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The Cyclic AMP-Epac1-Rap1 Pathway Is Dissociated from Regulation of Effector Functions in Monocytes but Acquires Immunoregulatory Function in Mature Macrophages

Tone Bryn,* Milada Mahic,* Jorrit M. Enserink,2* Frank Schwede,† Einar Martin Aandahl,* and Kjetil Taskén3**

Cyclic AMP regulates a wide range of cellular events, such as metabolism and gene expression, cell division, insulin secretion, exocytosis, and regulation of immune responses (1–6). Elevated levels of cAMP in immune cells generally lead to suppression of immune responses, including cytokine production, cell proliferation, chemotaxis, and phagocytosis (7–21). In lymphocytes, cAMP inhibits immune responses by a PKA-dependent mechanism that involves activation of Rap1 in response to cAMP. However, by using an Epac-specific cAMP analog (8-CPT-2′-O-Me-cAMP), we show that monocyte activation parameters such as synthesis and release of cytokines, stimulation of cell adhesion, chemotaxis, phagocytosis, and respiratory burst are not regulated by the Epac1-Rap1 pathway. In contrast, activation of PKA by a PKA-specific compound (6-Bnz-cAMP) or physiological cAMP-elevating stimuli like PGE2 inhibits monocyte immune functions. Furthermore, we show that the level of Epac1 increases 3-fold during differentiation of monocytes into macrophages, and in monocyte-derived macrophages cAMP inhibits FcR-mediated phagocytosis via both PKA and the Epac1-Rap1 pathway. However, LPS-induced TNF-α production is only inhibited through the PKA pathway in these cells. In conclusion, the Epac1-Rap1 pathway is present in both monocytes and macrophages, but only regulates specific immune effector functions in macrophages. The Journal of Immunology, 2006, 176: 7361–7370.

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3 Address correspondence and reprint requests to Dr. Kjetil Taskén, Center Director, Biotechnology Center of Oslo, University of Oslo, P.O. Box 1112, Blindern, N-0317 Oslo, Norway. E-mail address: kjetil.tasken@biotek.uio.no

4 Abbreviations used in this paper: PKA, protein kinase A; ABS, heat-inactivated human AB serum; AM, alveolar macrophage; FSC, forward light scatter; GDS, guanine exchange factor for the small GTPases Rap1 and Rap2; cGMP, cyclic GMP; GTPγS, guanosine 5′-O-(3-thiotriphosphate); H9252, H11011, H11032, H9251, H11034 cAMP analogues with specificity toward PKA (6-Bnz-cAMP) and Epac (8-CPT-2′-O-Me-cAMP), we show that the level of Epac1 increases 3-fold during differentiation of monocytes into macrophages, and in monocyte-derived macrophages cAMP inhibits FcR-mediated phagocytosis via both PKA and the Epac1-Rap1 pathway. However, LPS-induced TNF-α production is only inhibited through the PKA pathway in these cells. In conclusion, the Epac1-Rap1 pathway is present in both monocytes and macrophages, but only regulates specific immune effector functions in macrophages. The Journal of Immunology, 2006, 176: 7361–7370.

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Abbreviations used in this paper: PKA, protein kinase A; ABS, heat-inactivated human AB serum; AM, alveolar macrophage; FSC, forward light scatter; GDS, guanine nucleotide-dissociation stimulator; BBM, 3′-isobutyl-1-methylxantine; MDM, monocyte-derived macrophage; PDE, phosphodiesterase; SSC, side light scatter.
Materials and Methods

Reagents and Abs

LPS, derived from *Escherichia coli* serotype O26:B6, glutathione-agarose beads, PGE2, 3-isobutyl-1-methylxantine (IBMX), and rolipram were purchased from Sigma-Aldrich. H-89 was obtained from Calbiochem. Rp-8-Br-cAMPs, Sp-8-Br-cAMPs, 8-CPT-2′-O-Me-cAMP, and 6-Bnz-cAMP were obtained from BIOLOG Life Sciences Institute. TNF-α (detection level 4.4 pg/ml), IL-12 heterodimer (detection level 5.0 pg/ml), and MIP-1β (detection level 4.0 pg/ml) ELISA kits and human rIFN-γ were purchased from R&D Systems. Anti-CXCR4 (PE), anti-CCR5 (PE), anti-CD11a (PE), anti-CD11b (PE), anti-CD11c (PE), anti-CD62L (FITC), anti-TNF-α (PE), and Annexin V (FITC) Abs; propidium iodide; and Phagotest and Burst test were purchased from BD Pharmingen, whereas Vybrant Phagocytosis Assay Kit and *E. coli* Bioparticle opsonization reagent were obtained from Molecular Probes. Anti-CD11b CB1R/5 Ab was obtained from Biologend, Epac1 Ab was obtained from Upstate Biotechnology, Rap1 Ab was obtained from Santa Cruz Biotechnology, and PKA substrate phospho-specific Ab (ab-anti-RXXPS/PT) was obtained from Cell Signaling Technology. PKA R1α, PKA R1β, and PKA C Abs were from BD Transduction Laboratories. GST-tagged Ras binding domain of Ras-guanine nucleotide-dissociation stimulator (GST-Ral-guanine nucleotide-dissociation stimulator (GDS)-Ras binding domain) construct was a gift from J. Bos (University Medical Center, Utrecht, The Netherlands). The endothoxin content of all reagents was, when not declared endotoxin free from the manufacturer, determined by the Limulus amebocyte lysate assay (Cambrex) and was <20 pg/ml for the concentrations used in cell culture.

