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SHORT COMMUNICATION

Differences Between Macrophages and Dendritic Cells in the Cyclic AMP-Dependent Regulation of Lipopolysaccharide-Induced Cytokine and Chemokine Synthesis

DAVID M. ARONOFF,[1] JENNIFER K. CARSTENS,[1] GWO-HSIAO CHEN,[2] GALEN B. TOEWS,[2] and MARC PETERS-GOLDEN[2]

ABSTRACT

Cyclic adenosine monophosphate (cAMP) is an intracellular signaling molecule responsible for directing cellular responses to extracellular signals. Once believed to signal exclusively through its ability to bind protein kinase A (PKA), recent research has revealed alternative cAMP-binding targets involved in PKA-independent processes. In this study we addressed the hypothesis that the guanine nucleotide exchange protein directly activated by cAMP (Epac-1) and PKA differentially regulate inflammatory mediator production in distinct phagocytic cell types. To accomplish this, we compared the release of cAMP-regulated polypeptide inflammatory mediators in both macrophages (obtained from the lung and peritoneum) and bone marrow-derived dendritic cells (DCs) stimulated with bacterial endotoxin. Using the highly selective Epac-1 and PKA activating cAMP analogs 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP, respectively, we found that macrophages differ from DCs in the involvement of these distinct cAMP pathways in modulating inflammatory mediator release in response to endotoxin. Whereas the regulation of cytokine and chemokine production in macrophages by cAMP was solely dependent on PKA, we found that both Epac-1 and PKA activation could regulate mediator production in DCs. This finding may be important in the pharmacologic regulation of immune responses through manipulation of cAMP signaling cascades and contributes to our understanding of the differences between these cell types.

Since its discovery in the late 1950s, cyclic adenosine monophosphate (cAMP) has maintained a position as the archetypal second messenger responsible for directing cellular responses to extracellular signals. It is synthesized from adenosine triphosphate (ATP) by the enzyme adenylate cyclase following stimulation by G_s protein-coupled receptors and is catabolized to adenosine monophosphate (AMP) by cellular phosphodiesterases. Many physiologic agents signal through G_s protein-coupled receptors and effect changes in cell behavior through the actions of cAMP. These include epinephrine, histamine, serotonin, adenosine, and prostaglandin E_2 (PGE_2).

Importantly, cAMP signaling pathways modulate key aspects of host innate immunity. In general, elevations in intracellular cAMP depress innate immune responses.[1] For example, cAMP inhibits the elaboration of proinflammatory cytokines and lipids by macrophages and polymorphonuclear leukocytes (PMNs); these include tumor necrosis factor-α (TNF-α),[2] interleukin-1β (IL-1β),[3] IL-12,[4] and leukotriene (LT)B_4.[5,6] By contrast, cAMP enhances the production of the immunosuppressive cytokine IL-10.[7] Interestingly, cAMP enhances production of IL-6 in lipopolysaccharide (LPS)-stimulated macrophages[8] but inhibits its generation in LPS-treated dendritic cells (DCs).[9] This cytokine exerts either proinflammatory or anti-inflammatory effects depending largely on the context in which it is produced.[10]

The mechanisms whereby cAMP modulates cell function were thought for many years to depend exclusively on the activation of protein kinase A (PKA), an enzyme that phosphorylates specific Ser or Thr residues on myriad cellular proteins, including the transcription factor cAMP response element binding (CREB) protein.[11] Through the use of specific inhibitors of PKA, it became apparent that certain effects of cAMP once thought to rely on PKA activation were, in fact, PKA independent. Within the last several years, alternative intracellular targets for cAMP have been identified. These include cyclic nu-

Divisions of [1]Infectious Diseases and [2]Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Health System, Ann Arbor, MI 48109.

