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Cyclic Nucleotides Promote Monocyte Differentiation Toward a DC-SIGN\(^+\) (CD209) Intermediate Cell and Impair Differentiation into Dendritic Cells\(^1\)

Daniela Giordano,* Dario M. Magaletti,* Edward A. Clark,\(^2\)* and Joseph A. Beavo\(^\dagger\)

Recruitment of monocytes into tissues and their differentiation into macrophages or dendritic cells (DCs) depend on the microenvironment of the inflammatory site. Although many factors affecting this process have been identified, the intracellular signaling pathways implicated are poorly understood. We found that cyclic nucleotides regulate certain steps of monocyte differentiation into DCs. Increased levels of the cyclic nucleotides, cAMP or cGMP, inhibit differentiation of CD14\(^+\)/CD1a\(^++\) monocytes into CD14\(^-/\)CD1a\(^++\) DCs. However, DC-specific ICAM-3-grabbing nonintegrin (CD209) up-regulation was not affected by cyclic nucleotides, indicating that DC development was not blocked at the monocyte stage. Interestingly, Ag-presenting function was increased by cyclic nucleotides, as measured by the higher expression of MHC class II, CD86, and an increased ability to stimulate CD4\(^+\) T cell proliferation in allogeneic MLRs. Although cyclic nucleotides do not completely block DC differentiation, they do block the ability of DCs to be induced to mature by LPS. Treatment during DC differentiation with either cAMP or cGMP analogues hampered LPS-induced expression of CD83, DC-LAMP, and CCR7 and the ability of DCs to migrate toward CCL19/ macrophage-inflammation protein 3β. Interestingly, the induction of a CD16\(^+\) subpopulation of cells was also observed. Thus, signals causing an increase in either cAMP or cGMP levels during monocyte recruitment to inflammatory sites may restrain the activation of acquired immunity by blocking DC development and migration to lymph nodes. At the same time, these signals promote development of an active intermediate cell type having properties between those of macrophages and DCs, which might contribute to the innate immune response in the periphery. The Journal of Immunology, 2003, 171: 6421–6430.

Dendritic cells (DCs)\(^3\) are APCs mainly responsible for bridging innate and adaptive immunity. Upon activation in the periphery by pathogens and other inflammatory stimuli, they migrate to T cell areas of the lymph nodes and potentially activate naïve T cells (1–4). DCs originate from both myeloid and lymphoid precursors, giving rise to subsets with different functional features. The myeloid subset is the most heterogeneous and includes skin Langerhans cells and other tissue DCs. Human peripheral blood has a small number of myeloid DCs, but circulating monocytes may be a major source of precursors for this subset. Human ex vivo studies and in vivo studies in mice show that the interaction of monocytes with endothelial cells during reverse transmigration into lymphatic vessels promotes differentiation of monocytes into DCs (5–7). When cultured in vitro in the presence of GM-CSF and IL-4, monocytes acquire specific markers and features of immature myeloid DCs (e.g., CD1a), while losing markers typical of monocytes and macrophages (Mphs) such as CD14 (8, 9). Nevertheless, monocytes have a certain plas-ticity in the acquisition of a specific developmental program (reviewed in Ref. 10). Stimuli like M-CSF, IL-10, or IL-6 made by fibroblasts or epithelial cells redirect differentiation of monocytes into Mphs in the presence of GM-CSF and IL-4 (11–13). In contrast, fully differentiated Mphs exposed to GM-CSF/IL-4 acquire some DC phenotypes (9, 14). Langerhans cell-like DCs also can be generated from murine hemopoietic progenitor cells through a monocyte/macrophage differentiation pathway (15).

The specific intracellular mechanisms underlying these differentiating processes have not been fully defined. Cyclic nucleotide (CN) signaling pathways, cAMP in particular, have been implicated in the cell differentiation of many cell types (16–19). Ka-llinski et al. (20) suggested that cAMP might regulate DC differentiation. They showed that the presence of PGE\(_2\), which increases intracellular cAMP, during monocyte differentiation into DCs blocked both the down-regulation of CD14 and the up-regulation of an immature DC marker CD1a. However, the ability of DCs to present Ag and activate naïve T cell proliferation remained unchanged. Cells exposed to PGE\(_2\) during differentiation also lost the ability to produce IL-12, suggesting that CAMP inducers have the potential to skew the immune response toward Th2 immunity. Recently, Sombroek et al. (21) reported that high concentrations of PGE\(_2\) produced by primary tumors play a major role in tumor-induced inhibition of DC differentiation. Neither of these studies addressed the intracellular mechanism operating downstream of PGE\(_2\).