Isolation and culture of human monocytes

Human PBMC were isolated from heparinized whole blood or buffycoat from Isopaque-Ficoll (Lymphoprep; Nycodens) density gradient centrifugation. Monocytes were isolated from PBMC by positive selection using MACS CD14 microbeads (Miltenyi Biotech), and >95% CD14+ monocytes were routinely obtained, as assessed by flow cytometry analysis. Cells were cultured in RPMI 1640 containing 2 mM L-glutamine and 10% heat-inactivated human AB serum (RPMI 1640/10% ABS) (BioWhittaker), 5% CO2, 37°C. To induce monocyte differentiation to macrophages, CD14+ monocytes were cultured in RPMI 1640/10% ABS containing 25 mM HEPES buffer (RPMI 1640/HEPES/10% ABS) supplemented with 40 ng/ml M-CSF (PeproTech) for 6–7 days. Fresh medium supplemented with M-CSF was added on day 3.

Rap activation assay and phosphorylation of PKA

Positively selected monocytes were stimulated, as described in Results, and Rap1 activation assay was performed, as described previously (38). Briefly, cell lysates were incubated with Ras binding domain of Ras-GDS fused to GST. This fusion protein was precoupled to glutathione beads to specifically pull down the activated GTP-bound form of Rap1. Samples were analyzed by Western blotting using Rap1 Ab. As positive control of Rap activation, Rap was activated by tumbling of the monocyte suspension (33). Glutathione beads incubated with 10% AB serum before tumbling with cell lysate were used as negative control.

To assay cAMP activation of PKA, positively selected human monocytes were stimulated, as described in Results, and cell lysates were analyzed for proteins phosphorylated by PKA (anti-RXXPS/PT) by Western blotting.

Cytokine secretion

To stimulate production of TNF-α and MIP-1β, cells were stimulated with 100 ng/ml LPS. In the IL-12 assays, monocytes were primed with IFN-γ (300 U/ml) for 2 h before LPS (50 ng/ml) activation. When used, Rp-8-Br-cAMPs, Sp-8-Br-cAMPs, 8-CPT-2′-O-Me-cAMP, 6-Bnz-cAMP, PGE2 (1 μM), rolipram (10 μM), and IBMX (200 μM) were added 30 min before activation or priming. Cells were incubated (37°C, 5% CO2) (see Results) before cell-free supernatants were harvested, and TNF-α, MIP-1β, and IL-12 secretion were determined using ELISA (R&D Systems). The ELISAs (duplicate or triplicate cultures) were performed according to the manufacturer’s instructions. The standard curve was plotted as log (picograms per milliliter) to OD660 nm, and the curve was fitted to the formula

\[
y = y_0 + ax/(b + x)
\]

The concentration of each sample was calculated, and the average of the duplicate or triplicate cultures was determined.

Flow cytometry analyses of surface expression of CXCR4 and CCR5

Purified PBMC were activated with 100 ng/ml LPS for 24 h. When used, Rp-8-Br-cAMPs (1000 μM), Sp-8-Br-cAMPs (1000 μM), 6-Bnz-cAMP (300 μM), or 8-CPT-2′-O-Me-cAMP (100 μM) was added 30 min before LPS activation. After 24 h, the cells were washed in PBS with 1% BSA before incubation with Dvorochrom-conjugated Abs against CD33 and CCR5 or CD33 and CXCR4 for 30 min at 4°C in the dark. The cells were washed twice before resuspension in 1% paraformaldehyde, and subsequently, the samples were analyzed using a FACS Calibur instrument (BD Pharmingen). The monocyte population was gated for CD33+ cells and analyzed for CCR5 and CXCR4 expression.

