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# Human monocytes respond to extracellular cAMP through A2A and A2B adenosine receptors

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## ABSTRACT

**In this study, we test the hypothesis that cAMP, acting as an extracellular mediator, affects the physiology and function of human myeloid cells. The cAMP is a second messenger recognized as a universal regulator of several cellular functions in different organisms. Many studies have shown that extracellular cAMP exerts regulatory functions, acting as first mediator in multiple tissues. However, the impact of extracellular cAMP on cells of the immune system has not been fully investigated. We found that human monocytes exposed to extracellular cAMP exhibit higher expression of CD14 and lower amount of MHC class I and class II molecules. When cAMP-treated monocytes are exposed to proinflammatory stimuli, they exhibit an increased production of IL-6 and IL-10 and a lower amount of TNF-α and IL-12 compared with control cells, resembling the features of the alternative-activated macrophages or M2 macrophages. In addition, we show that extracellular cAMP affects monocyte differentiation into DCs, promoting the induction of cells displaying an activated, macrophage-like phenotype with reduced capacity of polarized, naive $CD4^+$ T cells into IFN-γ-producing lymphocytes compared with control cells. The effects of extracellular cAMP on monocytes are mediated by CD73 ecto-5′-nucleotidase and A2A and A2B adenosine receptors, as selective antagonists could reverse its effects. Of note, the expression of CD73 molecules has been found on the membrane of a small population of $CD14^+CD16^+$ monocytes. These findings suggest that an extracellular cAMP-adenosine pathway is active in cells of the immune systems. *J. Leukoc. Biol.* 96: 113–122; 2014.**

## Introduction

The role of cAMP as a second messenger in cells of the immune system has been widely described [1]. In particular, elevation of intracellular cAMP concentration has an inhibitory effect on proliferation and activation of T and B lymphocytes [2, 3]. Furthermore, cAMP actively modulates the differentiation and function of myeloid cells [4]. After the discovery of the role of cAMP as an intracellular second messenger, several studies reported the presence of the cyclic nucleotide in plasma and urine, indicating that cAMP could be exported out of the cells [5, 6]. The release of cAMP from different cell types is an active transport against a concentration gradient, and it seems to be strictly regulated according to the stimulus and to the tissue involved [7–10]. We have shown that $CD4^+$ T lymphocytes treated with cAMP-elevating agents are able to release cAMP in the extracellular compartment in the absence of cell death [11] and that extracellular cAMP has an inhibitory effect on T cell activation and proliferation. The stimulation of several membrane receptors on different cell types by hormones or neurotransmitters, such as cathecolamines, prostaglandins, or histamine, mediates the activation of adenylyl cyclases, which results in the increase of intracellular cAMP and the release of the cyclic nucleotides in the extracellular compartments. In addition, several bacteria produce cAMP-elevating toxins [12] or are able to release cAMP themselves [13, 14]. Therefore, whether extracellular cAMP can exert immunoregulatory functions is important for the understanding of the immune homeostasis and the modulation of immune responses against invading microbes. Here, we extended our previous study and investigated whether extracellular cAMP could be sensed by cells of the immune system other than T lymphocytes.

Circulating cells of the myeloid lineage, including monocytes, migrate to tissues, where they differentiate into macrophages or DCs, depending on the microenvironment of the

Abbreviations: AOPCP=adenosine 5′-(α,β-methylene) diphosphate ADP analog, BFA=brefeldin A, CSC=8-(3-chlorostyryl)caffeine, DPSPX=1,3,-dipropyl-8-(p-sulfophenyl)xanthine, FSK=forskolin, Ion=ionomycin, MRS1754=8-(4-{[(4-cyanophenyl) carbamoylmethyl]oxy} phenyl)-1,3-di(n-propyl)xanthine hydrate, NECA=5′-(*N*-ethylcarboxamido) adenosine

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inflammatory sites [15, 16]. The differentiation process is complex and regulated by cytokines [17–20] and also by the interaction with pathogens, such as viruses or bacteria [21–23]. In this study, we evaluated whether extracellular cAMP could be sensed by human monocytes and if it were able to affect their functions and differentiation. We found that extracellular cAMP modulates phenotype, function, and differentiation of monocytes through CD73 ecto-5′-nucleotidase and A2A and A2B adenosine receptors. It induces monocyte differentiation into DCs, which display an activated, macrophage-like phenotype with reduced capacity of polarize naive $CD4^+$ T cells into IFN-γ-producing lymphocytes compared with control cells. Furthermore, the expression of CD73 molecules has been found on the membrane of a small population of $CD14^+CD16^+$ monocytes. These findings suggest that extracellular cAMP exerts immunoregulatory functions, not only as an intracellular second messenger but also as extracellular modulator, acting on the adenosine receptor pathway.

## MATERIALS AND METHODS

### Media and reagents

RPMI 1640, supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA) and 10% FBS (HyClone Laboratories, Logan, UT, USA), was used as complete medium in all cell cultures. GM-CSF and IL-4 were purchased from Immunological Science (Rome, Italy). cAMPs were purchased from Biolog Life Science Institute (Bremen, Germany). Ion, PMA, BFA, FSK, LPS, DPSPX, NECA, MRS1754, AOPCP, and DMSO were purchased from Sigma Chemical (St. Louis, MO, USA). Final concentrations of DMSO did not exceed 0.1%.

### Monocyte purification and cell cultures

Human monocytes were purified from PBMC of healthy donors by positive selection using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The recovered cells were 95–99% $CD14^+$, as determined by flow cytometry using the FITC conjugate anti-human CD14 mAb (BD Biosciences, San Diego, CA, USA). Monocytes were triple-stained with anti-CD14, anti-CD16 and anti-CD73 mAb, and the expression of CD73 was evaluated on $CD14^+CD16^-$ and on $CD14^+CD16^+$ subpopulations by FACS analysis. Cells were cultured at $1 \times 10^6$/ml in RPMI-FBS, with or without GM-CSF (50 ng/ml) and IL-4 (35 ng/ml), in the presence or absence of cAMP (0.5 mM). After 6 days, cells were washed and stained with FITC or PE conjugate anti-human CD14, CD1a, HLA-DR, HLA-I, CD80, CD86, CD16, CD32, and CD89 (BD Biosciences) to analyze their phenotype, differentiation, and activation status. Alternatively, monocytes ($1\times10^6$/ml) were cultured in RPMI-FBS, supplemented with 50 ng/ml GM-CSF and 35 ng/ml IL-4 in the presence or absence of cAMP (0.5 mM) or increasing doses of NECA (0.001–10 μM). After 6 days, cells were collected, washed, and stained for the membrane phenotype. Cells were acquired on a FACSCalibur instrument, running CellQuest software. In the same experiments, 30 min before adding cAMP (0.5 mM), NECA (10 μM), or FSK (10 μM), a nonselective antagonist of the adenosine receptors DPSPX (0.1 mM) and adenosine receptor antagonists A2A (CSC; escalating doses from 10 to 0.1 μM) and A2B (MRS1754; escalating doses from 10 to 0.1 μM) or a CD73 inhibitor AOPCP (1 and 10 μM) were added. The phenotype was analyzed by a FACSCalibur instrument, running CellQuest software.