cleotide-gated ion channels involved in the transduction of olfactory and visual signals and the guanine nucleotide exchange proteins directly activated by cAMP, Epac-1 and Epac-2.[12] The Epac enzymes, on binding cAMP, catalyze the exchange of GTP for GDP and subsequently activate the small GTPases Rap1 and Rap2.[13] Although Epac-2 appears to have a limited tissue distribution, Epac-1 is widely distributed[14] and has been implicated in the control of integrin-mediated cell adhesion via Rap1 activation in a number of cells.[15] Studying the relative roles of PKA and Epac in cellular processes has been advanced by the recent development of stable, cell-permeable, phosphodiesterase-resistant cAMP analogs that selectively activate either PKA (e.g., 6-Bnz-cAMP) or Epac (e.g., 8-pCPT-2′-O-Me-cAMP).[16] These compounds demonstrate remarkable se-

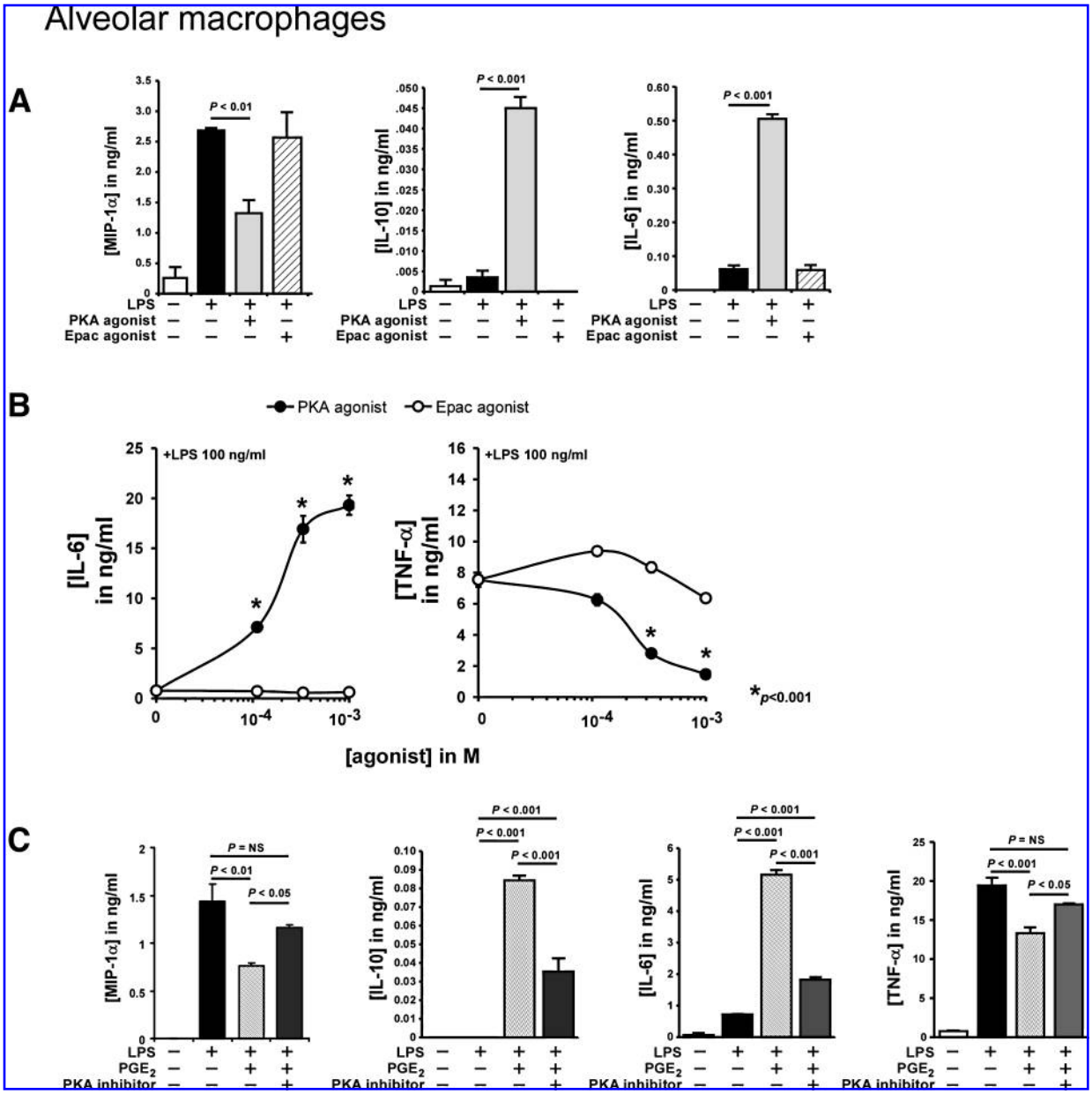

FIG. 1. Inflammatory mediator regulation by cAMP signaling cascades in AMs. (**A**) Rat AMs (1×10^5/well) were pretreated (60 min) with 6-Bnz-cAMP (PKA agonist, 1 mM) or 8-pCPT-2′-*O*-Me-cAMP (Epac-1 agonist, 1 mM) prior to overnight incubation with LPS (100 ng/mL) as described in the text. Levels of MIP-1α, IL-10, and IL-6 were measured by ELISA. (**B**) Dose-response relationship between the amount of LPS-stimulated IL-6 and TNF-α and the selective cAMP analogs 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP tested under the same conditions as in **A**. (**C**) Rat AMs were pretreated with the PKA inhibitor Rp-8-CPT-cAMPS (325μM) or vehicle (30 min), then incubated with PGE_2 (10^{-6} M) for 1 h before overnight treatment with LPS. 10^{-5} M PGE_2 was used to suppress TNF-α production. Mediators were quantified by ELISA. Shown are representative results of one experiment performed in triplicate from a total of $\geq$ three independent investigations. Results were analyzed by one-way ANOVA followed by a Bonferroni test of statistical significance.

lectivity in intact cells, even in the millimolar concentration range.[17,18]