Flow cytometry analyses of surface expression of LFA-1, p150,95, Mac-1, Mac-1 CBMR1/5, and CD62L

Heparinized whole blood was diluted 1/1 in RPMI 1640/20% ABS. Cells were transferred to ultralow attachment plates (Costar) and treated with PBS, activated with LPS (1 μg/ml), or preincubated with Sp-8-Br-cAMPs (1000 μM), 6-Bnz-cAMP (300 μM), or 8-CPT-2′-O-Me-cAMP (100 μM) for 30 min before LPS activation (1 μg/ml). To measure directly the effect of CAMP analogues, cells were stimulated with the compounds alone. After 2 (LFA-1, p150,95, Mac-1) or 3 (Mac-1 CBMR1/5 and CD62L) h, the cells were transferred to V-bottom plates and washed with PBS/1% BSA before incubation with fluorochrome-conjugated Abs (CD33-allophycocyanin and LFA-1-PE, p150,95-PE, Mac-1-PE, Mac-1 CBMR1/5, or CD62L-FITC) for 30 min at 4°C in the dark. RBC were lysed using cell lysis solution (BD Pharmingen) before subsequent washing, fixation, and flow cytometry analysis using a BD Biosciences FACS Calibur instrument. The monocyte population was gated in the CD33-side light scatter (SSC) diagram and analyzed for surface expression of LFA-1, p150,95, Mac-1, Mac-1 CBMR1/5, and CD62L. Incubation with CAMP analogues was performed in human whole blood samples, thereby keeping them close to the in vivo situation and preventing possible activation of monocytes by the isolation procedure.

Flow cytometric analyses of respiratory burst

Heparinized whole blood was left untreated or treated with Sp-8-Br-cAMPs (1000 μM), 6-Bnz-cAMP (300 μM), 8-CPT-2′-O-Me-cAMP (100 μM), or Rp-8-Br-cAMPs (1000 μM) for 30 min before stimulation with E. coli (10 min at 37°C and addition of a fluorogenic substrate, according to the manufacturer’s protocol). Subsequently, RBC were lysed, and the remaining cells were fixed and stained for DNA, followed by flow cytometry analyses. Duplicate samples were analyzed using a FACS Calibur instrument (BD Pharmingen). Monocytes were gated in the forward light scatter (FSC)-SSC diagram, and their green fluorescence histogram (FL-1) was analyzed.

Flow cytometric analyses of phagocytosis

Heparinized whole blood was left untreated or treated with Sp-8-Br-cAMPs (1000 μM), 6-Bnz-cAMP (300 μM), 8-CPT-2′-O-Me-cAMP (100 μM), or Rp-8-Br-cAMPs (1000 μM) for 30 min before addition of FITC-conjugated opsonized *E. coli* (duplicate or triplicate samples). After 10 min of incubation at 37°C, cells were washed, RBC was lysed, and the remaining cells were stained for DNA, followed by flow cytometry analyses using a FITC-conjugated *E. coli* (BD Pharmingen). Residual extracellular FITC (representing cell-adherent *E. coli*) was quantitated by addition of quenching solution. Monocytes were gated in the FSC-SSC diagram of FL-2-positive cells (leukocytes), and their green fluorescence histogram (FL-1) was analyzed.

Fluorometric phagocytosis assay

Paraformaldehyde-inactivated, FITC-labeled *E. coli* BioParticles were suspended in PBS and opsonized with specific rabbit polyvalent IgG, according to the manufacturer’s protocol. Opsonized *E. coli* (IgG-*E. coli*) was further diluted in RPMI 1600/HEPES/10% ABS for use in phagocytosis experiments. CD14+ monocytes were added to flat-bottom 96-well plates (Costar; 0.75 M/well, 1 M/ml) and either allowed to adhere for 2 h (for phagocytosis experiments on freshly isolated monocytes) or cultured in RPMI 1640/HEPES/10% ABS supplemented with M-CSF for 6 days (for experiments on MDM). Nonadherent cells were washed away with warm RPMI 1640 and the cells were preincubated with the compound of interest for 30 min before addition of IgG-*E. coli* (multiplicity of infection = 30:1). After 45 min, uningested bacteria were washed away with PBS and residual extracellular FITC (representing cell-adherent IgG-*E. coli*) were quantitated by tripyn blue for 1 min. Fluorescence was determined with a
fluorometer (485/535 nm, EnVision 2102 Multilabel Reader; PerkinElmer). Background fluorescence (wells containing only cells and medium) was subtracted before the average percentage of regulation was determined relative to PBS-treated control cells. Independent experiments were performed in quadruplicates.

The inhibitory effects of cAMP-elevating agents are not due to cell death

To investigate whether the inhibitory effects of cAMP on the various monocyte immune functions were due to apoptosis or cell death, PBMC and monocyte cultures were left unstimulated or activated with and without preincubation with the cAMP-elevating agents or the cAMP analogues. The inhibitory effects were not due to cell death, as assessed by annexin-V binding and propidium iodide exclusion by flow cytometry analyses (data not shown).

Results

Activation of Epac-Rap1 and the PKA pathways by cAMP in monocytes

Both PKA and Epac represent downstream effector pathways for cAMP. To determine whether PKA and Epac are present in human peripheral blood monocytes, positively selected monocytes were lysed and analyzed for the RIIα, RIIα, C subunits, and Epac1 by Western blotting. As shown in Fig. 1, A and B, monocytes contained predominantly PKA type I, as indicated by high levels of RIIα and C, and comparably lower levels of RIIα. Interestingly, the cells also contained significant levels of Epac1. To delineate different routes of signaling by cAMP, cAMP analogues with known specificity for PKA and Epac were used. As shown in Fig. 1C, positively selected human monocytes were treated with Sp-8-Br-cAMPS, 6-Bnz-cAMP, or 8-CPT-2'-O-Me-cAMP before Western blot analysis of proteins phosphorylated by PKA (anti-RXXPS/PT). Both the nonselective Sp-8-Br-cAMPS and the PKA-selective 6-Bnz-cAMP analogues induced phosphorylation by PKA, whereas the Epac-specific 8-CPT-2'-O-Me-cAMP did not.