### Cytokine production

Supernatants from monocytes, cultured with or without GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) in the presence or absence of cAMP for 6 days and further cultured for 48 h with LPS (200 ng/ml), were collected and stored at −80°C. The levels of IL-6, TNF-α, IL-10, and IL-12 were detected by Quantikine immunoassay kits (Pierce Endogen, Rockford, IL, USA) and were measured as absorbance (450 nm) on an ELISA reader.

### cAMP measurement

Supernatants from monocytes from five independent donors, cultured with or without GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) in the presence or absence of cAMP (0.5 mM), were collected at different time-points (at 0, 10 and 30 min, and 1, 3, 6, 12, 18, and 24 h), and its concentration was evaluated by ELISA assay (Assay Designs, Ann Arbor, MI, USA) following sample acetylation.

### Naive $CD4^+$ T cell purification

PBMCs were isolated from healthy donors by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. $CD4^+$ T lymphocytes were purified by negative selection using immunomagnetic cell sorting (Miltenyi Biotec). Briefly, PBMCs were labeled using a cocktail of the hapten-conjugated mAb anti-CD8, -CD14, -CD16, -CD19, -CD36, -CD56, -CD123, -TCR-γ/δ, and -glycophorin A, in combination with MACS microbeads coupled to an anti-hapten mAb. The magnetically labeled cells were depleted by retaining them on a column using a MidiMACS cell separator. $CD4^+$ T cells were purified further in $CD4^+CD45RO^+$ and $CD4^+CD45RA^+$ T cells by positive selection using anti-CD45RA microbeads (Miltenyi Biotec).

### Polarization assay

The ability of monocytes differentiated in the presence or absence of cAMP (0.5 mM) to stimulate and polarize naive T cells was evaluated. At Day 5, cells were washed, starved for 8 h, and cocultured with purified, naive $CD4^+CD45RA^+$ T cells (ratio of 1:5) in 48-well plates at 37°C for 11 days. IL-2 (20 UI/ml) was added to the culture at Days 5, 7, and 9 of the cocultures. On the 11th day, cells were harvested, washed, and analyzed for cytokine production by intracellular staining. In brief, cells were stimulated with 50 ng/ml PMA and 1 μg/ml Ion; after 1 h, 10 μg/ml BFA was added, and they were incubated for a further 5 h at 37°C. Cells were washed twice in PBS, 1% BSA, and 0.1% sodium azide and stained with anti-CD4 mAb for 15 min at 4°C, and then, they were fixed with lysing solution, permeabilized with permeabilizing solution, and stained with PE or FITC conjugate anti-human IL-4 and anti-IFN-γ (all from BD Biosciences). Samples were acquired and analyzed on a FACSCalibur instrument, running CellQuest software.

### Statistical analysis

Microsoft Excel (Microsoft, Redmond, WA, USA) was used for statistical analysis. Data were expressed as the mean ± SD, and statistical significance was determined by Student's *t*-test; $P < 0.05$ was considered statistically significant.

## RESULTS

### Exogenous cAMP affects human monocyte phenotype and functions

We have shown previously that $CD4^+$ T lymphocytes treated with cAMP-elevating agents are able to release cAMP in the extracellular compartment and that exogenous cAMP exerts inhibitory effects on T cell activation and proliferation [11]. To investigate whether cells of the immune system other than T lymphocytes could sense exogenous cAMP, we evaluated its effects on human monocytes. Cells were isolated from healthy donors and cultured in the presence or absence of cAMP (0.5 mM) for 6 days. Monocytes cultured with exogenous cAMP exhibit a higher cell-surface expression of CD14 molecules

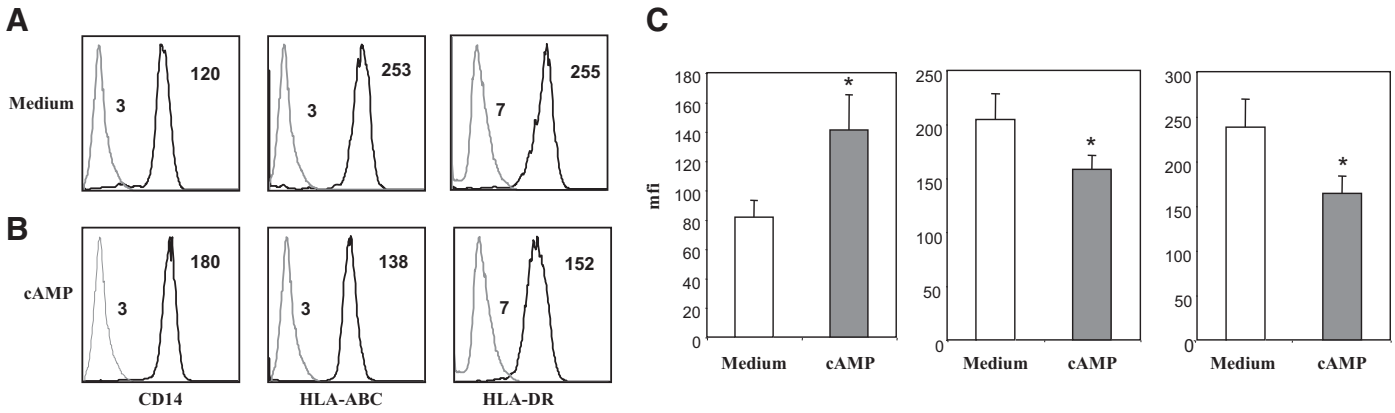



**Figure 1. Exogenous cAMP up-regulates CD14 and prevents the expression of MHC class I and class II molecules on human monocytes.** Histogram plots show the phenotype of human CD14 monocytes, isolated from the PBMC of healthy donors, cultured for 6 days with medium alone (A) or cAMP (0.5 mM; B). Cells were stained using anti-CD14, -HLA-ABC, and -HLA-DR mAb and analyzed by flow cytometry. Histogram plots are representative of five experiments performed. (C) The histogram bars show the means (+SE) of the relative mean fluorescence intensities (mfi) of the different markers analyzed from five independent experiments performed. $*P < 0.05$, significant differences between cells cultured in the presence or absence of cAMP.