Only recently was Epac-1 expression identified in cells involved in innate immunity. Aronoff et al.[6] first reported the presence of Epac-1 in macrophages and identified a role for this protein in downregulating the process of Fc receptor-mediated phagocytosis in alveolar macrophages (AMs). They found that the regulation by cAMP of certain processes (e.g., phagocytosis) was mediated by Epac-1, whereas that of others (e.g., the production of TNF-α and LTB_4) depended exclusively on PKA activation.[6] Interestingly, killing of intracellular bacteria following phagocytosis appeared to be regulated by both signaling cascades.[6]

A recent report from Jing et al.[19] suggests that this paradigm of distinct roles for Epac-1 and PKA in modulating cell function may not apply to all cell types. In their study, cAMP elevation inhibited the release of the inflammatory chemokines macrophage inflammatory protein-1α (MIP-1α) (CCL3) and MIP-1β (CCL4) from LPS-stimulated DCs in a PKA-independent, Epac-1-dependent manner.[19] This result was the opposite of the situation in AMs, where Epac-1 did not appear to regulate the production of TNF-α in response to LPS.[6] We, therefore, sought to address the hypothesis that Epac-1 and PKA differentially regulate inflammatory mediator production in distinct cell types. To accomplish this, we chose to compare the release of cAMP-regulated polypeptide mediators in both macrophages (obtained from the lung and peritoneum) and DCs (derived from bone marrow) stimulated with LPS. To our knowledge, this is the first head-to-head comparison of intracellular, regulatory cAMP signaling networks among distinct phagocyte populations.

We first assessed the relative impact of PKA vs. Epac-1 in regulating inflammatory mediator synthesis in AMs incubated overnight with LPS (100 ng/mL). Such experiments were an extension of the TNF-α results we published previously.[6] In light of the findings of Jing et al.,[19] we measured MIP-1α responses in AMs incubated with LPS in the presence or absence of specific activators of PKA (6-Bnz-cAMP, Biolog LSI, Bremen, Germany) or Epac-1 (8-pCPT-2′-O-Me-cAMP, Biolog LSI) (Fig. 1A). For these experiments, rat AMs were harvested by *ex vivo* lung lavage from pathogen-free 125–150-g female Wistar rats (Charles River Laboratories, Portage, MI) and cul-