To address whether Epac is activated by cAMP in monocytes, positively selected human monocytes were left untreated or stimulated with the Epac-specific cAMP compound, 8-CPT-2'-O-Me-cAMP, or the nonselective cAMP analog, Sp-8-Br-cAMPS, for 7 and 25 min (36). Rap1 activity was used as an indicator of Epac activation. Both 8-CPT-2'-O-Me-cAMP and Sp-8-Br-cAMPS activated Rap1 after 25 min of stimulation (Fig. 1D). Next, monocytes were treated with increasing concentrations of 8-CPT-2'-O-Me-cAMP, Sp-8-Br-cAMPS, or 6-Bnz-cAMP for 25 min before lysis of cells and subsequent analyses for Rap1 activation. As seen in Fig. 1E, 8-CPT-2'-O-Me-cAMP and Sp-8-Br-cAMPS activated Rap1 in a concentration-dependent manner, whereas 6-Bnz-cAMP did not. These results indicate that 8-CPT-2'-O-Me-cAMP specifically activates the Rap1 pathway through Epac and not PKA in human monocytes, whereas 6-Bnz-cAMP specifically activates PKA. In contrast, Sp-8-Br-cAMPS is a nonselective cAMP analog that activates both PKA and Rap1.

Regulation of TNF-α secretion by the cAMP/PKA signaling pathway in monocytes

LPS leads to strong activation of monocytes accompanied by increased TNF-α production. To obtain the optimal time point of LPS stimulation for measurements of TNF-α secretion, positively selected human monocytes were stimulated with LPS for 0, 1, 3, 6,
To investigate the effects of cAMP on LPS-induced TNF-α production in monocytes, positively selected human monocytes from normal blood donors were treated with a cAMP agonist (Sp-8-Br-cAMPS), PGE₂, or the phosphodiesterase (PDE) inhibitors rolipram and IBMX before activation with LPS. TNF-α was measured in the supernatants by ELISA. As shown in Fig. 2B, both direct and indirect elevation of intracellular level of cAMP led to a dramatic inhibition of LPS-induced TNF-α secretion from monocytes.

In T cells, we have demonstrated previously that cAMP through activation of PKA type I leads to a tonic inhibition of T cell activation and raises the threshold of signaling through the TCR/CD3 complex (22). To assess whether a similar mechanism operates in monocytes, the cells were treated with a PKA type I antagonist (Rp-8-Br-cAMPS) or with the PKA inhibitor H89 (data not shown) before LPS stimulation. None of these compounds altered TNF-α secretion (39). Our observations would therefore be compatible with the notion that PDE4B leads to a full degradation of endogenous cAMP upon LPS activation. Thus, no additional effect of blocking PKA function by cAMP antagonists would be observed as there is no cAMP left to inhibit TNF-α secretion after LPS stimulation.

To examine whether the cAMP-induced inhibition of TNF-α secretion was due to activation of the PKA pathway or activation of Rap1, analogues that specifically activate PKA or Rap1 were tested for their effects on LPS-induced TNF-α secretion. Sp-8-Br-cAMPS and the PKA-specific analog 6-Bnz-cAMP potently suppressed TNF-α secretion (Fig. 2, D and E), whereas the Epac-specific analog, 8-CPT-2’-O-Me-cAMP, did not have any inhibitory effect (Fig. 2F). These results clearly suggest that cAMP inhibits TNF-α expression in monocytes by activation of PKA and not through activation of Epac. Similar results were obtained when TNF-α expression was analyzed by intracellular flow cytometry (data not shown).

cAMP inhibits IL-12 secretion from human peripheral monocytes by a PKA-dependent mechanism

High levels of IL-12 are secreted from monocytes upon stimulation with a priming signal (i.e., IFN-γ or GM-CSF), followed by a second signal (e.g., LPS) (40, 41). Monocytes treated with IFN-γ or LPS alone did not secrete high levels of IL-12, whereas cells primed with IFN-γ following stimulation with LPS produced significant amounts of IL-12 (data not shown).

To find the optimal time of stimulation of monocytes for assessment of IL-12 secretion, positively selected monocytes were primed with IFN-γ before LPS stimulation for 0, 3, 6, 18, and 42 h.