($n$=5; $P$=0.02) and a lower expression of MHC class I ($n$=5; $P$=0.04) and class II ($n$=5; $P$=0.005) molecules compared with untreated cells (**Fig. 1**). No significant differences were observed for the expression of CD80 and CD86 costimulatory molecules and for FcRs CD16, CD32, and CD89 (data not shown). These data show that extracellular cAMP can be sensed by monocytes and that it affects their phenotype. Furthermore, to analyze whether exogenous cAMP had any effect on the function of monocytes, we analyzed how monocytes exposed to cAMP were able to respond to proinflammatory stimuli. Cells, cultured with or without cAMP for 6 days, were incubated with LPS (200 ng/ml) for a further 48 h, and the production of proinflammatory and regulatory cytokines was assessed in the culture supernatants by ELISA assay. Upon LPS stimulation, the production of IL-6, TNF$\alpha$, IL-10, and IL-12, although to different extents, was induced in untreated cells, as expected (**Fig. 2**). However, monocytes cultured in the presence of cAMP exhibit a further increase in production of IL-6 and IL-10 but a lower amount of TNF-$\alpha$ and IL-12 compared with control cells (Fig. 2), resembling the phenotype of the alternative-activated macrophages or M2 macrophages. Altogether, these data show that monocytes are able to sense and to respond to extracellular cAMP and that cells cultured in the presence of the cyclic nucleotide show the phenotype and functions resembling the anti-inflammatory M2 macrophages.

## Exogenous cAMP interferes with the differentiation of monocytes into DCs, giving rise to a macrophage-like population

It is well-established that monocytes are circulating precursor cells that can differentiate into macrophages or DCs depending on the microenvironment of the inflammatory sites [15,

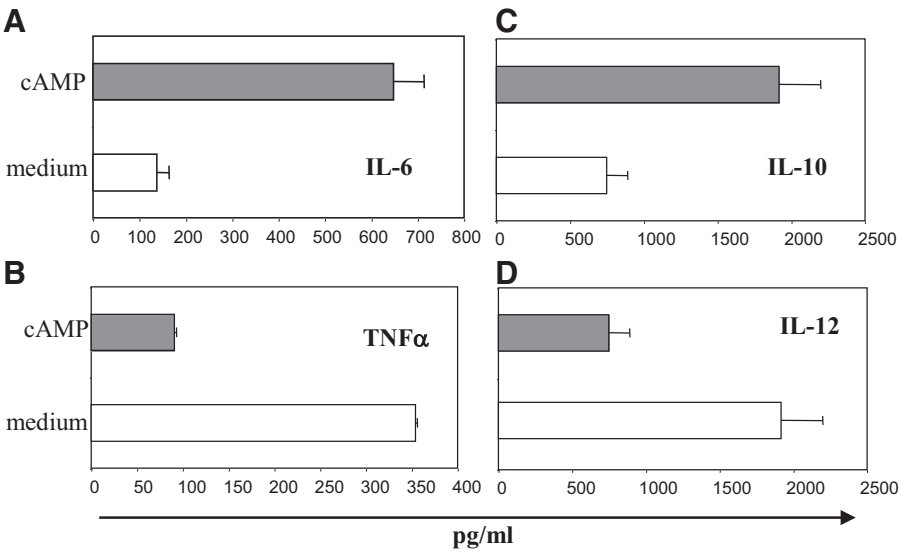



**Figure 2. Upon LPS stimulation, monocytes exposed to exogenous cAMP produce proinflammatory and regulatory cytokines.** Human CD14 monocytes, isolated from the PBMC of healthy donors, were cultured with medium alone or cAMP (0.5 mM). After 6 days, cells were incubated with LPS (200 ng/ml) for 48 h. The accumulation of IL-6 (A), TNF-$\alpha$ (B), IL-10 (C), and IL-12 (D) cytokines in culture supernatants was evaluated by ELISA assay. The data shown represent the mean (+SD) of three independent experiments.

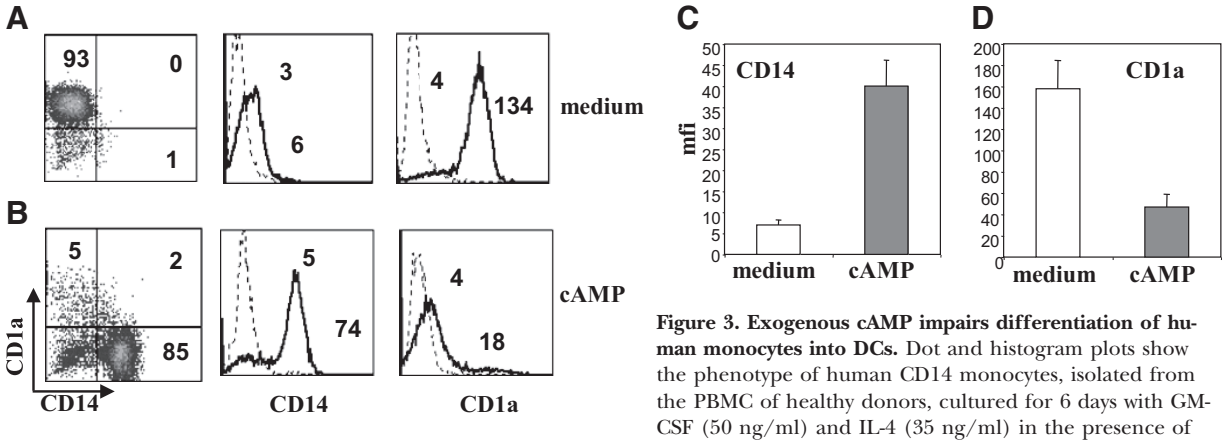



**Figure 3. Exogenous cAMP impairs differentiation of human monocytes into DCs.** Dot and histogram plots show the phenotype of human CD14 monocytes, isolated from the PBMC of healthy donors, cultured for 6 days with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) in the presence of medium alone (A) or cAMP (0.5 mM; B). Cells were double-stained using anti-CD14-FITC, anti-CD1a-PE mAb and analyzed by flow cytometry. Dot and histogram plots are representative of one experiment out of 12 performed. The histogram bars show the means (+SE) of the relative mfi of CD14 (C) and CD1a (D) expression from 12 independent experiments performed.

16]. The differentiation process is complex and regulated by cytokines [17–20] and also by the interaction with pathogens, such as viruses or bacteria [21–23]. As monocytes are able to sense and to respond to extracellular cAMP, we asked whether the exogenous nucleotide could affect their differentiation program toward DCs. Human monocytes were cultured with GM-CSF and IL-4 for 6 days, in the presence or absence of exogenous cAMP. In the absence of cAMP, monocytes differentiated into immature DCs, which are characterized by the expression of CD1a and the loss of CD14 molecules (**Fig. 3A**). In contrast, cells derived from cAMP-treated monocytes exhibited a reduced expression of CD1a but retained expression of CD14, suggesting that they acquired a monocyte/macrophage-like phenotype (Fig. 3B). This indicates that extracellular cAMP skews monocytes during their differentiation with GM-CSF and IL-4, toward a macrophage-like phenotype. To characterize better the phenotype of monocytes differentiated in the presence of exogenous cAMP, we analyzed the expression of different markers that are up-regulated during DC differentiation. We found that monocytes, differentiated in the presence of cAMP, up-regulated MHC class I and II, as well as CD80 and CD86 costimulatory molecules compared with control DCs, showing an activated phenotype (**Table 1**). In addition, the expression of CD16 and CD32 was enhanced by exogenous cAMP compared with untreated cells, leading to a cell type, phenotypically resembling a macrophage.