Most regulatory effects of PGE\(_2\) are exerted through the activation of the cAMP-dependent pathway (22, 23). This signaling pathway has been implicated in DC maturation and function (24–29), but the role of cAMP in DC differentiation from monocytes has not been addressed. Moreover, because many cAMP-elevating agents have been used as anti-inflammatory agents (30–33), we were interested in defining their ability to affect DC differentiation.
We also analyzed whether cGMP might play a role in DC differentiation. To assess whether cGMP or cAMP affects DC differentiation, we used the cell membrane-permeable CN analogues (8-bromo-cAMP (8B-cAMP); 8-bromo-cGMP (8B-cGMP)), and compounds that directly activate adenylate cyclase, e.g., forskolin (Fsk), as well as the wide range phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX). PDE inhibitors have been used to elevate cAMP and/or cGMP in cells as they prolong the increase in CN levels by inhibiting the enzymes responsible for turning down the signal (30, 34).

In addition to CD1a vs CD14 expression, we examined the effect of cAMP or cGMP on the expression of other specific DC markers, such as DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (CD209) (3), or LPS-induced maturation markers like CD83, the lysosomal marker DC-lysosomal-associated membrane protein (DC-LAMP) (35), and CCR7, the main chemokine receptor responsible for DC migration to lymph nodes (2, 36). Overall, our data show that an increase in CN levels during monocyte differentiation into DCs promotes differentiation into a CD14+/CD1a+/DC-SIGN+ intermediate cell type with increased ability to activate T cell proliferation compared with control DCs. CN treatment also induces a subpopulation of CD16+ cells resembling a monocyte subset with intermediate features between monocytes and monocyte-derived DCs (MoDCs). In contrast, by increasing intracellular cAMP or cGMP during differentiation, terminal development into mature DCs is strongly impaired, as shown by the inhibition of LPS-induced up-regulation of CD83, DC-LAMP, and CCR7, and the ability to migrate toward CCL19/ macrophage-inhibitory protein (MIP) 3β. Taken together, our data suggest that signals increasing CNs may block the activation of acquired immunity by arresting DC differentiation at an intermediate stage, unable to migrate to lymph nodes, but showing a potent T cell proliferation activity, therefore suggesting a potential role for this intermediate cell in the innate immune response in the periphery.

Materials and Methods

Generation of MoDCs and CD4+ T cells

MoDCs were generated from human PBMCs obtained either from buffy coat preparations or leukapheresis products from healthy donors. Monocytes purified from leukapheresis products by CD14+ immunomagnetic positive selection were purchased from the Cellular Therapy Laboratory at Fred Hutchinson Cancer Research Center (Seattle, WA). PBMCs obtained from buffy coat samples were isolated by centrifugation over Ficoll-Hypaque (Robbins Scientific, Sunnyvale, CA). After SRBC rosetting to deplete T cells, CD14+ cells were obtained by positive selection with magnetic anti-CD14 microbeads, according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). In both cases, CD14+ cells were 97–99% pure, as assessed by flow cytometry. Monocytes were either directly used for experiments or frozen in RPMI 1640 medium containing or not 200 ng/ml of recombinant human CCL19/MIP1β (RDI); Cells containing RPMI 1640 medium alone were used as control for spontaneous migration. A total of 2.5 × 106 cells in a total volume of 100 μl was added to the upper chamber and incubated at 37°C for 3 h. Cells that migrated into the lower chambers were harvested, concentrated to a volume of 200 μl, and counted by flow cytometry acquiring events for a fixed time period of 30 s with the use of CellQuest software (BD Biosciences). Each experiment was performed in duplicate. The number of spontaneously migrated cells was subtracted from the total number of migrated cells. Values are given as the mean number of migrated cells ± SD.