**FIGURE 2.** LPS-induced secretion of TNF-α from human peripheral blood monocytes is inhibited by cAMP via the PKA pathway. A, Positively selected human monocytes were left untreated or stimulated with LPS for 1, 3, 6, 18, and 42 h, and the concentrations of TNF-α in supernatants were assessed using ELISA. B, Human monocytes were left untreated or treated with PGE₂, Sp-8-Br-cAMPS (1000 μM), rolipram, or IBMX for 30 min before LPS stimulation for 24 h and TNF-α analyses. C, Human monocytes were left untreated or treated with Rp-8-Br-cAMPS (1000 μM) or IBMX for 30 min before LPS stimulation for 24 h and analyzed for TNF-α. When stimulated with both IBMX and Rp-8-Br-cAMPS, IBMX was added to cell cultures to increase cAMP levels. As seen from Fig. 2, 1 mM Rp-8-Br-cAMPS reversed the inhibitory effect of the PDE inhibitor. It has been shown previously that PDE4B is essential for LPS-induced TNF-α secretion (39). Our observations would therefore be compatible with the notion that PDE4B leads to a full degradation of endogenous cAMP upon LPS activation. Thus, no additional effect of blocking PKA function by cAMP antagonists would be observed as there is no cAMP left to inhibit TNF-α secretion after LPS stimulation.

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To find the optimal time of stimulation of monocytes for assessment of IL-12 secretion, positively selected monocytes were primed with IFN-γ before LPS stimulation for 0, 3, 6, 18, and 42 h,
and subsequent analyses of IL-12 by ELISA. Maximal IL-12 secretion was observed after 42 h (Fig. 3A). The effect of cAMP on regulation of IL-12 secretion from monocytes was examined using a cAMP agonist or agents known to elevate levels of cAMP. Positively selected monocytes from normal blood donors were treated with PGE\(_2\), Sp-8-Br-cAMPS, rolipram, or IBMX for 30 min before priming with IFN-\(\gamma\) and subsequent LPS stimulation. All agents inhibited IL-12 production (Fig. 3B). The cAMP antagonist, Rp-8-Br-cAMPS, did not have any effects on IL-12 production in line with observations made for TNF-\(\alpha\) (Fig. 3C).

To investigate whether the inhibitory effects of elevated cAMP were caused by activation of PKA or Rap1, monocytes were treated with Sp-8-Br-cAMPS, the PKA-specific compound 6-Bnz-cAMP, or the Epac-specific analog 8-CPT-2'-O-Me-cAMP before priming and LPS activation. Fig. 3D shows that Sp-8-Br-cAMPS and 6-Bnz-cAMP decreased IL-12 production, whereas 8-CPT-2'-O-Me-cAMP had no effects.

**Chemokines are regulated by cAMP through activation of the PKA pathway**

We have shown previously that CCR5, CXCR4, and MIP-1\(\beta\) are regulated by cAMP in T cells (9). In monocytes, maximal secretion of the chemokine MIP-1\(\beta\) occurred after 42 h of LPS stimulation (Fig. 4A). As observed with TNF-\(\alpha\) and IL-12, the cAMP analog Sp-8-Br-cAMPS, as well as PGE\(_2\), rolipram, and IBMX inhibited MIP-1\(\beta\) secretion, whereas Rp-8-Br-cAMPS did not affect the MIP-1\(\beta\) production (Fig. 4, B and C). Furthermore, the inhibitory effect of cAMP was mediated by PKA as 6-Bnz-cAMP suppressed MIP-1\(\beta\) secretion, whereas 8-CPT-2'-O-Me-cAMP had no effect (Fig. 3D).

To examine the effects of the PKA pathway on the regulation of chemokine receptors on monocytes, PBMC were next treated with the cAMP agonist Sp-8-Br-cAMPS or with the PKA inhibitor Rp-8-Br-cAMPS before LPS stimulation. Surface expression of chemokine receptors on monocytes was analyzed by flow cytometry. Interestingly, Sp-8-Br-cAMPS inhibited the expression of CCR5, while it increased the surface expression of CXCR4. The cAMP antagonist Rp-8-Br-cAMPS had no regulatory effect on surface expression of these chemokine receptors (Fig. 5, A and D). The 6-Bnz-cAMP, PGE\(_2\), rolipram, and IBMX had similar effect as Sp-8-Br-cAMPS on the surface expression of CXCR4 and CCR5 (data not shown). Again, the regulation by cAMP appeared to be

**FIGURE 3.** cAMP regulates IL-12 secretion from human peripheral blood monocytes via PKA pathway and not an Epac route. A, Positively selected human monocytes were left untreated or treated with IFN-\(\gamma\), followed by LPS stimulation for 3, 6, 18, and 42 h, and the concentrations of IL-12 in supernatants were assessed using ELISA. B, Positively selected human monocytes were left untreated or preincubated with PGE\(_2\), Sp-8-Br-cAMPS (125 \(\mu\)M), rolipram, or IBMX for 30 min before priming with IFN-\(\gamma\), followed by LPS stimulation for 20 h and subsequent IL-12 analyses. C, Human monocytes were left untreated or treated with Rp-8-Br-cAMPS (125 \(\mu\)M) for 30 min before priming with IFN-\(\gamma\) and LPS stimulation for 20 h. D, Human monocytes were stimulated with Sp-8-Br-cAMPS (125 \(\mu\)M), 6-Bnz-cAMP (100 \(\mu\)M), or 8-CPT-2'-O-Me-cAMP before priming with IFN-\(\gamma\) and LPS stimulation for 20 h. Results are given as mean of \(n = 2–8\) observations \(\pm\) SEM or half range.

