proliferation, was a general feature of AML cells, we further investigated both processes in KG-1a and HL-60 cell lines. In accordance with the results obtained in U937 cells in both cell lines, an increase in icAMP levels resulting from MRP inhibition by probenecid had a significant impact on cell proliferation.

The efflux of cAMP from cells was recognized for decades, but the transporters capable of mediating this process were

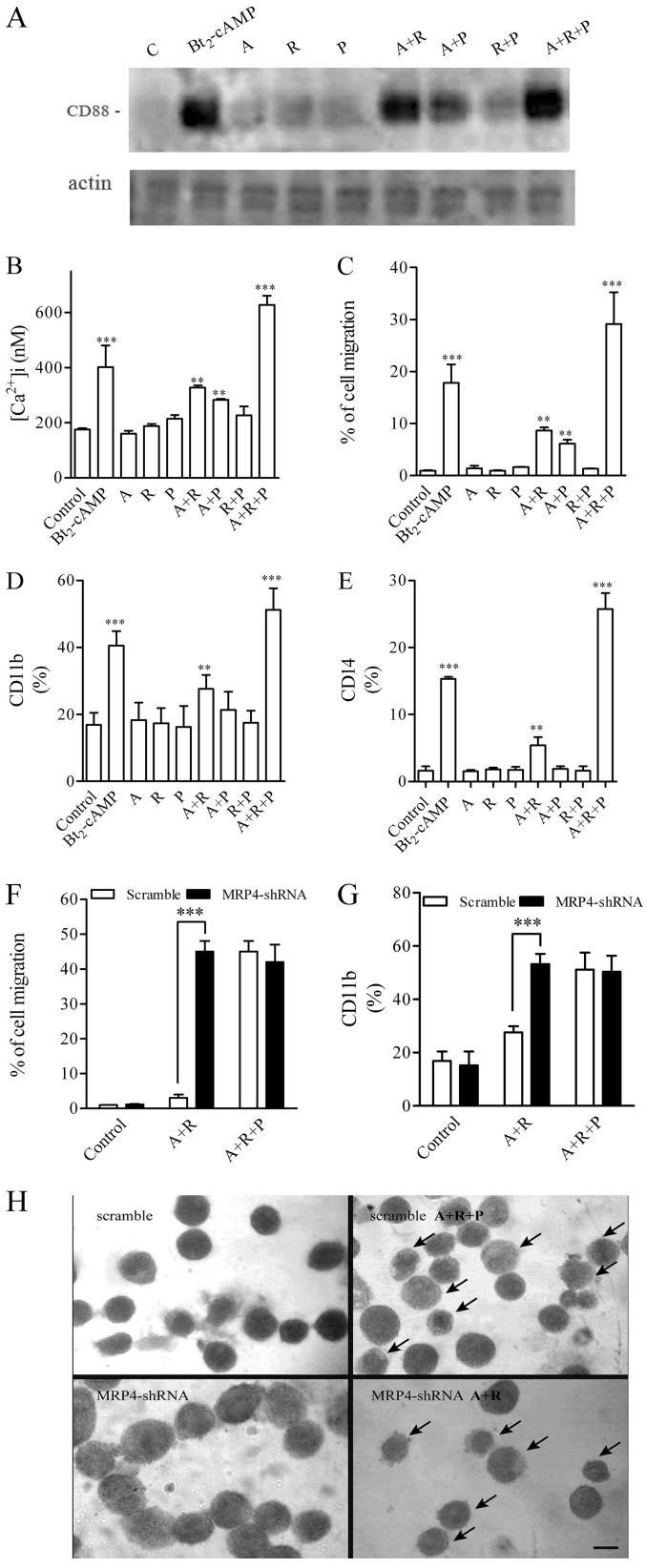

FIGURE 6. **Elevated icAMP levels resulting from MRP and PDE4 inhibition induce the expression of CD11b and CD88 in U937 cells, supporting their differentiation to monocyte-like cells.** *A*, Western blot analysis of CD88 expression (*lane C*) in U937 cells exposed to 10 μM amthamine (*A*), 500 μM probenecid (*P*), and 10 μM rolipram (*R*); 0.4 mM Bt_2cAMP was used as a positive control. *B–G*, CD88 functionality assays. *B* and *C*, mobilization of intracellular calcium (*B*) and cell migration mediated by C5a (*C*) in U937 cells exposed to 10 μM amthamine (*A*), 500 μM probenecid (*P*), and 10 μM rolipram (*R*); 0.4 mM Bt_2cAMP was used as a positive control. *D* and *E*, CD11b (*D*) and CD14 (*E*) antigen expression was evaluated by flow cytometry at day 3 following cell exposure to 10 μM amthamine (*A*), 500 μM probenecid (*P*), and 10 μM rolipram (*R*); 0.4 mM Bt_2cAMP was used as a positive control. *F* and *G*, cell migration (*F*) and CD11b antigen expression (*G*) were evaluated in scramble or MRP4 shRNA cells exposed to 10 μM amthamine and 10 μM rolipram (*A*+*R*) or 10 μM amthamine, 10 μM rolipram, and 500 μM probenecid (*A*+*R*+*P*). ***, $p < 0.001$, **, $p < 0.01$ *versus* control. Data represent mean ± S.E. ($n = 3$). The maximal expression of the differentiation markers was observed with combined blockade of MRPs and PDE4. *H*, cell morphology was evaluated after May Grünwald-Giemsa staining in untreated cells (*left panels*) and treated cells with 10 μM amthamine, 10 μM rolipram, and/or 500 μM probenecid (*right panels*). *Arrows* indicate differentiated cells. Each picture is representative of three independent experiments. *Bar*, 10 μm.

identified over the last years. Several reports show that MRP4, MRP5, and MRP8 are proteins unique in their ability to transport cyclic nucleotides as well as nucleoside analogs out of the cells (12–14). However, it is well known that MRP4 is considered a selective moderate high affinity transporter for cAMP, with a reported affinity of $K_m = 44.5$ μM (39), so we conducted studies focusing on this transporter. The genetic and protein expression of MRP4 was detected in KG-1a cells and confirmed in U937 and HL-60 cells (18).