Migration assays

Migration of MoDCs differentiated for 4 days in absence or presence of CN agonists was measured using the transwell system (24-well plates; 8.0 μm pore size; Costar, Corning, NY). To the lower chamber was added 600 μl of RPMI 1640 medium containing or not 200 ng/ml of recombinant human CCL19/MIP1β (RDI). Wells containing RPMI 1640 medium alone were used as control for spontaneous migration. A total of 2.5 × 106 cells in a total volume of 100 μl was added to the upper chamber and incubated at 37°C for 3 h. Cells that migrated into the lower chambers were harvested, concentrated to a volume of 200 μl, and counted by flow cytometry acquiring events for a fixed time period of 30 s with the use of CellQuest software (BD Biosciences). Each experiment was performed in duplicate. The number of spontaneously migrated cells was subtracted from the total number of migrated cells. Values are given as the mean number of migrated cells ± SEM.

Cytokine detection

Cytokine secretion by MoDCs differentiated in the absence or presence of CN agonists was measured by ELISA. IL-6, IL-12p70, TNF-α, and IFN-γ ELISA were performed on supernatants collected at day 2 or 4 of differentiation, or at day 4 plus 24 h of LPS stimulation. IL-12p70 ELISA (R&D Systems, Minneapolis, MN) was performed, according to the manufacturer’s instruction. IL-6, TNF-α, and IFN-γ were detected by capture immunoassay in triplicate using matched pairs of cytokine-specific mAbs (BD Pharmingen): capture anti-IL-6, MQ2-13A5; detection anti-IL-6, MQ2-39C3; capture anti-TNF-α, mAb1; detection anti-TNF-α, mAb11; capture anti-IFN-γ, NIH42; and detection anti-IFN-γ, 4B3. Concentrations of...
cytokines were extrapolated from a standard curve prepared with recombinant cytokine (BD Pharmingen). Detection limits of the assay were 94 pg/ml for IL-6, 0.5 ng/ml for TNF-α, and 0.5 ng/ml for IFN-γ.

Results
Elevating either cAMP or cGMP intracellular levels impairs monocyte differentiation into DCs

cAMP- or cGMP-elevating agents added to monocytes during their differentiation into MoDCs disrupted the normal changes in CD1a vs CD14 expression (Fig. 1). Treatment with graded doses of 8B-cGMP, a cGMP cell-permeable analog, inhibited both CD14 down-regulation and CD1a up-regulation (Fig. 1A). The 8B-cAMP, the cAMP analog, had the same effect, but 20-fold lower concentrations of 8B-cAMP than 8B-cGMP were required in this case (data not shown). The extent of inhibition of CD1a expression was donor dependent; in 30% of the donors, even at the highest concentration of CN analogues, some CD1a induction was still detected. DC differentiation was almost complete by 2 days; the blocking effect by either CN on CD14/CD1a expression was already evident at day 2 and was maintained. These changes in the expression of surface markers were also accompanied by inhibition of the classical morphological changes that occur during DC differentiation, e.g., increases in size and side light scatter typical of MoDCs (data not shown). Because external addition of CNs may not necessarily reflect physiologic changes, it was important to test whether other regulators of intracellular CNs could also affect DCs. Therefore, we examined both the adenylyl cyclase activator, Fsk, and a broad spectrum PDE inhibitor, IBMX, which can lead to increases in either cAMP or cGMP (Fig. 1B); the same inhibition of CD14 down-regulation and CD1a up-regulation, observed with the CN analogues, was seen with Fsk or IBMX. Taken together, these findings show that increased levels of either CN can block the differentiation of CD14<sup>+</sup>/CD1a<sup>−</sup> monocytes into CD14<sup>−</sup>/CD1a<sup>+</sup> DCs, suggesting that the same effect previously observed with PGE<sub>2</sub> treatment is likely to be mediated through an increase in cAMP (20).