**FIGURE 4.** LPS-induced secretion of MIP-1\(\beta\) from human peripheral blood monocytes is inhibited by cAMP via the PKA pathway and not the Epac route. A, Positively selected human monocytes were left untreated or stimulated with LPS for 3, 6, 18, and 42 h, and the concentrations of MIP-1\(\beta\) in supernatants were assessed using ELISA. The results are given as the mean \(\pm\) half range (\(n = 2\)). B, Human monocytes were left untreated or treated with PGE\(_2\), Sp-8-Br-cAMPS (1000 \(\mu\)M), rolipram, or IBMX for 30 min before LPS stimulation for 24 h and MIP-1\(\beta\) analysis. C, Positively selected human monocytes were left untreated or treated with Rp-8-Br-cAMPS (125 \(\mu\)M) for 30 min before LPS stimulation for 24 h and MIP-1\(\beta\) analyses. D, Human monocytes were stimulated with Sp-8-Br-cAMPS (125 \(\mu\)M), 6-Bnz-cAMP, or 8-CPT-2'-O-Me-cAMP before LPS stimulation for 20 h and measurements of MIP-1\(\beta\) in the supernatants. Data represent mean \(\pm\) SEM of \(n = 3–7\) observations.
mediated through activation of PKA and not by the Rap pathway as Sp-8-Br-cAMPS and 6-Bnz-cAMP had consistent regulatory effects, whereas no effect was observed with 8-CPT-2'-O-Me-cAMP (Fig. 5, B and D).

Regulation of surface adhesion molecules on monocytes by cAMP

Integrin activity is regulated through various mechanisms, including cell surface expression (change in number), redistribution at the cell surface (change in avidity), and conformational changes (change in affinity). The small GTPase Rap1 is involved in regulation of $\beta_2$ integrins in a variety of cells including J77.4.A1 macrophages, in which active Rap1 regulates functional activation of Mac-1 (CD11b/CD18, $\alpha_4\beta_2$) (29–34, 42). It has been reported recently that cAMP induces integrin-mediated cell adhesion through the Epac-Rap1 pathway independently of the PKA route (34, 35). We therefore wanted to investigate whether PKA or Rap1 affects relevant surface adhesion molecules such as the $\beta_2$ integrin receptors LFA-1 (CD11a/CD18, $\alpha_4\beta_2$), p150,95 (CD11c/CD18, $\alpha_4\beta_2$), Mac-1 (CD11b/CD18, $\alpha_4\beta_2$), and its active epitope CBRM1/5, or the L-selectin CD62L heparinized whole blood was diluted 1/1 in RPMI 1640/20% ABS and treated with PBS, Sp-8-Br-cAMPS, 6-Bnz-cAMP, or 8-CPT-2'-O-Me-cAMP, or activated with LPS and analyzed by flow cytometry. As shown in Fig. 6, the cell surface expression of LFA-1 (Fig. 6A), p150,95 (Fig. 6B), and Mac-1 (Fig. 6C) was suppressed by cAMP through the PKA pathway as Sp-8-Br-cAMPS and 6-Bnz-cAMP decreased, whereas 8-CPT-2'-O-Me-cAMP had no regulatory effect on these receptors. Next, we studied the regulation of the neo-epitope Mac-1

FIGURE 5. cAMP inhibits surface expression of CCR5 while increasing the surface expression of CXCR4 via the PKA pathway. A and C, Human PBMC were preincubated with Sp-8-Br-cAMPS (1000 $\mu$M) or Rp-8-Br-cAMPS (1000 $\mu$M) for 30 min before LPS stimulation for 24 h. The cell populations were then subjected to flow cytometry analysis. Monocyte populations were gated in the CD33-SSC diagram and analyzed for surface expression of CCR5 and CXCR4 receptors. Data are representative of three observations. B and D, Human PBMC were preincubated with Sp-8-Br-cAMPS (125 $\mu$M) or 8-CPT-2'-O-Me-cAMP (100 $\mu$M) for 30 min before LPS stimulation for 24 h and subsequent flow cytometry analyses. Receptor expressions on monocytes (gated in the CD33-SSC diagram) were measured as median fluorescence intensity on monocyte populations, and the data show receptor regulation by Sp-8-Br-cAMPS or 8-CPT-2'-O-Me-cAMP relative to untreated LPS-activated cells. Data represent mean of $n = 3–4$ observations $\pm$ SEM or half range in which $n = 2$.