## Monocytes differentiated in the presence of exogenous cAMP produce a distinct cytokine pattern and exhibit a reduced capacity to skew the immune response toward a Th1 profile

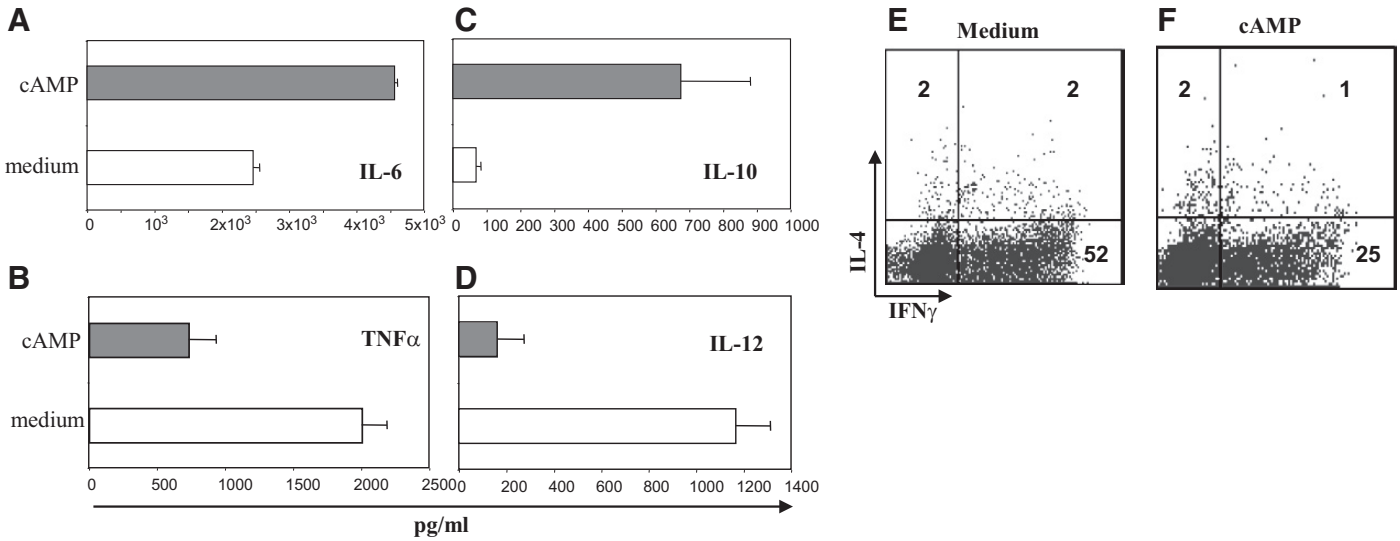
Next, we analyzed the production of the proinflammatory cytokines IL-6 and TNF-α by monocytes differentiated in the presence or absence of cAMP for 6 days and cultured with LPS for further 48 h. Upon LPS stimulation, the production of IL-6 and TNF-α was induced in untreated cells, as expected (**Fig. 4A** and **B**). The presence of cAMP during the differentiation of monocytes into DCs led to an increase of LPS-induced IL-6 but to a decreased production of TNF-α (Fig. 4A and B). The accumulation of IL-10 and IL-12, which are regulatory cytokines involved in directing the immune responses, was also measured. The production of IL-10 induced by LPS was strongly enhanced in cultures containing monocytes differentiated in the presence of cAMP (Fig. 4C). On the other hand, the release of IL-12, which was induced by LPS treatment, was strongly inhibited in cells differentiated in the presence of cAMP (Fig. 4D). These data show that monocytes differentiated in the presence of cAMP produce a distinct pattern of proinflammatory and regulatory cytokines compared with conventional DCs, suggesting that they might have a different capacity in directing immune responses.

Indeed, as DCs have the unique capacity to stimulate naive T lymphocytes and drive them into distinct classes of effector cells, we performed a polarization assay to evaluate whether DCs, differentiated in the presence of exogenous cAMP, had the capacity to support naive T cell differentiation. Cells differentiated in the presence or in the absence of cAMP were cultured with purified $CD4^+CD45RA^+$ T cells

**TABLE 1. Expression of Different Markers on DCs Differentiated in the Presence or Absence of Exogenous cAMP**

| | Medium | cAMP |
|---|---|---|
| HLA-I | 69 ± 11 | 111 ± 15[a] |
| HLA-DR | 41 ± 5.5 | 121 ± 17.4[a] |
| CD80 | 10 ± 1.2 | 16 ± 1.3[a] |
| CD86 | 6.6 ± 0.9 | 14.2 ± 2.4[a] |
| CD16 | 3.3 ± 1.4 | 8 ± 1.4[a] |
| CD32 | 12 ± 2.7 | 45 ± 9[a] |
| CD64 | 3.6 ± 1.4 | 5.5 ± 0.8 |

Human CD14 monocytes, isolated from PBMCs of healthy donors, cultured for 6 days with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml), in the presence of medium alone or cAMP (0.5 mM). Cells were stained using directly conjugated mAb and analyzed by flow cytometry. [a]$P < 0.05$, significant differences between cAMP-treated and untreated cells.



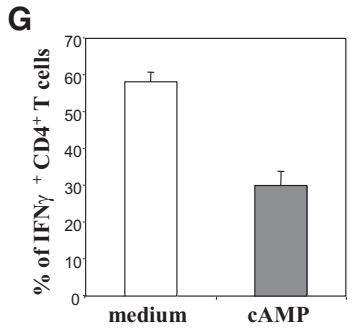




**Figure 4. Monocytes exposed to exogenous cAMP produce proinflammatory and regulatory cytokines and exhibit a reduced capacity to skew the immune response toward a Th1 profile.** Human CD14 monocytes, isolated from the PBMC of healthy donors, were cultured with medium alone or cAMP (0.5 mM). After 6 days, cells were incubated with LPS (200 ng/ml) for 48 h. The accumulation of IL-6 (A), TNF-α (B), IL-10 (C), and IL-12 (D) cytokines in culture supernatants was evaluated by ELISA. The data shown represent the mean (+SD) of three independent experiments. Alternatively, after 6 days, cells cultured in medium alone (E) or exogenous cAMP (F) were washed, starved for 8 h, and cocultured with purified $CD4^+CD45RA^+$ T cells at a 1:5 ratio. After 11 days of culture, the production of IL-4 and IFN-γ by $CD4^+$ T lymphocytes was analyzed by flow cytometry after intracellular staining, using anti-CD4-PerCP, -IFN-γ-FITC, and -IL-4-PE mAb. Dot plots show the percentage of cytokine-producing cells on $CD4^+$-gated populations. The data shown are from one representative experiment of three performed. (G) The histogram bars (+SD) show the means of the percentages of IFN-γ-positive $CD4^+$ T cell from six independent experiments performed.