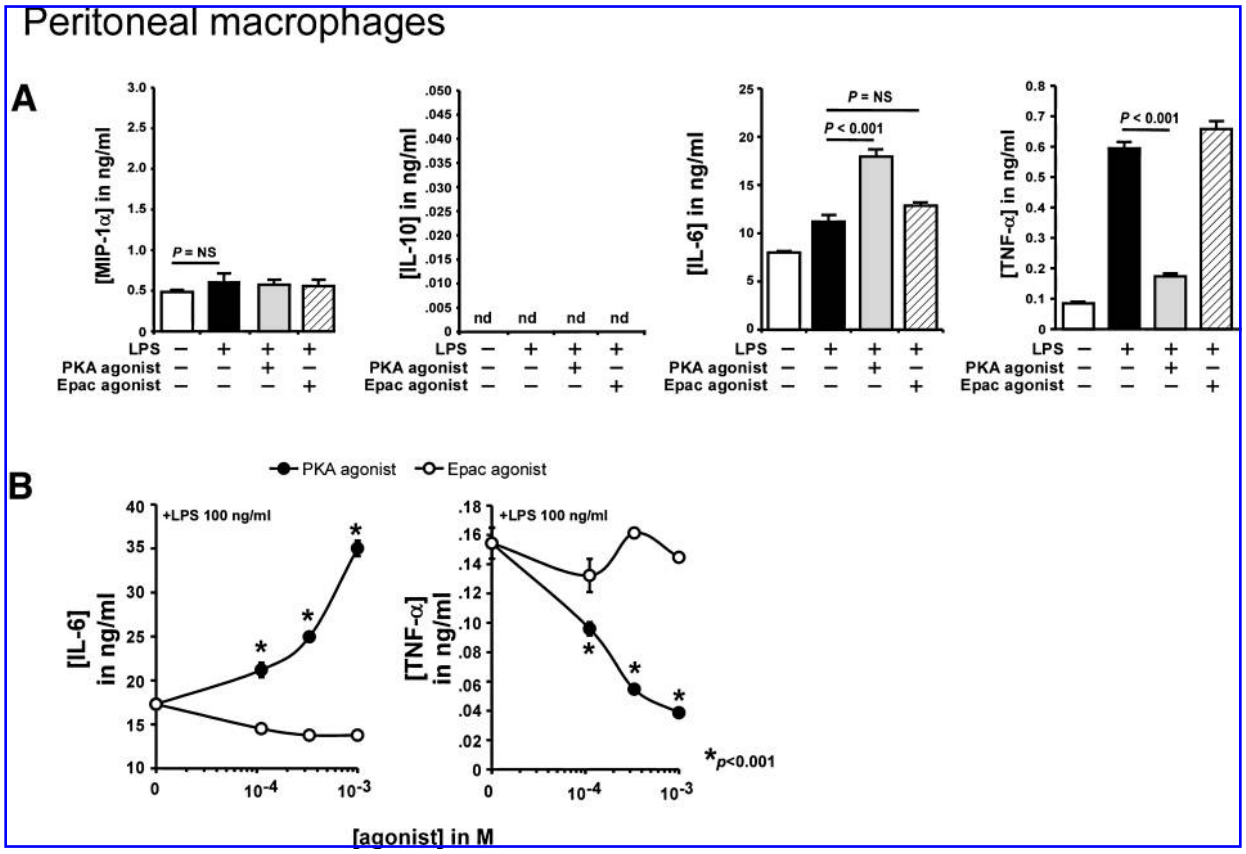

FIG. 2. Inflammatory mediator regulation by cAMP signaling cascades in PMs. (**A**) Rat PMs (1×10^5/well) were pretreated (60 min) with 6-Bnz-cAMP (PKA agonist, 1 mM) or 8-pCPT-2′-*O*-Me-cAMP (Epac-1 agonist, 1 mM) prior to overnight incubation with LPS (100 ng/mL) as described in the text. Levels of MIP-1α, IL-10, IL-6, and TNF-α were measured by ELISA. (**B**) Dose-response relationship between the amount of LPS-stimulated IL-6 and TNF-α and the selective cAMP analogs 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP tested under the same conditions as in **A**. Shown are representative results of one experiment performed in triplicate from a total of ≥ three independent investigations. Results were analyzed by one-way ANOVA followed by a Bonferroni test of statistical significance. nd, none detected.