Next we looked at the effect of CNs on surface expression of DC-SIGN (CD209), a DC-restricted C-type lectin involved in the early interaction between DCs and naive T cells and also DC trafficking and internalization of Ags (3, 37). DC-SIGN is up-regulated during differentiation of monocytes into DCs (38). This up-regulation was not inhibited by treatment with Fsk, 8B-cAMP, or 8B-cGMP (Fig. 2A). We also analyzed the expression of FcγRI (CD64), the high affinity IgG FcR, which is constitutively expressed on monocytes and down-regulated during DC differentiation. Again, CD64 down-regulation was not affected by CN treatment (Fig. 2A). Terminal differentiation of myeloid cells is characterized by a switch of expression from FcγRI/CD64 to the low affinity FcγRII/CD32 and other FcRs (e.g., for IgA and IgE) (12, 39). Therefore, we analyzed the effect of CNs on the low affinity IgG receptors, FcγRII/CD32 and FcγRII/CD16. CD32 is expressed on monocytes and up-regulated during either Mpf differentiation or to a lesser extent DC differentiation. Treatment with either CN analog during monocyte differentiation into DCs caused an increase in the expression of CD32 greater than on control MoDCs (Fig. 2A), suggesting a skew toward a more Mpf-like cell type. CD16 is expressed on two subsets of monocytes, CD14<sup>dim</sup>/CD64<sup>−</sup> and CD14<sup>high</sup>/CD64<sup>+</sup>, each representing <10% of the total monocyte population in human peripheral blood (40). Both subsets of CD16<sup>+</sup> monocytes display an intermediate phenotype between monocytes and MoDCs, e.g., share with MoDCs an increased expression of CD86 and MHC class II and higher alloreactivity compared with CD14<sup>−</sup>/CD16<sup>+</sup> monocytes. Interestingly, the percentage of CD16<sup>+</sup> cells, normally very low in MoDCs (2–9% of total cells), was increased up to 50% after treatment with the PDE inhibitor IBMX (Fig. 2B) or with the cAMP analog (data not shown) and also consistently increased by treatment with the cGMP analog (Fig. 2B). The two CNs differed in their ability to induce a subpopulation of CD16<sup>+</sup> cells. With some variability between donors, after treatment with 8B-cAMP the percentage of CD16<sup>+</sup> cells was 5- to 10-fold higher than control MoDCs, while upon 8B-cGMP treatment was increased by 2.5- to 5-fold (reaching respectively up to 20–50% of total cells for cAMP and 10–20% for cGMP). These CD16<sup>+</sup> cells are CD64<sup>+</sup> on the surface, have low forward and side scatter signal, and may be related to the previously described CD14<sup>dim</sup>/CD14<sup>+</sup>/CD64<sup>+</sup> subset (Fig. 2A and B, and data not shown). However, a higher level of DR and CD80 was observed by two-color labeling in CN-treated CD16<sup>+</sup> cells compared with the monocyte subset (data not shown).

FIGURE 1. CNs block differentiation of CD14<sup>+</sup> monocytes into CD1a<sup>+</sup> DCs. The percentages of each cell subset are indicated. A, CD1a/CD14 expression in monocytes (MO) at day 0, and after 4 days of differentiation with GM-CSF/IL-4 in absence (MoDC) or presence of increasing doses (from the left to the right panel) of 8B-cGMP (0.5–1–1.5 mM). B, CD1a/CD14 expression after 4 days of differentiation in absence (MoDC) or presence of increasing doses of Fsk (10–50–100 μM), or (lower panel) 100 μM IBMX. Data are representative of eight for 8B-cGMP separate experiments from different donors, and six for Fsk and IBMX.
termediate cells having properties between monocytes, Mphs, and DCs. In further discussion about these cells, we will use the term CN-DCs, to indicate monocytes differentiated into DCs in the presence of CN-increasing agents, as opposed to normal MoDCs.

**CNs up-regulate molecules involved in Ag presentation and enhance the ability to activate T cell proliferation**

Interestingly, even though some markers of DC differentiation were inhibited by CNs, the expression of markers involved in Ag presentation, like MHC class II and the costimulatory molecule CD86, was enhanced by treatment with CNs during differentiation (Fig. 3). Already by day 2 of culture, when untreated DC only slightly up-regulated surface expression of the MHC class II molecule DQ, the addition of 8B-cGMP strongly increased the expression of this marker (Fig. 3A). Although DQ was down-regulated in control MoDCs by day 4, cGMP-treated cells retained high levels of DQ. If control MoDCs were stimulated with LPS at day 4 for an additional 24 h, as expected, DQ was partially up-regulated again (Fig. 3A), while 100% of cells previously differentiated in the presence of 8B-cGMP remained DQhigh. Therefore, the increased levels of DQ were induced and maintained in 8B-cGMP-treated cells throughout DC differentiation and maturation. The same results were observed by increasing CAMP levels (data not shown). CN or IBMX also induced a significant increase in DR expression during DC differentiation (Fig. 3, B and D). Moreover, the costimulatory molecule CD86 underwent a similar up-regulation when DCs were differentiated in the presence of increased levels of cAMP or cGMP (Fig. 3C). However, no changes in the expression of CD80 were observed (data not shown). In parallel with the increases in MHC class II and CD86 expression, treatment with CN-inducing agents also induced the formation of large cell clusters (data not shown). Cluster formation is typical of DCs induced to mature.