at a 1:5 ratio. After 11 days of culture, the production of IL-4 and IFN-γ by $CD4^+$ T lymphocytes was analyzed by intracellular staining. A lower number of naive $CD4^+$ T cells cultured with cAMP differentiated into cells producing IFN-γ (25%±2) compared with cells cultured with untreated DCs (52%±3; $P=0.008$; Fig. 4E) was found. No differences were observed between cells differentiated in the presence or in the absence of cAMP in promoting the differentiation of $CD4^+$ T lymphocytes producing IL-4. These data indicate that cAMP-DCs are able to support naive T cell differentiation but with a reduced capacity to induce Th1-producing T lymphocytes compared with untreated cells.

## Adenosine receptors mediate the effect of extracellular cAMP on DC differentiation

It has been reported in different cell types and explored extensively in renal cells that extracellular cAMP can be converted into adenosine by extracellular phophodiesterases and ecto-5′-nucleotidases [24]. To investigate further the mechanisms by which monocytes sense extracellular cAMP, we measured the rate of disappearance of cAMP added to the cells over time by evaluating its concentration in the supernatants at different time-points (at 0, 10 and 30 min, and 1, 3, 6, 12, 18, and 24 h). We found that the concentration of cAMP, indicated as 100% at the beginning of culture, started to decrease substantially after 12 h and was not detectable after 24 h (**Fig. 5**), showing that the cAMP was metabolized rapidly.

To evaluate whether adenosine receptors were involved in mediating the effects of extracellular cAMP on DC differentiation, we first cultured monocytes with GM-CSF and IL-4 in the presence of exogenous cAMP or a stable cell-impermeable adenosine analog, NECA. We monitored phenotypical changes

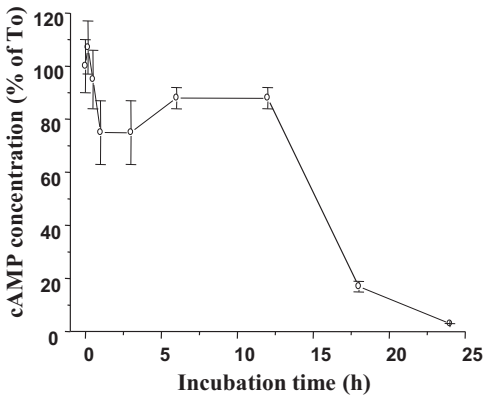



**Figure 5. Rapid metabolism of cAMP in the supernatants of monocytes induced to differentiate into DCs.** Monocytes were cultured in the presence of cAMP (0.5 mM), and its concentration was evaluated at different time-points (at 0, 10 and 30 min, and 1, 3, 6, 12, 18, and 24 h) by ELISA assay. The results are expressed as percentage, considering 100% as the concentration of cAMP measured at the beginning of culture (To). Means ± SEM of five analyzed donors run in duplicate are presented.

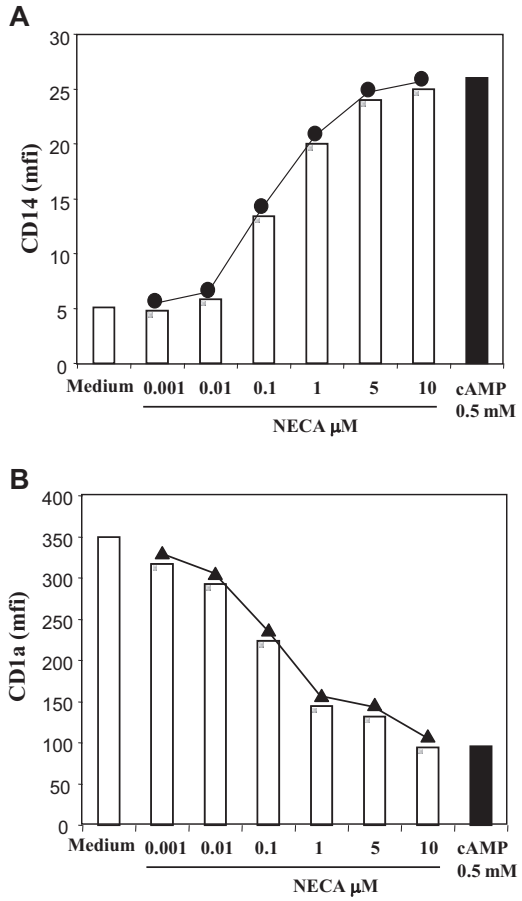



**Figure 6. Dose-dependent effects of NECA on DC differentiation.** Human CD14 monocytes, isolated from the PBMC of healthy donors, were cultured for 6 days with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) in the presence of medium alone, scalar doses of NECA (0.001–10 $\mu$M), or cAMP (0.5 mM). Cells were double-stained using anti-CD14-FITC and anti-CD1a-PE mAb and analyzed by flow cytometry. The histograms show the mean fluorescence intensity of CD14 (A) and CD1a (B) expression.

using CD1a and CD14 as molecular markers. We found that the differentiation of monocytes in the presence of NECA had a similar effect on DC differentiation as exogenous cAMP. Cells derived from NECA-treated monocytes did not express CD1a and retained the expression of CD14, and the effect was exerted in a concentration-dependent manner (**Fig. 6**). In addition, in these set of experiments, we identified the dose of NECA (10 $\mu$M), which gave rise to cells expressing the same amount of CD1a and CD14 induced by 0.5 mM extracellular cAMP (Fig. 6). Therefore, to address directly whether the effect of the extracellular cAMP on monocytes was mediated by the adenosine receptors, we induced monocytes to differentiate into DCs with exogenous cAMP in the presence or absence of DPSPX (0.1 mM), a nonselective antagonist of the adenosine receptors. Monocytes, induced to differentiate into DCs with NECA at a concentration of 10 $\mu$M in the presence or absence of the antagonist, were used as controls in the experiments. The adenosine receptor antagonist efficiently blocked the effects of cAMP and NECA on monocytes to DC differentiation (**Fig. 7**), and cells expressed levels of CD1a and CD14 comparable with untreated cells or cells treated with the antagonist alone (data not shown). Furthermore, as a control, we evaluated the effect of the adenosine receptor antagonist on cells cultured with FSK, a drug that directly activates adenylyl cyclase and induces an increase of intracellular cAMP. The antagonist only slightly affected the effect of FSK on the differentiation of monocytes into DCs, suggesting that the exogenous cAMP exerts its effects through an extracellular pathway and not by a passive or active uptake from the cells.