tured according to our previously published protocol.[20] They were then pretreated for 1 h with 1 mM 6-Bnz-cAMP (hereafter, PKA agonist) or 1 mM 8-pCPT-2′-O-Me-cAMP (hereafter, Epac agonist) prior to the addition of LPS-containing medium as described.[6] Cell culture supernatants were centrifuged to remove any cellular debris, then stored at −80°C until used. Mediators were quantified using ELISA kits for TNF-α, MIP-1α, MIP-1β, IL-6, and IL-10 (all from R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations.

As illustrated in Figure 1A, only selective activation of PKA inhibited LPS-induced MIP-1α production in AMs, whereas Epac-1 stimulation had no effect. The effect of the PKA agonist on MIP-1α production was dose dependent (data not shown), with maximal suppression seen at the concentration of 1 mM (Fig. 1A). We confirmed the dependence on PKA in this system using the PKA inhibitor Rp-8-CPT-cAMPS to block the suppressive effects of PGE_2 (Fig. 1C). Our results parallel the findings of Pozo et al.[21] that PKA inhibition prevented the inhibitory effects of cAMP on LPS-stimulated MIP-1α in the RAW 264.7 murine macrophage cell line.

cAMP is known to increase LPS-stimulated IL-10[7] and IL-6 expression by macrophages[22] in a PKA-dependent fashion. However, the ability of Epac-1 to regulate these cytokines has not been previously explored. As shown (Fig. 1A), concentrations of the Epac agonist as high as 1 mM failed to augment IL-6 in the presence of LPS stimulation, whereas the PKA agonist had a dramatic effect. Both the stimulation of IL-6 and the suppression of TNF-α generation by 6-Bnz-cAMP were clearly dose dependent (Fig. 1B). As with IL-6, we found a similar profile for PKA/Epac-1 activation in the regulation of IL-10 (Fig. 1A). It is interesting that rat AMs do not generate increased amounts of IL-10 in response to either LPS (Fig. 1A) or cAMP analogs alone (data not shown) but require priming by PKA activation to produce enhanced quantities after LPS treatment. The involvement of PKA in the regulation of these mediators was confirmed by experiments in which AMs were pretreated for 60 min with or without the cAMP-stimulating lipid mediator PGE_2 in the presence or absence of the selective PKA inhibitor Rp-8-CPT-cAMPS (Fig. 1C).

Shown in Figure 2A are the results of similar experiments using resident peritoneal macrophages (PMs) obtained from rats as previously described.[23] Like AMs, PMs express Epac-1.[24] Notably, LPS failed to enhance MIP-1α production by PMs, a phenomenon noted more than a decade ago.[25] In addition, rat PMs failed to generate detectable amounts of IL-10 under our experimental conditions. However, IL-6 and TNF-α regulation by cAMP in the PM recapitulated our findings with AMs, with effects shown only with selective PKA activation (Fig. 2). Thus, our results demonstrate that in primary tissue macrophages, the regulation of inflammatory mediators by cAMP depends on PKA and not Epac-1.

The ability of cAMP to suppress MIP-1α and MIP-1β in LPS-stimulated DCs has been reported to be PKA independent.[19] We sought to confirm this finding and extend such studies to other mediators in DCs in order to provide a broader comparison with other phagocytic cells. To accomplish this, bone marrow (BM) cells were harvested from the flushed marrow cavities of femurs and tibiae from CBA/J mice under aseptic conditions. BM DCs were generated from mouse BM cells (2 ×