To test whether CN-DCs could function as APCs, we examined their ability to activate T cell proliferation in allogeneic MLR assays. Monocytes differentiated in the presence of Fsk, 8B-cAMP, or 8B-cGMP displayed up to 5-fold higher stimulatory ability than MoDCs when added to allogeneic CD4 T cells (Fig. 4), consistent with their increased expression of MHC class II and CD86. Therefore, increasing the levels of CNs during DC differentiation produces a cell type (CN-DCs) with an increased ability to activate T cell proliferation.

**Upon LPS stimulation, cells differentiated in presence of CN do not up-regulate CD83, DC-LAMP, or CCR7 and have impaired migration toward CCL19/MIP3B**

Based on morphology and the expression of CD14/CD1a, DC differentiation is impaired by treating monocytes with CN (Fig. 1); in contrast, treated cells still develop into efficient APCs, typical of DCs (Figs. 3 and 4). Thus, it was unclear whether CN-DCs could be induced to mature. To test whether CNs affect maturation of DCs, we compared the ability of LPS to induce expression of specific mature DC markers in MoDCs vs CN-DCs. In further discussion about these cells, we will use the term CN-DCs, to indicate monocytes differentiated into DCs in the presence of CN-increasing agents, as opposed to normal MoDCs.

Mature MoDCs, cells differentiated into MoDCs for 4 days and then stimulated with LPS for 24 h, up-regulated expression of CD83 and DC-LAMP (Fig. 5, A and B, left panels). In contrast, CN-DCs, differentiated in the presence of increasing levels of CN inducers, show a decrease in the LPS-stimulated induction of CD83 (Fig. 5A) and DC-LAMP (Fig. 5B). At the highest concentration of CN inducers, CD83 and DC-LAMP expression was comparable to immature MoDCs, although a small percentage of mature CN-DCs still expressed high levels of DC-LAMP (Fig. 5B).
To verify the specificity of the effect of CNs on DCs, we examined LPS-treated CN-DCs for the expression of CD68, a lysosomal glycoprotein homologue to DC-LAMP, which is found in both Mφs and DCs. In contrast to DC-LAMP, CD68 is normally up-regulated during MO differentiation into DCs, and then slightly down-regulated upon DC maturation (35). LPS-matured CN-DCs still expressed as high levels of CD68 as control immature MoDCs and Mφs (data not shown). Thus, CNs only inhibit the expression of DC-restricted markers. LPS-induced expression of CCR7 was also affected in CN-DCs (Fig. 6, A and B). Unlike DC-LAMP, CCR7 expression was reduced in all treated cells. Furthermore, CCR7 induction was more readily inhibited compared with CD83 and DC-LAMP; a strong effect was already evident at the lowest concentration of CN inducers (Figs. 5 and 6, A and B). Consistent with the inhibition of CCR7 induction, LPS-treated CN-DCs were also impaired in their ability to migrate toward CCL19/MIP3β in transwell migration assays (Fig. 6C). However, 8B-cGMP in most experiments was less effective than IBMX or 8B-cAMP in the ability to inhibit migration, perhaps suggesting a different mechanism for the two CNs (e.g., expt. 2; Fig. 6C).

In summary, the effect of CN on CD83, DC-LAMP, and CCR7 expression by LPS-treated CN-DCs is consistent with the effects of CNs on CD14/CD1a expression. Therefore, CNs strongly impair differentiation of monocytes into MoDCs by affecting the regulation of certain DC-restricted markers and by disrupting the development of an important DC function: the ability to respond to CCL19/MIP3β, i.e., the chemotactic signal that normally triggers DCs to migrate to sites where they can activate naive T cells.