## A2A and A2B adenosine receptors mediate the effect of extracellular cAMP on monocyte differentiation toward DCs

To investigate further the role of adenosine receptors on the capacity of monocytes to sense and to respond to extracellular cAMP, we induced monocytes to differentiate into DCs with exogenous cAMP in the presence or absence of A2A- or A2B-selective antagonists, CSC, and MRS1754, respectively. Monocytes were induced to differentiate into DCs with exogenous cAMP (0.5 mM) or NECA (10 $\mu$M) in the presence or absence of A2A- or A2B-selective antagonists, and the modulation of CD14 and CD32 molecules was monitored after 6 days of culture. We found that A2A and A2B adenosine receptor antagonists neutralized the effects of extracellular cAMP on CD14

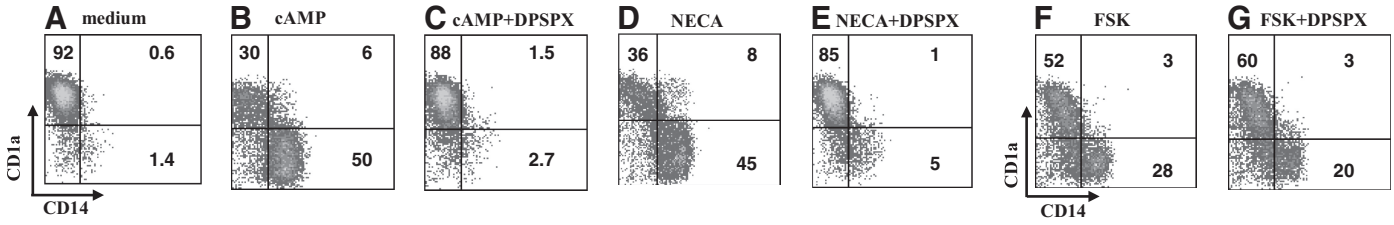



**Figure 7. Nonselective adenosine receptor antagonist prevents the effects of cAMP and NECA on DC differentiation from monocytes.** Dot plots show the phenotype of human CD14 monocytes, isolated from the PBMC of healthy donors and cultured for 6 days with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) in the presence of medium alone (A), cAMP (B), cAMP plus DPSPX (C), NECA (D), NECA plus DPSPX (E), FSK (F), or FSK plus DPSPX (G). Cells were double-stained using anti-CD14-FITC, anti-CD1a-PE mAb and analyzed by flow cytometry. Dot plots are representative of one experiment out of three performed.

(**Fig. 8A** and **B**) and CD32 (Fig. 8C and D) expression on cells during the differentiation toward DCs in a dose-dependent manner. Whereas, as reported previously [25], only A2B receptor-selective antagonist MRS1754, and not the A2A receptor-selective antagonist CSC, was able to block the maintenance of CD14 efficiently (Fig. 8E and F) and the up-regulation of CD32 (Fig. 8G and H) on NECA-exposed cells in a dose-dependent manner. These data suggest that extracellular cAMP is sensed by monocytes through A2A and A2B adenosine receptors, and selective antagonists could reverse its effect on monocytes.

### CD73 ecto-5′-nucleotidase inhibition partially prevents the effect of extracellular cAMP on monocyte differentiation toward DCs

It is known that AMP can be hydrolyzed rapidly to adenosine by membrane-bound 5′-nucleotidase CD73; therefore, to evaluate whether the effects of cAMP and AMP on monocytes were influenced by inhibitors of CD73, we performed experiments using AOPCP as CD73 inhibitor. We found that the effects of cAMP on monocyte differentiation into DCs were partially reverted in a dose-dependent manner by AOPCP (**Fig. 9**), showing that the metabolism of AMP into adenosine by CD73 activity is important in skewing the differentiation of monocytes into DCs.

Next, we evaluated the expression of CD73 on monocytes by flow cytometry, and in particular, we focused our attention on the expression of CD73 on $CD14^+CD16^-$ and $CD14^+CD16^+$ macrophage/monocyte subsets. $CD14^+CD16^+$ monocytes are a minor population of circulating monocytes (ranging between 2% and 10%; **Fig. 10C**), and we found that a small percentage of $CD14^+CD16^+$ monocytes expressed CD73 on their surface (Fig. 10). Altogether, these data suggest a link between expression of ectoenzymes and the functional outcome mediated via adenosine receptors on monocytes.

## DISCUSSION

The cAMP is a well-known second messenger recognized as a universal regulator of several cellular functions in organisms, including amoeba, plants, and humans [4]. After it was described that cAMP could be actively exported in the extracellular compartments, many studies have shown that exogenous cAMP exerts regulatory functions, acting as a first mediator in multiple tissues [6]. However, the impact of extracellular cAMP on cells of the immune system has not been fully investigated. In a previous work, we have shown that extracellular cAMP has an inhibitory effect on T cell activation and proliferation [11]. Here, we extended the study showing that extracellular cAMP modulates phenotype, function, and differentiation of human monocytes. The effects of extracellular cAMP on monocytes are mediated by A2A and A2B adenosine receptors, suggesting that an extracellular cAMP-adenosine pathway might be active in cells of the immune systems.

Monocytes are circulating precursors of macrophages and DCs, which can be recruited into tissues and differentiate depending on the microenvironment of the inflammatory sites [15, 16]. The differentiation process is complex and regulated by cytokines [17–20] and also by the interaction with pathogens, such as viruses or bacteria [21–23] or their derivatives