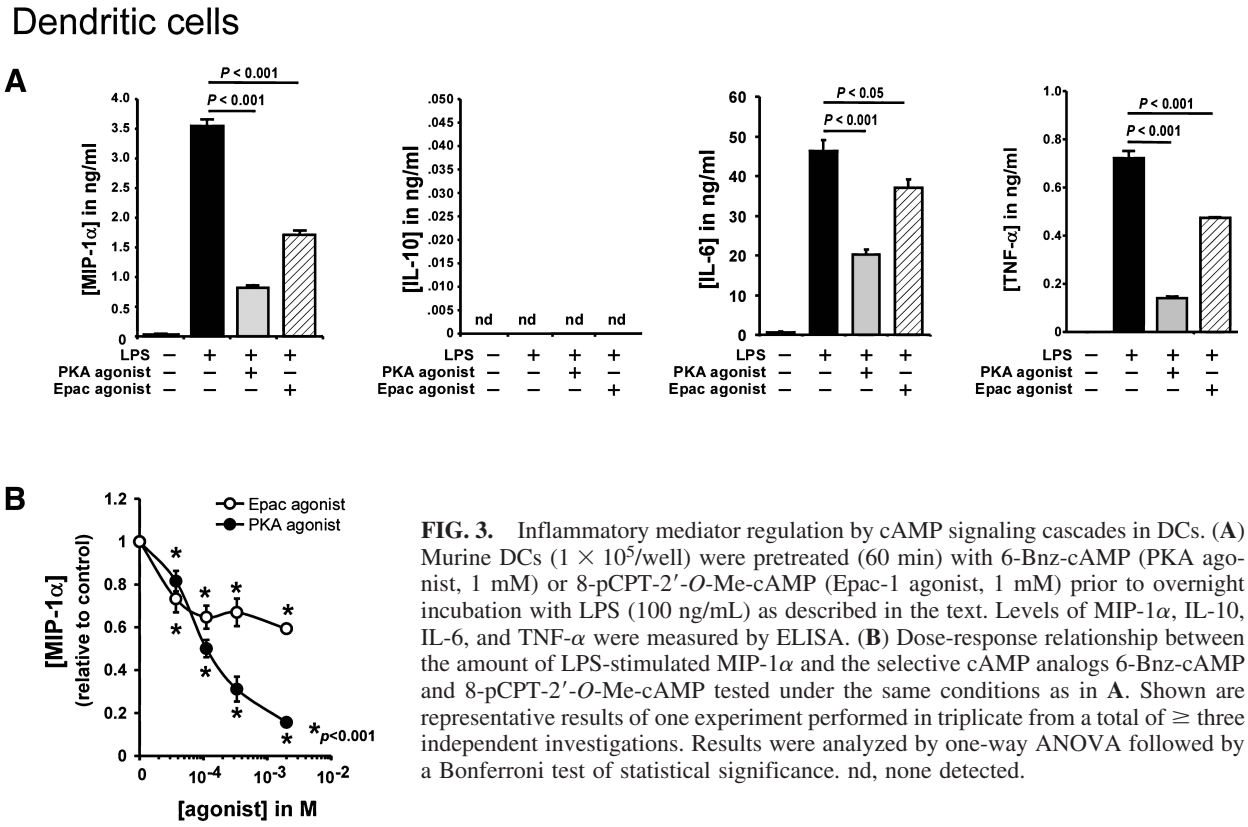

FIG. 3. Inflammatory mediator regulation by cAMP signaling cascades in DCs. (**A**) Murine DCs (1×10^5/well) were pretreated (60 min) with 6-Bnz-cAMP (PKA agonist, 1 mM) or 8-pCPT-2′-*O*-Me-cAMP (Epac-1 agonist, 1 mM) prior to overnight incubation with LPS (100 ng/mL) as described in the text. Levels of MIP-1α, IL-10, IL-6, and TNF-α were measured by ELISA. (**B**) Dose-response relationship between the amount of LPS-stimulated MIP-1α and the selective cAMP analogs 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP tested under the same conditions as in **A**. Shown are representative results of one experiment performed in triplicate from a total of ≥ three independent investigations. Results were analyzed by one-way ANOVA followed by a Bonferroni test of statistical significance. nd, none detected.