CNs affect spontaneous and induced cytokine production of MoDCs

We next tested whether cytokine production in CN-DCs was different compared with MoDCs. Because CN-DCs show an intermediate phenotype, we evaluated IFN-γ and TNF-α production, as they are inflammatory cytokines released in large amounts by activated Mφs. No release of IFN-γ or TNF-α could be detected in the first 4 days of differentiation in the absence or presence of CN analogues (data not shown). We then measured IL-12p70 and IL-6,
also normally produced by activated Mφs. Neither MoDCs nor CN-DC produced IL-12p70 throughout 4 days of differentiation (data not shown). Conversely, either 8B-cAMP or 8B-cGMP induced a dose-dependent induction of IL-6 release (Fig. 7A). However, the induced IL-6 release, although consistent, was always 100–250 times lower than the concentration described to affect DC differentiation by skewing toward a Mφ phenotype (13).

We then tested the ability of MoDCs and CN-DCs to release IL-12p70 and other cytokines upon LPS stimulation. As previously shown with PGE2 treatment (20), pretreatment during differentiation with 8B-cAMP potently inhibited LPS-induced IL-12p70 production, even at the lowest concentration tested (Fig. 7B). Interestingly, cGMP also exerted a similar effect (Fig. 7B). Similarly, the ability of DCs to produce high amounts of TNF-α after LPS stimulation was affected by CN pretreatment (Fig. 7B). These results are consistent with previous observations, showing that increases in CNs reduce the ability of activated monocytes, Mφs and DCs, to produce TNF-α (32, 33). In contrast, LPS-induced release of IL-6 was relatively refractory to CN treatment and only inhibited at the higher concentrations of CNs used (Fig. 7B).

Discussion

Monocytes are circulating precursors of both macrophages and DCs, which can be recruited into tissues and differentiate depending on the microenvironment of the inflammatory site (5, 6, 10). This differentiation process is complex and regulated by cytokines (9) and also can be regulated by viruses (42, 43). Although many factors affecting DC and Mφ differentiation have been identified, the intracellular signaling pathways that regulate these processes also need to be further elucidated.
are poorly understood. PGE\textsubscript{2}, which generally signals through activating the cAMP pathway, is known to affect DC differentiation by inhibiting CD14 down-regulation and CD1a up-regulation (20). In this study, we investigated whether CNs affect monocyte differentiation into DCs by using cell-permeable CN analogues, an adenylate cyclase agonist, and a broad spectrum PDE inhibitor. We found that both cAMP and cGMP regulate certain steps in DC differentiation. To our knowledge, this is the first study showing that increased levels of cGMP as well as cAMP affect DC differentiation. Increased levels of either CN block the reciprocal regulation of CD14/CD1a and morphologic changes induced by the

**FIGURE 6.** Inhibition of LPS induction of CCR7 and impaired migration toward CCL19/MIP3\textbeta in cells differentiated in presence of increased levels of CNs. Monocytes differentiated into DCs for 4 days and then stimulated (MoDC + 24-h LPS) or not (MoDC + 24-h medium) for 24 h with LPS were tested for surface expression of CCR7. A, MoDC differentiation performed in absence or presence of IBMX (10–25 \mu M) for 24 h. MoDC differentiation performed in absence or presence of 8B-cGMP (0.5–1 mM). Data are representative of nine separate experiments from different donors. B, Immature MoDC and LPS-treated MoDCs with or without pretreatment for 4 days with 50 \mu M 8B-cAMP (expt. 1), 25 \mu M IBMX (expt. 2), or 1 mM 8B-cGMP (expts. 1 and 2) were tested for their chemotactic response to CCL19/MIP3\textbeta. The data are shown as the mean of duplicate cultures ± SEM and are from two of four experiments from different donors. The mean numbers of spontaneously migrated cells were subtracted from the number of cells that migrated in response to chemokines.