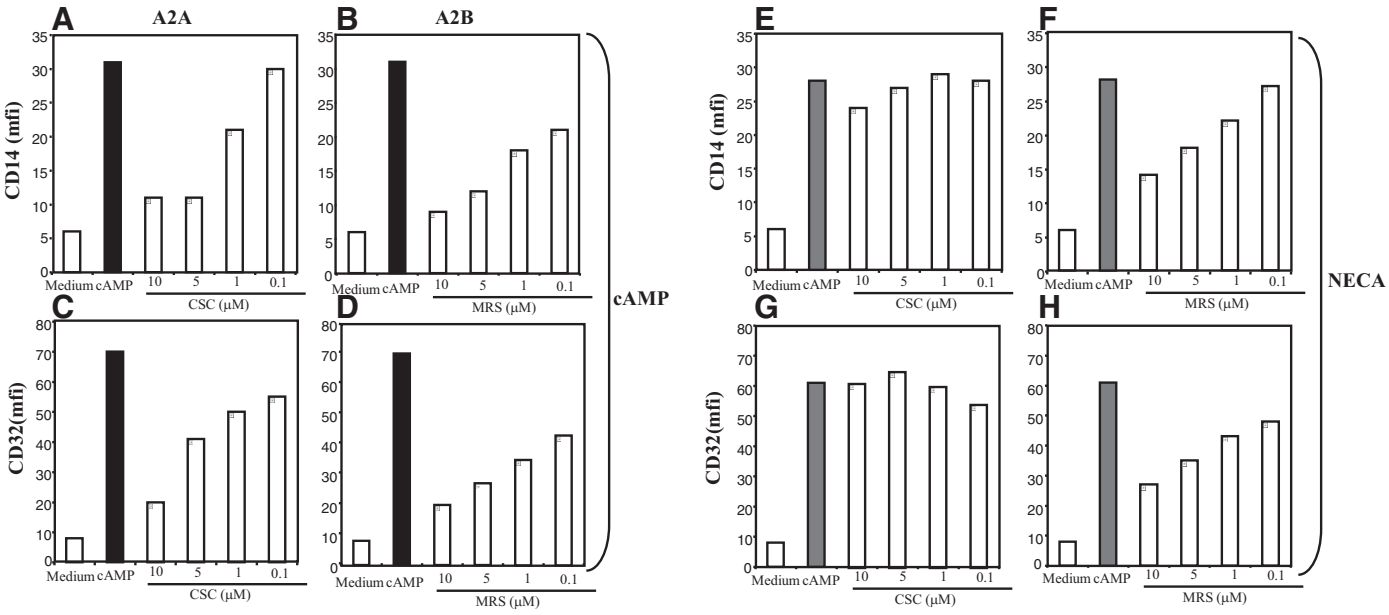



**Figure 8. Selective A2A and A2B adenosine receptor antagonists prevent the effects of cAMP on DC differentiation from monocytes.** Human CD14 monocytes, isolated from the PBMC of healthy donors, were cultured for 6 days with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) in the presence of medium alone, cAMP (0.5 mM), NECA (5 $\mu$M), or scalar doses of A2A antagonist CSC plus cAMP or plus NECA or scalar doses of A2B antagonist MRS1754 plus cAMP or plus NECA. Cells were stained using anti-CD14-FITC, or anti-CD32 mAb and analyzed by flow cytometry. The histograms show the mean fluoresce intensity of CD14 (A, B, E, and F) and CD32 (C, D, G, and H) expression.

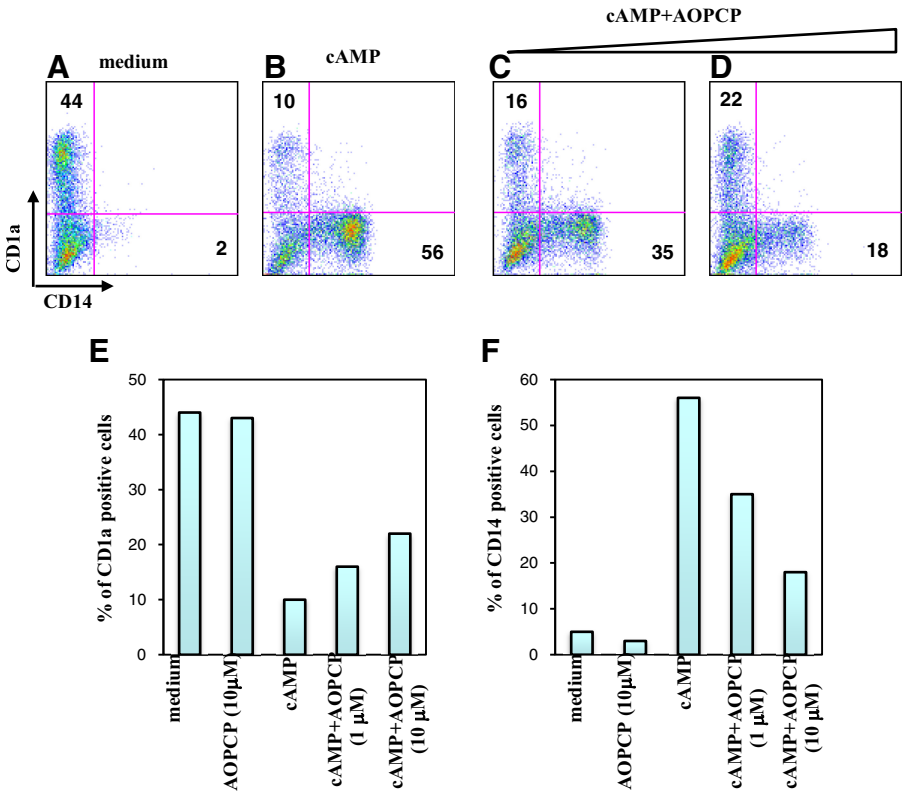



**Figure 9. Selective CD73 inhibitor partially prevents the effects of cAMP on DC differentiation from monocytes.** Human CD14 monocytes, isolated from the PBMC of healthy donors, were cultured for 6 days with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) in the presence of medium alone, cAMP (0.5 mM), or scalar doses of AOPCP (1 and 10 $\mu$M) plus cAMP. Cells were stained using anti-CD14-FITC and anti-CD1a-PE mAb and analyzed by flow cytometry. The histograms show the percentages of CD1a (E) and CD14 (F) expression.

[26]. In this study, we found that human monocytes exposed to exogenous cAMP exhibit a higher expression of CD14 molecules and a lower amount of MHC class I and class II molecules compared with untreated cells. In addition, when cAMP-cultured monocytes are treated with proinflammatory stimuli, they exhibit an increased production of IL-6 and IL-10 and lower amount of TNF-$\alpha$ and IL-12 compared with control cells, resembling the features of the alternatively activated macrophages or M2 macrophages [27, 28]. The anti-inflammatory effects of cAMP on monocytes and innate immune cells have been well-documented [29–31]. It has also been reported that cAMP is a key molecule in the resolution of inflammation by restraining M1-macrophage activation and favoring M2-like differentiation [32]. However, all of these effects are mediated by changes of intracellular cAMP levels and are consistent with the role of cAMP as an intracellular second messenger; in contrast, our study associates the induction of alternatively activated macrophage to extracellular cAMP, and this, to our knowledge, has not be described before.