Consistent with MRP pharmacological inhibition, similar results were obtained using the approach of shRNA for silencing MRP4 in U937 cells. MRP4 knockdown led to a pronounced decrease in cAMP efflux with the corresponding increase in icAMP levels, associated with a significant inhibition of cell proliferation. This effect was reversed by Rp-cAMPS, a selective antagonist of cAMP. Together, these findings strengthen the role of MRP4 in cAMP extrusion and the involvement of this cyclic nucleotide in the regulation of AML cell proliferation.

Based on our studies in cell proliferation, we further evaluated the expression and functionality of various differentiation markers and morphological changes. Pharmacological MRP inhibition as well as specific MRP4 knockdown, when combined with cAMP-stimulating agents, induced significant promonocytic maturation. These findings support that MRP4 is an important regulator of icAMP levels in U937 cells, leading to the inhibition of cell proliferation and promoting cell differentiation.

Resistance to drug therapy occurs by diverse mechanisms, including signal transduction impairment and active drug efflux from tumor cells. It has been demonstrated that MRP4-overexpressing cells are resistant to the cytotoxic effect of 6-mercaptopurine and 6-thioguanine, drugs currently used for leukemia treatment (40). As MRP4 confers resistance to nucleoside analog drugs and promotes the efflux of cyclic nucleotides, it has the potential to affect the treatment of AML. Furthermore, MRP4 expression in patients with AML, higher in blast cells than in monocytes (18), and the self-renewal capacity suppression of blast progenitors upon cAMP signaling activation (41), render MRP4 as a selective therapy target.

To our knowledge, this is the first study to provide evidence that in AML cells MRP4 has a relevant role in the regulation of icAMP levels, leading to the inhibition of cell proliferation and promoting cell differentiation. The present findings further

suggest that MRP4 may represent a new potential target for differentiation therapies.

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Multidrug Resistance Protein 4 (MRP4/ABCC4) Regulates cAMP Cellular Levels and Controls Human Leukemia Cell Proliferation and Differentiation

Sabrina Copsel, Corina Garcia, Federico Diez, Monica Vermeulem, Alberto Baldi, Liliana G. Bianciotti, Frans G. M. Russel, Carina Shayo and Carlos Davio

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