**FIGURE 7.** LPS-induced cytokine production affected in cells differentiated in presence of increased levels of cAMP or cGMP. A, Monocytes were cultured for 4 days in either medium containing cytokines (GM-CSF/IL-4) or medium containing cytokines plus increasing doses of 8B-cAMP (25–50–100 \mu M), or 8B-cGMP (0.5–1–2 mM). Supernatants were collected and analyzed for IL-6 by ELISA. B, After 4 days of DC differentiation in absence or presence of increasing doses of either 8B-cAMP or 8B-cGMP, cells were stimulated or not with LPS (0.5 \mu g/ml) for 24 h. Supernatants were collected and analyzed for IL-12p70, TNF-\alpha, and IL-6 by ELISA. Data represent the mean of triplicate wells ± SEM and are representative of four separate experiments from different donors. *, \( p < 0.002; **, \( p < 0.0001 \) compared with control LPS-treated MoDCs (■). Student’s \( t \) test.
DC-differentiating cytokines GM-CSF/IL-4. Yet, CNs do not inhibit the regulation of other markers such as DC-SIGN and FcγRII/CD64. Also, the up-regulation of the low affinity FcγRII/CD32, occurring during normal differentiation into DCs, was not impaired by CN treatment. Interestingly, the expression of CD32 was even enhanced by CN treatment compared with control MoDCs, leading to a cell type resembling a Mph. Similarly, during Mph development, a cAMP analog has been found to promote a switch from CD64 to CD32 expression (12, 39). Because CN inducers promoted development of an intermediate cell type between Mphs or DCs, we referred to these cells as CN-DCs.

To better characterize this intermediate cell, we tested the sensitivity of CN-DCs to LPS maturation compared with normal MoDCs. We found that differentiation in the presence of either cAMP or cGMP inhibited LPS-induced increases of some specific mature DC markers, such as CD83, DC-LAMP, and CCR7. DC-LAMP is a lysosome-associated membrane glycoprotein that belongs to a DC-specific apparatus for Ag processing (35). Ag goes through distinct endocytic routes in different APCs, and specific markers characterize each path (44). Both Mphs and DCs express another lysosomal marker, CD68, which is homologous to DC-LAMP, but is regulated differently than DC-LAMP during DC maturation. As DC-LAMP appears in Ag-processing compartments of DCs, CD68 progressively disappears (35). Contrary to the remarkable inhibition of DC-LAMP induction by CN treatment, CD68 was not affected. Therefore, treatment with CN during monocyte differentiation into DCs impairs the ability of these cells to acquire DC-specific machinery for processing Ags, although it does not inhibit Ag-processing pathways shared with other myeloid cells.

More important, we observed that CN-DCs upon LPS stimulation have impaired induction of CCR7 expression, and consequently, show impaired ability to migrate toward CCL19/MIP3β, a key chemokine leading DCs to lymph nodes (2, 36). We detected a difference in the ability of cGMP vs cAMP to affect migration; the mechanisms underlying this difference are currently under investigation. Taken together, our data suggest that signals causing an increase in CN level during monocyte recruitment to inflammatory sites might hold back the activation of acquired immunity by impairing development and migration of mature DCs to lymph nodes.

In contrast, contrary to many other specific immature and mature DCs markers, CN inducers do not impair the up-regulation of DC-SIGN during differentiation. This suggests that DC development from monocytes is not completely blocked by CNs. DC-SIGN has been considered a DC-specific marker, because of the high expression restricted to DCs, where it can stabilize the initial contact between DCs and naive T cells. However, DC-SIGN also promotes cell trafficking and internalization of Ags (3, 37). Recent reports show that DC-SIGN is expressed also by certain subsets of monocytes and Mphs (41, 45).