10^5 cells/mL) cultured for 7 days in culture medium (RPMI with 10% fetal bovine serum [FBS]) supplemented with 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ), as described by Jing et al.[26] $CD11c^+$ DCs were harvested and purified from the nonadherent cells by immunomagnetic sorting using anti-CD11c-coated magnetic beads and the autoMACS system (Miltenyi Biotec, Auburn, CA). The purity of the sorted cells was determined by FACS analysis (>94% $CD11c^+$).

As illustrated (Fig. 3A), LPS stimulation provoked MIP-1α production by DCs, and this was, in fact, inhibited ~50% by the Epac agonist (1 mM). This effect was dose dependent (Fig. 3B). Interestingly, however, we also found an even greater degree of inhibition of MIP-1α production by the PKA agonist, suggesting that PKA activation can also potently counterregulate MIP-1α production in DCs. Similar results were seen for MIP-1β (data not shown). In our hands, murine DCs did not secrete detectable quantities of IL-10 (Fig. 3), but both TNF-α and IL-6 were upregulated by LPS treatment. In the case of TNF-α, selective activation of either PKA or Epac suppressed TNF-α production (Fig. 3). These data accord with those of Vassiliou et al.,[27] who reported that the suppression of LPS-stimulated TNF-α in murine DCs by PGE_2 was only partially prevented by the PKA inhibitor H-89. In contrast to the observation with macrophages, intracellular cAMP elevation in DCs suppressed LPS-stimulated IL-6 production.[9] We witnessed profound inhibition of LPS-stimulated IL-6 with the PKA agonist but only modest suppression with the Epac-activating cAMP analog (Fig. 3).

To begin to elucidate the mechanistic basis whereby Epac-1 regulates inflammatory mediator production by DCs but not in macrophages, we performed immunoblot assays for Epac-1 and its proximal target, Rap1, in AMs, PMs, and DCs. For these experiments, AMs and resident PMs were harvested from CBA/J mice and cultured in 6-well dishes (2×10^6 cells/well). Rat cells were not used in these experiments to control for possible differences in antibody-binding affinities between rat and mouse proteins. Following adherence purification (for AMs and PMs), cells were collected in sterile PBS and lysed by sonication in the presence of protease inhibitors (complete protease inhibitor cocktail tablets, Roche, Indianapolis, IN) and sodium orthovanadate (2 mM). DCs were collected and lysed in a similar fashion. Western blots were performed as previously described.[28] Protein samples (100 μg) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with commercially available rabbit polyclonal Epac-1 (1:500) (Upstate, Lake Placid, NY), Rap1 (1:1000) (Pierce, Rockford, IL), or mouse monoclonal α-tubulin (1:1000) (Sigma, St. Louis, MO) antibodies, followed by horseradish peroxidase (HRP)-conjugated antirabbit secondary antibodies and ECL Plus chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ).

As shown in Figure 4, Epac-1 expression was confirmed in each of the three cell types. Although Epac-1 activity has been documented pharmacologically in DCs,[19] this is, to our knowledge, the first direct confirmation of its expression in the DC. Because there were no obvious differences in Epac-1 expression among AMs, PMs, and DCs, we turned our attention to the downstream Epac effector protein Rap1, a GTPase of the Ras subfamily of small GTP-binding proteins. Using an antibody that detects both isoforms of Rap1 (Rap1A and Rap1B), we found approximately equivalent amounts in AMs and PMs (Fig. 4). Surprisingly, Rap1 expression appeared to be diminished in unstimulated DCs in comparison to macrophages. How this translates into the immunoregulatory behavior of Epac-1 in these cells remains an important question. Whether the subcellular localization of Epac-1 or Rap1 differs between macrophages and DCs is under investigation by our group.