FIGURE 6. cAMP modulates the surface expression of adhesion molecules on monocytes. Heparinized whole blood was treated with PBS; activated with LPS; stimulated with Sp-8-Br-cAMPS, 6-Bnz-cAMP, or 8-CPT-2'-O-Me-cAMP (A, B, C, and E); or preincubated with PBS, Sp-8-Br-cAMPS, 6-Bnz-cAMP, or 8-CPT-2'-O-Me-cAMP for 30 min before LPS activation (D). The samples were incubated for 2 (A–C) or 3 (D and E) h before staining with Abs, lysis of RBC, and subsequent flow cytometry analyses. Monocytes were gated in CD33-SSC diagram and analyzed for surface expression of LFA-1 (A), p150,95 (B), Mac-1 (C), Mac-1 CBRM1/5 (D), and CD62L (E). The data show median fluorescence intensity relative to control cells of $n = 2$ observations $\pm$ half range.
CBRM1/5. cAMP, through activation of PKA, inhibited LPS-induced increase in the surface expression of the active integrin receptor (Fig. 6D).

The adhesion molecule CD62L is responsible for rolling of cells along the endothelium. In contrast to the other adhesion molecules, expression of L-selectin decreased significantly on monocytes following LPS activation (Fig. 6E). The nonspecific cAMP analog Sp-8-Br-cAMPS and the PKA-specific 6-Bnz-cAMP increased the surface expression of CD62L, whereas the Epac-specific analog 8-CPT-2′-O-Me-cAMP had a moderately inhibitory effect (Fig. 6E). None of the cAMP analogues modulated the L-selectin expression on LPS-activated cells (data not shown).

cAMP inhibits phagocytosis and respiratory burst activity in monocytes through PKA activation

Monocytes are phagocytic cells that play an important role in host defense against microorganisms. The phagocytic process can be separated into chemotaxis, phagocytosis, and intracellular killing by oxygen-dependent (oxidative burst) and oxygen-independent mechanisms. The effect of the cAMP pathway on phagocytosis and oxidative burst activity in monocytes was examined using heparinized whole blood stimulated with E. coli in the absence and presence of a cAMP analog. As seen from Fig. 7A, PGE2, Sp-8-Br-cAMPS, and 6-Bnz-cAMP inhibited phagocytosis of opsonized E. coli moderately (10%), whereas 8-CPT-2′-O-Me-cAMP and Rp-8-Br-cAMPS had no effect. The results were verified using another phagocytosis assay (data not shown; see Materials and Methods for assay).

Furthermore, we analyzed the respiratory burst activity in monocytes and found that cAMP through activation of PKA suppressed the burst activity, whereas 8-CPT-2′-O-Me-cAMP and Rp-8-Br-cAMPS had no effect (Fig. 7B).

**Discussion**

In this study, we have investigated the effects of cAMP on various immune responses by monocytes, such as cytokine and chemokine production, surface expression of chemokine receptors and adhesion molecules, as well as phagocytosis and respiratory burst activity. By using cAMP analogues specific for PKA (6-Bnz-cAMP) or Epac1 (8-CPT-2′-O-Me-cAMP), we have discerned whether immune responses that are regulated by cAMP are dependent on regulation by PKA or Epac1. In conclusion, we show that although Epac1 is expressed in human peripheral monocytes and activates Rap1, cAMP modulates most monocyte immune functions by activation of PKA and not via the Epac1-Rap1 route. However, upon monocyte differentiation into macrophages, the Epac1 expression level is increased 3-fold relative to circulating monocytes. In these cells, cAMP mediates suppression of FcR-mediated phagocytosis in a PKA-dependent manner as the Epac-specific analog 6-Bnz-cAMP potently suppressed TNF-α production. In contrast, the Epac analog 8-CPT-2′-O-Me-cAMP did not significantly reduce production of TNF-α. We conclude that cAMP regulation of FcR-induced phagocytosis is mediated via both the Epac1 and PKA pathways, whereas TNF-α secretion is suppressed by just the PKA pathway.
6-Bnz-cAMP. 

Mainly been studied in macrophages (42, 43). In rat AM, PKA and Epac1 play differential roles in the suppressive activity of cAMP on immune functions as cAMP-dependent inhibition of phagocytosis is mediated by Epac1, whereas PKA suppresses TNF-α production (42). In neutrophils, cAMP is shown to protect against TNF-α-induced apoptosis by activation of PKA, independently of Epac, again showing the discrepancy of cAMP regulation in cells (48).