Considering the observed pattern of markers expressed by CN-treated cells, we cannot exclude the possibility that the resulting cell type might correspond to another myeloid subpopulation, which cannot be strictly defined as a DC. In this regard, several studies describe two subsets of monocytes expressing CD16 with an intermediate phenotype between monocytes/Mphs and DCs (40, 41, 46, 47). In peripheral blood of normal individuals, less than 10% of monocytes are CD16⁺; interestingly, a CD14⁺/CD16⁺ subset is increased up to 40% in many pathological conditions such as HIV, autoimmune diseases, or sepsis (40, 46). A similar monocyte/DC subset, which also expresses DC-SIGN, may be responsible for HIV transmission in the blood where viral concentrations are low (41). Consistent with the possibility that a subpopulation of CN-DCs might be related to the intermediate CD14⁺/CD16⁺ monocytes, after differentiation of DCs in the presence of CNs, we observed an increase in the percentage of cells expressing CD16 (up to 50% with cAMP and 20% with cGMP analogues). A comparison with freshly isolated monocytes from the same donors shows that CD16⁺ CN-DCs obtained in this study closely resemble the phenotype of the CD14⁺/CD16⁺/CD64⁻ subset (40, 47). Indeed, after CN treatment, we observed an enrichment in the subpopulation of CD16⁺ cells that coexpress low CD14, do not express CD64, and have reduced granularity and cell size, as opposed to the CD16⁺/CD64⁺ subset of monocytes with higher expression of CD14, granularity, and cell size (47). CD16⁺ cells induced in our system also coexpress DC-SIGN, similarly to the CD14⁺/CD16⁺/DC-SIGN⁺ subset described by Engering et al. (41). Furthermore, the CD14⁺/CD16⁺ monocyte subset described in literature displays high levels of Ag-presenting and costimulatory molecules and higher alloreactivity, comparable to those expressed by DCs (46). Similarly, we found that increased levels of cAMP or cGMP during DC differentiation induced up-regulation of MHC class II and CD86, and that the subpopulation of CD16⁺ CN-DCs correlates with DRhigh and CD86high subpopulations, although the level of expression of these markers was even higher than that of the CD14⁺/CD16⁺/CD64⁻ subset. Ancuta et al. (46) found that the monocyte subset CD14⁺/CD16⁺ can also be obtained in vitro by culturing monocytes with GM-CSF/IL-4 plus IL-10. This is interesting because cAMP-elevating agents can induce IL-10 release from DCs (26). Also, TGF-β has been shown to expand the CD16⁺ monocyte subset (7). It might be interesting to test whether CN signaling might be part of the intracellular mechanisms used by physiological signals to modulate the expansion of the CD16⁺ subpopulation in pathological conditions.

The expression of MHC class II and CD86 was even higher on CN-DCs than control MoDCs, consistent with their increased ability to activate CD4 T cells in allogeneic MLRs. Therefore, CN-DCs, despite having lower levels of specific DC markers, do acquire potent APC capability, higher than monocytes, Mphs, and control immature MoDCs. In these regards, they resemble the stimulatory ability of mature MoDCs. These findings are consistent with recent studies showing that PGE₂ exerts the same effect on the ability of differentiated MoDCs to activate T cell proliferation (21, 28, 29).

Our results demonstrate that when the level of cAMP is increased during DC differentiation from monocytes, the resulting cells cannot be induced by LPS to become mature DCs able to migrate toward CCL19/MIP3β. In contrast, when PGE₂ is added to immature DCs during stimulation with LPS or CD40L, DC maturation and migration toward CCL19/MIP3β are increased (28, 29). Together these findings suggest that the timing of exposure to CN can influence whether DC precursors become intermediate DCs or mature DCs. It will be interesting to test whether the intermediate cell type described in this study is equivalent to a myeloid subset described in vivo.

Alternatively, CN-DCs might represent an intermediate stage in myeloid development, and CN treatment during DC differentiation might result in freezing the cells in this stage. On this matter, earlier studies about APC function and the role of cAMP in Mph differentiation are consistent with CN-DCs being different from Mphs and support the existence of an intermediate stage in myeloid differentiation. The differences in the phenotype of transitory DCs are discussed in this study (Fig. 8), correlated to a transitory increase in markers involved in Ag presentation (9). Our data suggest that CNs can arrest DC differentiation at this stage (Fig. 8). Only a further stimulation of MoDCs by maturing stimuli leads to induction of these markers.
therefore might exert a functional role during innate immune re-
activity, but the ability to stimulate T cell proliferation, which
intermediate stage or a myeloid subset with reduced in-
time, this environment may promote the enrichment of either an
spons in the periphery. Monocyte differentiation into Mphs and
DCs is a multistep complex process that regulates both innate and
acquired immune response. Defining the intracellular mechanisms
regulating this process may lead to more specific targets for drug
development.

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FIGURE 8. Model of macrophage and DC differentiation from monocytes. The effect of CNs on both Mph (according to Peters et al.; see Ref. 51) and DC differentiation is represented.