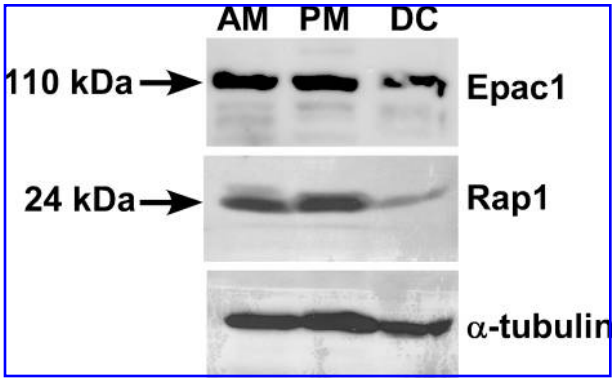

FIG. 4. Expression of Epac-1 and Rap1 in macrophages and DCs. AMs, PMs, and BM-derived DCs were isolated and cultured as described in the text. Protein samples from total cell lysates (100 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and then probed for the presence of Epac-1, Rap1, and α-tubulin (loading control). Shown is a representative blot of $n = 3$.

These data confirm the involvement of Epac described by Jing et al. in DCs,[19] but differ from their results by suggesting an even greater role for PKA in chemokine regulation. Jing et al.[19] concluded that PKA did not play a role in their system because the suppression of LPS-stimulated MIP-1α by PGE_2 was not prevented by H-89. However, the inability of H-89 to block clearly PKA-mediated phenomena has been observed,[29] and its specificity for PKA has been questioned.[29] In our hands, PKA inhibitors other than H-89 (Rp-8-CPT-cAMPS and PKI_{14-22}) only partially abrogated the effect of PGE_2 on MIP-1α release by LPS-stimulated DCs (data not shown). Although such data accord with the results of Jing et al.,[19] our investigations were strengthened by testing the effects of the highly PKA-specific agonist 6-Bnz-cAMP[16] and clearly demonstrate an ability for PKA to regulate chemokine release in DCs. Additionally, as we employed a different strain of mouse in our studies than did Jing et al., this may also contribute to the differences in findings.

In this paper, we have compared the release of mediators known to be regulated by cAMP in LPS-stimulated phagocytes to address the hypothesis that Epac-1 and PKA regulate inflammatory mediator production differently in distinct phagocytic cell types. To our knowledge, these studies are the first to do so and provide novel insights into the regulatory cascades governing cAMP signaling in inflammatory cells. We have confirmed an important role for PKA in regulating cytokine and chemokine expression in two primary macrophage populations and have ruled out a contributory role for Epac-1 in this system. Our data extend our previous observation that TNF-α and

LTB_4 production by AMs could be regulated by cAMP in a PKA-dependent, Epac-1-independent manner. Recently, similar findings for TNF-α were reported for human peripheral blood monocytes.[2]

Regulation of polypeptide mediator generation by DCs differs from that in macrophages, as a role for Epac-1 is demonstrable. The molecular mechanisms underlying the differential regulation of inflammatory mediators by PKA or Epac-1 in leukocytes remain unclear. It is notable that the selective activation of Epac-1 did not modify inflammatory mediator production in DCs to nearly the same extent as did PKA activation. How Epac-1 activation modulates cytokine and chemokine generation in DCs remains speculative. Jing et al.[19] present a model in which Epac-1 activation ultimately provokes the DNA binding of the transcriptional repressor CCAAT displacement protein (CDP), which in turn suppresses MIP-1α and MIP-1β transcription. A role for CDP in regulating either TNF-α or IL-6 production has not been shown, however, and our finding that Epac-1 also regulates these cytokines implies that other mechanisms may be operative.

In conclusion, we have shown that regulation of several polypeptide mediators of inflammation by cAMP in the macrophage follows activation of PKA, whereas in DCs both Epac-1 and PKA appear to play a regulatory role. This finding may be important in the pharmacologic regulation of immune responses through manipulation of cAMP signaling cascades and contributes to our understanding of the differences between these cell types.

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Address reprint requests or correspondence to:
Dr. David M. Aronoff
Division of Infectious Diseases
Department of Internal Medicine
University of Michigan Health System
5220 MSRB III
1150 W. Medical Center Drive
Ann Arbor, MI 48109

Tel: (734) 647-1786
Fax: (734) 764-4556
E-mail: daronoff@umich.edu

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