Integrin activity can be regulated by changes in integrin surface expression, integrin surface distribution (avidity), or induction of conformational changes that increase integrin ligand affinity (49). Functional activation converts the inactive integrin into a ligand-binding heterodimer, a process that involves major conformational changes in the extracellular domains of both α- and β-chains (50, 51). Several studies have demonstrated that Rap1 has an important role in the regulation of integrins (29, 30, 42, 52). Rap1 activation is induced by various extracellular stimuli and is strongly connected with cAMP signaling through Epac. In Oc1r3 cells, Epac is involved in controlling integrin-mediated cell adhesion to fibronectin (35). cAMP-mediated cell adhesion and spreading on laminin-5 are independent of PKA, but rather Rap1 dependent and mimicked by 8-CPT-2′-O-Me-cAMP (34). Studies using activation-specific Abs show that Rap1 regulates both the avidity and affinity, but not the cell surface expression of integrin receptors (29, 30, 52). Elevated levels of cAMP, induced by treatment with rolipram and forskolin, have been shown previously to inhibit cell migration and Mac-1 expression (19, 53). In our studies on human monocytes, activation of the Epac1-Rap1 pathway using 8-CPT-2′-O-Me-cAMP did not have any regulatory effects on β2 integrins. However, PKA activation reduced the surface expression of the LFA-1, p150,95, and Mac-1, and also the number of the active epitope of this latter integrin (Mac-1 CBRM1/5).

FcγRs as well as Mac-1 (also known as the complement receptor 3) are involved in the uptake of opsonized microorganisms during infection. Mac-1 binds complement receptor 3b on complement-opsonized targets, whereas FcγRs bind to IgG-coated targets to mediate phagocytosis. In J774.A1 macrophages, Rap1 activates Mac-1, allowing phagocytosis of complement-opsonized targets (42). In this study, Rap1 overexpression, or inhibition, did not affect FcR-mediated phagocytosis. However, activation of Epac1 suppressed phagocytosis in a different system using rat AM and NR8383 rat AM cells, whereas no effect of the PKA-specific agonist 6-Bnz-cAMP was found even at 2 mM concentrations (43). This is in contrast to our findings using human MDM in which activation of both Epac1 and PKA showed inhibitory effects on FcR-mediated phagocytosis. Our results demonstrate that concentrations as low as 30 μM 8-CPT-2′-O-Me-cAMP suppressed phagocytosis to the same level as PGE2 and 6-Bnz-cAMP. In a very recent report, myelin phagocytosis was inhibited by both the PKA-specific analog 6-Bnz-cAMP and the Epac-specific 8-CPT-2′-O-Me-cAMP in mouse microglia and macrophages (54), supporting our data that cAMP inhibits phagocytosis through both PKA and Epac activation. Furthermore, our data show that cAMP-mediated TNF-α secretion from MDM is only regulated through the PKA pathway in line with observations made in rat AM (43). However, we did not experience any inhibitory regulation of phagocytosis by cAMP on freshly isolated monocytes, indicating that cAMP elicit different downstream signaling events dependent on the differentiation status of the cells, which may relate to the tissue-specific role of macrophages as opposed to circulating monocytes.

In conclusion, we have shown that Epac1 is present in human peripheral monocytes. Despite this, none of the cAMP-mediated effects on the various immune functions investigated in this study

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**FIGURE 8.** Epac1 expression is increased in MDM and exerts a specific immunoregulatory function. A, CD14+ monocytes were cultured in RPMI 1640/10% ABS/HEPES supplemented with 40 ng/ml M-CSF for 0, 3, and 7 days before lysis of cells and analysis by Western blot using Epac1 Ab. The protein concentration was measured to ensure equal loading. The Western blot shown is representative of four independent cultures. Densitometry scanning revealed a 3-fold induction in Epac1 expression level (average, 3.0 ± 0.4 (3 days) and 2.8 ± 0.5 (7 days)) (n = 4). B, CD14+ monocytes were cultured in RPMI 1640/10% ABS/HEPES supplemented with 40 ng/ml M-CSF for 6 days. Cells were preincubated with PGE2 (1 μM), 8-CPT-2′-O-Me-cAMP, or 6-Bnz-cAMP before addition of FITC-labeled IgG E. coli and subsequent determination of fluorescence intensity. Data represent mean ± SEM (n = 4) or mean ± half range (n = 2) for 30 μM 8-CPT-2′-O-Me-cAMP and 100 μM 6-Bnz-cAMP. *, Denotes p < 0.02, by two-tailed paired samples t test. C, CD14+ monocytes were cultured in RPMI 1640/10% ABS/HEPES supplemented with 40 ng/ml M-CSF for 6 days before preincubation with PGE2 (1 μM), 8-CPT-2′-O-Me-cAMP, or 6-Bnz-cAMP, followed by LPS activation. TNF-α in supernatants was measured after 20 h of incubation. The data show mean ± half range (n = 2) or mean ± SEM (n = 3) for control, 100 μM 8-CPT-2′-O-Me-cAMP, and 300 μM 6-Bnz-cAMP. *, Denotes p < 0.025, by two-tailed paired samples t test.
was addressed to Epac1. However, the expression of Epac1 increases during the differentiation process of monocytes into macrophages, and Epac1 acquires a specific immunoregulatory function in mature macrophages, suggesting a specific effect of Epac in fully differentiated cells.

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Disclosures

The authors have no financial conflict of interest.

References