It has been established that monocytes are circulating precursors, which in response to infectious stimuli, migrate to peripheral lymph nodes and acquire the key properties of DCs, becoming important cells in initiating the adaptive immune responses [33]. However, the differentiation program of monocytes to DCs is sensitive to a variety of environmental stimuli; therefore, the nature of the innate and adaptive immune responses results from the integration of multiple environmental cues. Here, we observed that monocytes exposed to exogenous cAMP in DC-differentiating medium, give rise to a phenotypically and functionally distinct population. This population exhibits an activated, macrophage-like phenotype, has a distinct anti-inflammatory cytokine profile, and exhibits a lower capacity to polarize naive $CD4^+$ T lymphocytes into a Th1 phenotype compared with control cells. Once again, the phenotype and functions of monocyte-derived DCs, cultured in the presence of exogenous cAMP, resemble previous findings, showing that an increase of intracellular cAMP impairs the differentiation of monocytes into DCs and gives rise to a cell population that exhibits a macrophage-like population and restrains the Th1-induced polarization [26, 34–37]. Altogether, our results suggest that exogenous cAMP activates the intracellular cAMP signaling pathway; however, the cellular mechanisms by which extracellular cAMP exerts its effects need further investigation. The results could be explained by a passive or active influx of cAMP directly into cells, and this may account for the increase of intracellular cAMP. Alternatively, the binding of cAMP to specific membrane receptors could deliver the signals to the cells. In support of the first hypothesis, it has been shown that the influx of cAMP into smooth muscle cells involves a membrane transporter [38]. Conversely, although cAMP receptors have not been identified in mammals, they have been well-characterized in lower eukaryotes. Four different cAMP receptors have been described in the amoeba *Dictyostelium discoideum* [39, 40]. They belong to the superfamily of seven-transmembrane domain GPCRs, which modulate the levels of intracellular cAMP. It is interesting that these receptors exhibit certain homology with the eukaryotic secretin receptor family [41]; however, cAMP receptors have not been found in mammalian cells. Another possible mechanism by which exogenous cAMP could mediate its

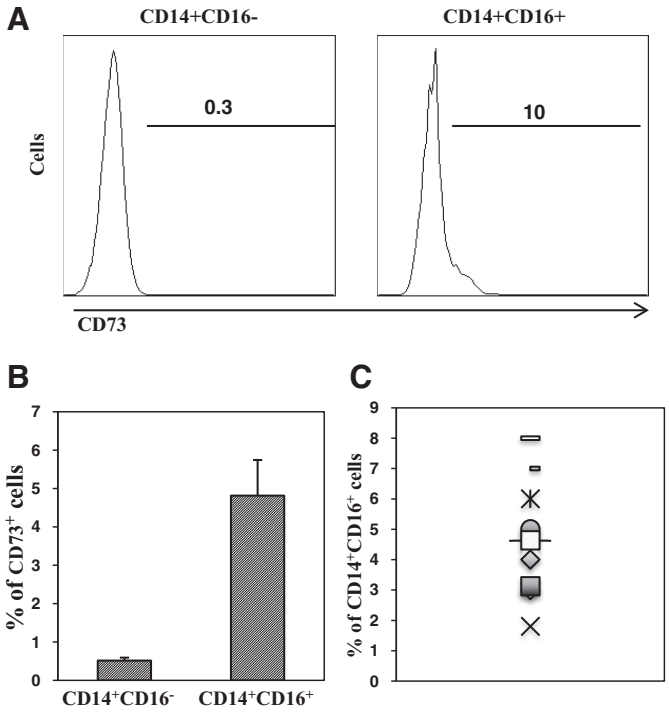



**Figure 10. Expression of CD73 on $CD14^+CD16^-$ and $CD14^+CD16^+$ macrophage/monocyte subsets.** Human CD14 monocytes, isolated from the PBMC of healthy donors, were purified by positive selection, using anti-CD14-conjugated magnetic microbeads. Cells were triple-stained with anti-CD14, -CD16, and -CD73 mAb, and the expression of CD73 was evaluated on $CD14^+CD16^-$ (A) and on $CD14^+CD16^+$ (B) subpopulations. The percentages of $CD14^+CD16^+$ in different healthy donors is reported in panel C. The histograms show the percentages of CD73 expression on $CD14^+CD16^-$ and on $CD14^+CD16^+$ subpopulations, and the graph shows the frequency of $CD14^+CD16^+$ in analyzed donors.

effects on monocytes is through the extracellular cAMP-adenosine pathway [24]. By extracellular phophodiesterases and ecto-5′-nucleotidases, extracellular cAMP is converted into adenosine, which activates adenylyl cyclases via A2A and A2B adenosine receptors, leading to an increase of intracellular cAMP. By using nonselective or specific adenosine receptor antagonists, we found that the effects of exogenous cAMP on monocyte differentiation were blocked, suggesting that A2A and A2B adenosine receptors mediate the effects of extracellular cAMP on monocytes. On the other hand, adenosine antagonists only slightly affected the effect of FSK—a drug that directly activates adenylyl cyclases and induces an increase of intracellular cAMP—on the differentiation of monocytes into DCs, suggesting that the exogenous cAMP exerts its effects through an extracellular pathway and not by a passive or active uptake from the cells. However, we cannot exclude that intracellular cAMP, generated by adenosine receptor engagement at the beginning of the culture, could be exported and replenishes cAMP outside of the cells, affecting the kinetics of cAMP disappearance.

These findings suggest that an extracellular cAMP-adenosine pathway, which has been described in different cell types and tissues, including kidney, brain, smooth muscles [6, 7, 24, 42, 43], could also be active in human monocytes, having a profound impact on shaping the innate and adaptive immune responses to endogenous stimuli or invading microbes. However, recently, it has been shown that exogenous AMP can also activate A1 and A2a receptors directly, without being transformed in adenosine [44]. Therefore, we cannot rule out the possibility that cAMP could be enzymatically degraded to AMP, which could directly mediate the effects on monocytes. Indeed, with the use of the CD73 inhibitor, we found that the effects of cAMP on monocyte differentiation into DCs were partially reverted, indicating that the metabolism of AMP into adenosine by CD73 activity is important but does not fully take into account the skewing of the differentiation of monocytes into DCs. Interestingly, we found that a small percentage of $CD14^+CD16^+$ monocytes expressed CD73 on their surface. $CD14^+CD16^+$ monocytes are a minor population of circulating monocytes, ranging from 2% to 10% in healthy donors, which is increased in the course of diseases [45, 46]. Therefore, the expression of CD73 on this population suggests a possible role of CD73 in regulating the response in the course of diseases. It would be interesting to evaluate the percentage of CD73 in the course of diseases in future studies. In the present work, we used 3′,5′-cAMP, which was discovered originally by Sutherland and colleagues [47], and the effects were likely a result of metabolism of 3′,5′-cAMP to 5′-AMP and adenosine, but it is conceivable that 2′,3′-cAMP, discovered recently by Jackson [42], would have similar effects, as it too is ultimately metabolized to adenosine. However, the identification of mechanisms and molecules associated with the capacity of monocytes to sense and to respond to extracellular nucleotides needs further investigation, but it could provide a basis for monocyte-targeted therapeutic strategies.

## AUTHORSHIP

E.S., A.R., V.G., and E.C. performed the experiments and collected and analyzed data. E.S., R.L., and M.P. contributed to the design of the experiments and to the writing of the manuscript. M.T.D.M. and S.V. conceived of the project, supervised the study, and wrote the manuscript.

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### DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
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